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Plasma Membrane Processes in Smooth Muscle: Characterization of Ca$^{2+}$ Transport and Muscarinic Cholinergic Receptors: A Thesis

Pamela A. Lucchesi
University of Massachusetts Medical School

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Plasma Membrane Processes in Smooth Muscle:

Characterization of Ca$^{2+}$ Transport and Muscarinic Cholinergic Receptors

A Thesis Presented

By

Pamela A. Lucchesi

Submitted to the Faculty of the University of Massachusetts Medical School in partial fulfillment of the requirements for the degree of

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PLASMA MEMBRANE PROCESSES IN SMOOTH MUSCLE:
CHARACTERIZATION OF Ca²⁺ TRANSPORT AND
MUSCARINIC CHOLINERGIC RECEPTORS

A Thesis
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Pamela A. Lucchesi

Approved as to style and content by:

Peter Grigg, Ph.D., Chairman

Martin Marinus, Ph.D.

Daniel Kilpatrick, Ph.D.

Cheryl R. Scheid, Ph.D.

Thomas Honeyman, Ph.D., Advisor

Cheryl R. Scheid, Ph.D., Advisor

Hiroshi Yamaguchi, Ph.D., Advisor

Hiroshi Yamaguchi, Ph.D.

Thomas B. Miller, Ph.D.
Dean of Graduate School
of Biomedical Sciences
Department of Physiology
April 1989
DEDICATION

This thesis is dedicated to my parents John and Janet Lucchesi for their love, continuous support and encouragement. More importantly, I am grateful to them for teaching me to work hard to achieve my goals and to never sacrifice my integrity.
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I sincerely thank Dr. Thomas Honeyman and Dr. Hiroshi Yamaguchi for their guidance and encouragement. I would also like to express my appreciation for the training I received from Greta Carraway and Dr. Martin Marinus.

There are several people who deserve special thanks for their help and guidance throughout my thesis research. I am grateful to Chris Mangsen-Baker and Rachel Cooney for their excellent technical assistance and for putting up with my many compulsions. Debra Mullikin-Kilpatrick and Judy Luber-Narod were of invaluable help in discussing the receptor studies, and I am indebted to them for their insights and expertise. A special thanks to Carol Enterline for her extreme patience and flawless skills in typing this thesis.

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ABSTRACT

The thesis research was designed to study the characteristics of two important physiological processes in smooth muscle: Ca$^{2+}$ transport mediated by the plasmalemmal Ca$^{2+}$-ATPase and muscarinic receptor-G protein interactions. In resting smooth muscle, several Ca$^{2+}$ extrusion or sequestration processes offset the passive inward leak of Ca$^{2+}$. Although biochemical evidence suggests that the plasmalemmal Ca$^{2+}$ pump plays a key role in this process, the precise role of this enzyme could not be proven until a reliable estimate of the inward Ca$^{2+}$ leak was measured. Recent studies using dispersed smooth muscle cells from the toad stomach provided an estimate of the basal transmembrane Ca$^{2+}$ flux rate; thus, we examined the transport capacity of the plasmalemmal Ca$^{2+}$ pump in this tissue. Gastric smooth muscle tissue was disrupted by homogenization and nitrogen cavitation. Membranes enriched 20-fold for plasma membrane markers were obtained using differential centrifugation and purification by flotation on discontinuous sucrose gradients.

The membrane vesicles exhibited an ATP-dependent $^{45}$Ca uptake that was insensitive to azide or oxalate but sensitive to stimulation by calmodulin or inhibition by orthovanadate and the calmodulin antagonists trifluoperazine (TFP) or calmidazolium (CMZ). $^{45}$Ca accumulated in the
presence of ATP was rapidly released by Ca\textsuperscript{2+} ionophore but not by agents that stimulate Ca\textsuperscript{2+} release from the sarcoplasmic reticulum (caffeine, inositol trisphosphate, GTP). However, both CMZ and TFP evoked a Ca\textsuperscript{2+} release that was comparable to that observed in the presence of Ca\textsuperscript{2+} ionophore, suggesting that these compounds have profound effects on membrane Ca\textsuperscript{2+} permeability.

\textsuperscript{45}Ca transport exhibited a high affinity for Ca\textsuperscript{2+} (K\textsubscript{D} 0.2 \textmu M) and a high transport capacity, producing a > 12,000-fold gradient for Ca\textsuperscript{2+} and a transmembrane flux rate at least 3-fold greater than that observed in resting smooth muscle cells.

As a first step toward understanding the biochemical basis for the diversity of muscarinic cholinergic actions on smooth muscle, we examined the distribution of muscarinic receptor subtypes and coupling to guanine nucleotide-binding (G) proteins in airway and gastric smooth muscle. Receptor subtypes were classified in membranes prepared from bovine trachea and toad stomach based on the relative abilities of the selective antagonists pirenzepine (M\textsubscript{1}), AF-DX 116 (M\textsubscript{2}) and 4-DAMP (M\textsubscript{3}) to displace the binding of nonselective antagonist \([\textsuperscript{3}H]\)QNB (quinuclidinyl benzilate). Based on the binding profiles for these antagonists, it was concluded that both smooth muscle types contain a mixture of M\textsubscript{2} and M\textsubscript{3} subtypes. In trachea the majority of receptors (86%) were M\textsubscript{2}, whereas in stomach the majority of receptors (88%) were M\textsubscript{3}. 
The displacement of $[^3]H$QNB binding by the agonist oxotremorine indicated a mixed population of high affinity ($K_D = 4 \text{ nM}$) and low affinity ($K_D = 2-4 \text{ mM}$) binding sites. The addition of GTPyS abolished all high affinity agonist binding, suggesting that coupling of the receptors to G proteins may confer high affinity. Reaction of membranes with pertussis toxin in the presence of $[^32]P$NAD caused the $[^32]P$-labelling of a ~41 kD protein in both gastric and tracheal smooth muscle. Pretreatment of the membranes with pertussis toxin and NAD completely abolished high affinity agonist binding in gastric smooth muscle, but produced little if any decrease in high affinity agonist binding in trachea. We conclude that, although muscarinic receptor activation leads to the elevation of intracellular Ca$^{2+}$ and contraction of both airway and gastric smooth muscle, the dissimilar distributions of receptor subtypes and distinct patterns of coupling to G proteins may indicate that each smooth muscle type uses different receptor-G protein interactions to regulate intracellular signalling pathways.
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CHAPTER I.
INTRODUCTION TO SMOOTH MUSCLE Ca\(^{2+}\) HOMEOSTASIS

The level of cellular calcium (Ca) is considered to be the primary determinant regulating the contractile state in smooth muscle. Although the detailed mechanisms by which Ca regulates smooth muscle contraction are not fully understood, it is generally considered that a rise in intracellular Ca\(^{2+}\) activates the regulatory protein calmodulin, resulting in a stimulation of myosin light chain kinase with a concomitant increase in actomyosin ATPase activity and tension development. The messenger role of Ca\(^{2+}\) requires its maintainence within the cytoplasm at a low (submicromolar) concentration, and the cell has evolved mechanisms to maintain intracellular Ca\(^{2+}\) at a level that is approximately 10,000 fold lower than the extracellular fluid. (Carafoli, 1986). The resultant large electrochemical gradient for Ca\(^{2+}\) ensures that even minor changes in the Ca\(^{2+}\) permeability of the plasma membrane in response to physiological stimuli could result in significant changes in its intracellular concentration.

The major goal of studies of Ca\(^{2+}\) handling by smooth muscle has been to elucidate the mechanisms by which the Ca\(^{2+}\) concentration within smooth muscle cells are maintained at rest and in response to relaxing or contractile stimuli. A large variety of preparations have been employed in such studies including intact smooth muscle strips, subcellular membrane fractions prepared from smooth muscle or suspensions of enzymatically dispersed smooth muscle cells. Initial efforts focused on determining the source of cellular Ca\(^{2+}\)
(extracellular vs. intracellular) that was involved in the contractile response of intact tissues. These studies indicated that two different reservoirs of Ca$^{2+}$ could serve as activator Ca$^{2+}$: an extracellular pool of Ca$^{2+}$ and a pool of Ca$^{2+}$ that is tightly bound or sequestered within the cell (Hurwitz and Suria, 1971). The role of extracellular Ca$^{2+}$ in contraction was initially demonstrated in studies in smooth muscle strips in which contraction was measured as a function of extracellular Ca$^{2+}$ concentration (Edman and Schild, 1962; Hurwitz et al., 1962; Brading and Sneddon, 1980). Such studies showed that Ca$^{2+}$ removal from the external medium led to the gradual loss of contractile responsiveness of the smooth muscle strips (Hiroaka et al., 1968), although there was considerable tissue to tissue variability in the rate of decline of responsiveness. In addition, there was considerable variability as to the relative importance of extracellular Ca$^{2+}$ for different contractile agents: responses to electrical stimuli and KCl depolarization were much more dependent on extracellular Ca$^{2+}$ than were responses to pharmacological stimuli. Such results led to the observation initially made by Edman and Schild (1962) that Ca$^{2+}$ may also be released from an intracellular store by pharmacological agents. The role of these intracellular Ca$^{2+}$ stores in the regulation of smooth muscle contractility have been studied primarily in vascular smooth muscle, in which responses to norepinephrine were shown to persist in Ca$^{2+}$-free solutions (Hudgins and Weiss, 1968; Devine et al, 1972). Similar results were later demonstrated with smooth muscle from the trachea using histamine and acetylcholine (Kirkpatrick, 1975) and from the taenia coli (Brading and Sneddon, 1980).
Further evidence for the role played by extracellular and cellular Ca\textsuperscript{2+} stores was sought by following the unidirectional and net movements of 45\textsuperscript{Ca} between the smooth muscle tissue and its external environment. 45\textsuperscript{Ca} fluxes at rest and in response to contractile agents (KCl depolarization, norepinephrine, acetylcholine) were extensively investigated, but the outcome of such experiments did not lead to a unified concept. In initial studies of 45\textsuperscript{Ca} efflux from smooth muscle, Schatzmann (1961) was unable to correlate changes in 45\textsuperscript{Ca} washout with net changes in Ca content of the guinea pig taenia coli. Moreover, studies of 45\textsuperscript{Ca} efflux from intact tissue by Bozler (1963) led to the conclusion that the observed efflux was too large to be accounted for by an ATP-dependent Ca\textsuperscript{2+} pump, since the energy required by this pump would be considerably greater than the entire metabolism of the tissue. In addition, van Breeman and Daniel (1966) demonstrated that K\textsuperscript{+}-induced depolarization of rat uterus, which contracts smooth muscle presumably by promoting Ca\textsuperscript{2+} entry, caused a decrease in 45\textsuperscript{Ca} uptake and a net reduction in Ca content of the tissue. These results were not confirmed by Schatzmann (1964), however, who found that K\textsuperscript{+} depolarization of guinea pig taenia coli caused an increase in 45\textsuperscript{Ca} efflux with no net change in Ca content.

Reports on agonist-induced Ca\textsuperscript{2+} flux were equally contradictory. Schatzmann (1961) noted a slight stimulation of 45\textsuperscript{Ca} efflux with acetylcholine (ACh) in taenia coli whereas van Breeman and Daniel (1966) found no effect of this agonist on 45\textsuperscript{Ca} efflux from the uterus. Potter et al. (1970) found both an increase in 45\textsuperscript{Ca} uptake and efflux induced by ACh in toad stomach smooth muscle. Similarly, some studies
showed an epinephrine-induced increase in $^{45}$Ca efflux from the aorta (Hudgins, 1969), while others showed an epinephrine-induced decrease in $^{45}$Ca efflux from the same tissue (Seidel and Bohr, 1971). Thus, there was little correlation between contractility and flux studies. The principal reason underlying these apparent discrepancies is that the transport of Ca$^{2+}$ across the smooth muscle cell membrane represents only a small fraction of the total exchange that occurs between the external medium and the various extracellular binding sites within the smooth muscle tissue. Thus, the complex geometry of the extracellular space within the tissue (and resultant diffusion delays) and the prevalence of $^{45}$Ca binding sites within the extracellular matrix posed considerable difficulties in the analysis and interpretation of the results (van Breeman and McNaughton, 1979).

Several methods were subsequently developed to circumvent the problems of extracellular $^{45}$Ca binding and exchange, including the addition of La$^{3+}$ (Lanthanum) or Ca$^{2+}$ EGTA [ethyleneglycol-bis (β-aminoethylether)-N,N'- tetracetic acid], in order to displace or deplete the extracellular-bound $^{45}$Ca (van Breeman et al., 1973; van Breeman and Castels, 1974). The principle behind the "La$^{3+}$ method" is that this trivalent cation displaces the labelled Ca$^{2+}$ from extracellular sites and blocks further transmembrane $^{45}$Ca exchange. Using this method Deth and van Breeman (1974) were able to show that the rapid initial phase of aortic contractions induced by norepinephrine and angiotensin II were dependent on a common intracellular store while the slow phase of the contractions were dependent on Ca$^{2+}$ influx. In addition, they were able to observe relaxation of the tissue even though
La$^{3+}$ blocked Ca$^{2+}$ efflux across the plasma membrane, a finding which suggested that intracellular sequestration was able to bring about cellular relaxation. Finally, by exposing tissues to metabolic inhibitors to deplete cellular ATP and monitoring uptake using the "La$^{3+}$ method", Casteel and van Breeman (1975) obtained an estimate of the passive inward leak of Ca$^{2+}$ in resting smooth muscle (~ 0.02 pmol/cm$^2$ surface area·sec) and demonstrated that Ca$^2+$ extrusion from smooth muscle was markedly sensitive to depletion of cellular ATP but insensitive to changes in the transmembrane Na$^+$ gradient.

Although these approaches provided some insights into Ca$^{2+}$ handling in smooth muscle, technical problems (secondary changes in ion contents and in the permeability and stability of the plasma membrane, etc.) have compromised their usefulness. The use of isolated subcellular membranes from smooth muscle represented an alternative method to assess the complex interactions between Ca$^{2+}$ ions and smooth muscle membranes and circumvented some of the intrinsic difficulties associated with intact preparations. Studies using such preparations were able to demonstrate ATP-dependent accumulation by these membrane fractions which was initially presumed to reflect uptake by the sarcoplasmic reticulum (e.g. Carsten, 1969). It was later recognized that these crude microsomal fractions consisted primarily of plasma membrane vesicles. The presence of an active, ATP-dependent Ca$^{2+}$-transport system localized in the plasma membrane was first demonstrated in vascular (Fitzpatrick, 1972) and in visceral smooth muscle (Hurwitz et al., 1973) using membrane vesicles prepared by homogenization and differential centrifugation. These vesicles accumulated Ca$^{2+}$ in an
ATP-dependent, azide-insensitive manner that was associated with a 
(Ca$^{2+}$ + Mg$^{2+}$)-dependent ATPase activity. With more extensize 
purification of the membrane fractions it was possible to demonstrate 
two types of Ca$^{2+}$ pumps within smooth muscle. Janis et al. (1977) 
confirmed the presence of Ca$^{2+}$ pumps in the plasmalemmal and 
sarcoplasmic reticulum fractions of myometrium that exhibited similar 
affinities for Ca$^{2+}$ and similar maximum velocities ($V_{\text{max}}$). The 
presence of a second Ca$^{2+}$ transport ATPase was also suggested by 
phosphorylation experiments with smooth muscle microsomes. If membranes 
were phosphorylated in the absence of La$^{3+}$, the most prominent 
Ca$^{2+}$-dependent phosphointermediate corresponded to a protein of MW ~ 
100,000, similar to that reported for the skeletal muscle sarcoplasmic 
reticulum Ca$^{2+}$ pump. In the presence of La$^{3+}$, the most prominent 
phosphointermediate corresponded to a protein of MW ~ 138,000 similar 
to that observed for the erythrocyte plasmalemmal Ca$^{2+}$ ATPase.

The use of subcellular fractions also allowed for the direct 
demonstration of a Na$^{+}$/Ca$^{2+}$ exchange mechanism in smooth muscle. In 
addition to an active Ca$^{2+}$ transport system, it was postulated that 
smooth muscles also may be able to control intracellular Ca$^{2+}$ levels 
by an Na$^{+}$/Ca$^{2+}$ exchange system that, under physiological 
circumstances, lowers cytosolic Ca$^{2+}$ by utilizing the potential energy 
of the Na$^{+}$ gradient. Earlier attempts to demonstrate this transporter 
in intact smooth muscle (by removing Na$_o$ and observing changes in 
contractility or changes in Ca$^{2+}$ flux) had been compromised by 
technical problems such as the possible competition between Na$^{+}$ and 
Ca$^{2+}$ for membrane binding sites (Daniel et al., 1982). Using isolated
membrane fractions, however, Grover et al. (1981) were able for the first time to obtain direct evidence for the existence of a Na\(^+\)/Ca\(^{2+}\) exchange mechanism in smooth muscle plasma membranes isolated from rat myometrium (Grover et al., 1981). Such studies demonstrated that an outwardly directed Na\(^+\) gradient caused net \(^{45}\)Ca uptake and that abolition of the Na\(^+\) gradient with monensin, a Na\(^+\)-selective ionophore, abolished the effect of the Na\(^+\) gradient on Ca\(^{2+}\) uptake. Similar results have subsequently been reported for guinea pig ureter (Aickin et al., 1984) rat mesenteric artery (Matlib, 1985), tracheal smooth muscle (Slaughter, et al., 1987) and arterial smooth muscle cells (Smith et al., 1987).

The results from these types of studies have led to a better understanding of the regulation of intracellular Ca\(^{2+}\) ions in smooth muscle cells. It is now clear that contractile agents can increase intracellular Ca\(^{2+}\), either by stimulating the release of Ca\(^{2+}\) from internal stores in the sarcoplasmic reticulum or by stimulating the entry of Ca\(^{2+}\) from the extracellular medium via voltage-sensitive or receptor-operated channels. Although the sarcoplasmic reticulum represents only a small percentage of total cell volume (2-5% or roughly 0.5 pl; see Eggermont et al., 1988), it may nevertheless play an important role in regulating cytosolic Ca\(^{2+}\). For example, assuming an average resting intracellular Ca\(^{2+}\) level of 100-200 nM (Williams and Fay, 1986; Yamaguchi, 1986) and assuming that the sarcoplasmic reticulum Ca\(^{2+}\) pump generates a 10,000 fold gradient for Ca\(^{2+}\), the total Ca\(^{2+}\) in the sarcoplasmic reticulum would approach 1-2 mM, similar to measured estimates of total cellular Ca\(^{2+}\) in isolated smooth muscle
cells (Scheid and Fay, 1984). This concentration of Ca\textsuperscript{2+} within the sarcoplasmic reticulum represents approximately 1.0 fmol of Ca\textsuperscript{2+} (assuming a volume of ~ 0.5 pl). Therefore, a release of 10% of the Ca\textsuperscript{2+} from the sarcoplasmic reticulum into an average cell volume of 10 pl could raise cytosolic Ca\textsuperscript{2+} to ~ 10 \mu M, a value 10-fold higher than that required for smooth muscle contraction (Williams and Fay, 1986; Yamaguchi, 1986). Thus, the sarcoplasmic reticulum represents a potentially important storage and release site for Ca\textsuperscript{2+}.

Due to the constant inward leak of Ca\textsuperscript{2+} down its electrochemical gradient, inhibition alone of Ca\textsuperscript{2+} influx or release is not sufficient to allow relaxation to occur. Thus, an outward extrusion or inward sequestration mechanism must be operative to maintain the low cytoplasmic Ca\textsuperscript{2+} concentrations observed at rest. The studies using subcellular membrane fractions indicated that three cellular transport systems act to lower cytoplasmic Ca\textsuperscript{2+}: the Ca\textsuperscript{2+} pump located in the sarcoplasmic reticulum, the Ca\textsuperscript{2+} pump on the plasma membrane and a Na\textsuperscript{+}/Ca\textsuperscript{2+} antiporter on the plasma membrane. Although the precise contributions of these Ca\textsuperscript{2+}-transporting systems are not completely understood, there is abundant evidence that each system can contribute to the regulation of intracellular Ca\textsuperscript{2+} in smooth muscle. For example, the plasma membrane Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger has been characterized as a large capacity, low affinity (K_m = 1-10 \mu M) system that presumably functions to remove excess cytosolic Ca\textsuperscript{2+} that occurs following activation (Kwan, 1986), whereas both the plasmalemmal and sarcoplasmic reticulum pumps serve as lower capacity but higher affinity (K_m = 200-500 nM) transporters.
The Ca\textsuperscript{2+} pumps of the sarcoplasmic reticulum and plasma membrane differ in their molecular mass, immunological properties, stoichiometry between ATP hydrolyzed and calcium transported, and sensitivity to a number of inhibitors and activators. The sarcoplasmic reticulum Ca\textsuperscript{2+} pump has an affinity (\(K_m\)) for Ca\textsuperscript{2+} below 0.5 \(\mu\text{M}\), is inhibited by vanadate (50 \(\mu\text{M}\)) and transports Ca\textsuperscript{2+} with a stoichiometry of 2 Ca\textsuperscript{2+}:1 ATP (Carafoli, 1988). Its activity appears to be modulated by a proteolipid, phospholamban which can be phosphorylated by cAMP and calmodulin-dependent protein kinases (Meissner, 1987). The plasma membrane Ca\textsuperscript{2+} pump has a \(K_m\) for Ca\textsuperscript{2+} in the 0.1 to 0.5 \(\mu\text{M}\) range, is inhibited by lower concentrations of vanadate (0.1 - 1 \(\mu\text{M}\)) and transports Ca\textsuperscript{2+} with a stoichiometry of 1 Ca\textsuperscript{2+}:1 ATP. An important feature of the plasmalemmal Ca\textsuperscript{2+} pump is its regulation by calmodulin. The stimulatory effect of calmodulin was originally observed by Bond and Clough (1972) who found that a protein in human erythrocytes activated the Ca\textsuperscript{2+}-ATPase activity of the plasma membrane. Jarret and Penniston (1977) later demonstrated that purified calmodulin mimicked the effects of this soluble protein on the erythrocyte membrane.

The plasmalemmal smooth muscle Ca\textsuperscript{2+} pump is also a target for calmodulin and this interaction has been exploited to purify the ATPase on calmodulin affinity columns (Wuytack et al., 1981). The purified enzyme is a single polypeptide of MW 138,000. Quantitative analysis of the effects of calmodulin were measured using purified Ca\textsuperscript{2+} pumps reconstituted into liposomes. The most clear-cut effects of calmodulin on the overall ATPase reaction and Ca\textsuperscript{2+} transport are an increase in
the apparent affinity for Ca\textsuperscript{2+} activation and an increase in the maximal rate of the reaction (Garrahan, 1986). In intact membrane systems, calmodulin interactions with the plasmalemmal Ca\textsuperscript{2+} pump are often assessed by measuring the effects of calmodulin antagonists on ATP-dependent Ca\textsuperscript{2+} transport or on Ca\textsuperscript{2+} Mg\textsuperscript{2+} stimulated ATPase activity.

The direct involvement of calmodulin in the activation of the plasmalemmal Ca\textsuperscript{2+}-ATPase suggests the existence of a self-regulatory mechanism for intracellular Ca\textsuperscript{2+} homeostasis in smooth muscle. Calmodulin is fully activated when the cytosolic Ca\textsuperscript{2+} levels reach or exceed micromolar concentrations (Cheung, 1982), thereby activating Ca\textsuperscript{2+} extusion by the pump. As Ca\textsuperscript{2+} returns to the submicromolar steady-state level, the active calmodulin-Ca\textsuperscript{2+} pump complex dissociates, thereby decreasing enzymatic activity to the basal level (Popescu and Ignat, 1983). In addition to calmodulin regulation, the Ca\textsuperscript{2+} pump may also be regulated by cAMP-dependent phosphorylation in certain smooth muscle types (Kwan, 1986) and by protein kinase C (Furukawa et al., 1989).

The exact role of the smooth muscle sarcoplasmic reticulum Ca\textsuperscript{2+} pump is unclear, in part due to the scarcity of sarcoplasmic reticulum in smooth muscle and its variable distribution among the different smooth muscle types. It is generally considered that this enzyme plays a role in the rapid removal of increased cytosolic Ca\textsuperscript{2+} that occurs after activation by contractile agents (Eggermont et al., 1988). On the other hand, the plasmalemmal Ca\textsuperscript{2+} pump, because of its high affinity for Ca\textsuperscript{2+} and high translocation velocity, is generally considered to
be the primary regulator of steady-state cytosolic $\text{Ca}^{2+}$ levels. However, its precise contribution to cellular $\text{Ca}^{2+}$ homeostasis has been difficult to assess due to several limitations. For one, the magnitude of the basal transmembrane leak rate for $\text{Ca}^{2+}$ that must be opposed by the plasmalemmal $\text{Ca}^{2+}$ pump has not, until recently, been precisely determined due to the complexities of intact tissue preparations (diffusion delays, extensive extracellular $\text{Ca}^{2+}$ binding, etc.). Thus it has been difficult to determine whether the plasma membrane $\text{Ca}^{2+}$ pump has sufficient transport capacity to offset this leak. This difficulty was circumvented using suspensions of enzymatically dispersed gastric smooth muscle cells such that it was possible to obtain reliable estimates of basal and agonist-stimulated unidirectional transmembrane $\text{Ca}^{2+}$ fluxes (Scheid and Fay, 1984a,b).

Another limitation associated with defining the contribution of the $\text{Ca}^{2+}$ pump to $\text{Ca}^{2+}$ homeostasis involves difficulty in translating biochemical estimates of enzymatic activities to estimates of transport function in vivo. For example, estimates of maximal transport rates in membrane fractions, expressed as moles of $\text{Ca}^{2+}$ transported per mg protein, are highly dependent on the purity of the membrane preparation and on the initial velocity of $\text{Ca}^{2+}$ transport. The contention from biochemical studies that the plasmalemmal $\text{Ca}^{2+}$ pump plays a dominant role in maintaining low resting levels of cellular $\text{Ca}^{2+}$ had thus not been directly proven.

The major goal for the first portion of my thesis research was to evaluate the hypothesis that the smooth muscle plasmalemmal $\text{Ca}^{2+}$ ATPase plays an essential role in cellular $\text{Ca}^{2+}$ homeostasis. The
specific aims were designed to document the functional role of the plasma membrane Ca$^{2+}$ pump by assessing the transport capacity of the toad gastric smooth muscle enzyme under conditions which are thought to prevail in resting cells.

To this end, we:

1) developed a plasma membrane preparation from toad gastric smooth muscle. The purity of the preparation was assessed by determining the activity of several marker enzymes for the plasma membrane, mitochondria, or sarcoplasmic reticulum.

2) defined the characteristics and kinetic properties of ATP-dependent $^{45}$Ca uptake and compared them with those measured in membrane fractions prepared from pig antral smooth muscle, a preparation that has been extensively studied. The studies with porcine muscle allowed us to assess the effectiveness of our membrane preparative procedures and compare our observed Ca$^{2+}$ transport characteristics to those observed previously in this tissue by other investigators. More specifically, we measured the sensitivity to vanadate, oxalate and azide, and assessed the calmodulin sensitivity of $^{45}$Ca transport in order to determine whether the observed ATP-dependent $^{45}$Ca uptake was due to the operation of a plasmalemmal ATPase. Finally, in order to determine if the membranes were contaminated by sarcoplasmic reticulum, the effects of agents known to stimulate sarcoplasmic reticulum Ca release on $^{45}$Ca efflux from preloaded vesicles were examined.

3) determined if the plasmalemmal Ca$^{2+}$ pump was capable of
generating a transmembrane calcium gradient similar to that observed in intact smooth muscle cells.

4) converted the measured velocity of the Ca\(^{2+}\) pump at Ca\(^{2+}\) levels observed in resting smooth muscle cells (100-200 nM Ca\(^{2+}\)) to a flux rate per surface area and compared this rate to the value of the passive transmembrane flux rate observed in intact smooth muscle cells.
Chapter II.
Assessment of the Transport Capacity of the Plasmalemmal Ca\textsuperscript{2+} Pump in Smooth Muscle

ABSTRACT

In resting smooth muscle, a variety of Ca\textsuperscript{2+} extrusion processes offset the inward Ca\textsuperscript{2+} leak. Biochemical studies suggest that the plasmalemmal Ca\textsuperscript{2+} pump dominates this process; however, this contention could not be proven without a reliable estimate of the inward Ca\textsuperscript{2+} leak that must be opposed by active transport. Recent studies using dispersed cells from the toad stomach provided such an estimate; thus, we examined the capacity of the plasmalemmal Ca\textsuperscript{2+} pump in this tissue. Membranes were prepared using nitrogen cavitation, high salt extraction and flotation on discontinuous sucrose gradients. These membrane vesicles were enriched 16 - 24 fold for plasma membrane markers and exhibited an ATP-dependent uptake of \textsuperscript{45}Ca that was insensitive to azide or oxalate, but sensitive to orthovanadate inhibition and calmodulin stimulation. \textsuperscript{45}Ca accumulated in the presence of ATP was rapidly released by Ca\textsuperscript{2+} ionophore but not by caffeine, IP\textsubscript{3}, or GTP. Uptake exhibited a high affinity for Ca\textsuperscript{2+} (K\textsubscript{m} 0.2 \textmu M) and a high transport capacity, producing > 12000 fold gradient for Ca\textsuperscript{2+} and a transmembrane flux rate greater than that observed in resting smooth muscle cells. Thus, this enzyme is capable of maintaining steady state Ca\textsuperscript{2+} levels in smooth muscle.

The results from these studies were accepted for publication:

INTRODUCTION

It has long been recognized that the plasma membrane of smooth muscle plays a key role in maintaining the low levels of intracellular Ca\(^{2+}\) that prevail under resting conditions. This semipermeant barrier regulates Ca\(^{2+}\) entry through voltage- and/or receptor-gated Ca\(^{2+}\) channels and Ca\(^{2+}\) extrusion via various transport processes. Thus far at least two Ca\(^{2+}\) extrusion processes have been identified in the smooth muscle plasmalemma: an ATP-independent exchange process in which the extrusion of cellular Ca\(^{2+}\) is coupled to the entry of extracellular Na\(^+\) (Na\(_o\)/Ca\(_i\) exchange) and an ATP-dependent Ca\(^{2+}\) pump which actively extrudes cellular Ca\(^{2+}\) (see Daniel et al., 1983 for review).

Of these processes, the plasmalemmal Ca\(^{2+}\) pump has been considered the primary regulator of cellular Ca\(^{2+}\) homeostasis in smooth muscle based on the high affinity of the enzyme and its high translocation capacity (Wibo et al., 1981; Wuytack et al., 1981; Raeymakers et al., 1983; Grover, 1985; Sharma and Bhalla, 1986). Demonstrating the precise contribution of this enzyme to steady state Ca\(^{2+}\) homeostasis in vivo has been difficult, however, because of uncertainty regarding the magnitude of the basal transmembrane Ca\(^{2+}\) flux that must be opposed by the Ca\(^{2+}\) pump. Estimates of this transmembrane leak rate for Ca\(^{2+}\) cannot readily be obtained in intact smooth muscle because of technical problems associated with diffusion delays in the extracellular matrix and extracellular Ca\(^{2+}\) binding. However, recent studies using suspensions of enzymatically dispersed smooth muscle cells from the toad
stomach measured this flux rate in resting cells (Scheid and Fay, 1984). This passive leak averages 0.1 pmoles/cm²·sec in these cells; thus, one would predict that the plasmalemmal Ca²⁺ pump—in the presence of prevailing Ca²⁺ levels of 100 - 200 nM (see Williams and Fay, 1986 and Yamaguchi, 1986)—must be capable of producing an opposing outward Ca²⁺ flux equal to or greater than this leak rate if indeed the enzyme plays a dominant role in smooth muscle Ca²⁺ homeostasis.

The present studies were designed to document the functional role of the plasma membrane Ca²⁺ pump in smooth muscle by assessing the transport capacity of the toad smooth muscle enzyme under conditions that are thought to prevail in resting smooth muscle cells. To this end, plasma membrane fractions were prepared from toad gastric smooth muscle and a number of the kinetic properties of ATP-dependent uptake were defined. These characteristics were compared to those of membrane fractions prepared from pig antral smooth muscle, a preparation that has been extensively characterized by other investigators (Wuytack et al., 1981) and that served as a benchmark for the present studies. Other characteristics, including vanadate sensitivity, calmodulin regulation, and insensitivity to azide and oxalate, were used to confirm that the observed ATP-dependent uptake was due to the presence of a plasmalemmal Ca²⁺ ATPase. The transport capacity of the plasmalemmal Ca²⁺ pump in membrane vesicles, expressed as moles/sec·cm² cell membrane, was 2.5 fold higher than the passive transmembrane flux rate observed in isolated smooth muscle cells. Thus, the low levels of cytosolic Ca²⁺ that prevail in resting smooth muscle could be maintained solely by the
operation of the plasmalemmal Ca\textsuperscript{2+} pump. A preliminary report of these findings has appeared (Lucchesi et al., 1987).

METHODS

Preparation of Membrane Fractions. Preparation of plasmalemmal vesicles from toad stomach muscle was carried out as indicated in Figure 1. All procedures were carried out at 4°C. For each preparation, stomachs from 25 toads (Bufo marinus) were excised, trimmed of mucosa and connective tissue and minced with scissors in ice cold homogenization buffer consisting of 50 mM MOPS ([N-Morpholino]propanesulfonic acid), pH 7.1, 150 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol (DTT), 17 mg/L of PMSF (phenylmethylsulfonyl fluoride), and 20 mg/L of soybean trypsin inhibitor. The minced tissue was then homogenized in a Waring blender (2 x 30 sec at low speed) in 20 volumes of the above buffer. Further disruption was achieved using an Ultraturrax Tissuemizer (an initial 25 sec burst using the SDT-1B2EN probe followed by a 25 sec burst using the smaller SDT-100 EN probe). The crude homogenate was then filtered and disrupted twice by nitrogen cavitation. Cellular debris was removed by two low speed centrifugations, a 20 min spin at 5,000 x g in a GSA rotor followed by a 10 min centrifugation at 10,000 x g in a GSA rotor. The supernatant from this spin was centrifuged at 30,000 x g for 90 min in a SS34 rotor. The resultant crude microsomal pellets were then suspended in homogenization buffer containing 0.6 M NaCl and centrifuged at 30,000 x g for 60 min in a SS34 rotor. The treatment with a buffer of high salt concentration served to remove contaminating myofilaments.
Figure 1. Flow chart for preparation of membrane vesicles from toad stomach muscle.

Membrane fractions were further enriched by a sucrose flotation method modified from Jones (1986). The salt-extracted microsomal pellets were resuspended in a buffer containing 150 mM NaCl, 50 mM MOPS (pH 7.1), 25 mM Na pyrophosphate, and 1.0 M sucrose. Ten ml aliquots of this solution were transferred to polycarbonate centrifuge tubes. This suspension was overlayed with seven ml of buffer containing 0.6 M sucrose, 50 mM MOPS (pH 7.1), 300 mM NaCl and 25 mM Na pyrophosphate. The tubes were filled to volume with a buffer containing 0.3 M sucrose and 50 mM MOPS (pH 7.1). The samples were centrifuged using a Beckman Ti 60 rotor for 70 min at 370,000 x g. Two translucent, membrane-rich bands were obtained: an upper band at the 0.3/0.6 M sucrose interface.
and a lower band at the 0.6/1.0 M sucrose interface. The bands were carefully extracted with a Pasteur pipet in order to avoid removing media (containing soluble contractile proteins) immediately surrounding the fractions. The collected fractions were then diluted 5-fold with ice cold buffer containing 100 mM NaCl and harvested by centrifugation for 90 min at 161,000 x g in a Ti 60 rotor. The sedimented membranes were resuspended by sonication in storage buffer (250 mM sucrose, 40 mM MOPS (pH 7.1), 1 mM DTT, and 10 μg/ml leupeptin), rapidly frozen in liquid nitrogen, and stored at -80°C. Aliquots of the various fractions were assayed for protein content according to the method of Lowry (1951).

For the preparation of membranes from pig antral smooth muscle, pig stomachs (usually 1 or 2 for each batch of membranes) were obtained from a local slaughterhouse, transferred on ice to the laboratory and processed as described for the toad stomach. One additional centrifugation step was utilized in processing pig tissue: an additional 10 min, 5000 x g centrifugation between the nitrogen cavitation steps. Other steps in the preparative procedure were identical to those described for the toad.

Membrane Characterization

a. Cholinergic Receptor Binding. The presence of muscarinic cholinergic receptors was quantitated by assaying for specific binding of [3H]-quinuclidinyl benzilate (QNB). Reactions were carried out in a total volume of 1.0 ml at 31°C in a buffer containing: 40 mM MOPS, pH 7.1, 10 mM MgCl₂, 2 mM EDTA, 50 mM NaCl, 3 nM [3H]-QNB (specific activity 35.2 Ci/mmol). Binding was initiated by the addition of 50-100
μg protein. After a 60 min incubation period, the reaction was terminated by rapid filtration through Whatman GF/F filters using a Hoeffer filtration apparatus. The filters were washed 5 times with 3 mls of ice cold buffer containing 50 mM MOPS (pH 7.1), 25 mM NaCl, and 5 mM MgCl₂. The filters were then dissolved in counting fluid (Liquiscint, National Diagnostic Reagents) and counted in a liquid scintillation counter. Specific binding of [³H]-QNB was calculated as the difference in binding observed in the presence and absence of 100 µM atropine, a specific antagonist for muscarinic cholinergic receptors. Nonspecific binding was <1% in these studies.

b. Marker Enzyme Assays. The activity of the plasma membrane marker 5' nucleotidase was measured in a medium containing 50 mM glycine-NaOH, pH 9.1, 5 mM 5'AMP and 10 mM MgCl₂. Reactions were initiated by the addition of 50-60 μg of protein and nucleotidase activity was detected by measuring the amount of inorganic phosphate that was released during a 30 minute incubation at 31°C. Inorganic phosphate was quantitated by the malachite green assay of Itaya and Ui (1966).

Assays for NADPH cytochrome c reductase, a putative marker for the endoplasmic reticulum (Matlib et al., 1979), and NADH cytochrome c reductase, a marker for mitochondrial membranes (Matlib et al., 1979), were carried out spectrophotometrically according to Sottocasa et al. (1967) in a buffer containing 50 mM potassium phosphate, pH 7.7, 1 mM KCN, 47 nmoles cytochrome c and either 0.15 mM NADPH or 0.1 mM NADH. Reactions were initiated by the addition of 60-90 μg protein and the progress of the reaction was monitored as an increase in the absorbance at 550 nm.
K\(^+\) stimulated, oubain-sensitive \(\rho\)-nitrophenylphosphatase (PNPPase) activity was determined according to the procedure of Kostka and Kwan (1986). The standard assay medium contained 50 mM imidazole, pH 7.8, 5 mM MgCl\(_2\), and 5 mM PNPP. The reaction was initiated by the addition of 50-70 \(\mu\)g of protein at 31\(^\circ\)C (toad) or 37\(^\circ\)C (pig) and was terminated after 30 min of incubation by the addition of 1 ml of ice-cold 1N NaOH. Samples were then centrifuged to remove precipitated proteins. Enzyme activity was quantitated spectrophotometrically by monitoring the absorbance of \(\rho\)-nitrophenol at 400 nm using an extinction coefficient of 18,000/M\(\cdot\)cm for the enzyme (Kostka and Kwan, 1986). K\(^+\)-activated PNPPase activity was defined as the difference between activities observed in the presence and absence of 10 mM KCl. For those assays in which K\(^+\)-activated PNPPase activity was employed to assess the sidedness of the vesicles, the amount of imidazole in the reaction mixture was increased to 250 mM to maintain isotonic conditions. Enzyme activity was then determined for control membranes and for membranes that had been rendered permeant by a 30 min exposure at 4\(^\circ\)C to 70 \(\mu\)g/ml of saponin. The difference in activity between permeant vesicles (total activity) and in control membranes (right side out membranes) is assumed to reflect the activity associated with inside-out vesicles.

c. Measurement of \(^{45}\)Ca Transport in Membrane Vesicles. \(^{45}\)Ca uptake by membrane vesicles was determined by a Millipore filtration technique. This involved incubating plasmalemmal vesicles in the following standard reaction mixture: 20 mM HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethane sulfonic acid), pH 7.1, 5 mM Na\(_3\), 100 mM KCl,
3 mM MgCl₂, 10 µM Ca²⁺ and ~1 µCi/ml ⁴⁵Ca. When indicated, ATP (5 mM) was added along with a regenerating system consisting of 5 mM creatine phosphate and 8 U/ml creatine phosphokinase. Under these conditions, the levels of MgCl₂ and CaCl₂ were increased to 8.066 mM and 17 µM, to maintain the levels of free Mg²⁺ and Ca²⁺ at 3 mM and 10 µM, respectively. In experiments in which calmodulin was added to the reaction mixture, the calmodulin was added as a Ca²⁺-calmodulin complex at a final concentration of 2 µg/ml. Uptake experiments were carried out at room temperature such that the data obtained for vesicular uptake in toad vesicles could be compared to the transmembrane fluxes observed previously in suspensions of toad smooth muscle cells (Scheid and Fay, 1984a). Experiments were initiated by adding 30-35 µg of membrane protein to the reaction mixture. At various times after membrane addition, duplicate 1 ml aliquots were removed and filtered through 1.2 µm Millipore filters. Filters were washed with 5 ml of ice-cold buffer containing 1 mM LaCl₃ and 150 mM NaCl, dissolved in counting fluid and counted using a liquid scintillation counter. ATP-dependent Ca²⁺ uptake was defined as the uptake in the presence of ATP minus the uptake in the absence of ATP.

In some experiments, the level of ionized Ca²⁺ in the reaction mixture was varied using EGTA buffers. In these instances, total Ca²⁺ was maintained at 100 µM, and the amount of EGTA required to achieve the desired concentrations of ionized Ca²⁺ and a final [Mg²⁺] of 1.2 mM was calculated using Fabiato's stability constants for EGTA, ATP, and creatine phosphate (Fabiato, 1981). All other constituents of the reaction buffer remained the same.
For assessing $^{45}$Ca release from membrane vesicles, vesicles were loaded with $^{45}$Ca for 60 min at room temperature in the standard uptake buffer (see above) containing 5 mM ATP. Efflux was initiated by a 10-fold dilution of preloaded vesicles with buffer containing 100 mM KCl, 20 mM HEPES, pH 7.1, and either 1.0 mM EGTA or 0.1 mM EDTA. In some experiments, caffeine (20 mM), D-myoinositol 1, 4, 5-trisphosphate (IP$_3$, 10 μM), GTP (10 μM), or IP$_3$ (10μM) + GTP (10 μM) were included in the efflux buffer. At various times after dilution, duplicate 1 ml aliquots of the reaction mixture were removed, filtered and counted as described above.

**Electron Microscopy.** The relative purity of membranes at various steps of the preparative procedure was assessed by electron microscopic examination of thin sections of pelleted material. Purified membranes (both upper and lower fractions) and material obtained at the initial 30,000 x g spin were fixed in 2% glutaraldehyde, postfixed in 1% osmium tetroxide and embedded in epon. Sections were stained with uranyl acetate and lead citrate and examined on a JEOL 100 ZX microscope.

Since the membrane vesicles used for transport studies were subjected to a single sonication prior to their use, whereas the vesicles used to monitor intravesicular volume (from $^3$H-inulin trapping studies, see below) were subjected to a second sonication before use, we examined the vesicle size of both populations. This involved measurement of over 500 vesicle profiles in at least six electron micrographs for each group to obtain an estimate of the average vesicle diameter $d$. This initial estimate was then corrected for errors produced by sectioning vesicles above or below their midpoint; thus, the
value for the true vesicle diameter $D$ was calculated as $D = d(4/\pi)$ (see Weibel, 1979).

**Electrophoresis.** The relative purity of various membrane fractions was also assessed using sodium dodecyl sulphate polyacrylamide gel electrophoresis. A linear 6-16% gradient of polyacrylamide and a Laemmli buffer system (pH 6.8, see Laemmli, 1980) were used for these studies. Gels were stained with Coomassie brilliant blue.

**Intravesicular Volume Measurements.** To assess intravesicular volumes, 0.75 mg of membrane vesicles (toad lower fraction) were disrupted by sonication (2 x 10 sec burst at 65 Watts with a Sonifer Cell Disrupter) in the presence of [$^3$H]-inulin (1.0 µCi/ml). The amount of labelled inulin trapped within the vesicles was then determined by filtering the vesicles through 1.2 µm filters, washing the filters with ice cold isotonic saline containing 10 mM inulin and counting the filters. Total counts associated with the filters were corrected for nonspecific binding of [$^3$H]-inulin (ie. for label bound to filters in the absence of added membranes).

**Materials.** Reagents employed in these studies were of the highest purity available. Those used for uptake buffers were dissolved in ultrapure water (18 MΩ resistivity) prepared by filtering distilled, deionized water through a Gelman-Water I filtration apparatus.

Calmidazolium, calmodulin (bovine brain), GTP, PNPP (ditris salt) and ATP were purchased from Sigma Chemical Co. Ionomycin was obtained from Calbiochem, and D-myoinositol 1,4,5-trisphosphate (IP$_3$) was purchased from both Sigma Chemical Co. and Calbiochem. Caffeine was obtained from Aldrich Chemical Co. $^{45}$CaCl$_2$, [$^3$H]-inulin and


$[^3\text{H}]$-QNB were obtained from New England Nuclear Co.

Statistics. Statistical analysis to determine the significance levels of differences employed either a simple Student's t-test (for comparing a single experimental condition with its control) or a Dunnett's test (for multiple comparisons with a single control, see Winer, 1962). Differences were considered significant if the probability of chance occurrence was $< 0.05$.

RESULTS

The last step in the preparative procedure described above provides two membrane vesicle fractions, an upper fraction in which vesicle diameters averaged $0.157 \pm 0.008$ in the pig (Mean ± SE for 130 measurements) and a lower fraction in which the vesicle diameters averaged $0.158 \pm 0.004$ and $0.23 \pm 0.012 \mu m$ in the toad and pig, respectively (Mean ± SE for 513 and 120 measurements, respectively). The total yield of protein in these two fractions averaged ~12.0 mg per 70 g tissue in both species. Some of this protein is contributed by residual filamentous material associated with the vesicles; however, as can be seen in the electron micrograph of toad lower fraction (Figure 2) and in comparable micrographs of pig vesicles (not shown), the bulk of the material in these bands is comprised of closed membrane vesicles that are homogeneous in size and shape. In contrast, the pellet from the $30,000 \times g$ spin from either toad or pig contains substantial amounts of mitochondria, collagen, and filamentous material (not shown). Further evidence for the effectiveness of our current purification
Figure 2. Plasmalemmal vesicles from toad stomach muscle. Vesicles in this electron micrograph were collected from the sucrose gradient at the interface between the layers of 0.6 and 1.0 M sucrose. X 66,000. Bar = 0.1 μm.

procedures can be seen in Figure 3. For this figure, samples were removed after various steps in the purification procedure and subjected to gel electrophoresis. As is evident in this gel, both actin and myosin are progressively removed during purification (compare Lane 3 with Lanes 7 and 8), although there is still detectable actin contamination remaining in the purified membrane fractions.

The distribution of membrane marker enzymes in crude and purified membrane fractions is shown in Table 1. Specific binding of QNB was
Figure 3. SDS-polyacrylamide gel electrophoresis of the fractions obtained during the isolation of pig antral plasma membrane vesicles. 80µg of sample or 7 µg of purified actin or calmodulin were applied to a linear 6%-16% polyacrylamide gel. Lane 1. Mr markers- carbonic anhydrase (29 kD), egg albumin (45 kD), bovine albumin (66 kD), phosphorylase b (97.4 kD), β-galactosidase (116 kD), and myosin (205 kD). Lane 2. Buffer. Lane 3. Crude homogenate. Lane 4. Pellet from 5000 x g spin. Lane 5. purified skeletal muscle actin and bovine brain calmodulin. Lane 6. pellet from 30,000 x g spin. Lane 7. upper membrane fraction isolated from the 0.3/0.6 M sucrose interface. Lane 8. lower membrane fraction isolated from the 0.6/1.0 M sucrose interface. Lane 9. Mr markers- α Lactalbumin (14.2 kD), soybean trypsin inhibitor (20 kD), glyceraldehyde-3-phosphate dehydrogenase (36 kD), egg albumin (45 kD), and bovine albumin (66 kD).

assessed in membrane fractions from both species. In the toad, the relative enrichment for specific QNB binding was ~13 fold in the lower fraction and ~17 fold in the upper fraction. In the pig, the enrichment for specific binding of this muscarinic antagonist was
Table 1: Relative Enrichment of Various Marker Enzymes in Toad and Pig Gastric Smooth Muscle Membranes.

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<tr>
<th>Marker Enzymes</th>
<th>Toad Crude Membranes</th>
<th>Purified Membranes</th>
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<tr>
<td></td>
<td>Upper Fraction</td>
<td>Lower Fraction</td>
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<tr>
<td>NADH cyt f. reductase</td>
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<td>NADPH cyt f. reductase</td>
<td>2.0 fold (2)</td>
<td>1.5 fold (2)</td>
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<td>K-stimulated PNPPase</td>
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<td>NADPH cyt f. reductase</td>
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<td>NADPH nucleotidase</td>
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Specific QNB binding in crude homogenate of the toad = 35.9 ± 1.4 fmoles/mg protein (mean ± SEM from 9 experiments).

K-stimulated PNPPase activity in the crude homogenate averaged 83 ± 2.7 fmoles/min-mg (mean ± SEM from 4 experiments).

NADPH cyt f. reductase activity averaged 6.7 mol/min-mg in the crude homogenate of the toad and 4.2 mol/min-mg in the crude homogenate of the pig (2 experiments for each).

NADH cyt f. reductase activity averaged 23 mol/min-mg in the crude homogenate of the toad and 4.2 mol/min-mg in the crude homogenate of the pig (2 experiments for each).

NADPH nucleotidase activity averaged 0.16 mol/min-mg in the crude homogenate was 0.016 mol/min-mg in this experiment.
somewhat higher than in the toad, but the relative enrichment for each fraction was similar, averaging 21 fold in the upper fraction and 24 fold in the lower. The other plasma membrane marker employed for toad plasma membranes was 5' nucleotidase. The activity of this enzyme was purified to a similar extent in both fractions in the toad, with an average enrichment of 16 to 18-fold. In the pig, 5'nucleotidase activity did not appear to be a suitable marker for plasma membranes because a large fraction of the activity in the crude homogenate was apparently soluble (readily lost after high salt extraction). Thus, in the pig the activity of K\textsuperscript+ -stimulated, ouabain-sensitive PNPPase was used as an alternative plasma membrane marker. PNPPase activity in pig antral smooth muscle membranes was enriched to a slightly lower extent than QNB binding, averaging 10-fold in the upper fraction and 15-fold in the lower fraction.

The activity of NADPH cytochrome c reductase, a putative marker for sarcoplasmic reticulum, was enriched only slightly in the membrane fractions from both species as compared to the crude homogenate (~1.6-fold in the pig fractions and ~1.4-fold in the toad). Similarly, NADH cytochrome c reductase activity, a mitochondrial enzyme marker, was slightly enriched in the purified membrane fractions showing an enrichment of ~ 5-fold over homogenate in the pig and ~ 2-fold in the toad. Note, however, that for both of these enzymes the relative activities were similar in both the crude microsomes and in the purified membrane fractions whereas for the plasma membrane markers the activities were considerably higher in purified membranes than in crude microsomes.
The gastric smooth muscle plasmalemmal vesicles exhibited a time- and ATP-dependent uptake of $^{45}$Ca as evidenced by the data presented in Figure 4. In this experiment, addition of 5 mM ATP produced a marked

![Figure 4. Effect of ionomycin on ATP-dependent uptake by membrane vesicles from toad stomach muscle. Results from a single experiment in which calcium uptake was carried out in the standard reaction mixture in the presence (X's) or absence (circles) of 5 mM ATP. At indicated times, duplicate 1 ml aliquots were removed and filtered to halt exchange. Ionomycin (10 µM) was added to both groups at t = 5 min (arrow). Note that exposure to ionomycin rapidly released the label accumulated in the presence of ATP but had little effect on ATP-independent $^{45}$Ca accumulation.](image-url)
increase in $^{45}$Ca uptake by vesicles derived from toad stomach muscle. Pooled data from 7 such experiments indicated that this stimulation averaged 8.4 fold in membranes from toad upper fraction (uptake increased from $1.5 \pm 0.2$ to $12.6 \pm 1.0$ nmoles/mg protein within 2 min, Mean ± SE from 7 experiments, $p < 0.01$ ) and 5.0 fold in membranes from toad lower fraction$^1$ (an increase from $1.5 \pm 0.2$ to $6.7 \pm 0.7$ nmoles/mg membrane protein within 2 min, Mean ± SE from 7 experiments, $p < 0.01$)$^1$. $Ca^{2+}$ transport in pig vesicles showed a comparable time- and ATP-dependent uptake of $^{45}$Ca (data not shown). ATP-dependent accumulation of $^{45}$Ca by these vesicles appeared to reflect vesicular uptake rather than binding since the label was rapidly lost on addition of the $Ca^{2+}$ ionophore ionomycin (Figure 4). Exposure to ionomycin caused a rapid loss of label accumulated in the presence ATP but had no effect on label accumulated in the absence of ATP.

In order to further characterize ATP-dependent transport in these vesicles and discern the source (eg. mitochondria, sarcoplasmic reticulum or plasma membrane) of the $Ca^{2+}$ pumps, we examined ATP-dependent $^{45}$Ca uptake in the presence of various inhibitors. In one series of experiments we assessed the efficacy of orthovanadate as an inhibitor of ATP-dependent $^{45}$Ca uptake since this inhibitor is reportedly extremely effective at inhibiting the plasmalemmal $Ca^{2+}$ pump (apparent $K_i$ of $0.1 - 0.6 \mu M$, see Sharma et al., 1987 and Wibo

$^1$The reduced uptake in the lower membrane fraction may be due to greater contamination by actomyosin in earlier preparations; in more recent experiments in which more care was used in extracting the lower band, the uptake/mg protein was identical in the two membrane fractions.
et al., 1981) but less effective at inhibiting the sarcoplasmic reticulum Ca\textsuperscript{2+} pump (apparent $K_i \approx 50$ $\mu$M, see Sharma et al., 1987). These studies indicated that the Ca\textsuperscript{2+} uptake in both toad and pig membrane vesicles was extremely sensitive to orthovanadate inhibition with half maximal inhibition obtained at vanadate concentrations less than 0.1 $\mu$M (Figure 5). Azide, an inhibitor of mitochondrial Ca\textsuperscript{2+} transport (Daniel et al., 1983; Grover et al., 1983; Sottocasa, 1967), had no effect on this transport.

![Figure 5. Effect of vanadate on ATP-dependent 45Ca uptake. The initial 45Ca uptake rate (that observed during the first 30 seconds of uptake) was measured in the presence of various concentrations of vanadate. The plot of the reciprocal of the velocity versus the corresponding vanadate concentration yielded an apparent $K_i$ of < .1 $\mu$M.](image-url)
ATP-dependent Ca\(^{2+}\) uptake averaged 2.01 nmoles/mg-min in the presence of azide (15 mM) and 1.97 nmoles/mg-min in the absence of azide (Means from 3 paired experiments using membranes derived from the toad; comparable results were obtained for the pig). Similarly, addition of oxalate, an anion that has been used to trap Ca\(^{2+}\) and enhance the uptake of \(^{45}\)Ca in purified vesicles from the sarcoplasmic reticulum (Grover et al., 1980; Wibo et al., 1981; Raeymakers et al., 1983; Grover et al., 1985), had no effect on uptake by any of the membrane fractions tested (eg. toad upper or lower fractions, pig upper or lower fractions; data not shown).

The studies using various inhibitors and activators of Ca\(^{2+}\) uptake suggested that the observed Ca\(^{2+}\) accumulation was attributable to sequestration in plasmalemmal vesicles. This conclusion was further supported by studies of \(^{45}\)Ca release from prelabelled vesicles. A variety of agents that have been reported to release Ca\(^{2+}\) from the sarcoplasmic reticulum failed to stimulate \(^{45}\)Ca release from these vesicles. Exposure of the vesicles to caffeine (20 mM), to IP\(_3\) (10 \(\mu\)M), to GTP (10 \(\mu\)M) or to IP\(_3\) plus GTP (10 \(\mu\)M each, not shown) had no effect on \(^{45}\)Ca release (Figure 6). On the other hand, label was rapidly released after the addition of the Ca\(^{2+}\) ionophore ionomycin (Figure 6). Basal \(^{45}\)Ca release from these vesicles was relatively slow, \(K_D\) averaged \(6.0 \pm 0.8 \times 10^{-3}\) min\(^{-1}\) in the toad and \(0.020 \pm 0.003\) min\(^{-1}\) in pig membrane vesicles within 5 to 10 min after dilution (Mean ± SE for 8 and 10 experiments, respectively). Thus, the results from efflux studies indicated that the membrane vesicles were tightly sealed and primarily of plasmalemmal origin.
Figure 6. $^{45}$Ca efflux from plasmalemmal vesicles. In this experiment, membranes were prelabelled for 60 min with $^{45}$Ca in the presence of 10 μM free Ca$^{2+}$ and 5 mM ATP. Efflux was initiated by diluting the vesicles 10 fold with Ca$^{2+}$-free/EGTA buffer. At various times after dilution, control aliquots (circles) were removed and filtered. At the arrows, drugs were added to other aliquots of diluted membranes. Note that label was lost rather slowly from the untreated vesicles, a finding that suggests that the vesicles are tightly sealed. Exposure to 10 μM IP$_3$ (x's), 10 μM GTP (triangles), or 20 mM caffeine (squares) had no apparent effect on $^{45}$Ca efflux. Exposure to 1 μM ionomycin (inverted triangles) led to a rapid release of labelled Ca, however.

Additional studies examined the kinetic characteristics of ATP-dependent Ca$^{2+}$ transport to assess the affinity and translocation capacity of this process and to examine its sensitivity to exogenous calmodulin. These studies indicated that the rate of ATP-dependent
Ca uptake in both pig and toad membranes was dependent on pCa. The apparent $K_m$ for $Ca^{2+}$ was 0.2 $\mu$M in toad membranes and 0.4 $\mu$M in pig membranes, while the apparent $V_{max}$ for this transport was 14.3 nmoles/min·mg in the toad and 13.6 nmoles/min·mg in the pig (Figure 7).

![Graph](image)

Figure 7. $Ca^{2+}$ dependence of ATP-dependent uptake in smooth muscle plasmalemma. The initial velocity of ATP-dependent $^{45}Ca$ uptake (that observed during the first 30 seconds of uptake) was monitored at varying pCa's in the presence of exogenous calmodulin. A plot of the initial velocity ($V$) vs $V/S$ (where $S = [Ca^{2+}]$ in nmoles) is shown for plasmalemmal vesicles from toad (circles) and pig (X's) gastric smooth muscle. The apparent $K_m$ for $Ca^{2+}$ was 0.2 $\mu$M in toad vesicles and 0.4 $\mu$M in pig vesicles. The apparent $V_{max}$ for ATP-dependent $Ca^{2+}$ transport was 14.3 nmoles/min·mg in the toad and 13.6 nmoles/min·mg in the pig.

Addition of exogenous calmodulin at a given free $[Ca^{2+}]$ produced a small but consistent stimulation of ATP-dependent $Ca^{2+}$ uptake in both the toad (upper panel, Figure 8) and the pig (not shown) with no effect.
on ATP-independent $\text{Ca}^{2+}$ accumulation. This stimulation averaged ~1.4 fold in the toad and ~1.5 fold in the pig ($p < 0.05$ in both cases, paired analysis of data from 13 and 25 experiments, respectively) and involved a stimulatory effect on both the $K_m$ and $V_{\text{max}}$ of the transport system. No attempts were made to deplete the membranes of endogenous calmodulin for these studies, however; thus, these results presumably underestimate the ability of calmodulin to stimulate $\text{Ca}^{2+}$ uptake by these preparations.

The regulation of ATP-dependent $\text{Ca}^{2+}$ transport by calmodulin was further explored using the specific calmodulin antagonist, calmidazolium (Gietzen et al., 1981; Van Belle, 1981). Exposure of the vesicles to calmidazolium led to an inhibition of the calmodulin-stimulated $^{45}\text{Ca}$ uptake (Figure 8, lower panel) with no effect on ATP-independent uptake (data not shown). This inhibitory effect of calmidazolium was concentration-dependent, with statistically significant inhibition obtained at a concentration of 5 $\mu$M in the toad and 1.0 $\mu$M in the pig (data not shown).

In order to determine whether the plasmalemmlal $\text{Ca}^{2+}$ pump in toad membrane vesicles has a sufficient transport capacity to offset the measured transmembrane leak rate for $\text{Ca}^{2+}$ in resting smooth muscle cells, we examined ATP-dependent $^{45}\text{Ca}$ uptake under conditions that approximate those in resting smooth muscle cells (free $\text{Ca}^{2+} \leq 200 \text{nM}$, see Williams and Fay, 1986 and Yamaguchi, 1986). The observed uptake rate (expressed in nmoles/min-mg) was then converted to a transmembrane flux rate (expressed in pmoles/sec-cm$^2$ cell membrane) as follows (see also Table 2). The estimate of average vesicle diameter
Figure 8. Calmodulin dependence of ATP-dependent $^{45}$Ca uptake in toad plasmalemmal vesicles. Upper panel: Stimulation of ATP-dependent $^{45}$Ca uptake on addition of exogenous calmodulin. Results from a representative experiment in which ATP-dependent $^{45}$Ca uptake was monitored as a function of time in our standard uptake buffer (7 nM free Ca$^{2+}$) in the presence (X's) and absence (circles) of added calmodulin (2 nM/ml). Lower panel: Concentration-dependent inhibition of ATP-dependent $^{45}$Ca uptake by the specific calmodulin antagonist calmidazolium (CMZ). Asterisks indicate a statistically significant effect of calmidazolium ($^* = p < 0.05$, $^{**} = p < 0.01$).
Table 2. Calculation of intravesicular volumes and surface areas

<table>
<thead>
<tr>
<th>Average Vesicle Radius</th>
<th>0.085 µm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Vesicle Volume</td>
<td>2.57 x 10^-15 cm^3</td>
</tr>
<tr>
<td>Average Vesicle Surface Area</td>
<td>9.10 x 10^-10 cm^2</td>
</tr>
<tr>
<td>Intravesicular Volume per Mg</td>
<td>1.47 x 10^-3 cm^3</td>
</tr>
<tr>
<td>Number of Vesicles per Mg</td>
<td>5.72 x 10^{11}/mg</td>
</tr>
<tr>
<td>Total Surface Area per Mg</td>
<td>520 cm^2/mg</td>
</tr>
</tbody>
</table>

In toad lower fraction obtained from morphometric analysis was used to calculate the average volume/vesicle \((V = 2.57 \times 10^{-15} \text{ cm}^3)\) and the average surface area/vesicle \((S.A. = 9.1 \times 10^{-10} \text{ cm}^2)\). From our estimate of the trapped intravesicular volume/mg membrane protein (Total volume = 1.47 ± 0.08 µl/mg; Mean ± SE for 4 determinations), we calculated the total number of vesicles of volume \(V\) per mg of membrane protein and the total membrane surface area/mg membrane protein \((S.A. = 520 \text{ cm}^2/\text{mg membrane protein})\). Using this value we could convert the observed ATP-dependent uptake at pCa ≤ 6.6 \((2.16 \times 10^{-9} \text{ moles/30 sec•mg}; \text{Mean from 8 experiments})\) to a flux rate \((1.39 \times 10^{-13} \text{ moles/sec•cm}^2; \text{see Table 3})\). The latter underestimates the transmembrane flux rate produced by ATP-dependent Ca^{2+} transport, however, in that it assumes all vesicles contribute to the observed uptake. Presumably, only inside-out vesicles are capable of ATP-dependent Ca^{2+} accumulation, and estimates of vesicle orientation

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2Note that vesicular diameter averaged 0.169 ± 0.004 µm (Mean ± SE for 513 measurements) for vesicles sonicated twice prior to fixation - the conditions used in ^3H-inulin trapping studies - and 0.158 ± 0.005 (Mean ± SE for 507 measurements) for vesicles sonicated once. Thus, the second sonication had no appreciable effect on intravesicular volumes, surface areas, etc., nor did it appear to increase the number of broken vesicles evident in electron micrographs.
Table 3. Membrane Transport in Vesicles and Isolated Cells From Toad Stomach Muscle.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Flux (x10^-13 mol/cm^2·sec)</th>
<th>Transport (x10^-23 mol QNB/receptor)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolated Cells</td>
<td>1.0</td>
<td>2.55</td>
</tr>
<tr>
<td>Membrane Vesicles*</td>
<td>1.39</td>
<td>3.99</td>
</tr>
<tr>
<td>Membrane Vesicles†</td>
<td>3.47</td>
<td>10.0</td>
</tr>
</tbody>
</table>

*Uncorrected for vesicle sidedness
†Corrected for vesicle sidedness assuming that only inside-out vesicles contribute to uptake and that 40% of the vesicles are inside-out.

(based on K^+ -stimulated, ouabain-sensitive PNPPase activity before and after vesicle permeabilization) indicate that only 40% of the vesicles were inside-out. Thus, the actual transmembrane flux rate produced by ATP-dependent Ca^{2+} transport under these conditions is 3.47 x 10^-13 moles Ca/sec·cm^2 cell membrane.

We also compared the Ca^{2+} transport of membrane vesicles to the Ca^{2+} flux of intact cells by a measurement that did not rely on estimates of vesicular surface area. This involved normalizing the transport data from both cells and vesicles relative to the distribution of another plasma membrane protein, the muscarinic cholinergic receptor. Density of muscarinic cholinergic receptors in both preparations was determined from studies of ^3H-QNB binding. In intact cells, receptor density averaged ~123,000 sites/cell (Data from 4 experiments) or 3.92 x 10^9 receptors/cm^2 cell membrane (membrane surface area per cell calculated for cells assuming that the cells are oblate spheroids with average dimensions of 100 μm x 10 μm). Thus,
the flux per muscarinic receptor in cells with a transmembrane Ca$^{2+}$
flux of 0.1 pmoles/cm$^2$·sec was 2.55 x 10$^{-23}$ moles
Ca/receptor·sec. In membrane vesicles from toad lower fraction, the
density of muscarinic receptors averaged 1.806 x 10$^{12}$ receptors/mg
(Mean from 4 determinations). Since ATP-dependent uptake (corrected for
vesicle orientation) by these vesicles averages 1.8 x 10$^{-10}$
moles/sec·mg at pCa ≤ 6.6, the Ca$^{2+}$ transport per QNB binding site
in vesicles is 1.0 x 10$^{-22}$ moles Ca/receptor·sec, a value nearly
4-fold higher than that for intact cells.

**DISCUSSION**

The present studies were designed to assess the transport capacity
of the plasmalemmal Ca$^{2+}$ pump in membrane fractions from toad stomach
muscle. To this end, we developed simple, relatively rapid procedures
to obtain high yields of intact, tightly-sealed membrane vesicles.
Membranes were prepared from both toad stomach muscle and from pig
antral smooth muscle to validate our preparative procedures and allow
for a direct comparison of our data with published reports on mammalian
smooth muscles. These procedures yielded two purified membrane
fractions for each species that were functionally identical with respect
to the distribution of various marker enzymes and with respect to the
characteristics of ATP-dependent Ca$^{2+}$ transport. The explanation for
the different mobilities of these fractions on the discontinuous sucrose
gradients is not entirely clear. The difference could simply reflect
variation in vesicle size since the average diameter of vesicles in the
lower fraction (at least for pig smooth muscle membranes) was ~30%
larger than that of vesicles in the upper fraction. Alternatively, the
fractions may differ in their permeability to sucrose, in the extent of contamination by nonmembrane proteins or in the relative enrichment for different regions of the plasma membrane (eg. regions rich in dense bodies or caveolae). At present we cannot distinguish among these possibilities.

Both of the membrane fractions appeared to consist primarily of vesicles derived from the plasma membrane. These fractions were enriched 13-24 fold for plasma membrane marker enzymes; and although there was some residual contamination by nonplasmalemmal membranes (based on the activity of marker enzymes for mitochondria and sarcoplasmic reticulum), essentially all of the observed ATP-dependent $^{45}$Ca uptake could be attributed to plasmalemmal vesicles. Azide, an inhibitor of mitochondrial Ca$^{2+}$ transport had no effect on $^{45}$Ca uptake. Nor was uptake stimulated by oxalate, a Ca$^{2+}$ trapping anion that potentiates uptake by sarcoplasmic reticulum$^3$. Moreover, release of $^{45}$Ca from prelabelled vesicles was not affected by caffeine, IP$_3$ or GTP, agents that purportedly release Ca$^{2+}$ from the sarcoplasmic reticulum (Endo et al., 1977; Obara and Yabu, 1982; Carsten and Miller, 1985; Seumatsu et al., 1985; Wolf et al., 1987).

Other characteristics of ATP-dependent Ca$^{2+}$ transport by these fractions were also consistent with the involvement of a plasmalemmal Ca$^{2+}$ transport ATPase. For one, uptake was inhibited by low concentrations of orthovanadate. The apparent $K_i$ for vanadate inhibition was less than 0.1 μM, a value similar to that required for

$^3$The use of oxalate sensitivity as a criterion for the presence of sarcoplasmic reticulum membranes is controversial, see reference 2.
inhibition of plasmalemmal Ca\(^{2+}\) pumps and considerably lower than that required for inhibition of sarcoplasmic reticulum Ca\(^{2+}\) pumps (Wibo et al., 1981; Raeymakers et al., 1983; Sharma et al., 1987). In addition, ATP-dependent Ca\(^{2+}\) uptake by these fractions was regulated by calmodulin. Addition of exogenous calmodulin produced a significant stimulation of ATP-dependent \(^{45}\)Ca uptake in these vesicles and exposure of the membranes to the specific calmodulin antagonist calmidazolium inhibited ATP-dependent \(^{45}\)Ca uptake.

The accumulation of \(^{45}\)Ca observed in these vesicles appears to reflect uptake by tightly sealed vesicles. Accumulated label was lost only slowly from these vesicles under control conditions (half time for tracer loss averaged 385 min) but was rapidly lost after exposure to a Ca\(^{2+}\) ionophore (>80% of accumulated label lost within 1 min). Moreover, the total intravesicular Ca\(^{2+}\) accumulated was appreciable as shown by the following calculation. For vesicles exposed to 250 nM Ca\(^{2+}\), ATP-dependent uptake averaged 4.52 ± 0.74 nmoles/mg during the first minute (Mean ± SE from 8 experiments). Since the total intravesicular volume for such vesicles averaged 1.47 µl per mg, the transmembrane gradient produced by this transport would approach 12,000 fold assuming all vesicles contribute to the observed uptake or 30,000 fold if uptake data are corrected for sidedness (~40% of the vesicles appear to be inside-out and only those vesicles can utilize ATP to transport Ca\(^{2+}\)). Note, however, that an appreciable fraction of the transported Ca\(^{2+}\) may be bound within the vesicles, such that the final intravesicular [Ca\(^{2+}\)] or the transmembrane Ca\(^{2+}\) gradient cannot be determined precisely.
The ATP-dependent \( \text{Ca}^{2+} \) transport systems observed in the present studies on toad and pig smooth muscle had an affinity similar to that reported previously for plasmalemmal \( \text{Ca}^{2+} \) pumps (Matlib et al., 1979; Grover et al., 1980; Wibo et al., 1981; Grover et al., 1983; Raeymakers et al., 1983;). The apparent \( V_{\text{max}} \) for uptake, expressed as an uptake rate per mg membrane protein, also resembled that in a number of other preparations (Matlib et al., 1979; Grover et al., 1980; Grover et al. 1983;) but was considerably lower than that reported for reconstituted transport systems (Wuytack, 1981) and for more highly purified preparations (Sharma and Bhalla, 1986; Sharma et al., 1987), since estimates of \( V_{\text{max}} \) largely reflect the extent of enrichment or purity of the different preparations. Our goal in these studies was not simply to purify plasma membranes from smooth muscle but rather to obtain a membrane fraction in which essentially all of the observed uptake could be attributed to ATP-dependent \( ^{45}\text{Ca} \) accumulation in plasmalemmal vesicles. Once this was achieved we could reliably convert uptake rate to transport capacity or to transmembrane \( \text{Ca}^{2+} \) flux by expressing the data as an uptake rate per cm\(^2\) membrane. Unlike velocity, the calculated transport capacity is not affected by the presence of nonmembrane proteins since both the estimates of velocity and membrane surface area are normalized per mg protein such that the influence of nonmembrane protein contaminants cancels out.

Calculation of the transport capacity of the plasmalemmal \( \text{Ca}^{2+} \) pump in toad membrane fractions and comparison with the known rate of \( \text{Ca}^{2+} \) flux in intact smooth muscle cells indicates that this transport system is capable of playing a key role in determining the free
cytosolic $Ca^{2+}$ levels in smooth muscle. At prevailing intracellular $Ca^{2+}$ concentrations, the calculated transport capacity of the enzyme, after correction for the percentage of inside-out vesicles, is $3.47 \times 10^{-13}$ moles/cm$^2$·sec, more than 3 fold higher than the basal transmembrane flux rate ($1.0 \times 10^{-13}$ moles/cm$^2$·sec) observed in isolated smooth muscle cells (Scheid and Fay, 1984). In fact this calculation may underestimate the actual transport capacity in vesicles slightly, since some vesicles may be capable of trapping inulin but may be leaky to $Ca^{2+}$. This population of vesicles would not contribute to $Ca^{2+}$ uptake, but would contribute to surface area measurement and, as a result, the actual transport rate per surface area would be minimized.

The transport capacity of the plasmalemmal $Ca^{2+}$ pump can be also be compared to the transmembrane $Ca^{2+}$ flux in intact cells using an independent approach that does not rely on estimates of vesicular volumes and membrane surface areas but which instead relates the $Ca^{2+}$ transport in both cells and vesicles to the distribution of muscarinic receptors in the two preparations. For isolated smooth muscle cells, the flux per muscarinic receptor averages $2.55 \times 10^{-23}$ moles $Ca^{2+}$/receptor·sec. For vesicles, this flux is considerably higher, $10.0 \times 10^{-23}$ moles $Ca^{2+}$/receptor·sec after correction for vesicle orientation. Thus, two independent assessments indicate that the transport capacity of the plasmalemmal $Ca^{2+}$ pump is at least 3 fold higher than that required to offset the inward leak of $Ca^{2+}$ measured in smooth muscle cells at rest.
In summary, the present report provides an estimate of the transport capacity of the plasmalemmal Ca$^{2+}$ pump at prevailing intracellular Ca$^{2+}$ concentrations and indicates that the transport system is capable of playing a dominant role in maintaining cytosolic Ca$^{2+}$ levels in resting smooth muscle. Moreover, since the prevailing cytosolic Ca$^{2+}$ levels in resting smooth muscle are close to the $K_m$ of this enzyme (Williams and Fay, 1986, Yamaguchi, 1986) and since the transport capacity of the enzyme appears to exceed the passive Ca$^{2+}$ leak rate, operation of the plasmalemmal Ca$^{2+}$ pump should be capable of buffering small changes in cellular Ca$^{2+}$ resulting from increased Ca$^{2+}$ entry. The data also indicate that this "transport reserve" is somewhat limited, however, and thus predict the observed increases in cytosolic Ca$^{2+}$ that occur during exposure to contractile stimuli (Williams and Fay, 1986, Yamaguchi, 1986).
Chapter III.

EFFECTS OF THE ANTI-CALMODULIN DRUGS CALMIDAZOLIUM AND TRIFLUOPERAZINE ON $^{45}$CA TRANSPORT IN PLASMALEMMAL VESICLES FROM GASTRIC SMOOTH MUSCLE

ABSTRACT

The anticalmodulin drugs calmidazolium (CMZ) and trifluoperazine (TFP) were shown to have a number of effects on $^{45}$Ca transport by plasmalemmal vesicles from gastric smooth muscle. Although these compounds produced the expected dose-dependent inhibition of the plasmalemmal ATP-dependent Ca$^{2+}$ transport system, they also evoked a Ca$^{2+}$ release comparable to that observed in the presence of the Ca$^{2+}$ ionophore, ionomycin. This increased transmembrane Ca$^{2+}$ flux was so large that it accounted for much of the apparent decrease in $^{45}$Ca uptake produced by these agents. Thus, direct effects of CMZ and TFP on ATP-dependent $^{45}$Ca uptake could only be reliably assessed for brief ($\leq 30$ seconds) drug exposures. The explanation for the observed effects of CMZ and TFP on membrane Ca$^{2+}$ permeability is unclear. The increased transmembrane Ca$^{2+}$ flux may reflect nonspecific effects on membrane permeability or it may reflect a specific interaction of the anticalmodulin drugs with a Ca$^{2+}$ release channel or with the Ca$^{2+}$ transport ATPase. In any case, these results suggest the need for caution in the design and interpretation of studies using both CMZ and TFP as anticalmodulin agents.

This work has been accepted for publication:
INTRODUCTION

Calmodulin (CaM) plays a pivotal role in regulating a variety of Ca^{2+} dependent cell enzymes including phosphodiesterase, adenylate cyclase, the plasmalemmal Ca^{2+} transport ATPase and myosin light chain kinase (Cheung, 1980; Means and Dedman, 1980). Because of the importance of this regulator, investigators have utilized a variety of anticalmodulin drugs to identify calmodulin-dependent processes in various tissues. Phenothiazines such as trifluoperazine have been used most extensively in such studies, but recent evidence suggests that these drugs exert calmodulin-independent effects which compromise their usefulness in intact systems. For example, it was found that the phenothiazines inhibit neurotransmitter binding at concentrations less than those required for antagonism of calmodulin-dependent processes (Blackmore et al., 1981; Gietzen et al., 1981). These problems led to a search for better calmodulin antagonists, one of the most promising of which is calmidazolium (CMZ, formerly known as R 24571). This drug is considerably more potent than other available anticalmodulin drugs, does not affect receptor binding and exhibits high specificity for calmodulin-regulated enzymes including the plasmalemmal Ca^{2+} transport ATPase (Gietzen et al., 1981; van Belle, 1981). Because of these apparent advantages calmidazolium was employed in recent studies of the calmodulin dependence of Ca^{2+} transport in gastric smooth muscle plasmalemmal membranes (Lucchesi et al., 1988). These studies indicated that while this drug is an effective inhibitor of calmodulin-stimulated Ca^{2+} transport, it also exerts large effects on membrane permeability...
to Ca$^{2+}$ that seriously compromise its usefulness.

METHODS

Isolation of purified plasmalemma. Plasmalemmal vesicles were prepared from the stomach muscle of the pig and the toad *Bufo marinus* as described previously (Lucchesi et al., 1988). Briefly, this involved homogenization of the gastric smooth muscle with an Ultraturrax Tissuemizer, further disruption of the tissue by nitrogen cavitation, and centrifugation of the homogenate at low speed to remove cellular debris. The crude microsomal pellet was then treated with high salt to solubilize contractile proteins, and purified membranes were collected by flotation in a discontinuous sucrose gradient (Jones, 1986). Membranes were rapidly frozen in liquid nitrogen and stored at -80°C prior to their use in these studies.

Assays for the activity of various marker enzymes indicated that the membrane vesicles were largely derived from the plasma membrane. The purified membrane fractions from both pig and toad were enriched 15-18 fold for the plasma membrane markers 5'-nucleotidase and K$^+$-stimulated PNPPase, and 13-24 fold for the specific binding of $^3$H-QNB (quinuclidinyl benzilate), a muscarinic cholinergic antagonist; whereas these membranes were enriched less than 1.5 fold for the sarcoplastic reticulum marker, NADPH cytochrome c reductase. In addition, the characteristics of ATP-dependent Ca$^{2+}$ transport by these membrane fractions were consistent with those of a plasmalemmal Ca$^{2+}$ pump (Lucchesi et al., 1988). ATP-dependent $^{45}$Ca uptake was unaffected by
azide, a mitochondrial Ca\textsuperscript{2+} transport inhibitor, or by oxalate, an Ca\textsuperscript{2+} trapping anion that reportedly potentiates uptake by the sarcoplasmic reticulum (see Daniel et al., 1983, for review). Moreover, 45\textsuperscript{Ca} uptake was stimulated (1.4 - 2.0 fold) by exogenous calmodulin and inhibited by low concentrations of orthovanadate (apparent K\textsubscript{i} < 0.1 \textmu M).

**Assay of 45\textsuperscript{Ca} uptake.** Studies of ATP-dependent 45\textsuperscript{Ca} uptake were carried out as described previously (Lucchesi et al., 1988). Briefly, this involved incubating membrane vesicles (30-35 \textmu g membrane protein/ml reaction mixture) at room temperature in a standard uptake buffer containing 20 mM HEPES, pH 7.1, 100 mM KCl, 3 mM MgCl\textsubscript{2}, 5 mM NaN\textsubscript{3}, 10 \textmu M free Ca\textsuperscript{2+}, and 0.1 \textmu Ci/ml 45\textsuperscript{Ca}. For buffers containing 5 mM ATP, the amounts of MgCl\textsubscript{2} and Ca\textsuperscript{2+} were increased to 8.066 mM and 17 \textmu M, respectively, and a regenerating system consisting of 5 mM creatine phosphate and 8 U/ml creatine phosphokinase was included. In experiments in which exogenous calmodulin was added to the reaction mixture, bovine brain calmodulin was added as a Ca\textsuperscript{2+}-calmodulin complex at a final concentration of 2 \textmu g/ml (~120 nM).

For uptake studies, ATP-independent and ATP-dependent uptake were monitored separately and uptake was initiated by the addition of membranes to the reaction mixture. Uptake was halted at predetermined intervals by filtering duplicate 1 ml aliquots of the reaction mixture through 1.2 \textmu m Millipore filters. Filters were washed with 5 ml of ice-cold buffer containing 1 mM LaCl\textsubscript{3} and 150 mM NaCl, dissolved in counting fluid and counted in a liquid scintillation counter. ATP-dependent Ca\textsuperscript{2+} uptake was defined as the uptake in the presence of
ATP minus the uptake in the absence of ATP. Separate studies were carried out to verify that the effects of CMZ on ATP-dependent or ATP-independent $^{45}$Ca uptake could not be explained by effects of the solvent DMSO employed for CMZ stock solutions. In all cases the final concentration of DMSO in the assay buffer was 0.1-0.5% (v/v).

**Assays of $^{45}$Ca Release.** To examine the acute effects of calmidazolium on $^{45}$Ca transport, vesicles were allowed to accumulate $^{45}$Ca in the presence of ATP for several minutes and were then exposed to either 10 μM calmidazolium or to the $\text{Ca}^{2+}$ ionophore ionomycin (1 μM).

Vesicles were filtered at various times after drug addition and the effects of these agents on accumulated label was determined. Alternatively, we examined the effects of CMZ or TFP on $^{45}$Ca efflux from prelabeled vesicles. In these experiments, vesicles were allowed to accumulate $^{45}$Ca in the presence of ATP for 60 min, and efflux was initiated by diluting the vesicles 10-20 fold with unlabelled buffer containing 100 mM KCl, 20 mM HEPES, pH 7.1, and 0.1 mM EGTA. In some experiments, aliquots of loaded vesicles were diluted in buffer containing calmidazolium (1-10 μM), TFP (10 or 100 μM) or in buffer containing the $\text{Ca}^{2+}$ ionophore ionomycin (1 or 10 μM). In other experiments, vesicles were diluted with control buffer and aliquots of diluted vesicles were exposed to drugs during the course of efflux. In either case, duplicate 1 ml aliquots were removed at various times after dilution, and filtered as described above for uptake studies.

**Statistics.** Statistical analysis employed either a simple Student's t-test for comparing a single experimental condition with its control or a Dunnett's test for multiple comparisons with a single control (Winer,
1962). Differences were considered significant if the probability of chance occurrence was < 0.05.

**Materials.** Stomach muscle from the domestic pig was obtained from a local slaughterhouse. Toads (Bufo marinus) were imported from the Dominican Republic and maintained in the university animal facilities until sacrifice.

Reagents employed for the transport studies were of the highest purity available (Aristar grade) and were dissolved in ultrapure water (18 MΩ resistivity) prepared by filtering distilled, deionized water through a Gelman-Water I filtration apparatus. Calmidazolium, trifluoperazine and calmodulin (bovine brain) were purchased from Sigma Chemical Co. Ionomycin was obtained from Calbiochem. $^{45}\text{CaCl}_2$ was obtained from New England Nuclear Co.

**RESULTS**

**Effects of CMZ on $^{45}\text{Ca}$ release.** The effect of CMZ on $^{45}\text{Ca}$ release was initially demonstrated by experiments like that presented in Figure 9, in which we examined the acute effects of CMZ on ATP-dependent $^{45}\text{Ca}$ uptake in membrane vesicles derived from pig antral smooth muscle. These experiments demonstrated that exposure of the vesicles to 10 μM CMZ during the course of an uptake experiment caused a marked, rapid release of $^{45}\text{Ca}$ accumulated in the presence of ATP. This release of $^{45}\text{Ca}$ resembled that observed in vesicles exposed to 10 μM ionomycin, a Ca$^{2+}$ ionophore. Brief exposure to CMZ caused only a slight release of $^{45}\text{Ca}$ accumulated in the absence of ATP (data not shown). However,
Figure 9. Results from a typical study of the acute effects of CMZ and ionomycin on 45Ca uptake in the presence (closed circles) and absence (open circles) of ATP. Exposure of pig plasmalemmal vesicles to 10 µM CMZ (squares) or 1 µM ionomycin (X's) (at arrow) led to a marked loss of label.

Prolonged exposure to CMZ produced a marked reduction in ATP-independent 45Ca accumulation. In some of our initial studies in which vesicles were preincubated with CMZ for 10-12 min prior to initiation of uptake, ATP-independent uptake was reduced by 35% in vesicles derived from the pig (10 µM CMZ reduced uptake from 1.06 ± 0.20 nmoles/mg·min in controls to 0.64 ± 0.15 nmoles/ mg·min, Mean ± SEM for 7 paired experiments; p < 0.01 for paired analysis), and by 36% in the toad (controls reduced from 1.54 ± 0.36 to 0.88 ± 0.26 nmoles/mg·min in the presence of 10 µM CMZ, Mean ± SEM for 5 experiments; p < 0.05 for paired analysis). These results could not be explained by an effect
of the solvent DMSO employed for calmidazolium. The levels of DMSO employed in these studies (0.2%) had no effect on either ATP-dependent or ATP-independent $^{45}$Ca uptake (data not shown).

The effect of CMZ on $^{45}$Ca release was further confirmed in studies of $^{45}$Ca efflux from prelabeled vesicles as shown in Figure 10.

Figure 10. Results from a representative $^{45}$Ca efflux study. Prelabeled toad vesicles were diluted 10-fold with control buffer. At predetermined times, aliquots were removed and either filtered without further treatment (controls, filled circles) or exposed to the following drugs: 1.0 $\mu$M CMZ (X's), 2.5 $\mu$M CMZ (squares), 5.0 $\mu$M CMZ (triangles), 10 $\mu$M CMZ (stars), or 10 $\mu$M ionomycin (open circles). Note that the effect of CMZ on efflux increased with increasing CMZ concentrations and that 10 $\mu$M CMZ produced a greater enhancement of $^{45}$Ca release than did exposure to Ca$^{2+}$ ionophore.
Exposure of toad plasmalemmal vesicles to varying concentrations of CMZ increased the rate of $^{45}$Ca loss from prelabeled vesicles in a concentration dependent manner such that at the highest dose tested (10 µM), the efflux rate was comparable to that observed in the presence of 10 µM ionomycin, a Ca$^{2+}$ ionophore. Exposure to 100 µM TFP produced similar increases in $^{45}$Ca efflux from preloaded vesicles while 10 µM TFP had no effect (see Table 4 below). Experiments using plasmalemmal vesicles derived from pig antral smooth muscle produced similar results (Table 4).

Table 4. Effect of CMZ, TFP and Ionomycin on the rate of $^{45}$Ca efflux from smooth muscle plasmalemma.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Pig Efflux rate (min$^{-1}$)</th>
<th>Toad Efflux rate (min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.017 ± 0.003 (11)</td>
<td>0.0061 ± 0.0005 (6)</td>
</tr>
<tr>
<td>CMZ (0.1 µM)</td>
<td>0.035 (2)</td>
<td>N.D.</td>
</tr>
<tr>
<td>CMZ (1.0 µM)</td>
<td>0.040 ± 0.011 (5)</td>
<td>0.052 ± 0.018 (3)</td>
</tr>
<tr>
<td>CMZ (2.5 µM)</td>
<td>N.D.</td>
<td>0.078 ± 0.018 (3)*</td>
</tr>
<tr>
<td>CMZ (5.0 µM)</td>
<td>0.113 (2)</td>
<td>0.284 ± 0.088 (3)</td>
</tr>
<tr>
<td>CMZ (10 µM)</td>
<td>0.318 ± 0.050 (7)**</td>
<td>1.457 ± 0.264 (7)**</td>
</tr>
<tr>
<td>Ionomycin†</td>
<td>0.491 ± 0.132 (5)**</td>
<td>0.694 ± 0.103 (6)**</td>
</tr>
<tr>
<td>TFP (10 µM)</td>
<td>0.008 ± 0.004 (4)</td>
<td>N.D.</td>
</tr>
<tr>
<td>TFP (100 µM)</td>
<td>0.235 ± -0.062 (5)**</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

†Both 1 µM and 10 µM ionomycin produced similar effects on $^{45}$Ca release; thus data using either concentration were pooled for this table. Data presented are Means ± SEM for the number of experiments in parentheses. Levels of significance, determined by a Dunnett's test, are indicated by asterisks: * = p < 0.05; ** = p < 0.01; *** = p < 0.001. N.D.= not determined.

Pooled data from several efflux experiments are presented in Table 4. Note that 10 µM CMZ caused a 240-fold increase in the efflux rate constant for the toad and a 20-fold increase in the rate constant for
the pig. As mentioned above, the effects were concentration-dependent with statistically significant increases observed at concentrations greater than 1.0 μM in both species. High doses of TFP (100 μM) also produced a significant increase in the rate of $^{45}$Ca efflux, whereas 10 μM TFP had no effect.

**Effects of CMZ and TFP on ATP-dependent $^{45}$Ca uptake.**

CMZ produced a concentration-dependent inhibition of ATP-dependent uptake in both toad and pig plasmalemmal vesicles. Since CMZ also produced a marked increase in $^{45}$Ca efflux, it was possible that this apparent inhibition of Ca$^{2+}$ uptake was due to increased Ca$^{2+}$ efflux rather than reduced ATP-dependent uptake. Thus, we reexamined the uptake data after correcting for backflux of label (see Scheid and Fay, 1980 for details). This correction is somewhat imprecise for non-steady state conditions (Keynes, 1954) such that we focused on the effects of brief exposure (30 seconds) to the drug and found that CMZ still produced a clear concentration-dependent inhibition of uptake in membranes derived from either pig or toad gastric smooth muscle (Fig. 11 and 12). For longer exposure times, however, the corrections were so large that the apparent inhibition of uptake, even at high concentrations of CMZ (10 μM), could be completely accounted for by the increased backflux (efflux) of label.

The data presented in Figure 11 illustrate the effects of CMZ on ATP-dependent $^{45}$Ca uptake in toad plasmalemmal vesicles. In this species, a brief (30 second) exposure to CMZ produced a dose-dependent inhibition with significant reductions in ATP-dependent $^{45}$Ca uptake observed at concentrations ≥ 5 μM. These studies were carried out in the absence of exogenous calmodulin (CaM).
Figure 11. Effect of varying concentrations of CMZ on ATP-dependent 45Ca uptake in toad plasmalemma. Bars indicate the percent inhibition of uptake at 30 sec. All data were corrected for backflux of Ca2+ using the correction factor \((kt/1-e^{-kt})\) where \(k\) = rate constant determined from efflux experiments (Keynes, 1954). Data presented are the Mean ± SEM for the number of experiments in parentheses. Significant differences are indicated by asterisks (* = p < 0.05, ** = p < 0.01).

In membrane vesicles derived from pig antral smooth muscle, brief exposures (30 seconds) to CMZ also produced a concentration-dependent inhibition of uptake, and these effects were more pronounced in the presence than in the absence of exogenous CaM. In these studies, the addition of exogenous CaM caused a 1.7-fold increase in ATP-dependent uptake measured at 30 seconds, from 1.63 ± 0.20 nmol Ca2+/mg protein in the absence of added CaM to 2.80 ± 0.22 nmol Ca2+/mg protein in
the presence of exogenous calmodulin (p < 0.01, Figure 12A). (Note, however, that membrane vesicles were not depleted of endogenous CaM prior to these studies such that the uptake observed in the "controls" does not necessarily reflect CaM-independent ATP-dependent 

\[^{45}\text{Ca}\] uptake).

![Figure 12. Effects of various concentrations of CMZ and TFP on ATP-dependent 

\[^{45}\text{Ca}\] uptake in plasmalemmal vesicles derived from pig antral smooth muscle. A. CMZ. Open bars indicate uptake in the absence of exogenous CaM. Hatched bars represent uptake in the presence of bovine brain CaM (2 μg/ml). The effects of 10 μM CMZ were not determined in the presence of CaM. B. TFP. Open bars indicate uptake in the absence of CaM. Hatched bars indicate uptake measured in the presence of exogenous CaM. All data were obtained 30 sec after the initiation of uptake and were corrected for backflux. Data presented are the Mean ± SEM for the number of experiments indicated above the bars. Significant differences are indicated by asterisks (* = p < 0.05, ** = p < 0.01).
When increasing amounts of CMZ were added to the uptake medium, a significant inhibition of ATP-dependent uptake was observed at 10 μM CMZ in the absence of exogenous calmodulin. In the presence of added CaM, significant inhibition was obtained for concentrations ≥ 1 μM. The inhibitory effects of CMZ in the presence of exogenous CaM were largely attributable to an attenuation of the CaM-induced stimulation of ATP-dependent $^{45}$Ca uptake. However, at a concentration of 5 μM, CMZ completely abolished the effects of CaM and reduced ATP-dependent uptake to levels below those observed in controls in the absence of exogenous CaM ($0.1 < p > 0.05$). The differing sensitivities of ATP-dependent $^{45}$Ca uptake to CMZ in the presence vs the absence of exogenous CaM may indicate that the drug has differing efficacies for transport stimulated by endogenous CaM (which is presumably tightly associated with the membrane vesicles and the Ca$^{2+}$ ATPase) vs that stimulated by exogenous CaM, and/or it may indicate that at higher concentrations, the drug can produce additional CaM-independent effects on Ca$^{2+}$ transport.

Studies were also carried out to assess the effects of TFP on ATP-dependent $^{45}$Ca uptake. These studies indicated that exposure of pig plasmalemmal vesicles to TFP (in the absence of exogenous calmodulin) produced a concentration-dependent inhibition of uptake, with 10 μM TFP inhibiting uptake by $18.9 \pm 5.7\%$ (Mean ± SEM for 6 experiments, $p < 0.05$) and 100 μM inhibiting uptake by $63.8 \pm 14.5\%$ (Mean ± SEM for 6 experiments, $p < 0.01$). In contrast to the results with CMZ, the addition of 2 μg/ml of CaM completely reversed the effects of TFP at both concentrations tested (Figure 12B).
DISCUSSION

The results from the present studies indicate the need for extreme caution in the use of both calmidazolium and trifluoperazine as anticalmodulin agents. The levels of calmidazolium (10 μM) and of trifluoperazine (100 μM) that caused profound effects on $^{45}\text{Ca}$ release by gastric smooth muscle plasmalemmal vesicles have been used routinely in many studies, often with no controls to assess for effects of these agents on membrane $\text{Ca}^{2+}$ permeability. At these levels, however, we observed a number of actions of these agents that complicate the interpretation of experimental data. For one, consistent with other findings (Gietzen et al., 1981; Van Belle, 1981; Gietzen, 1983) we found that prolonged exposure (10 min) to relatively low concentrations of CMZ (10 μM) produced a significant inhibition of ATP-independent $^{45}\text{Ca}$ uptake. Secondly, exposure of plasmalemmal vesicles to CMZ or TFP (at 10 and 100 μM, respectively) cause a marked increase in membrane permeability to $\text{Ca}^{2+}$ (a finding which may also explain the inhibition of ATP-independent $^{45}\text{Ca}$ uptake). The increase in membrane $\text{Ca}^{2+}$ permeability was so large that one could not reliably detect an inhibitory effect on ATP-dependent transport unless exposure times were brief (30 sec or less). With longer exposures to CMZ ($\geq$ 1 min) the increased backflux of label was sufficient to account for essentially all of the observed reduction in $^{45}\text{Ca}$ accumulation even at the highest concentration tested (10 μM). Thus, it would seem that CMZ and TFP may not be suitable for studies of calmodulin-regulated $\text{Ca}^{2+}$ transport, except in isolated cells or membrane vesicles in which brief
exposure times (≤ 30 sec) are feasible.

Studies of the interactions between calmodulin and the anticalmodulin agents CMZ and TFP on ATP-dependent \(^{45}\)Ca uptake also produced some surprising results. We found that the addition of exogenous CaM increased the efficacy of CMZ for inhibiting ATP-dependent uptake, whereas similar additions of CaM abolished the inhibitory effects of TFP on this process. The latter results were expected based on the evidence that TFP can compete with CaM at its effector site on various enzymes (Vincenzi, 1981). The interaction between CMZ and CaM suggests a different type of competition at the CaM/enzyme complex, however. CMZ attenuated the stimulatory effects of CaM on the transport system (reducing uptake to levels seen in the absence of exogenous CaM), whereas CaM abolished the inhibitory effects of TFP on ATP-dependent transport. These findings suggest a different mode of interaction between CMZ and TFP with CaM and the \(\text{Ca}^{2+}\) transport ATPase: CMZ appears to interact with the CaM/\(\text{Ca}^{2+}\) ATPase complex in the cell membrane whereas TFP appears to compete directly with CaM for its binding site on the \(\text{Ca}^{2+}\) ATPase.

The precise manner by which CMZ and TFP evoked the observed changes in ATP-dependent \(\text{Ca}^{2+}\) transport and in membrane permeability is not entirely clear. These compounds may elicit changes in the membrane transport or permeability that are not mediated by CaM. This possibility was not ruled out by the present studies since the membranes were not depleted of endogenous calmodulin. However, there are numerous examples of CaM-independent actions of these compounds in the literature. Previous studies have shown that CMZ can produce direct
CaM-independent inhibition of a variety of enzymes including myosin light chain kinase (Mazzei, et al., 1984; Zimmer et al., 1984), calmodulin-depleted \( \text{Ca}^{2+} \) transport ATPase (Van Belle, 1981; Vincenzi et al., 1982), phospho-diesterase (Gietzen, 1983), proteolysis-activated \( \text{Ca}^{2+} \) transport ATPase (Adunyah et al., 1982; Gietzen et al., 1982) and the sarcoplasmic reticulum Ca-ATPase (Anderson et al., 1984; Tuana and MacLennan, 1984). Thus, the effects of CMZ and TFP on membrane \( \text{Ca}^{2+} \) transport and permeability may reflect a calmodulin-independent action of these compounds.

The manner by which these compounds produced the observed increase in membrane \( \text{Ca}^{2+} \) permeability is also unclear. The increased transmembrane \( \text{Ca}^{2+} \) flux may reflect a nonspecific effect on membrane integrity. However, these compounds do not produce similar effects in the permeability of sarcoplasmic reticulum vesicles (Tuana and MacLennan, 1984). Moreover, the clear dose-dependency of the changes in \( \text{Ca}^{2+} \) efflux make this explanation less likely. Alternatively, the increased permeability may reflect a direct interaction with a \( \text{Ca}^{2+} \) release channel in the membrane vesicles and/or with the \( \text{Ca}^{2+} \) ATPase. Recent studies in isolated sarcoplasmic reticulum have suggested that the \( \text{Ca}^{2+} \) transport system can function as a \( \text{Ca}^{2+} \) channel (McWhirter et al., 1987), thus it is possible that the drugs shift the transport system to this mode of operation.

In summary, while we were able to define conditions in which we could detect a direct, calmodulin-dependent effect of CMZ and TFP on ATP-dependent \( ^{45} \text{Ca} \) uptake, the profound effects of these compounds on membrane \( \text{Ca}^{2+} \) permeability made these conditions very restrictive.
Thus, although these compounds might be quite useful in biochemical studies on isolated enzymes and/or in systems in which short drug exposures are feasible, they may not be useful in other systems.
Chapter IV
Discussion of Ca$^{2+}$ Transport Studies

The primary goal of this portion of my thesis was to determine the relative contribution of the plasmalemmal Ca$^{2+}$ pump to Ca homeostasis in smooth muscle. As suggested in the Introduction, at least two transport systems contribute to Ca$^{2+}$ extrusion in this tissue: Na$^+$/Ca$^{2+}$ exchange and the plasmalemmal Ca$^{2+}$ pump. Biochemical evidence suggests that the former is a low affinity, high capacity system which might be more important in nonsteady-state conditions (e.g., when cellular Ca$^{2+}$ is high, as during activation), whereas the plasmalemmal ATPase is a high affinity, low capacity system. These findings led to a general agreement that the plasmalemmal Ca$^{2+}$ pump was the primary determinant of resting Ca$^{2+}$ levels, but it has been difficult to demonstrate that the characteristics of the Ca$^{2+}$ transport processes established under in vitro conditions can be directly applied to the in vivo situation. For example, it was difficult to assess whether a given transport rate determined in membrane vesicles was of sufficient magnitude to offset completely the passive leak rate measured in vivo. Part of the difficulty lies in the fact that estimates of this leak rate were unavailable. However, this problem was resolved in at least one preparation: enzymatically dispersed toad gastric smooth muscle cells. In order to assess the transport capacity of the plasmalemmal Ca$^{2+}$ pump in this tissue, it was necessary to correlate the velocity of the pump at physiological Ca$^{2+}$ concentrations with the observed estimate of the inward Ca$^{2+}$ leak rate in intact smooth muscle cells.
The study of the transport capacity of the Ca\(^{2+}\) pump required the development of the necessary biochemical tools to evaluate its translocation velocity in toad gastric smooth muscle. This included the development of suitable methods for the preparation of plasma membrane fractions as well as methods for assessing membrane purity. Then, to ensure that the observed Ca\(^{2+}\) transport in these membranes could be attributable to a Ca\(^{2+}\) pump of plasmalemmal origin, the characteristics of the measured Ca\(^{2+}\) transport had to be carefully evaluated.

The methodology that was developed for preparing plasma membrane fractions from smooth muscle involved several refinements of existing published techniques. These refinements included a modification of the homogenization procedure to include a nitrogen cavitation step to disrupt the tissue more fully. This procedure involves the sudden decompression of the homogenate after it has been equilibrated with nitrogen at 1250 psi. The rapid change in pressure causes a thorough disruption of the tissue fragments and cells and is especially effective for smooth muscle tissue because of its high content of connective tissue. A second refinement was the use of high salt to solubilize the actomyosin that heavily contaminates the crude microsomal fraction. This procedure not only increases the purity of the membrane fraction (see Figures 2 and 3) but also serves to remove the (Ca\(^{2+}\) + Mg\(^{2+}\))-dependent ATPase activity associated with the myofilaments. The last procedural modification was the use of sucrose flotation gradients, in which the vesicle were suspended in 1 Molar sucrose and allowed to equilibrate to their buoyant density during centrifugation.
In this procedure, only tightly sealed vesicle with entrapped buffer of low ionic strength would be able to float; any leaky vesicles would become equilibrated with 1 Molar sucrose and would be unable to reach the inherent buoyant density (determined by their phospholipid content) during the short centrifugation step. Thus, this separation technique enriches for membrane vesicles that are tightly sealed.

In order to use the isolated membrane fractions for the study of the transport capacity of the plasmalemmal Ca\textsuperscript{2+} pump, it was necessary to first demonstrate that the majority of the vesicles were largely derived from the plasma membrane. This involved the measurement of the activity of various membrane markers: 5' nucleotidase activity and cholinergic receptor binding for plasma membranes, NADH cytochrome c reductase activity for mitochondria and NADPH-cytochrome c reductase activity for sarcoplasmic reticulum. The results from these experiments indicated that the membrane preparation was enriched 13-24 fold for plasma membrane markers and enriched only a minimal extent for mitochondrial and sarcoplasmic reticulum markers.

These results suggested that most of the membranes were in fact derived from the plasmalemma. Using these preparations from both toad and porcine gastric muscle, we were then able to demonstrate that the smooth muscle membranes could accumulate \textsuperscriptr{45}Ca in an ATP-dependent manner (Figure 4). It could be concluded that this ATP-dependent \textsuperscriptr{45}Ca uptake reflected the presence of an active transport system rather than ATP-dependent binding because exposure to the Ca\textsuperscript{2+} ionophore ionomycin caused an instantaneous release of the accumulated \textsuperscriptr{45}Ca.

It was then necessary to determine whether this ATP-dependent uptake
was due to the presence of a plasmalemmal Ca\(^{2+}\) pump. According to Penniston (1983), two fundamental criteria can be used to define the presence of a plasma membrane Ca\(^{2+}\) pump: 1) the observed Ca\(^{2+}\) uptake should exhibit characteristics expected for a pump of plasmalemmal origin and 2) the ATP-dependent uptake should possess a high affinity for Ca\(^{2+}\). The first criterion was evaluated by examining the sensitivity of \(^{45}\)Ca uptake to agents that modulate Ca\(^{2+}\) transport by intracellular organelles. Our results indicated that ATP-dependent \(^{45}\)Ca uptake was unaffected by azide, a mitochondrial transport inhibitor, or oxalate, a Ca\(^{2+}\) precipitating anion that enhances uptake by skeletal muscle sarcoplasmic reticulum (Hasselbach, 1978). It should be noted, however, that the assumption that oxalate stimulation of uptake reflects transport into smooth muscle sarcoplasmic reticulum has been brought into question given the results of several recent studies (Kwan, 1985). In some tissues (e.g. rat mesentary) oxalate produced no stimulation of uptake in microsomal fractions enriched in sarcoplasmic reticulum (Kwan et al., 1981), while in other tissues (e.g. dog aorta) oxalate stimulated \(^{45}\)Ca accumulation by a fraction enriched in plasma membranes (Kwan et al., 1984).

More rigorous evidence that sarcoplasmic reticulum contamination did not contribute to the observed \(^{45}\)Ca fluxes in our vesicle preparation was obtained from the \(^{45}\)Ca efflux experiments (see Figure 6). These results indicated that \(^{45}\)Ca release from preloaded vesicles was not affected by agents that stimulate sarcoplasmic reticulum Ca\(^{2+}\) release, including caffeine, IP\(_3\) and GTP (Endo et al., 1977; Obara and Yabu, 1982; Carsten et al., 1985; Suematsu et al., 1985; Wolf et al., 1987).
Other studies demonstrated that the ATP-dependent $^{45}$Ca uptake in both pig and toad stomach membranes exhibited properties expected for a plasmalemmal Ca$^{2+}$ pump. Vanadate at low concentrations has been shown to inhibit plasma membrane Ca$^{2+}$ + Mg$^{2+}$-dependent ATPase activity and Ca$^{2+}$ uptake by blocking a step in the Ca$^{2+}$-pump reaction cycle in which the phosphorylated form of the pump protein undergoes a conformational change required for the translocation of Ca$^{2+}$ across the membrane (Schatzmann et al., 1986). In our study, vanadate inhibited $^{45}$Ca uptake in a concentration-dependent manner with an apparent $K_i < 0.1 \, \mu M$, similar to the values for vanadate inhibition of the plasmalemmal Ca$^{2+}$ pump in guinea pig ileum (Sharma et al., 1987) and in porcine stomach muscle (Raeymakers, 1983).

The second criterion for establishing the existence of a plasmalemmal Ca$^{2+}$ pump - high Ca$^{2+}$ affinity - was fulfilled by demonstrating that ATP-dependent Ca$^{2+}$ uptake had an apparent $K_m$ for Ca$^{2+}$ of 0.2 $\mu M$ in toad vesicles and 0.4 $\mu M$ in pig vesicles. These values are well within the physiological range of Ca$^{2+}$ reported in resting smooth muscle and are comparable to values reported for the smooth muscle plasmalemmal Ca$^{2+}$ ATPase (Matlib et al., 1979; Grover et al., 1980; Wibo et al., 1981; Raeymakers et al., 1983).

These data led us to conclude that most of the Ca$^{2+}$ transport appeared to be plasmalemmal in origin. Further support for this contention was provided by studies examining the sensitivity of the transport system to calmodulin. Calmodulin has been reported to stimulate the ATP-dependent Ca$^{2+}$ transport by plasma membrane fractions from pig antral smooth muscle (Wuytack et al., 1981), canine
gastric smooth muscle (Grover et al., 1983) and intestinal smooth muscle (Wibo et al., 1981). It is generally considered that the binding of Ca-CaM to the Ca^{2+} pump induces a conformational change in the enzyme (see Figure 13) removing a 4-kDa inhibitory sequence from the region of the active site (Carafoli, 1988).

![Figure 13. A scheme of the architecture of the plasma membrane Ca^{2+} pump as derived from studies of limited proteolysis. The 9-kDa calmodulin-interacting domain is visualized to contain a 4-kDa calmodulin binding sequence, and a 5-kDa sequence that is necessary for activation by calmodulin, i.e., for the removal of the 4-kDa "inhibitory" sequence from the active site. (from Carafoli, 1988).](image)

Sensitivity to calmodulin was tested in two ways: by examining the effects of exogenous calmodulin addition and the effects of calmodulin antagonists on ATP-dependent Ca^{2+} uptake. The addition of exogenous CaM produced a 1.4 ~ 1.5 fold stimulation of ATP-dependent ^{45}Ca uptake by pig and toad plasmalemmal vesicles, increasing both the affinity for Ca^{2+} and the maximum velocity. These modest effects of calmodulin are probably an underestimate since the membrane preparation
was not depleted of endogenous calmodulin. This conclusion is supported by results from Enyedi et al. (1988), who studied the effects of calmodulin on plasmalemmal Ca\textsuperscript{2+} ATPase in rat myometrium. Their data indicated that exogenous calmodulin caused only a 20% increase in Ca transport in untreated membranes, but a 4-fold increase if the membranes were first depleted of endogenous calmodulin (Enyedi et al., 1988). The depletion of endogenous calmodulin was not attempted in the present studies since we felt that the rather drastic procedures necessary for calmodulin extraction (including hypotonic and hypertonic washes with EGTA) would compromise the integrity of the plasmalemmal vesicles.

Instead, we sought evidence for the calmodulin dependence of ATP-dependent Ca\textsuperscript{2+} transport using calmodulin antagonists. A variety of chemically unrelated substances have been shown to antagonize the effects of calmodulin on target enzymes in vitro. These include phenothiazines (e.g. TFP), naphthalene sulfonamides (e.g. W7) and calmidazolium, a complex imidazolium compound. Despite their variable structures, most if not all of these compounds are cationic amphiphiles and exhibit structural regions complementary to calmodulin. It has been proposed that these agents bind to the hydrophobic regions on calmodulin that are exposed upon its binding to Ca\textsuperscript{2+}, competitively blocking the interaction of calmodulin with target enzymes (Gietzen, 1982). Most of the antagonists have other actions apart from blocking the effect of calmodulin, however. Some exert direct effects on the target enzyme, producing, for example, an inhibition of the purified Ca\textsuperscript{2+} ATPase and an attenuation of the effects of other activators of the pump (acidic lipids and limited proteolysis; Adunyah et al., 1982). Therefore,
extreme caution must be exercised if inhibition of $^{45}$Ca uptake by calmodulin antagonists is to be taken as reliable evidence for regulation by calmodulin.

In the present studies we were able to define conditions under which we could demonstrate a calmodulin-dependent inhibition of ATP-dependent $^{45}$Ca uptake in gastric smooth muscle by both TFP and CMZ. These conditions involved brief exposure of the membranes to relatively low concentrations of the antagonists and produced an inhibition of calmodulin-stimulated, ATP-dependent uptake that could not be explained by nonspecific changes in membrane permeability. At higher concentrations of TFP and CMZ (100 μM and 10 μM, respectively) or at longer exposure times (greater than 1 min), we could not demonstrate selective effects of these drugs on Ca$^{2+}$ transport. The increase in membrane Ca$^{2+}$ permeability could account for essentially all the observed decrease in $^{45}$Ca accumulation. Therefore, any decrease in ATP-dependent uptake observed at these moderate concentrations would be a composite of two simultaneous events: a decrease in ATP-dependent uptake and an increase in Ca$^{2+}$ leakage. Note that these concentrations and exposure times have been extensively used in previous studies to test the role of a calmodulin-sensitive Ca$^{2+}$ pump in smooth muscle Ca$^{2+}$ homeostasis with no recognition of these secondary problems.

The profound effects of TFP and CMZ on Ca$^{2+}$ efflux can be explained in several ways. Due to the highly lipophilic nature of these compounds, it is possible that the changes in $^{45}$Ca could result from a nonspecific effect on membrane integrity. Anderson et al.(1984)
reported that binding of 1-10 μM calmidazolium to sarcoplasmic reticulum membranes produced morphological changes including membrane thickening and loss of resolution of the surface detail. However, the results may be artifacts due to the high concentration of DMSO (the solvent for CMZ) that was added to the membranes. In fact, other reports demonstrated that neither TFP or CMZ, in concentrations as high as 100 μM, produced any effects on the membrane permeability of sarcoplasmic reticulum vesicles (Tuana and MacLennon, 1984). This evidence along with the clear dose-dependency of the effects on Ca$^{2+}$ permeability in our own system makes this explanation less likely.

Alternatively, the increased loss of $^{45}$Ca from vesicles in the presence of calmodulin antagonists could result from an interaction of these agents with a Ca$^{2+}$ release channel located within the plasma membrane and/or within the Ca$^{2+}$ pump. Support for this possibility has also been provided from studies using sarcoplasmic reticulum membranes. Although camodulin regulation is not a property of the sarcoplasmic reticulum Ca$^{2+}$ pump per se, CMZ has been shown to inhibit Ca$^{2+}$ uptake by skeletal muscle sarcoplasmic reticulum fractions without affecting Ca$^{2+}$ ATPase activity (Tuana and MacLennon, 1984). This effect was attributed to interaction with a Ca$^{2+}$ release channel or some other calmodulin-dependent Ca$^{2+}$ translocation site. The latter speculation is of particular interest since it has been suggested that under certain conditions the sarcoplasmic reticulum Ca$^{2+}$ pump can function as a Ca$^{2+}$ pore or channel. McWhirter et al. (1987) were able to observe a fast Ca$^{2+}$ efflux from reconstituted phospholipid vesicles containing the sarcoplasmic reticulum Ca$^{2+}$ ATPase as the sole protein.
The results from these studies have also provided some insight into the interaction of CMZ or TFP with calmodulin. As expected, we found that the addition of exogenous calmodulin reversed the inhibitory effects of TFP on $^{45}\text{Ca}$ uptake. This is consistent with observations from many studies suggesting a competitive interaction between TFP and the Ca$^{2+}$ pump (i.e. the target enzyme) for the hydrophobic binding region on calmodulin (for a review see Garrahan, 1986). However, the inhibitory action of CMZ on ATP-dependent $^{45}\text{Ca}$ uptake was enhanced in the presence of added calmodulin. This result suggests that CMZ binds to the calmodulin-Ca$^{2+}$ pump complex and may indicate an interaction of CMZ with an allosteric site on the Ca$^{2+}$ pump that is exposed upon calmodulin binding. Support for this mechanism can be inferred from studies demonstrating that CMZ attenuates the effects of treatments that enhance Ca$^{2+}$ ATPase activity by mimicking calmodulin's actions (exposure to acidic phospholipids or mild proteolytic digestion; Adunyah et al., 1982).

It was concluded from the results described above that essentially all of the observed Ca$^{2+}$ uptake could be attributed to the presence of a plasmalemmal Ca$^{2+}$ ATPase. We were then able to determine that the plasmalemmal Ca ATPase is capable of maintaining steady-state levels of Ca$^{2+}$ in smooth muscle. More specifically we were able to show that: 1) the $K_m$ for Ca$^{2+}$ of the toad gastric smooth muscle Ca$^{2+}$ pump was 0.2 μM, similar to the resting intracellular Ca$^{2+}$ levels in toad stomach smooth muscle measured with Quin 2 (Williams and Fay, 1986) or with an intracellular Ca$^{2+}$ electrode (Yamaguchi, 1986); 2) vesicles exposed to 250 nM Ca$^{2+}$ were capable of generating at least a 10,000
fold gradient for Ca\textsuperscript{2+}, similar to the physiological transmembrane gradient for Ca\textsuperscript{2+} that is thought to exist across most cell membranes; 3) two independent assessments indicate that the transport capacity of the plasmalemmal Ca\textsuperscript{2+} pump is at least 3-fold higher than that required to offset the inward leak of Ca\textsuperscript{2+} measured in smooth muscle at rest.

These results indicate that operation of the plasmalemmal Ca\textsuperscript{2+} pump in smooth muscle plays a dominant role in maintaining low steady-state cytosolic Ca\textsuperscript{2+} levels. In addition, since the K\textsubscript{m} of the pump for Ca\textsuperscript{2+} is close to the prevailing resting Ca\textsuperscript{2+} concentration, it is likely that this enzyme can buffer any small changes in cytosolic Ca\textsuperscript{2+} resulting from increased Ca\textsuperscript{2+} entry. Moreover, from the point of view of cellular Ca\textsuperscript{2+} homeostasis, the interrelationship between cytosolic Ca\textsuperscript{2+} concentrations, calmodulin, and the activity of the plasmalemmal pump provides an elegant autoregulatory system for the control of cytosolic Ca\textsuperscript{2+} levels. For example, an abrupt rise in the Ca\textsuperscript{2+} concentration inside the cell would activate calmodulin, which in turn would increase the activity of the Ca\textsuperscript{2+} pump to lower the Ca\textsuperscript{2+} back to resting levels.

The values measured for the transport capacity of the plasmalemmal Ca\textsuperscript{2+} pump predict that the operation of this transport system alone would not be able to remove completely the rise in free cytoplasmic Ca\textsuperscript{2+} produced in response to contractile agents. During contraction, the free cytoplasmic Ca\textsuperscript{2+} in smooth muscle may rise to levels approaching 1 \textmu M (Williams and Fay, 1986; Yamaguchi, 1986). Under these conditions, restoration of Ca\textsuperscript{2+} back to resting levels would necessitate an additional extrusion mechanism, such as Na\textsuperscript{+}/Ca\textsuperscript{2+}
exchange, or sequestration into internal stores.

The relative contribution of each $\text{Ca}^{2+}$ transport system (Na$^+$/Ca$^{2+}$ exchange and the plasmalemmal and sarcoplasmic reticulum Ca$^{2+}$ pumps) under these condition remains unknown. However, procedures similar to those used in these studies could be applied to determine transport capacity of the plasmalemmal Na$^+$/Ca$^{2+}$ exchange system. The way in which intracellular Ca$^{2+}$ homeostasis is achieved may vary in different types of smooth muscle. For example, many investigators have argued that the sarcoplasmic reticulum may be relatively more important in vascular smooth muscle than in visceral.

This hypothesis is in agreement with morphological data showing a well-developed (up to 7.5% of cell volume) sarcoplasmic reticulum occurs in vascular smooth muscle whereas this organelle comprises only 2% of the cell volume in visceral smooth muscle (Eggermont et al., 1988a). Another interesting difference found between vascular and visceral smooth muscle is that the Na$^+$/K$^+$-ATPase activity is higher and the (Ca$^{2+}$ + Mg$^{2+}$) ATPase activity is lower in pulmonary arteries than in gastric smooth muscle (Eggermont, et al., 1988b). Since the activity of the Na$^+$/K$^+$-ATPase sets the upper limit to Ca$^{2+}$ efflux that can be mediated by the Na$^+$/Ca$^{2+}$ exchanger, the conditions present in vascular smooth muscle may allow the Na$^+$/Ca$^{2+}$ exchanger to play a more important role in Ca$^{2+}$ extrusion.

The relative contribution of each Ca$^{2+}$ transport system to the restoration of resting Ca$^{2+}$ levels after contraction may also be determined by the relative levels of other intracellular mediators (cAMP and diacylglycerol). Scheid and Fay (1984b) observed a 13-fold increase
in $^{45}$Ca efflux from toad gastric smooth muscle cells in response to the beta-adrenergic agonist isoproterenol. From the observations that the effects of isoproterenol were mimicked by dibutyryl cAMP and blocked by β receptor, anyagonists and by ouabain (an inhibitor of the Na$^+/K^+$ ATPase), they were able to conclude that the increase in Ca$^{2+}$ efflux reflected an indirect activation of Na$^+/Ca^{2+}$ exchange, secondary to β adrenergic stimulation of Na$^+,K^+$ pump activity.

Other investigators have suggested that both the ATP-dependent Ca$^{2+}$ transport and the (Ca$^{2+}$ + Mg$^{2+}$)-dependent ATPase activity of the pump are stimulated by cAMP or by the catalytic subunit of cAMP dependent protein kinase (Bhalla et al., 1978; Hisayama and Takayanagi, 1983; Katternburg and Daniel, 1984). Others, however, found no effect of either compound on the activity of the plasmalemmal enzyme (Sands et al., 1977). The reasons for these inconsistent findings is not clear but may depend on the experimental conditions used, since the most consistent effects of cAMP were found in studies where the membranes were first pretreated with phosphatase to remove any native phosphorylation of the Ca$^{2+}$ pump. In our own studies, the effects of cAMP and the kinase were inconclusive perhaps because we did not attempt to dephosphorylate the membranes. Other studies demonstrated that activation of protein kinase C by phorbol esters or 1-oleoyl-2-acetyl glycerol stimulated the plasmalemmal Ca$^{2+}$ pump in vascular smooth muscle cells (Furukawa, 1989).

The above discussion indicates that many factors may dictate the relative contribution of each transport system to the removal of the increased cytosolic Ca$^{2+}$ levels that are observed in response to
contractile stimuli. These factors may include the abundance of each transport system, their affinity for Ca\(^{2+}\), the maximal translocation velocity, their stoichiometry of Ca\(^{2+}\) transport, and their regulation by second messengers. Thus, the precise role of the smooth muscle plasmalemmal Ca\(^{2+}\) pump under which Ca\(^{2+}\) levels are elevated by contractile agents remains to be resolved.

In summary, the results from this portion of my thesis research provide the first quantitative measurement of the transport capacity of the smooth muscle plasmalemmal Ca\(^{2+}\) ATPase and demonstrated that this enzyme was capable of completely offsetting the inward Ca\(^{2+}\) leak observed in intact smooth muscle cells. The methodologies developed could be applied to future studies of the transport capacity of the Na\(^+\)/Ca\(^{2+}\) exchanger and/or to studies of the regulation of each transport system by intracellular second messengers. This methodology could also be used to study the mechanisms that underlie alterations in cellular Ca\(^{2+}\) handling that are observed in certain disease states such as hypertension. In addition, these studies provided the first demonstration of the profound effects of the calmodulin inhibitors TFP and CMZ on the permeability of the plasma membrane to Ca\(^{2+}\) and seriously questioned the use of these agents to examine the calmodulin regulation of plasmalemmal Ca\(^{2+}\) transport.
Chapter V.
Introduction to Muscarinic Receptors

The study of the mechanisms by which the parasympathetic nervous system regulates smooth muscle contraction has long been an important aspect of smooth muscle physiology and pharmacology. It is generally accepted that acetylcholine is the major excitatory neurotransmitter that regulates the contractility of smooth muscle in the airways, the gastrointestinal tract and the urogenital tract. Evidence for the postsynaptic localization of muscarinic receptors is well established, having been provided by a variety of functional and binding studies. In binding studies, Burgen et al. (1974), using the muscarinic antagonist propylbenzilylcholine mustard, were among the first to demonstrate saturable binding to specific muscarinic acetylcholine receptors on longitudinal muscle of the guinea pig ileum. In functional studies, it was demonstrated that the exogenous addition of acetylcholine or carbachol caused contraction of bronchial or gastric smooth muscle (Yamamura and Snyder, 1974). The contractile response was mediated by muscarinic receptors since it was antagonized by low concentrations of the muscarinic-selective antagonist atropine (for a review, see Mitchelson, 1988).

The first radioligand binding studies of muscarinic receptors were performed by Paton and Rang (1965) who demonstrated that the binding of $[^3]H$-atropine could be used to identify receptors in guinea pig intestinal smooth muscle. The development of radioligands with higher specific activity (and thus a greater signal to noise ratio) allowed for
a more detailed identification of smooth muscle muscarinic receptors. The antagonist \[^{3}H\]-dextemide was used to label receptors in homogenates of bovine tracheal smooth muscle (Beld and Ariens, 1974) and the high affinity antagonist \[^{3}H\]-quinuclidinyl benzilate (QNB) was used to identify receptors in guinea pig ileum (Yamamura and Snyder, 1974). The excellent agreement between the values of the affinity constants determined by binding measurements and by antagonism of smooth muscle contraction indicated that the sites labelled by the muscarinic antagonists represent functionally active receptors.

The interaction of muscarinic agonists with smooth muscle muscarinic receptors was determined by competition with labeled antagonists for the binding sites. In contrast to the apparent homogeneity of antagonist binding, agonist binding was complex and deviated significantly from that expected for a simple bimolecular reaction of the ligand with one population of receptors (Nathanson, 1987). Studies of the binding of radiolabelled agonists to intestinal smooth muscle indicated that the observed heterogeneity in agonist binding could be explained by the presence of both high and low affinity sites (Roeske, et al., 1983). Moreover, other evidence indicated that conversions between the high and low agonist affinity sites could be induced by guanine nucleotides, suggesting that smooth muscle muscarinic receptors were coupled to GTP-binding regulatory (G) proteins.

While the results from these early binding studies demonstrated the distribution of muscarinic receptors within smooth muscle and their affinities for agonists and antagonists, the relationship between receptor occupancy and contraction induced by muscarinic agonists was
poorly understood. However, it is now generally accepted that activation of smooth muscle muscarinic receptors leads to contraction by increasing the availability of cytosolic $\text{Ca}^{2+}$ to the contractile machinery. Cholinergic control of intracellular $\text{Ca}^{2+}$ levels, and hence the contractile state of smooth muscle, is now thought to result from the actions of cholinergic agonists on a number of cellular processes. Many studies have indicated that muscarinic receptors in smooth muscle and other excitable tissues modulate ion channel activity, intracellular $\text{Ca}^{2+}$ release; and $\text{Ca}^{2+}$ extrusion; and these actions of cholinergic agents may be mediated by the generation of second messengers via the phosphoinositide and adenylyl cyclase pathways. However, the detailed mechanisms by which muscarinic receptors regulate these diverse biochemical responses in smooth muscle is unclear.

In other systems, the effects of muscarinic agents on both second messenger systems are thought to be mediated by receptors coupled to guanine-nucleotide binding regulatory (G) proteins. For example muscarinic receptors in brain (Nathanson, 1987) and exocrine tissues (Merritt et al., 1986) are thought to regulate phospholipase C activity in a GTP-dependent manner. Phospholipase C hydrolyzes phosphatidylinositol 4,5 bisphosphate (PIP$_2$), generating two second messengers: inositol 1,4,5 trisphosphate (IP$_3$) which mobilizes $\text{Ca}^{2+}$ from intracellular stores, and 1,2 diacylglycerol which activates protein kinase C (Berridge, 1987). There appears to be more than one type of G protein (termed Gp) that couples muscarinic receptors to phospholipase C, as the muscarinic stimulation of phosphoinositide hydrolysis is blocked by pertussis toxin in some, but not in other cell
types (Ashkenazi et al., 1989). Thus, at least two types of Gp-coupled muscarinic receptors can be distinguished by their sensitivity to ADP-ribosylation catalyzed by pertussis toxin.

Muscarinic agents in airway smooth muscle (Takuwa et al., 1986) have been shown to regulate polyphosphoinositide metabolism, indicating that IP$_3$ and diacylglycerol can be generated in response to receptor activation. IP$_3$ in turn can mobilize Ca$^{2+}$ from storage sites within the sarcoplasmic reticulum, as is evident by its ability to release Ca from permeabilized smooth muscle (Somlyo et al., 1985) or from microsomes derived from uterine sarcoplasmic reticulum (Carsten and Miller, 1985). In addition, Salmon and Honeyman (1979) demonstrated that carbachol stimulated the formation of phosphatidic acid (a metabolite of diacylglycerol) in toad gastric smooth muscle cells; and diacylglycerol has been shown to regulate a voltage-sensitive Ca$^{2+}$ channel in the same preparation, presumably via activation of protein kinase C (Vivaudou et al., 1988). The involvement of G proteins in the muscarinic-induced changes in phospholipid metabolism has not yet been demonstrated in smooth muscle.

Muscarinic regulation of levels of the second messenger cAMP is also mediated by coupling to G proteins. Muscarinic receptors in many tissues inhibit adenylyl cyclase activity through interaction with the pertussis-sensitive G protein, G$_i$. Studies in tracheal smooth muscle indicated that muscarinic agonists can inhibit cAMP production induced by the β-adrenergic agonist isoproterenol, although it was not determined if the decrease in intracellular cAMP was due to an inhibition of adenylyl cyclase or stimulation of a cAMP-dependent
phosphodiesterase (Torry, et al., 1985). The first direct demonstration of muscarinic inhibition of adenyl cyclase in smooth muscle was provided by the studies of Jones et al. (1987) in which muscarinic agonists were shown to decrease cAMP levels in the presence of added phosphodiesterase inhibitors. Again the involvement of G proteins in mediating the cholinergic effects on cAMP levels has not yet been demonstrated in smooth muscle.

Given these diverse mechanisms of signal transduction mediated by muscarinic receptors in smooth muscle, a major question in the study of cholinergic control of smooth muscle contractility relates to the molecular mechanisms that underlie the biological diversity of muscarinic activity. One proposed explanation for this tremendous diversity is the presence of multiple receptor subtypes, with each receptor subtype mediating a distinct biochemical response. On the other hand this heterogeneity may reflect diversity in G proteins that transduce muscarinic receptor occupancy to the activation of specific biochemical responses (i.e. a single class of receptor may couple to different effector systems).

The existence of different muscarinic receptor subtypes was initially inferred from studies which showed that selective muscarinic antagonists exhibit different affinities for receptors in various tissues. Hammer et al. (1980) were the first to demonstrate that the ligand binding properties of muscarinic receptors could be differentiated by the antagonist pirenzepine. In contrast to the homogeneous binding seen for classical muscarinic antagonists such as atropine, competition curves for pirenzepine indicated the presence of
multiple affinity states. Cardiac and smooth muscle had a single class of low affinity binding sites while glands and neural tissues possessed varying proportions of an additional site with approximately a 50-fold higher affinity. Receptors with high affinity were termed $M_1$ while those with lower affinity were termed $M_2$. In general, functional studies examining the inhibition of agonist-induced responses by pirenzepine supported this distribution of muscarinic receptor subtypes. For example, low doses of pirenzepine could inhibit cholinergic stimulation of gastric secretion from glandular tissue but much higher doses were needed to inhibit contraction of cardiac muscle and of smooth muscle lining the stomach wall (Hammer and Giachetti, 1982). It was later found that pirenzepine inhibited gastric secretion indirectly, by antagonizing the release of excitatory neurotransmitters from enteric nerve within the stomach tissue (Nathanson, 1987).

Further studies indicated that the $M_2$ receptor population consisted of a mixed population of receptors. In functional tests comparing receptors on atrial versus ileal tissue, for example, the compound 4-diphenylacetoxy- N-methylpiperidine (4-DAMP) proved more potent for the muscarinic receptors on ileal smooth muscle than on cardiac tissue (Doods et al., 1987). Moreover, the binding characteristics of 4-DAMP and hexyhydro-siladifenidol differed in cardiac versus glandular or smooth muscle tissue. Thus it was suggested that a third subtype of muscarinic receptors may exist.

The development of the compound AF-DX 116, a muscarinic antagonist structurally related to pirenzepine, provided more conclusive evidence of an additional class of muscarinic receptor. AF-DX 116 bound with
high affinity to $M_2$ receptors in the heart but with an approximately 30-fold lower affinity to $M_2$ receptors in exocrine glands (Giachetti et al., 1986; Micheletti et al., 1986). In pharmacological tests, AF-DX 116 also displayed a 10-fold higher potency for antagonism of muscarinic agonist-induced decrease in heart rate than for antagonism of salivary secretion (Giraldo et al., 1987). Based on both the functional and binding data, Doods et al. (1987) have suggested that cardiac muscarinic receptors with high affinity for AF-DX 116 be classified as $M_2$ receptors and that glandular muscarinic receptors which have low affinity for AF-DX 116 and high affinity for 4-DAMP be classified as $M_3$ receptors.

Evidence for the existence of multiple receptor subtypes in functional and binding studies has been verified in genetic studies by the isolation and cloning of multiple genes encoding for different muscarinic receptor subtypes. Muscarinic receptor genes have been cloned and sequenced from porcine brain and heart cDNA libraries as well as from human genomic libraries (see Kerlavage et al., 1987). The amino acid compositions of the purified porcine brain ($M_1$) and heart ($M_2$) receptors suggested differences in their primary sequences. These differences have been confirmed by the analysis of the primary sequence of each receptor based on their respective cDNA's. Recent studies have identified at least five distinct but highly homologous genes encoding for muscarinic receptors (Bonner et al., 1988; Peralta et al., 1988). The amino acid sequence of each subtype reflects a structure consisting of seven highly conserved transmembrane sequences, a structure common to all $G$ protein-coupled receptors, and a large intracellular region.
between the fifth and sixth transmembrane loops that is unique to each subtype and may constitute the effector-coupling domain (Ashkenazi et al., 1989). Thus, both pharmacological and genetic evidence support the existence of multiple receptor subtypes.

Muscarinic receptor heterogeneity in smooth muscle has been indicated by several studies. In competition experiments against \(^{3}\text{H}\)-N-methylscopolamine, AF-DX 116 recognized a heterogeneous receptor population in membranes prepared from both longitudinal and circular smooth muscle of the guinea pig ileum (Giraldo et al., 1987). The majority of the receptors (~ 80%) were identified as M\(_2\) receptors (\(K_D = 100\) nM, characteristic of "cardiac M\(_2\)" receptors) while the remainder were classified as M\(_3\) (\(K_D = 1-3\) \(\mu\)M, characteristic of "glandular M\(_2\)" receptors). Similar findings have also been reported for membranes from rat urinary bladder smooth muscle (Ladinsky et al., 1988) and bovine tracheal smooth muscle (Roffel et al., 1987, 1988). In addition, Collins and Crankshaw (1986) demonstrated that 4-DAMP recognized a heterogeneous population of muscarinic receptors on smooth muscle cells isolated from canine stomach. Further evidence for muscarinic receptor heterogeneity in smooth muscle has been gained from the determination of the tissue distribution of the mRNA's encoding for each receptor subtype by blot hybridization analysis with subtype-specific mRNA probes (Maeda et al., 1988). This study indicated that smooth muscle from the trachea, intestine and urinary bladder contained both M\(_2\) and M\(_3\) mRNA's.

The data summarized above support the possibility that different receptor subtypes may mediate each of the biochemical pathways involved
in muscarinic actions on smooth muscle contractility. Further support for this notion has been provided by studies correlating the occupancy of a particular subtype by agonist with the preferential activation of a specific cellular effector. Initial studies with pirenzepine suggested the preferential coupling of rat brain \( M_1 \) receptors to the activation of phosphoinositide hydrolysis and rat cardiac \( M_2 \) receptors to the inhibition of adenylyl cyclase (Peralta et al., 1988). More direct information concerning the selective coupling of receptor subtypes to cellular responses has been revealed from experiments in which the DNA for each individual subtype was transfected and expressed in different cell lines. The results indicate that the transfected \( M_1 \) and \( M_3 \) receptor are strongly coupled to phosphoinositide hydrolysis in CHO cells, *Xenopus* oocytes and NG108-15 neuroblastoma x glioma cells, and \( M_2 \) receptors are preferentially coupled to adenylyl cyclase (Peralta et al., 1988; Ashkenazi et al., 1987). Other cDNA expression studies using *Xenopus* oocytes indicated that \( M_1 \) receptor occupancy induced an activation of a \( \text{Ca}^{2+} \)-dependent \( \text{Cl}^- \) current, whereas \( M_2 \) receptor occupancy evoked an activation of \( \text{Na}^+ \) and \( \text{K}^+ \) currents in a \( \text{Ca}^{2+} \)-independent manner (Fukuda et al., 1988). Finally, \( M_1 \) and \( M_3 \) receptors expressed in NG108-15 cells efficiently stimulated phosphoinositide metabolism, eliciting an activation of a \( \text{Ca}^{2+} \)-dependent \( \text{K}^+ \) current and an inhibition of the \( M \)-current (Fukuda et al., 1988).

These observations indicate that different muscarinic receptor subtypes have the capability of preferentially coupling to different biochemical effector systems. However, this coupling may not be
exclusive. For example, M$_2$ receptors expressed in CHO cells were not only coupled to the inhibition of adenylyl cyclase through a pertussis sensitive G protein, but were also coupled to phosphoinositide hydrolysis though a pertussis insensitive G protein if the receptor number was increased to $\sim 1 \times 10^6$ sites/cell (Ashkenazi et al., 1987). Therefore, a single muscarinic receptor subtype may be coupled to multiple biochemical effector systems by interacting with different G proteins. This contention has been confirmed in reconstitution experiments using purified G proteins and purified brain muscarinic receptors. Studies by Florio and Sternweiss (1985) indicated that a single receptor subtype was capable of coupling to both G$_{i}$ and G$_{o}$, as measured by agonist stimulated GTP-binding to reconstituted vesicles and by GTP-induced shifts in agonist binding. These results raise the possibility that the complex actions of cholinergic agonists in smooth muscle could be mediated by a single muscarinic receptor subtype interacting with several G proteins, each coupling to distinct cellular responses.

The major goal for this portion of my thesis research was to determine whether the diverse biochemical responses observed upon cholinergic activation of smooth muscle were due to a heterogeneity in muscarinic receptor subtypes and/or different stimulus-effector coupling mechanisms. The specific aims were to: 1). define the muscarinic receptor subtype(s) in smooth muscle and 2). determine whether or not these receptors were coupled to G proteins. These studies were undertaken in both toad gastric smooth muscle and bovine tracheal smooth muscle since both preparations exhibit multiple cellular responses to
cholinergic agents. In toad gastric smooth muscle, cholinergic stimuli evoke changes in phospholipid metabolism (Salmon and Honeyman, 1979), activate a voltage-sensitive Ca\(^{2+}\) channel (Vivaudou et al., 1988) and suppress a time- and voltage-dependent K\(^+\) current termed the M-current (Sims et al., 1985). In tracheal smooth muscle, cholinergic activation leads to changes in adenylyl cyclase activity and phospholipid metabolism (Jones et al., 1987; Takuwa et al., 1986). The parallel studies on both smooth muscle types enabled us to determine if each smooth muscle used the same muscarinic receptor subtypes and G proteins to regulate the biochemical effector systems that ultimately lead to the same biological endpoint, contraction.
Chapter VI

Interaction of Agonists and Selective Antagonists with Gastric Smooth Muscle Muscarinic Receptors

SUMMARY

The interaction of cholinergic agonists and antagonists with smooth muscle muscarinic receptors has been investigated by measurement of displacement of the muscarinic antagonist $[^{3}H]QNB$ (quinuclidinyl benzilate) in membranes prepared from toad stomach. The binding of $[^{3}H]QNB$ was saturable, reversible and of high affinity ($K_D = 423$ pM). The muscarinic receptor subtypes present in gastric smooth muscle were classified by determining the relative affinities for the selective antagonists pirenzepine ($M_1$), AF-DX 116 ($M_2$) and 4-DAMP ($M_3$). The results from these studies indicate the presence of a heterogeneous population of muscarinic receptor subtypes, with a majority (87%) exhibiting characteristics of $M_3$ receptors and a much smaller population (13%) exhibiting characteristics of $M_2$ receptors. The binding curve for the displacement of $[^{3}H]QNB$ binding by the agonist oxotremorine was complex and was consistent with presence of two affinity states: 24% of the receptors had a high affinity ($K_D = 4.7$ nM) for oxotremorine and 76% displayed nearly a 1000-fold lower affinity ($K_D = 4.4$ µM). When oxotremorine displacement of $[^{3}H]QNB$ binding was determined in the presence GTPγS, high affinity binding was abolished, indicating that high affinity agonist binding may represent receptors coupled to G proteins. Moreover, pertussis toxin pretreatment of membranes also abolished high affinity agonist binding, indicating
that the muscarinic receptors are coupled to pertussis toxin-sensitive G proteins. Reaction of smooth muscle membranes with pertussis toxin in the presence of $^{32}$P-NAD caused the $^{32}$P-labelling of a 40 kD protein that may represent the $\alpha$ subunit(s) of G proteins that are known to be NAD-ribosylated by the toxin. We conclude that both $M_3$ and $M_2$ receptors may be coupled to G proteins in a pertussis-sensitive manner.

This work has been accepted for publication.

INTRODUCTION

There is now considerable experimental evidence suggesting that there are multiple types of muscarinic cholinergic receptors. These subtypes have been distinguished pharmacologically based on differences in the ability of various muscarinic cholinergic antagonists to bind to muscarinic receptors and/or on differences in their ability to block muscarinic actions (see Mitchelson, 1988 and Nathanson, 1987 for reviews). In addition, receptor subtypes have been defined genetically from the identification of discrete (albeit closely related) cDNA and mRNA species (see Kerlavage et al., 1987 for a recent review).

Initially, muscarinic receptors were divided into two classes based on the relative affinities of the receptors for the antagonist pirenzepine. Receptors which exhibited high affinity for pirenzepine were localized predominantly in the brain and termed M₁. Receptors which exhibited low affinity for pirenzepine were localized in heart, smooth muscle and glandular tissue and termed M₂ (Hammer et al., 1980; Hammer and Giachetti, 1982). Subsequent studies using the newly developed muscarinic antagonists AF-DX 116 and 4-DAMP have suggested that the receptors initially designated as M₂ also represent a heterogeneous population of receptors. AF-DX 116 showed a higher affinity for receptors in the heart than for those in exocrine glands (Giachetti et al., 1986; Hammer et al., 1986; Micheletti et al., 1986), while 4-DAMP showed a higher affinity for receptors in exocrine glands (de Jonge et al., 1986; Doods et al., 1987). Tissues such as smooth muscle on the other hand appeared to possess a mixed population of
"M₂" receptors (Giraldo et al., 1987; Giraldo et al., 1988; Ladinsky et al., 1988). Because of these recent findings, Doods et al., (1987) proposed a classification scheme in which muscarinic receptors were subdivided into three subtypes, M₁, M₂, and M₃, based on the relative binding affinities of pirenzepine, AF-DX 116 and 4-DAMP. M₁ receptors were defined as those exhibiting high affinity for pirenzepine and 4-DAMP (Kᵰ = 1-20 nM) and intermediate affinity for AF-DX 116 (Kᵰ = 700-800 nM). M₂ receptors were defined as those receptors exhibiting high affinity for AF-DX 116 (Kᵰ = 50-100 nM) and low affinity for pirenzepine and 4-DAMP (Kᵰ = 200-600 nM). M₃ receptors were defined as those exhibiting high affinity for 4-DAMP (Kᵰ = 1-10 nM) and low affinity for pirenzepine and AF-DX 116 (Kᵰ = 3-5 μM). A similar classification scheme has also been proposed by de Jonge et al., (1986) and reviewed by Mitchelson (1988).

In addition to the diversity in receptor subtypes that recognize muscarinic cholinergic agents, there appears to be considerable diversity in the biological responses to these agents. Muscarinic stimuli have been shown to regulate adenylate cyclase, phosphoinositide phospholipase C and membrane ion conductances in various tissues (Ashkenazi et al., 1987; Christie and North 1988; Mitchelson 1988). In addition, these agents appear capable of producing multiple responses within a single tissue type. In smooth muscle for example, in which muscarinic activation elicits contraction, muscarinic agonists produce changes in diacylglycerol, inositol phosphate and cyclic AMP metabolism as well as changes in specific ion conductances in the membrane (Nathanson, 1987; Takuwa et al., 1986; Akhtar et al., 1987; Benham, et
The manner by which muscarinic agents elicit such diverse responses in smooth muscle is as yet unknown. This diversity could reflect a heterogeneity in muscarinic receptor subtypes since recent reports (Giraldo et al., 1987; Giraldo et al., 1988; Ladinsky et al., 1988) indicate that smooth muscle from the gastrointestinal tract possesses both $M_2$ and $M_3$ receptors (using the classification scheme for receptor subtypes described above). It is also possible that the diversity in biological responses of smooth muscle to muscarinic agonists reflects diversity in stimulus-effector coupling mechanisms. In other tissues, the coupling of muscarinic receptors to biological responses seems to involve one or more of the family of guanine nucleotide binding proteins termed G proteins (Merritt et al., 1986; Nathanson, 1987), and indirect evidence suggests that different subtypes of muscarinic receptors may preferentially interact with different G proteins and regulate different biochemical processes.

The present studies were designed to examine the biological basis for the heterogeneity of muscarinic responses in smooth muscle. These studies employed toad gastric smooth muscle, a preparation in which muscarinic agonists have been shown to elicit changes in phospholipid metabolism (Salmon and Honeyman, 1980), in the ion conductance of the plasma membrane (Sims et al., 1986; Vivaudou et al., 1988) and in intracellular $Ca^{2+}$ levels (Williams et al., 1987). Using this preparation we looked for possible heterogeneity in muscarinic receptor subtype(s) on the smooth muscle membranes by examining the relative affinities of the receptors for the muscarinic antagonists pirenzepine, AF-DX 116 and 4-DAMP. We examined the involvement of GTP binding
proteins in muscarinic actions by assessing the effects of guanine nucleotides and pertussis toxin on agonist binding. These studies indicate that at least two types of muscarinic receptors are present in toad gastric smooth muscle and that both subtypes may be coupled to G proteins in a manner sensitive to pertussis toxin.

METHODS

Membrane Preparation.

Smooth muscle plasma membranes were prepared from the stomach of the toad (Bufo marinus) as previously described (Lucchesi et al., 1988). Briefly, the muscle layer was dissected free of mucosa and homogenized with an Ultraturrax Tissuemizer. The tissue homogenate was further disrupted by nitrogen cavitation and membrane fractions were collected by centrifugation. Crude membrane fractions were then extracted with 0.6 M NaCl to remove contractile proteins and further purified by flotation on discontinuous sucrose gradients. The plasmalemmal vesicles were then resuspended by sonication in storage buffer consisting of 250 mM sucrose, 40 mM MOPS ([N-morpholino]- propane sulfonic acid, pH 7.1), 1 mM DTT (dithiothreitol) and 10 μg/ml of leupeptin), rapidly frozen in liquid nitrogen, and stored at -80° C. Protein content was measured with the bicinchoninic acid (BCA) protein assay reagent described by Redinbaugh and Turley (1986). The purified membranes were enriched 15-20 fold for the membrane markers 5' nucleotidase and K⁺-stimulated ω-nitrophenyl-phosphatase and contained minimal contamination by mitochondria or sarcoplasmic reticulum (Lucchesi et al., 1988).
Muscarinic cholinergic receptor assays.

All binding assays were carried out in a final volume of 2 ml in a buffer containing 50 mM MOPS, pH 7.1, 10 mM MgCl₂, 50 mM NaCl and 2 mM EDTA. For saturation binding experiments, 0.01 to 30 nM [³H]QNB (quinuclidinyl benzilate) was added. Nonspecific binding was defined as the amount of [³H]QNB bound in the presence of 100 µM atropine. For displacement experiments, membranes were incubated in the presence of 1 nM [³H]QNB and varying concentrations of unlabelled ligands. All binding reactions were initiated by the addition of 50 µg of membranes and were allowed to incubate for 60 min at 31°C. Bound [³H]QNB was separated from free by filtration through Whatman GF/F filters that were presoaked in 0.1% BSA. The filters were washed 5 times with 3 ml aliquots of ice-cold buffer containing 50 mM MOPS, pH 7.1, 25 mM NaCl, and 5 mM MgCl₂. The filters were then placed in vials containing scintillation fluid (Optiflour, Hewlett-Packard) and ³H was determined by liquid scintillation counting at 40-47% efficiency.

ADP-Ribosylation of Membranes by Pertussis Toxin.

[³²P]ADP ribosylation reactions were performed using a modification of the method described by Ribiero-Neto et al., (1987). Incubations were carried out for 60 min at 30°C in a final volume of 60 µl containing 35 µg of membranes, 1 µg/ml BSA, 10 µM [³²P]NAD (~ 5 x 10⁶ cpm/assay), 10 mM thymidine, 20 mM Tris-HCl, pH 7.5, 1 mM ATP, 1.5 mM DTT, in the presence or absence of 10 µl of 100 µg/ml pertussis toxin. Pertussis toxin was preactivated by incubation with 20 mM DTT for 10 min at 30°C. NADP (0.5 mM) was included in the reaction mixture to decrease the hydrolysis of [³²P]NAD by glycohydrolases.
Reactions were initiated by the addition of \( ^{32}P \)NAD and stopped by the addition of 500 \( \mu l \) of ice-cold 0.1 mM NAD. The samples were then centrifuged 7 min at 14,000 rpm in an Eppendorf microfuge. The pellets were resuspended in 120 \( \mu l \) of Laemmli's sample buffer and the proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% polyacrylamide gel slabs (Laemmli 1980). After electrophoresis, the gel slabs were dried under vacuum and subjected to autoradiography at \(-80^\circ C\) for 24 hrs using Kodak XR-5 film.

For experiments measuring the effects of pertussis toxin on agonist binding, membranes were incubated in a reaction mixture consisting of 20 mM glycine-phosphate, pH 7.5, 1 mM NAD, 1 mM ATP, 2 mg/ml ovalbumin, 2.5 mM DTT, and 1 mM EDTA, in the presence or absence of 3 \( \mu g/ml \) of preactivated pertussis toxin. After incubation for 30 min at 30\(^\circ C\), the reaction was stopped by the addition of 5 volumes of ice-cold glycine-phosphate buffer and the membranes were collected by centrifugation in a Ti50 rotor at 4\(^\circ C\) for 90 min at 40,000 rpm. The membranes were then resuspended in the buffer used for \( ^{3}H \)QNB binding.

Data Analysis

Data from all binding experiments were analyzed using a weighted, nonlinear least squares curve fitting program, LIGAND (Munson and Rodbard, 1980). For analysis of displacement curves, data were also analyzed using the ALLFIT program (De Lean et al., 1978), an analysis using a four-parameter logistics equation, in order to obtain estimates of the IC\(_{50}\) (the concentration of ligand required to inhibit 50% of \( ^{3}H \)QNB binding) and the slope factor (pseudo-Hill coefficient, a
parameter that indicates the complexity of the interaction(s) between ligands and receptors. Kinetic values are presented as Mean ± SE. However, these errors are only approximations and are not useful for traditional statistical analysis. A two-site binding model was chosen over a one-site binding model when a significant reduction in the residual sum of squares was obtained (F-test, P < 0.05 or lower).

MATERIALS

\(^3\)H]QNB (35-45 Ci/mmol) and \(^{32}\)P]NAD (800 Ci/mmol) were purchased from New England Nuclear (Boston, MA). AF-DX 116 (11-((diethylamino)-methyl)-1-piperidiny1) acetyl)-5,11-dihydro-6H-pyrido(2,3-b) (1,4) benzodiazepine-6-one) and pirenzepine were generous gifts from Dr. Karl Thomae, supplied through Boehringer Ingelheim (Ridgefield, CT). 4-DAMP (4-diphenylacetoxy-N-methyl-piperidine methobromide) was purchased from Research Biochemicals (Natick, MA). Pertussis toxin was purchased from List Biologicals (Campbell, CA). GTP\(\gamma\)S (guanosine 5'-o-(3-thiotriphosphate) was purchased from either Boehringer-Mannheim (Indianapolis, IN) or Sigma Chemicals (St. Louis, MO). Oxotremorine, leupeptin and DTT were from Sigma. Atropine was purchased from Mallinckrodt (Swedesboro NJ). All other chemicals were of reagent grade. The LIGAND program was obtained from the National Institutes of Health (Bethesda, MD) and was modified for use with an IBM PC by P.J. Munson and M. Beveridge.
RESULTS.

Studies of the binding of $[^3\text{H}]$QNB to toad gastric smooth muscle membranes indicated that the binding of this nonselective antagonist was specific and saturable (panel A of Figure 14). Nonspecific binding, which constituted only 2-3% of total binding at the equilibrium dissociation constant ($K_D$) for $[^3\text{H}]$QNB, was nonsaturable and increased linearly with increasing concentrations of $[^3\text{H}]$QNB. Simultaneous analysis of the data obtained from five separate experiments with the LIGAND program indicated that the binding of QNB could best be described by its interaction with a single population of receptors with a $K_D$ of 423 ± 63 pM and a $B_{\text{max}}$ of 941 ± 25 fmol/mg protein. A Scatchard representation of the data is shown in panel B of Figure 14. There was no evidence for a second class of saturable sites with lower affinity for QNB.

Muscarinic receptor density in these membrane fractions was estimated to be $1.10 \times 10^9$ sites/cm$^2$ based on the observed $B_{\text{max}}$ and our previous estimates of membrane surface area/vesicle (see Lucchesi et al., 1988 for details of the estimation of surface area). For comparison, we also determined the density of muscarinic receptors on isolated smooth muscle cells prepared by enzymatic dispersion of toad stomach muscle. Saturation binding studies of $[^3\text{H}]$QNB to suspensions of smooth muscle cells revealed that the density of muscarinic receptors on the isolated cells averaged $1.18 \times 10^5$ sites/cell (mean of 5 experiments) or $3.77 \times 10^9$ sites/cm$^2$ (assuming a cell surface area of $3.13 \times 10^{-5}$ cm$^2$/cell, see Lucchesi et al., 1988). The close
Figure 14. Concentration dependence of $[^3H]$QNB binding to Toad Gastric Smooth Muscle Membranes.

Panel A: representative experiment in which varying amounts of $[^3H]$QNB were incubated with 45 μg of toad membranes for 60 min at 31°C. Specific binding of $[^3H]$QNB (squares) was defined as the difference between total binding (circles) and binding observed in the presence of 100 μM atropine (X's). Nonspecific binding (X's) was linear and represented less than 2-3% of total binding at concentrations equal to the dissociation constant ($K_D$). Panel B: Scatchard transformation of the data in panel A. Analysis of the data with the LIGAND program indicated a $K_D$ of 557 pM and a $B_{max}$ of 1.08 pmol/mg protein for this experiment. Similar data were obtained in 4 additional experiments.

agreement between the receptor density calculated for membrane vesicles and for isolated cells suggests that the binding characteristics observed in membranes derived from intact tissue may be largely attributable to the smooth muscle cells within the tissue.

To characterize muscarinic receptor subtypes in these membranes, we examined $[^3H]$QNB binding in the presence of several muscarinic antagonists including the non-selective antagonist atropine, the
M₁-selective antagonist pirenzepine, the M₂-selective antagonist AF-DX 116 and the M₃-selective antagonist 4-DAMP. Specific QNB binding was inhibited in a concentration-dependent manner by all antagonists tested (Fig. 15). Analysis of the atropine displacement curve indicated that this antagonist interacted with a single class of binding sites (pseudo-Hill coefficient of 1.04 ± 0.13) with a Kₐ of 4.31 ± 0.43 nM. Analysis of the displacement curve for the M₁-selective antagonist pirenzepine indicated interaction with a single class of sites (pseudo-Hill coefficient of -0.93) with a Kₐ of 267 ± 32 nM. The displacement curves for AF-DX 116 and 4-DAMP, however, indicated that these antagonists exhibited a complex interaction with the muscarinic receptors. The binding isotherms for both AF-DX 116 and 4-DAMP both had pseudo-Hill coefficients less than 1 suggesting that these agents may interact with a heterogeneous population of receptors (coefficients were -0.46 and -0.73, respectively). Analysis of these data with the LIGAND program indicated that the binding of AF-DX 116 could best be fitted by assuming two populations of receptors; 13% having a high affinity, Kₐ = 7.37 ± 9.21 nM and 87% having a low affinity Kₐ = 3.50 ± 0.35 μM. Analysis of the data obtained with 4-DAMP indicated that the curve was best fit to a two-site model with 88% of the receptors having a high affinity (Kₐ = 7.00 ± 1.4 nM) and 12% of the receptors displaying a low affinity (Kₐ = 600 ± 332 nM) (see Table 5). Since all of the antagonists that we employed displaced QNB to the same extent, it appeared that all of the ligands were interacting with the same total population of receptors, but that at least two subtypes of receptors
Displacement of [³H]QNB binding by selective muscarinic antagonists in toad gastric smooth muscle.

Representative binding curves for the displacement of 1 nM [³H]QNB by selective antagonists. Panel A. Squares: Displacement curve for the nonselective antagonist atropine. Atropine interacted with a single class of high affinity receptors (Kᵦ = 4.48 nM, pseudo-Hill slope = -1.04; n=2). Circles: Displacement curve for the M₁-selective antagonist pirenzepine. Pirenzepine interacted with a single class of low affinity receptors (Kᵦ = 330 nM, pseudo-Hill slope = -0.95; n=3). Panel B. Squares: Displacement curve for the M₃-selective antagonist 4-DAMP. 4-DAMP binding was best described by an interaction with a mixed population of receptors: 83% of the receptors displayed a high affinity (Kᵦ = 4.92 nM) and 17% of the receptors had a low affinity (Kᵦ = 204 nM) for the antagonist (pseudo-Hill slope = -0.58; n=3). Circles: Displacement curve for the M₂-selective antagonist, AF-DX 116. AF-DX 116 interacted with two classes of receptors, a small population (20%) that bound with a high affinity (Kᵦ = 3.88 nM) and a larger population (80%) that bound with low affinity (4.41 μM, pseudo-Hill slope = -0.45; n=3).
Table 5. Binding Characteristics of Selective Muscarinic Receptor Antagonists.

<table>
<thead>
<tr>
<th></th>
<th>Pirenzepine (n = 3)</th>
<th>AF-DX 116 (n = 3)</th>
<th>4-DAMP (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50 (nM)</td>
<td>769 ± 327</td>
<td>4250 ± 2340</td>
<td>27.9 ± 4.6 nM</td>
</tr>
<tr>
<td>Slope</td>
<td>-0.93 ± 0.08</td>
<td>-0.46 ± 0.08*</td>
<td>-0.73 ± 0.06*</td>
</tr>
<tr>
<td>KH (nM)</td>
<td>-</td>
<td>7.37 ± 9.21**</td>
<td>7.00 ± 1.4**</td>
</tr>
<tr>
<td>KL (nM)</td>
<td>267 ± 32</td>
<td>3500 ± 350</td>
<td>600 ± 332</td>
</tr>
<tr>
<td>RH</td>
<td>-</td>
<td>13%</td>
<td>88%</td>
</tr>
<tr>
<td>RL</td>
<td>100%</td>
<td>87%</td>
<td>12%</td>
</tr>
</tbody>
</table>

Values are the Mean ± SE for the number of experiments indicated in parentheses. IC50 and slope values were obtained by individual analysis of each curve using the ALLFIT curve fitting program. KH, RH; KL, RL: dissociation constants and percentage of receptors in the high and low affinity states, respectively, determined by a non-linear least squares curve fitting program, LIGAND.

* Statistically significant difference from unity (P < 0.05).
** A two-state model was accepted only if it significantly improved the fit over a one-state model using the F-test (P < 0.001).

were present. The majority of receptors exhibited high affinity for 4-DAMP and low affinity for both AF-DX 116 and pirenzepine and thus were classified as M3. The remaining small population of receptors exhibited high affinity for AF-DX 116 and low affinity for both 4-DAMP and pirenzepine and thus were classified as M2.

To examine the coupling of muscarinic receptors to G proteins, the binding of the muscarinic agonist oxotremorine was determined in the absence and presence of the non-hydrolyzable GTP analog, GTPγS (Figure 16). In the absence of guanine nucleotide, the oxotremorine displacement
**Figure 16.** Oxotremorine Displacement of $[^3\text{H}]$QNB in the Presence or Absence of GTPyS.

Plasma membranes from toad stomach muscle were incubated for 60 min at 31°C in the presence of 1 nM $[^3\text{H}]$QNB and the indicated concentrations of oxotremorine. Representative experiment illustrating oxotremorine displacement curves in the absence (squares) and presence (X's) of 100 µM GTPyS. In the absence of GTPyS, the oxotremorine displacement curve could best be described by assuming the agonist interacted with two species of muscarinic receptors, a small population (29%) of high affinity receptors ($K_D = 11.7$ pM), and a larger population (71%) of low affinity receptors ($K_D = 3.42$ µM, pseudo-Hill slope = -0.44). In the presence of GTPyS, essentially all of the receptors were converted to the low affinity state ($K_D = 3.67$ µM, pseudo-Hill slope = -0.86). Similar results were obtained in 5 additional experiments.

curves exhibited a pseudo-Hill coefficient of -0.49 while in the presence of GTPyS the coefficient was not different from unity (Table 6). Analysis of the binding isotherms with the LIGAND program indicated
Table 6. Effects of Guanine Nucleotides and Pertussis Toxin on Oxotremorine Binding.

<table>
<thead>
<tr>
<th></th>
<th>Oxotremorine (n = 6)</th>
<th>+ GTPγS (n = 5)</th>
<th>+ Pertussis Toxin (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50 (μM)</td>
<td>6.47 ± 2.45</td>
<td>3.32 ± 1.39</td>
<td>12.25 ± 0.6</td>
</tr>
<tr>
<td>Slope</td>
<td>-0.49 ± 0.03*</td>
<td>-0.86 ± 0.13</td>
<td>-0.93 ± 0.08</td>
</tr>
<tr>
<td>KH (μM)</td>
<td>4.7 ± 4.11 nM**</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>KL (μM)</td>
<td>4.4 ± 0.66</td>
<td>3.01 ± 0.27</td>
<td>2.93 ± 0.2</td>
</tr>
<tr>
<td>RH</td>
<td>24%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>RL</td>
<td>76%</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>

Values represent Mean ± SE for the number of experiments indicated in parentheses. See the legend to Table 1 for explanation of the terms and details of the analysis.

* Statistically significant difference from unity (P < 0.05).
** A two-site model was accepted only if it significantly improved the fit over a one-site model, using the F-test (P < 0.001).

that the binding of oxotremorine in the absence of GTPγS could best be described by the interaction with two populations of receptors: 24% exhibiting a high affinity, $K_D = 4.7 ± 4.11$ nM and 76% exhibiting a low affinity, $K_D = 4.4 ± 0.66$ μM. In the presence of GTPγS, oxotremorine binding could be best described by interaction with a single class of receptors with low affinity ($K_D = 3.01 ± 0.27$μM). Since the nucleotide had no effect on the affinity of QNB or on the total number of binding sites (data not shown), the GTP-dependent loss of high affinity agonist binding may reflect the conversion of a high affinity receptor state to a low affinity state. This sort of shift in receptor affinity is a hallmark for receptors coupled to G proteins.
The coupling of gastric smooth muscle muscarinic receptors to G proteins was also investigated by examination of the effects of pertussis toxin on agonist binding. As shown in Figure 17A, pertussis toxin pretreatment of the membranes caused the \([^{32}\text{P}]\text{NAD}\) ribosylation of a single, 37-41 kD protein band detected by one-dimensional SDS-gel electrophoresis of membrane proteins. To examine the effects of pertussis toxin on agonist binding, membranes were incubated with NAD in the absence and presence of the toxin. In control membranes, the oxotremorine displacement curve had a pseudo-Hill coefficient of less than 1 and could best be described by interaction with high and low affinity receptors (Figure 17 and Table 6). Conversely, the binding of oxotemorine to membranes reacted with pertussis toxin was best described as an interaction with one, low affinity site (Figure 17 and Table 6). Since the toxin did not alter the total number of binding sites, these results indicate that the toxin, like GTP, may shift the state of receptors from high to low affinity.

**DISCUSSION**

The present studies describe the characteristics of muscarinic receptors and their coupling to G proteins in toad gastric smooth muscle. Nearly all of the binding of the nonselective muscarinic antagonist \([^3\text{H}]\text{QNB}\) could be displaced by atropine and was therefore termed specific. The binding of \([^3\text{H}]\text{QNB}\) was best described by an interaction with a homogeneous population of receptors with a dissociation constant of 423 pM. This estimate for the \(K_D\) value
Figure 17. Effects of Pertussis Toxin on Gastric Smooth Muscle
Muscarinic Receptors.
Membranes were pretreated with pertussis as described in Methods. Panel
A. Autoradiograph of smooth muscle membranes reacted with [32P]NAD in
the absence (Lane 1) and presence (Lane 2) of pertussis toxin for 60 min
at 30°C. Labelled proteins were separated by SDS-PAGE on a 10% gel.
Molecular weight markers: 35 kD – porcine stomach pepsin; 45 kD – egg
albumin. Pertussis toxin catalyzed the ribosylation of a single
distinct band with a molecular weight of 37-41 kD. Panel B.
Oxotremorine displacement of [3H]QNB binding in control (squares) and
pertussis-treated (circles) membranes. In untreated membranes,
oxotremorine recognized two classes of receptors: a small population
(19%) with a high affinity (3.22 nM) and a larger population (81%) with
a low affinity (3.62 μM) for the agonist (pseudo-Hill slope = -0.54).
Pertussis toxin pretreatment converted all of the receptors to the low
affinity state (Kd = 3.66 μM, pseudo-Hill slope = -1.08). Similar
results were obtained in four additional experiments.
agreed well with those reported previously for smooth muscle and other tissues, as did our estimate for receptor density (Akhtar et al., 1987; Rinner et al., 1988). The displacement of specific $[^3H]$QNB binding by the selective muscarinic receptor antagonists pirenzepine, AF-DX 116 and 4-DAMP was used to characterize further the binding properties of muscarinic receptors in gastric smooth muscle. Pirenzepine, which binds with high affinity to $M_1$ receptors but with low affinity to $M_2$ and $M_3$ receptors, interacted with a single population of receptors with low affinity ($K_D = 267$ nM, Figure 15 and Table 5). The lack of high affinity pirenzepine binding indicates that the receptors are either $M_2$ or $M_3$. The conclusion that smooth muscle contains few $M_1$ receptors is in agreement with the results obtained with porcine gastric smooth muscle (Herawi et al., 1988), rabbit iris smooth muscle (Akhtar et al., 1987), and with the IC$_{50}$ values reported for pirenzepine's inhibition of acetylcholine-induced contraction of guinea pig longitudinal smooth muscle (Grider et al., 1987).

Displacement of $[^3H]$QNB binding with the $M_2$-selective antagonist AF-DX 116 and the $M_3$-selective antagonist 4-DAMP demonstrated the presence of a heterogeneous population of receptors. The majority of receptors displayed a low affinity for AF-DX 116 and a high affinity for 4-DAMP, and on this basis could be classified as $M_3$ receptors (see Doods et al., 1987). The remaining receptors (11-12 %) displayed high affinity for AF-DX 116 and low affinity for 4-DAMP and could therefore be classified as $M_2$ receptors (Figure 15 and Table 5). It is noteworthy that both 4-DAMP and AF-DX 116 detected a nearly identical proportion of $M_3$ and $M_2$ receptors.
While a heterogeneous population of muscarinic receptors has been reported for guinea pig ileum (Giraldo et al., 1987; Giraldo et al., 1988; Ladinsky et al., 1988;), rat urinary bladder (Ladinsky et al., 1988) and pig coronary smooth muscle (Rinner et al., 1988), these tissues exhibit a much higher proportion of \( M_2 \) receptors than \( M_3 \) receptors. The explanation for this difference in receptor distribution is unknown but may reflect differences amongst species or tissue types. It would be of interest to establish whether significant differences in physiological responses are manifest in cell types with different subtypes of receptors.

Clearly, most of the muscarinic receptors in toad stomach muscle are of the \( M_3 \) subtype. The location of the small population of \( M_2 \) receptors is not well defined. These receptors may exist in low numbers on all smooth muscle cells; they may occur in relatively high numbers on a small subpopulation of smooth muscle cells (eg. pacemaker cells); or they may be localized on nonmuscle cellular elements within the tissue (eg. presynaptic nerve endings, Kilbinger and Nafziger, 1985). The latter possibility seems unlikely for several reasons. For one, a heterogeneous population (70% \( M_3 \) and 30% \( M_2 \)) of muscarinic receptors was also observed in binding studies using homogeneous suspensions of dispersed canine gastric smooth muscle cells (Collins and Cranshaw, 1986). Secondly, \( M_2 \) receptors constitute the majority of receptors present in a number of other smooth muscle types.

A heterogeneity in agonist as well as antagonist binding was also observed in toad stomach muscle membranes, a finding that likely reflects the state of interaction of receptors with endogenous G
proteins. In other tissues, receptors coupled to G proteins exhibit high affinity for agonist while uncoupled receptors exhibit low affinity. The addition of GTP or its analogs leads to the dissociation of receptors from G proteins, thereby shifting those receptors to the low affinity state. This GTP-dependent shift in agonist affinity has been demonstrated for virtually all types of receptors coupled to G proteins, including muscarinic receptors in rat forebrain and ileum (Birdsall et al., 1978), and rat heart (McMahon and Hosey, 1985). A similar GTP-dependent shift in agonist affinity was observed in the present studies using toad gastric smooth muscle. In the absence of added guanine nucleotide, approximately 25% of the receptors exhibited high affinity for the agonist oxotremorine (Fig. 16, Table 6). The addition of GTPyS abolished all high affinity binding and produced a proportional increase in the number of receptors with low affinity.

Studies using pertussis toxin provided additional evidence for the existence of G proteins coupled to muscarinic receptors in toad gastric smooth muscle. In other cell types, pertussis toxin has been shown to ADP-ribosylate the α subunits of several G proteins, including G₁ and G₀ (Nathanson, 1987). This ribosylation prevents the interaction of receptors with the G proteins and therefore shifts these receptors to the low affinity state for agonist. In toad gastric smooth muscle membranes, incubation of membranes with pertussis toxin and [³²P]NAD led to the ribosylation of a protein band with a molecular weight similar to the α subunits of G₁ and G₀ (see Figure 17). Moreover, this treatment shifted receptors with high affinity to low affinity (Fig. 17). Since pertussis toxin and GTPyS treatment produced similar
effects on the affinity of muscarinic receptors, it appears likely that 
(a) GTP binding protein(s) couple(s) to muscarinic receptors in toad 
gastric smooth muscle.

The exact identity of the G protein(s) coupled to muscarinic 
receptors in these membranes is as yet unknown, but it is interesting to 
note that all of the receptors were shifted to the low affinity state by 
these treatments. This suggests that M₂ and M₃ receptors may both 
be coupled to G protein(s) in a pertussis sensitive manner, although 
some uncertainty remains as to the coupling state of the M₂ receptors, 
which constitute only 12% of the total receptor population. In any 
case, the data provide the most convincing evidence to date for the 
coupling of M₃ receptors to G proteins. (Note, however, that one 
previous report by Merritt et al., 1986, did demonstrate the coupling of 
G proteins to a muscarinic receptor that has been subsequently 
identified as an M₃ receptor).

The conclusion that toad smooth muscle contains two subtypes of 
muscarinic receptors both of which appear to be coupled to G proteins in 
a pertussis-sensitive manner leaves unresolved the biological basis for 
the diversity of responses to muscarinic agonists. As mentioned above, 
muscarinic activation of toad gastric smooth muscle elicits changes in 
both phospholipid metabolism (Salmon and Honeyman, 1980) and in membrane 
permeability to K⁺ and Ca²⁺ (Sims et al., 1986; Vivaudou et al., 
1988); and available data in the literature have not resolved the 
selectivity of M₂ versus M₃ receptors for these physiological 
responses. Changes in phospholipid metabolism were reportedly mediated 
by M₂ receptors in rabbit iris smooth muscle (Akhtar et al., 1987) and
rat parietal cells (Pfeiffer et al., 1988) but appeared to be mediated by $M_3$ receptors in neuroblastoma cells (Fisher and Heacock 1988). There also remains some question as to the involvement of G proteins in mediating all of the observed physiological responses to muscarinic agents. Data obtained in cardiac tissue suggested that free (uncoupled) $M_2$ receptors mediate the observed activation of phospholipase C by muscarinic agents (McMahon and Hosey, 1985). There is also some question as to whether the muscarinic-induced changes in ion permeability are direct or secondary to changes in phospholipid metabolism since diacylglycerol mimicks the effects of acetylcholine on the Ca$^{2+}$ current in toad gastric smooth muscle cells (Vivaudou et al., 1988). While questions such as these remain, the present study provides the information necessary for designing experiments to delineate the receptor subtypes and G proteins that are responsible for each of the known physiological responses to muscarinic stimuli.
Chapter VII
Muscarinic Receptor Subtypes and G Protein Coupling
in Airway Smooth Muscle

INTRODUCTION

It has long been recognized that the activation of muscarinic cholinergic receptors in smooth muscle, including airway smooth muscle (Nadel, 1984), leads to contraction, presumably by increasing Ca\textsuperscript{2+} availability to the contractile machinery. This increase in intracellular Ca\textsuperscript{2+} results from a concerted action of cholinergic agonists on a number of biochemical and physiological processes including inositol phospholipid and cyclic AMP metabolism, ion channel conductance, intracellular Ca\textsuperscript{2+} release and Ca\textsuperscript{2+} transport (see Nathanson, 1987 for a review).

The manner by which cholinergic stimuli produce such diverse physiological and biochemical responses remains unclear. Several possibilities have been suggested. Multiple subtypes of the muscarinic receptor have been described in many tissues including airway and other types of smooth muscle (Mitchelson, 1988). It is possible that these distinct subtypes regulate different biochemical and physiological signalling pathways (Ashkenazi et al., 1987, 1989; Peralta et al., 1987; Fukuda et al., 1988). Thus, the diversity of response in smooth muscle may reflect the presence of multiple receptor subtypes. Alternatively, since muscarinic receptors can exert effects through a number of different GTP binding proteins (G proteins), it is possible that the diversity of response in smooth muscle reflects the interaction of the
muscarinic receptor with more than one type of G protein.

The present studies were initiated to define some of the characteristics of the muscarinic receptors in airway smooth muscle and their coupling to G proteins. Previous studies have suggested that more than one subtype of muscarinic receptor may be present in trachea (Roffel, et al., 1987, 1988), but because the studies utilized membranes prepared from tissues, the exact cellular distribution of these receptors (eg. on smooth muscle cells and/or on other cellular elements within the tissue) was not clear. Furthermore, the extent and nature of the coupling of receptors to G proteins in airway smooth muscle has not been defined. Thus, the present studies examined the subtypes of muscarinic receptors and their coupling to G proteins in membranes and isolated cells prepared from bovine trachea. The results of these studies indicate that at least two subtypes of muscarinic receptor are present and that these receptors may interact with more than one type of G-protein.

Preliminary reports of some of these results have been presented (Lucchesi, et al., 1989). In addition, this work has been submitted for publication to the American Journal of Physiology.
METHODS

Fresh bovine trachea were obtained from a local abattoir and transported to the laboratory in ice cold mammalian Krebs-Ringer (MKR) containing 118 mM NaCl, 4.6 mM KCl, 24 mM NaHCO₃, 1.21 mM KH₂PO₄, 1.16 mM MgSO₄, 2.5 mM CaCl₂ and 11 mM glucose. The smooth muscle layer was dissected from the cartilage, trimmed of mucosa, connective tissue and fat and minced with scissors in ice cold homogenization buffer containing 50 mM 3-(N-morpholino)propane-sulfonic acid (MOPS), pH 7.1, 150 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol (DTT), 17 µg/ml of phenylmethylsulfonyl fluoride and 10 µg/ml soybean trypsin inhibitor. All subsequent procedures were performed at 4°C. The tissue was then homogenized in 20 volumes of buffer in a Waring blender (2 x 30 sec at low speed) and further disrupted with an Ultraturrax tissuemizer (an initial 25 sec burst with a SDT-182EN probe followed by a 25 sec burst with a SDT-100EN probe). The crude homogenate was filtered through gauze and disrupted twice by nitrogen cavitation (20 min at 1400 psi). Cellular debris and mitochondria were removed by two, low speed centrifugation steps: 20 min at 5000 x g followed by 10 min at 10,000 x g. The supernatant from this spin was centrifuged at 30,000 x g for 90 min. The resultant microsomal pellet was resuspended in homogenization buffer containing 0.6 M NaCl (to solubilize and effectively remove contaminating contractile proteins, see Lucchesi et al, 1988) and the membranes again harvested by centrifugation at 30,000 x g for 90 min. The membrane pellet was then resuspended in storage buffer (250 mM sucrose, 40 mM MOPS, pH 7.1, 1 mM DTT and 10 µg/ml leupeptin), rapidly
frozen in liquid nitrogen, and stored at -80°C. Protein content was measured by the bicinchoninic acid (BCA) protein assay reagent described by Redinbaugh and Turley (1986).

Cell Preparation.

Suspensions of isolated tracheal smooth muscle cells were prepared by modification of procedures previously described (Yamaguchi and Grayce, 1989). Briefly, the smooth muscle layer of the trachea was dissected free of cartilage, mucosa, connective tissue and fat, and cut into small segments with scissors. This mince was then incubated at 32°C for 60 min in MKR with Ca^{2+} reduced to 0.625 mM at a ratio of 1 g mince / 5 ml buffer. The buffer contained collagenase (1.6 mg/ml, Boehringer Mannheim, Type D or Sigma Chemical, Type V), elastase (0.67 mg/ml, Sigma Chemical, Type II), and hyaluronidase (1.5 mg/ml, Sigma Chemical). The partial digest was collected by filtration on a 1 mm mesh Teflon filter, rinsed with reduced Ca^{2+}-MKR containing 0.1% albumin and suspended in reduced Ca^{2+}-MKR containing 0.4 mg/ml ATP at a ratio of 5 ml buffer/g of original mince. After incubation at 32°C for 15 min, the isolated smooth muscle cells were harvested by filtering the digest through a 1 mm Teflon filter. This procedure yielded a suspension of approximately 2 X 10^5 cells/ml. About 75% of the cells were long and thin and appeared to be relaxed. Preparations in which less than 50% of the cells were relaxed were discarded.

As described in detail elsewhere (Yamaguchi, 1988), isolated tracheal smooth muscle cells reversibly contracted in response to muscarinic agonists, histamine or elevated extracellular [K^+]}. The cells had resting membrane potential and intracellular [Ca^{2+}] similar
to values determined in intact strips of muscle.

Muscarinic Cholinergic Receptor Assays.

Muscarinic cholinergic binding was determined from the binding of a non-selective antagonist, [3H]quinuclidinyl benzilate (QNB) to cells and membranes as described previously for gastric smooth muscle (Lucchesi et al., 1989). The apparent affinity for QNB was calculated from curves relating the amount of [3H]QNB bound as a function of QNB concentration (over a range from 0.01 to 7.0 nM). Nonspecific binding was defined as the amount of [3H]QNB bound in the presence of 100 µM atropine and generally represented less than 5% of total binding.

The binding constants for unlabelled ligands were estimated from curves relating the displacement of [3H]QNB by increasing concentrations of the ligands. For these studies QNB was added at a concentration near the estimated K_d (0.3 nM). Binding assays in membranes were carried out in a final volume of 3 ml in a buffer consisting of 50 mM MOPS, pH 7.1, 10 mM MgCl_2, 50 mM NaCl and 2 mM EDTA. Binding reactions were initiated by adding 15 µg membrane protein and were terminated after incubating for 60 min at 31°C. For cells, the binding reactions were carried out in a total volume of 3 ml in MKR supplemented with 25 mM HEPES, pH 7.4. These reactions were initiated by the addition of 2-3 x 10^4 cells and were terminated after a 30 min incubation at 20-22°C. These conditions were chosen to minimize the amount of cell-associated [3H]QNB that could come about as a consequence of receptor internalization. In both membrane and cell experiments, bound [3H]QNB was separated from free by filtration through Whatman GF/F filters presoaked in 0.1% BSA. The filters were
then washed four times with 3 ml aliquots of ice-cold HEPES-buffered MKR (cells) or ice-cold buffer containing 50 mM MOPS, pH 7.1, 5 mM MgCl$_2$ or 25 mM NaCl (membranes). The filters were placed in vials containing 5 mls of scintillation fluid (Optifluor, Packard Instrument Co.) and the radioactivity was quantitated by liquid scintillation counting at 45% efficiency.

ADP-Ribosylation by Pertussis Toxin.

$[^{32}P]$NAD ribosylation of membranes catalyzed by pertussis toxin was determined by a modification of the procedure described by Ribiero-Neto et al. (1987). Incubations were carried out for 60 min at 32°C in a final volume of 60 µl containing 35 µg protein, 20 mM Tris, pH 7.5, 10 mM thymidine, 1 mM EDTA, 30 µg/ml BSA, 1.75 mM DTT, 2 mM ATP, and 10 µM $[^{32}P]$NAD (5 x 10$^6$ cpm/assay) in the absence and presence of 1 µg pertussis toxin. NADP (0.5 mM) was included in the reaction to decrease the nonspecific hydrolysis of $[^{32}P]$NAD by glycohydrolases. Pertussis toxin was preactivated by incubation at a concentration of 10 µg/ml for 20 min at 32°C in the presence of 25 mM DTT. Reactions were initiated by the addition of $[^{32}P]$NAD and were stopped by the addition of 500 µl of ice-cold 0.2 mM NAD. Membranes were harvested by centrifugation for 10 min at 14,000 rpm in an Eppendorf microfuge, dissolved in 100 µl of Laemmli's sample buffer and separated by one dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in 10% gel slabs. The gel slabs were stained with 0.2% Coomassie blue, dried under vacuum and the $^{32}$p labelled proteins were detected by autoradiography (24-48 hours using Kodak XR-5 film).
To determine the effects of pertussis toxin pretreatment on agonist binding, membranes were incubated for 2 hr at 32°C in a reaction mixture containing 20 mM Tris, pH 7.5, 1.2 mM thymidine, 1 mM ATP, 16 μg/ml BSA, 2.5 mM DTT, 1 mM EDTA, and 1 mM NAD in the absence or presence of activated pertussis toxin (20 μg/mg protein). The reaction was stopped by addition of 10 volumes of ice-cold buffer (250 mM sucrose, 40 mM MOPS, pH 7.1), and the membranes were collected by centrifugation in a Ti60 rotor at 40,000 rpm for 90 min at 4°C and resuspended in the buffer for binding studies as described above.

Data Analysis.

Data from binding experiments were analyzed using the LIGAND program (Munson and Rodbard, 1980), an analysis that employs an iterative nonlinear least squares approach for curve fitting, and the ALLFIT program (De Lean et al. 1978), an analysis that uses a four-parameter logistics equation to obtain estimates of the IC_{50} and the pseudo-Hill coefficient (n_H). If binding curves had pseudo-Hill coefficients significantly less than unity (p < 0.05 one-tailed Student's t-test), it was assumed to reflect interaction of the ligand with more than one class of binding sites. Evidence for the interaction of the ligand with more than one class of binding sites was also obtained by analyzing the data with the LIGAND program. From this analysis, a two-site binding model was selected over a one-site model when a significant reduction in the residual sum of squares (F test, p < 0.05) was obtained. This analysis also provided estimates for binding constants and proportions of each receptor class which best fit the observed binding curves. Estimates for K_D and proportion of each class of binding site are
presented as means ± SEM but, because both the binding affinity and the relative proportion of receptor classes are highly interdependent and log normally distributed, these calculated errors are only approximations and are not useful for traditional statistical analysis.

Materials

[\(^3\)H]QNB (45 Ci/mmol) and [\(^{32}\)P]-NAD (800 Ci/mmol) were purchased from New England Nuclear. Pirenzepine and AF-DX 116 ([11-[(diethylamino-methyl]-1-piperidinyl) acetyl -5,11-dihydro-6H- pyrido(2,3,6)-(1,4 benzodiazepine-6-one) were generous gifts of Dr. Karl Thomae, GmbH, supplied through Boehringer Ingelheim. 4-DAMP (4-diphenyl-acetoxy-N-methyl-piperidine methobromide) was purchased from Research Biochemicals. Pertussis toxin was obtained from List Biologicals. GTPyS [guanosine-5'-o-(3-thiotriphosphate)] was purchased from either Boehringer-Mannheim or Sigma Chemical. Oxotremorine, leupeptin and DTT were from Sigma. Atropine was purchased from Mallinckrodt. All other chemicals were of reagent grade. We obtained a version of the LIGAND program compatible with an IBM PC from P.J. Munson and M. Beveridge.

RESULTS

The binding of \([\(^3\)H]QNB\) to membranes and isolated cells prepared from tracheal smooth muscle are shown in Fig. 18 and Fig. 19. In both cells and membranes, the saturation isotherms were best described by an interaction of \([\(^3\)H]QNB\) with a single class of binding sites. In
Figure 18. [3H]QNB binding to membranes prepared from tracheal smooth muscle.

Representative experiment in which various concentrations of [3H]QNB were incubated with 15 μg of membrane protein for 60 min at 31°C. Specific binding (squares) was defined as the difference between total binding (circles) and nonspecific binding (X's) observed in the presence of 100 μM atropine. Nonspecific binding varied as a direct function of the concentration of QNB, and represented less than 2-3% of total binding at QNB concentrations equal to the equilibrium dissociation constant (K_D). Inset: Scatchard transformation of the specific binding. Analysis of the data with the LIGAND program indicated that QNB binding could be described by interaction with a single class of binding sites with a K_D of 232 pM and a B_max of 2.86 pmol/mg protein. Similar data were obtained from three additional experiments.

membranes the average equilibrium dissociation binding constant (K_D) was 176 ± 33 pM and the number of binding sites (B_max) was 4.31 ± 0.3 pmol/mg (n=4). In isolated cells, the K_D averaged 234 ± 42 pM and the density of sites was 2.61 ± 0.65 x 10^6 sites/cell (n=4). Nonspecific binding represented 2-3% of the total binding at the K_D in both cells and membranes.
Figure 19. $[^3H]QNB$ binding to isolated smooth muscle cells prepared from bovine trachea.

Representative experiment in which various concentrations of $[^3H]QNB$ were incubated with 26,000 cells for 30 min at room temperature. Specific binding (squares) was defined as the difference between total binding (circles) and nonspecific binding (X's) observed in the presence of 100 pM atropine. Nonspecific binding varied as a direct function of the concentration of QNB and represented ~2% of total binding at QNB concentrations equal to the $K_D$. Inset: Scatchard transformation of the specific binding. Analysis of the data with the LIGAND program indicated that QNB binding could be described by interaction with a single class of binding sites with a $K_D$ of 175 pM and a $B_{max}$ of $1.94 \times 10^6$ sites/cell. Similar data were obtained in three additional experiments.

To characterize muscarinic receptor subtypes in tracheal smooth muscle we examined the displacement of $[^3H]QNB$ binding by the non-selective muscarinic antagonist, atropine and the selective antagonists, pirenzepine, AF-DX 116 and 4-DAMP. These selective antagonists have been used to characterize the subtypes of the muscarinic receptor in previous functional and receptor binding studies.
(Hammer et al., 1980; Hammer and Giachetti, 1982; Giachetti et al., 1986). For example, Doods et al. (1987) and others (1986) have classified muscarinic receptors as $M_1$, $M_2$ and $M_3$ based on studies with the selective antagonists, pirenzepine, AF-DX 116 and 4-DAMP. $M_1$ receptors, characteristic of muscarinic receptors in brain, have a higher affinity for pirenzepine ($K_D$ of 1-20 nMolar) than for AF-DX 116 ($K_D$ of 700-800 nM). $M_2$ receptors, characteristic of muscarinic receptors in cardiac tissue, have a higher affinity for AF-DX 116 ($K_D$ of 50-100 nM) and 4-DAMP ($K_D$ of 20 nM) than for pirenzepine ($K_D$ of 1 µM). $M_3$ receptors, characteristic of muscarinic receptors in glandular tissues, have a higher affinity for 4-DAMP ($K_D$ of 1-2 nM) than for AF-DX 116 ($K_D$ of 2-3 µM) or pirenzepine ($K_D$ of 0.7-1 µM). Thus pirenzepine exhibits a 50 fold selectivity for $M_1$ over $M_2$ and $M_3$ receptors. AF-DX 116 exhibits a 30 selectivity for $M_2$ over $M_1$ and $M_3$ receptors and 4-DAMP exhibits a 10 fold selectivity for $M_3$ over $M_2$ receptors. Representative displacement curves for atropine, pirenzepine, AF-DX 116 and 4-DAMP in membranes are found in Figure 20. The estimates of the binding constants and pseudo-Hill coefficients obtained from composite analysis of 3-6 such experiments are presented in Table 7. Analysis of the composite displacement curve for atropine indicated a pseudo-Hill coefficient not significantly different from 1.0 ($n_H = -0.99$), thus indicating an interaction with a single class of binding sites. When analysed with the LIGAND program, the atropine displacement curve was best described by an interaction with a single class of sites with an apparent $K_D$ of $3.8 \pm 0.3$ nM.
Figure 20. Displacement of \(^{3}H\)QNB binding by muscarinic antagonists in tracheal smooth muscle membranes.

**Top panel.** Circles: Representative displacement curve for the nonselective antagonist atropine. The atropine displacement curve had a pseudo-Hill coefficient near unity (\(n_H = -0.98\)) and was best described by an interaction with a single class of binding sites with a \(K_D\) of 4.36 nM. Squares: Representative displacement curve for the \(M_1\)-selective antagonist, pirenzepine. The displacement curve for pirenzepine had a \(n_H = -0.94\) and was best described by an interaction with a single class of binding sites with a \(K_D\) of 1.17 \(\mu\)M. **Bottom panel.** Circles: Displacement curve for the \(M_3\)-selective antagonist, 4-DAMP. The displacement curve for 4-DAMP had a \(n_H\) of -0.95 and was best described by an interaction with a single class of binding sites with a \(K_D\) of 14.3 nM. Squares: Displacement curve for the \(M_2\)-selective antagonist, AF-DX 116. The displacement curve for AF-DX 116 had a \(n_H\) significantly less than 1 (\(-0.74\)) and could best be described by an interaction with two classes of binding sites, with 76% of the total binding having high affinity (\(K_D = 113\) nM) and 24% having low affinity (\(K_D = 1.02\) \(\mu\)M). The data are representative of 3-6 experiments for each antagonist.
Table 7. Binding characteristics of muscarinic receptor antagonists to tracheal smooth muscle membranes.

<table>
<thead>
<tr>
<th></th>
<th>Atropine (n=3)</th>
<th>Pirenzepine (n=3)</th>
<th>AF-DX 116 (n=6)</th>
<th>4-DAMP (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IC₅₀ (nM)</strong></td>
<td>10.1 ± 0.1</td>
<td>2970 ± 400</td>
<td>563 ± 64</td>
<td>43.4 ± 3</td>
</tr>
<tr>
<td><strong>Slope</strong></td>
<td>-0.99 ± 0.06</td>
<td>-0.88 ± 0.12</td>
<td>-0.77 ± 0.05*</td>
<td>-0.95 ± 0.1</td>
</tr>
<tr>
<td><strong>Kᵥ (nM)</strong></td>
<td>3.8 ± 0.3</td>
<td>1,110 ± 60</td>
<td>129 ± 35**</td>
<td>16.4 ± 3</td>
</tr>
<tr>
<td><strong>Rᵥ</strong></td>
<td>100%</td>
<td>100%</td>
<td>85%</td>
<td>100%</td>
</tr>
<tr>
<td><strong>Kₗ (µM)</strong></td>
<td>-</td>
<td>-</td>
<td>2.7 ± 1.5</td>
<td>-</td>
</tr>
<tr>
<td><strong>Rₗ</strong></td>
<td>-</td>
<td>-</td>
<td>15%</td>
<td>-</td>
</tr>
</tbody>
</table>

Values are the Mean ± SEM for the number of experiments indicated in parentheses. IC₅₀ and slope values were obtained by simultaneous analysis of each curve using the ALLFIT program. KH, RH, KL, RL: dissociation constants and percentage of receptors in the high and low affinity sites, respectively, determined by composite analyses using the nonlinear least square curve fitting program, LIGAND.

* Statistically significant difference from unity (p < .05) using one-tailed Student's t test.
** A two site model was accepted only if it significantly improved the fit over a one site model using the F test (p < 0.001).

The displacement curves for the M₁-selective antagonist pirenzepine were also best described as an interaction with a single class of binding sites (nᵥ = -0.88) and the composite curve could be fit to a single site model with an apparent Kᵥ = 1.11 ± 0.06 µM. Similarly, the displacement curves for the M₃ selective antagonist 4-DAMP were consistent with an interaction with a single class of sites (nᵥ = -0.95) and the composite curve could be fit to a single site model with an apparent Kᵥ of 16.4 ± 3 nM.
The shape of displacement curves obtained with the $M_2$-selective antagonist AF-DX 116 suggested the presence of more than one class of binding sites for this antagonist. A curve representative of 4 of 6 experiments is presented in Figure 20 (lower panel). This curve has a $n_H$ significantly less than 1.0 (-.74) indicating that the ligand may interact with more than a single class of binding sites. The AF-DX 116 displacement curve was best fit by assuming interaction with two sites; 76% of the sites with a $K_D$ of 113 nM and the remainder with a $K_D$ of 1.02 μM. In the remaining two experiments the displacement curves could be fit to a model assuming a single class of binding sites. When the data from all 6 experiments were analysed, the $n_H$ was significantly less than unity (Table 7) and the composite curve could best be described by interaction of the ligand with two classes of binding sites, 84% having high affinity ($K_D = 129 \pm 35$ nM) and the remainder having lower affinity ($K_D = 2.72 \pm 1.5$ μM).

In intact cells, the displacement curves for $[^3H]QNB$ by 4-DAMP and AF-DX 116 were similar in character to those observed in membranes. Representative curves for individual experiments are presented in Figure 21 and estimates of binding parameters calculated from composites of all experiments are presented in Table 8. The binding of 4-DAMP to intact cells could be explained by interaction with a single class of receptors ($n_H = -0.88$) with an apparent $K_D$ similar to that observed for interaction with tracheal membranes (20 nM vs 16 nM). As was the case for membranes, the displacement curves for AF-DX 116 binding to cells could not always be fit by assuming interaction with a single class of binding sites. In three of six experiments conducted with isolated
Figure 21. Displacement of [³H]QNB binding by the muscarinic antagonist, 4-DAMP and AF-DX 116 in isolated tracheal smooth muscle cells.

Circles: Representative displacement curve for the M₃-selective antagonist 4-DAMP. The displacement curve for 4-DAMP had a nH not different from 1 (-0.88) and was best described by an interaction with a single class of receptors with a KD of 21.5 nM.

Squares: Representative displacement curve for the M₂-selective antagonist, AF-DX 116 that was best fit to a one-site model. The displacement curve had a nH not different from 1 (-1.03) and was best described by an interaction with a single class of binding sites with a KD of 89 nM.

Triangles: Representative displacement curve for AF-DX 116 that was best fit to a two-site model. The displacement curve had a nH significantly less than 1 (-0.66) and was best described by an interaction with two classes of binding sites; a large population (80%) having a KD of 54 nM and a small population (20%) having a KD of 1.2 μM.

cells, the AF-DX 116 displacement curves had pseudo-Hill coefficients significantly less than 1.0 and these curves were best fit as the sum of binding to a large number of high affinity sites and a small number of low affinity sites. In the remaining experiments, nH values were less
Table 8. Binding characteristics of selective muscarinic receptor antagonists to tracheal smooth muscle cells.

<table>
<thead>
<tr>
<th></th>
<th>AF-DX 116 (n=6)</th>
<th>4-DAMP (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC₅₀ (nM)</td>
<td>197 ± 20</td>
<td>45.1 ± 6</td>
</tr>
<tr>
<td>Slope</td>
<td>-0.84 ± 0.04*</td>
<td>-0.88 ± 0.1</td>
</tr>
<tr>
<td>Kₑ (nM)</td>
<td>93.6 ± 11 nM**</td>
<td>-</td>
</tr>
<tr>
<td>Rₑ</td>
<td>97%</td>
<td>-</td>
</tr>
<tr>
<td>Kᵣ (nM)</td>
<td>3,290 ± 2,040</td>
<td>19.6 ± 1.4</td>
</tr>
<tr>
<td>Rᵣ</td>
<td>3%</td>
<td>100%</td>
</tr>
</tbody>
</table>

Values represent mean ± SEM for the number of experiments indicated in parentheses. See the legend to Table 1. For explanation of the terms and details of the analyses.

* Statistically significant difference from unity (p < .05) using one-tailed Student's t test.

** The curve obtained from composite analysis is best fit to a 2 site model (p < .001, F test). Curves from individual experiments were best fit to a 1-site model (3 of 6 experiments) or a 2-site model with 10-20% of the sites having low affinity for agonist.

than unity but not significantly so and the displacement curves could be fit to a single site model. Examples of each type of result are presented in Fig. 21. When data from all experiments were analysed, the composite nₑ value was significantly less than 1.0 (-0.84), and the composite curve was best fit as a sum of binding to a large number of sites with a Kᵣ of 94 nM and a small number of sites with a Kᵣ of 3.3 μM. As shown in Figure 21, since the second class of binding sites represents only a small fraction of the total, their contribution to the overall binding curve is corresponding small. Furthermore, the ability to detect such sites will be variable and estimates as to their
number and affinity will be imprecise because of the large signal to noise ratio inherent in measurements of specific $[^3\text{H}]$QNB binding at the $\mu$M concentrations of AF-DX 116 necessary to interact with the low affinity sites.

In order to determine the interaction of receptors to G proteins, we examined the effects of guanine nucleotides on the ability of a muscarinic agonist, oxotremorine, to displace $[^3\text{H}]$QNB. Representative curves obtained from individual experiments are shown as Fig. 22 and analyses of composite data are shown in Table 9. Composite analysis of the data indicates that in the absence of GTP$\gamma$S, the displacement curve had a $n_H$ significantly less than 1.0 and could best be described as a sum of binding to two sites: 34% having high affinity ($K_D = 3.74$ nM ) and the remainder having low affinity ($K_D = 2.21$ $\mu$M). Although the total number of binding sites was not changed by incubation with 100 $\mu$M GTP$\gamma$S, the curve could be described by interaction of oxotremorine with a single class of low affinity binding sites with a $K_D$ of 2.53 $\mu$M. Since the total number of binding sites was not altered by the nucleotide, the GTP-dependent loss of high affinity agonist binding may be explained as a shift of the high affinity agonist binding sites to the low affinity form. This sort of GTP-dependent shift is a hallmark of receptor coupling to G proteins.

In order to determine the type(s) of G protein(s) coupled to the muscarinic receptor, we examined the effects of pertussis toxin on agonist binding affinities. In other cell types, pertussis toxin catalyzes the incorporation of ADP-ribose from NAD into certain G proteins, notably those termed $G_1$ and $G_9$. This covalent
Figure 22. Effects of GTPyS agonist binding to tracheal smooth muscle membranes.
Representative experiment in which membranes were incubated for 60 min at 31°C with 0.2 nM [3H]QNB and the indicated concentrations of the muscarinic agonist oxotremorine in the absence (circles) or presence (squares) of 100 μM GTPyS. In the absence of GTPyS, the oxotremorine displacement curve had a nH significantly less than 1 (-0.31) and was best described by an interaction with two classes of binding sites; a large population (63%) having low affinity (K_D of 1.3 μM) and a smaller population (37%) with high affinity (K_D = 0.55 nM). In the presence of GTPyS, the oxotremorine displacement curve had a nH not different from 1 (-0.93) and was best described by an interaction with a single class of binding sites with low affinity (K_D = 2.2. μM). The effects of the nucleotide can be described as converting high affinity binding sites to low affinity binding sites.

Modification disrupts the interaction of the G proteins with receptors and reduces the number of receptors exhibiting high affinity for agonist. As shown in Fig. 23, reaction of tracheal membranes with pertussis toxin and [32P]NAD generated a [32P]-labelled protein of 40 kD. This is the same molecular weight noted for the α subunits of G_i and/or G_0. To determine when the all available sites were
Table 9. Effects of guanine nucleotides and pertussis toxin on agonist binding to tracheal membranes.

<table>
<thead>
<tr>
<th></th>
<th>-GTPγS (n=6)</th>
<th>+ GTPγS (n=5)</th>
<th>- toxin (n=5)</th>
<th>+ toxin (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50 (nM)</td>
<td>1880 ± 620</td>
<td>5550 ± 500</td>
<td>1170 ± 200</td>
<td>1880 ± 400</td>
</tr>
<tr>
<td>Slope</td>
<td>-0.32 ± 0.04*</td>
<td>-0.89 ± 0.1</td>
<td>-0.43 ± 0.02</td>
<td>-0.47 ± 0.02</td>
</tr>
<tr>
<td>KH (nM)</td>
<td>3.74 ± 2.4**</td>
<td>-</td>
<td>12.1 ± 9.6**</td>
<td>8.04 ± 10*</td>
</tr>
<tr>
<td>RH</td>
<td>34%</td>
<td>-</td>
<td>40%</td>
<td>31%</td>
</tr>
<tr>
<td>KL (nM)</td>
<td>2210 ± 0.22</td>
<td>2530 ± 200</td>
<td>2880 ± 800</td>
<td>2300 ± 600</td>
</tr>
<tr>
<td>RL</td>
<td>66%</td>
<td>100%</td>
<td>60%</td>
<td>69%</td>
</tr>
</tbody>
</table>

Values represent Mean ± SEM for the number of experiments indicated. See the Legend to Table 1 for explanation of terms and details of the analyses. * Statistically significant difference from unity (p < .05) using one-tailed Student's t test. ** A two site model was accepted only if it significantly improved the fit over a one site model using the F test (p < 0.005).

reacted with the toxin and NAD, membranes were incubated with toxin and unlabelled NAD for one or two hours then reacted again with fresh toxin and [32P]NAD. Although reacting the vesicles with toxin in this manner produced almost complete ribosylation (as indicated by the relative lack of incorporation of 32P during the subsequent toxin reaction) the toxin had only a slight effect on high affinity agonist binding. In the experiment presented as Fig. 23, the displacement curves for membranes incubated with NAD in the absence of toxin had a nH of -0.43 and were best fit by assuming interaction with two classes of receptors, with 39% having a high affinity. The displacement curve for membranes treated with NAD and pertussis toxin had a nH of -0.46.
Figure 23. Effects of pertussis toxin treatment on tracheal smooth muscle membranes.

Panel A. Autoradiograph of $^{32}$P-labelled proteins produced by reaction of membranes with $[^{32}\text{P}]\text{NAD}$ and pertussis toxin as indicated below. Equal amounts of protein (35 µg) were applied to all lanes. **Lane 1:** Membranes were incubated for 60 min at 32°C with $[^{32}\text{P}]\text{NAD}$ in the absence of pertussis toxin. **Lane 2:** Membranes were incubated for 60 min at 32°C with $[^{32}\text{P}]\text{NAD}$ in the presence of pertussis toxin (13 µg/ml). **Lane 3:** Membranes reacted for 60 min with pertussis toxin and unlabelled NAD followed by reaction with $[^{32}\text{P}]\text{NAD}$ and toxin as in Lane 2. **Lane 4:** Membranes reacted for 120 min with pertussis toxin and unlabelled NAD followed by reaction with $[^{32}\text{P}]\text{NAD}$ as in Lane 1. The slight degree of labelling may be due to incomplete removal of pertussis toxin. **Lane 5:** Membranes reacted for 120 min with pertussis toxin and unlabelled NAD followed by reaction with $[^{32}\text{P}]\text{NAD}$ and toxin as in Lane 2.

Panel B. Representative experiments for oxotremorine displacement of $[^{3}\text{H}]\text{QNB}$ binding in control (circles) and pertussis-treated (squares) membranes. In untreated membranes, the oxotremorine displacement curve had a $n_H$ significantly less than 1 ($n_H = -0.34$) and was best described by an interaction with two classes of binding sites: a large population (61%) having low affinity ($K_D$ of 1.56 µM) and a smaller population (39%) with high affinity ($K_D = 3.8$ nM). In pertussis-treated membranes, the oxotremorine displacement curve had a $n_H$ significantly less than 1 ($n_H = -0.49$) and was best described by an interaction with two classes of binding sites: a large class (76%) having low affinity ($K_D$ of 2.74 µM) and a smaller class (24%) with high affinity ($K_D = 1.6$ nM).
and only a slightly smaller percentage of receptors with high affinity (24% vs 39%). The \( K_D \) values for control and toxin-treated groups were similar and the addition of guanine nucleotide abolished high affinity binding in both preparations. When data from 5 separate experiments were analyzed, the slight decrease in high affinity agonist binding produced by reaction with pertussis toxin was not statistically significant (Table 9). Thus, although the membranes contain a substrate for pertussis-dependent ADP ribosylation, conditions which produced nearly complete ribosylation of the substrate proteins produced only a minor effect on high affinity agonist binding. These results suggest that the G protein which confers the high affinity state of the receptor(s) for agonist is not affected by pertussis toxin-catalysed ribosylation.

**DISCUSSION**

The present studies have defined a number of characteristics of muscarinic receptors in airway smooth muscle. The binding to the antagonist QNB in both membranes and cells prepared from bovine trachea was highly specific (nonspecific or atropine-insensitive binding constituted less than 5% of total binding) and was described by interaction with a single binding site. The apparent \( K_D \) for QNB binding (~ 200 nM) is similar to values determined for muscarinic receptors in a number of other tissues and cell types. Because QNB binding could for the first time be assessed in isolated smooth muscle cells of airway origin, the receptor density could also be calculated.
The density of muscarinic receptors (1-2 X 10^6 sites/cell) is at least 10 fold higher than the values we observed in gastric smooth muscle cells (see Chapter VI).

Information derived from the displacement of [3H]QNB by the selective antagonists pirenzepine, AF-DX 116 and 4-DAMP indicates that the predominant subtype of muscarinic receptor in tracheal smooth muscle has the pharmacological characteristics of the M_2 subtype. Furthermore, in agreement with previous studies (Roffel et al., 1987, 1988) tracheal smooth muscle may also contain an additional population of M_3 subtype receptors. This conclusion is based on the following observations. Pirenzipine interacted with a single population of receptors and exhibited a K_D very much lower than that reported for interaction with M_1 receptors but near that reported for interaction with M_2 and/or M_3 subtypes. The displacement curves for AF-DX 116 and 4-DAMP indicate that the majority of receptors can be classified as the M_2 subtype since the K_D calculated for 4-DAMP (~ 20 nM) and for the majority of sites interacting with AF-DX 116 (~ 100 nM) are near values determined for the interaction with classical M_2 receptors (e.g. cardiac muscle). The conclusion that tracheal smooth muscle lacks M_1 receptors and contains M_2 receptors is in agreement with previous studies in airway smooth muscle (Roffel et al., 1988).

The composite displacement curves for AF-DX 116 in both membranes and isolated cells had n_H significantly less than 1. The observation that the binding of AF-DX 116 cannot be described by a simple bimolecular interaction is in agreement with previous studies with membranes and extends the observation to isolated cells. The
displacement curves for AF-DX 116 can be explained if the ligand interacted with more than one population of receptors or if the ligand bound allosterically in a negatively cooperative manner. The former explanation seems the more likely since we found no evidence of allosteric interaction in the binding of other antagonists. It follows that the best explanation for the binding curves is that AF-DX 116 interacted with two binding sites of differing affinities. In membranes, the composite AF-DX 116 displacement curve was best fit by the sum of binding to a population of two receptors: 85% having a $K_D$ of 129 nMolar (a value near that demonstrated in cell types containing $M_2$ receptors) and the remainder having a $K_D$ of 2.72 µMolar (a value near that demonstrated in cell types containing $M_3$ receptors). Thus the tracheal membranes contain a high proportion of $M_2$ versus $M_3$ receptors.

The composite AF-DX 116 displacement curve from experiments with isolated cells, like that for studies with membranes, exhibited a $n_H$ significantly less than 1; and the curve was best fit as the sum of binding to a relatively large number of sites with an estimated $K_D$ of 93 nMolar (i.e. $M_2$ subtype) and a small population of sites with an estimated $K_D$ of 3.3 µMolar (i.e. $M_3$ subtype). Since the composite analysis indicates that the binding of AF-DX 116 could not be explained by interaction with a single class of binding sites, the isolated smooth muscle cells also contain a mixed population of muscarinic receptor subtypes.

The conclusion that airway smooth muscle contains both $M_2$ and $M_3$ receptors is in agreement with the studies of Maeda et al. (1988) from
analysis of mRNA species encoding for muscarinic receptor subtypes). The presence of both subtypes in smooth muscle is in agreement with our findings in gastric smooth muscle which, interestingly, contains a predominance of $M_3$ receptors rather than $M_2$ receptors.

The proportion of $M_3$ receptors estimated from studies with isolated cells was less than that estimated from studies with isolated membranes. Although the membranes were prepared from muscle strips that were carefully dissected free of mucosal and epithelial layers (limiting the contamination of these cell types), it is possible that some of the $M_3$ receptors detected in membrane preparations are contributed by nonmuscle cells or elements embedded within the muscle layer. For example, Gabella (1987) observed that there were approximately 20 nerve terminals present for each 100 muscle cells. Receptors present in the nerve terminals could thus contribute to the receptor population in the membrane vesicle preparation. However, since the density of receptors is far greater on the muscle cells than on the mucosal or epithelial cells (50 times fewer in mucosa [Madison et al., 1987]; and 10 times fewer in epithelia [Scott and McMahon, 1988]), the membrane preparation would have to be very heavily contaminated to account for the findings. Furthermore, neural cells have a preponderance of $M_2$ receptors (Kilbinger and Nafziger, 1985) and would not contribute substantially to the observed $M_3$ population. Most likely the apparent difference in the relative numbers of $M_2$ versus $M_3$ receptors calculated for membranes and isolated cells reflects only the imprecision inherent in estimating the relative number of the two classes of binding sites from curve fitting routines.
The binding of 4-DAMP could be explained by interaction with a single site although this antagonist binds to M₃ receptors with higher affinity than to M₂ receptors. The finding that 4-DAMP apparently interacts with a single site while AF-DX 116 interacts with at least two sites agrees with previous studies (Ladinsky et al., 1988; Roffel et al., 1988), but would seem to be inconsistent with the conclusion that two subtypes of muscarinic receptors are present. However, the ability to resolve classes of binding sites with selective ligands is dependent on both the ratio of the Kᵤ values for the different sites (i.e. the selectivity of the ligand) and the relative proportions of the different classes of binding sites (De Lean et al., 1981). The selectivity of 4-DAMP for M₃ and M₂ receptors calculated from binding to other cell types which contain a predominance of each of these subtypes of receptor (Doods et al., 1987) is approximately 5 to 15 fold. Given the low selectivity of this ligand, it would be impossible to resolve binding to M₃ receptors in the presence of a large number of M₂ receptors, and therefore the displacement curve would be readily described by an interaction with a single class of binding sites.

The displacement curves for the agonist oxotremorine were complex and could best be explained by assuming interaction with low and high affinity binding sites. Based on information derived from other systems, it is reasonable to assume that the high affinity state corresponds to receptors coupled to G proteins (~35% of the total receptor pool) and the low affinity state corresponds to uncoupled (free) receptors. Consistent with this interpretation, we found that the addition of the GTP analogue GTPγS had no effect on the the total
number of binding sites but rather abolished the high affinity binding, i.e. the nucleotide shifted high affinity receptors to a low affinity form. This GTP-dependent shift in agonist affinity is characteristic of receptors that interact with G proteins.

As shown in Fig. 23, tracheal membranes contain protein(s) which serve as substrates for ADP ribosylation catalysed by pertussis toxin. The molecular weight of the protein (~ 41 kD) is near that of the \( \alpha \)-subunits of the G proteins, \( G_i \) and \( G_o \), which have been shown to be ribosylated in other cell types. However, reaction of the membranes with the toxin affected high affinity agonist binding only slightly, indicating that the muscarinic receptors in the trachea are not coupled in a pertussis-sensitive manner. While it is possible that some receptors do interact with pertussis-sensitive G proteins, (e.g. it may be that the \( M_3 \) subtype interacts with a toxin-sensitive G protein as was noted in gastric smooth muscle), this small population was not consistently detected.

In other cell types, notably cardiac muscle, the \( M_2 \) subtype is coupled to the toxin-sensitive G protein, \( G_i \) (McMahon and Hosey, 1985; Ladinsky et al., 1988). Since trachea contains both a large number of \( M_2 \) receptors and a protein ribosylated by pertussis toxin, it was surprising to find that the receptors are not coupled in a pertussis-sensitive manner. It follows that neither the receptor subtype nor the G protein complement of a cell is necessarily predictive of their interaction. In this regard, the results of transfection studies using cloned \( M_2 \) receptors are of interest (Ashkenazi et al., 1987). When \( M_2 \) receptors were expressed in CHO cells at low receptor density, they
appeared to be coupled exclusively to pertussis-sensitive G proteins. However, when expressed to a higher receptor density (~ 1.5 x 10^6 sites/cell), the receptors appeared to couple to pertussis-insensitive G proteins. It is perhaps significant then that tracheal smooth muscle cells have a density of receptors greater than that needed for toxin insensitive coupling (2.61 x 10^6 sites/cell).

The present results indicate that the major receptor subtype present in trachea is M₂, that this class of receptors is coupled to G proteins but in a largely pertussis toxin-insensitive manner. However, the trachea also contains M₃ receptors and proteins similar in size to the α subunits of pertussis-sensitive G proteins. Thus the trachea contains at least 2 subtypes of muscarinic receptors and at least 2 classes of G proteins (pertussis-sensitive and insensitive). It follows that the variety of cholinergic actions on smooth muscle (i.e. changes in cyclic AMP or inositol phospholipid metabolism, activation or inhibition of membrane channels or ion transporters) may be controlled by selective interaction between receptor subtypes and species of G protein. The results from the present study provide information essential for defining conditions for the selective occupation of receptor subtypes and inactivation of pertussis-sensitive G proteins and establish a basis for studies correlating specific receptor transduction mechanisms with specific cholinergic responses.
Chapter VIII
Discussion of Smooth Muscle Muscarinic Receptors

The foregoing studies were undertaken as an initial step towards understanding the cellular mechanisms that regulate the diverse physiological responses of smooth muscle to muscarinic stimulation. We performed experiments in both bovine tracheal and toad gastric smooth muscle in order to determine the extent to which they share common biochemical signalling systems to control contractility. These studies reveal a heretofore unappreciated degree of complexity in muscarinic regulation of contractility in different smooth muscle tissues. The two types of smooth muscle differed in their density of muscarinic receptors, as well as in the proportion of M₂ and M₃ receptors, and G protein coupling. Each of these aspects will be described below.

In both smooth muscle preparations analysis of the saturation binding isotherms for [³H]QNB indicated an interaction with a homogeneous population of receptors, as is expected for a nonselective antagonist. The Kᵢ of 175 pM for [³H]QNB exhibited by tracheal smooth muscle muscarinic receptors agrees well with values reported for other preparations (Yang et al., 1986; Akhtar et al., 1987; Giraldo et al., 1988; Rinner et al., 1988). However, the affinity of the amphibian gastric receptors for QNB, although somewhat lower than the average values reported for mammalian tissues, was in agreement with the Kᵢ values reported for QNB in canine gastric smooth muscle cells. Thus, a lower affinity for QNB may be a general property of gastric smooth muscle cells. An alternative, intriguing possibility is that the lower
affinity for QNB in toad gastric smooth muscle is attributable to the prevalence of $M_3$ receptors, since comparison of antagonist binding properties of cloned receptors expressed in cell lines indicated that $M_3$ receptors displayed a 3-5 fold lower affinity for QNB than other subtypes (Peralta et al., 1987).

The muscarinic receptor density in each smooth muscle type was markedly different. The density of binding sites for [${}^3$H]QNB to toad smooth muscle averaged 940 pmol/mg protein for purified membranes or $\sim 1.2 \times 10^5$ sites/cell for suspensions of enzymatically dispersed cells. These values are very similar to those reported for membranes prepared from ileum (Yamamura and Snyder, 1974), coronary smooth muscle (Rinner et al., 1988) and for suspensions of canine gastric smooth muscle cells (Collins and Crankshaw, 1986). The receptor density in tracheal smooth muscle was significantly higher averaging 4.3 pmol/mg protein for crude membranes or $2.6 \times 10^6$ sites/cell for suspensions of enzymatically dispersed cells. The density in tracheal membranes is quite similar to values reported by others for bovine (Madison et al., 1987; Cheng and Townley, 1982), porcine (Yang et al., 1986) and canine trachea (Murlas et al., 1982).

The functional significance of the nearly 10-fold higher receptor density in tracheal smooth muscle cells is unknown. Murlas et al. (1982) have speculated that the high density of muscarinic receptors reflects the important role played by the parasympathetic nervous system in regulating airway smooth muscle. On the other hand, the high density of receptors could also represent a spare receptor capacity that would allow the cell to respond rapidly but transiently to the low levels of
acetylcholine released from parasympathetic nerves. This would occur because the large receptor pool would provide a mechanism for obtaining a response at low concentrations of an agonist even when the affinity of the receptor for this agonist was low. The low affinity, in turn, would ensure a rapid dissociation of the agonist from the receptor and termination of the response. Support for this possibility is provided from studies by Delhaye et al., (1984) who demonstrated that the concentration of acetylcholine needed to fully inhibit adenylate cyclase in the heart required occupancy of only a few percent of the total muscarinic receptors. Alternatively, the occurrence of a large receptor pool may allow for the preferential coupling between a particular receptor subtype and a specific effector system, as was shown for \( M_2 \)-phospholipid coupling in transfection experiments (Ashkenazi et al., 1987). Ashkenazi and colleagues demonstrated that \( M_2 \) receptors coupled to a pertussis-sensitive G protein when expressed in CHO cells at low density (\( \sim 100,000 \) sites per cell), but that these \( M_2 \) receptors could also couple to a pertussis-insensitive G protein when expressed at a 10-fold higher density.

Information derived from binding profiles of the selective antagonists pirenzepine, AF-DX 116 and 4-DAMP indicated that a heterogeneous population of \( M_2 \) and \( M_3 \) receptors were present in both toad gastric and bovine tracheal smooth muscle. However, the relative proportion of each of these subtypes was completely opposite (see Table 10). In toad gastric smooth muscle, 85-90% of the receptors were \( M_3 \) and the remainder were \( M_2 \); in tracheal smooth muscle 85-90% of the receptors were \( M_2 \) and the remainder were \( M_3 \). It is interesting to
Table 10. Comparison of tracheal and gastric smooth muscle muscarinic receptors and G protein coupling.

<table>
<thead>
<tr>
<th>Receptor Subtypes</th>
<th>Tracheal Smooth Muscle</th>
<th>Gastric Smooth Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M₂ 85-90%</td>
<td>M₂ 12%</td>
</tr>
<tr>
<td></td>
<td>M₃ 10-15%</td>
<td>M₃ 88%</td>
</tr>
<tr>
<td>Interaction with G Protein</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Presence of Pertussis Toxin Substrate</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Pertussis Sensitivity of Agonist Binding</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

note that the small fraction of M₃ receptors in trachea, which has a higher receptor density, constitutes nearly the same total number of M₃ receptors as were found in gastric smooth muscle. Therefore, M₃ receptors may have equally important physiological roles in both tissues.

Whereas Roffel et al. (1987) concluded that the M₃ receptors found in tracheal membranes were localized on contaminating submucosal gland membranes, we concluded that this possibility was unlikely for several reasons. For one, based on published reports of the muscarinic receptor density in tracheal submucosal glands (Yang et al., 1986; Scott and MacMahon, 1988), our preparation of membranes from tracheal tissue would have to consist entirely of glandular membranes in order to account for the population of M₃ receptors. Moreover, our studies using suspensions of enzymatically dispersed cells confirmed that M₃ receptors are indeed located on the smooth muscle cells. However, it is
possible that our estimates of the proportion of $M_2$ versus $M_3$ receptors will change slightly as more selective antagonists become available. One such agent that appears promising in this regard is the antagonist methoctramine, a compound that exhibits a 50-75 fold selectivity for $M_2$ receptors versus $M_3$ and $M_1$ receptors (Michel and Whiting, 1988).

We were initially surprised at our findings that both AF-DX 116 and 4-DAMP provided a better resolution of $M_2$ versus $M_3$ receptors in toad gastric smooth muscle than in tracheal smooth muscle. In trachea, displacement curves for 4-DAMP could only be fit to a one site model ($n_H \sim 1$) in which the binding site exhibited an affinity characteristic of $M_2$ receptors. However, in retrospect, we realized that the inability to resolve 4-DAMP binding to $M_3$ receptors was due to a combination of factors: the limited 5-10 fold selectivity of this agent toward $M_3$ receptors and the fact that $M_3$ receptors represented only a minor component of the total receptor population. Similar difficulties in resolving mixed receptor pools were reported by Roffel (1988) in studies on the trachea and by Ladinsky et al. (1988) for the 80% $M_2$/20% $M_3$ mixture of receptors in ileal smooth muscle. Despite these difficulties, however, the results from composite analysis of the data in trachea for AF-DX 116, 4-DAMP and pirenzepine strongly suggested that both $M_2$ and $M_3$ receptors were present in this tissue.

In toad gastric smooth muscle the majority of receptors exhibited affinities for the selective antagonists that are characteristic of $M_3$ receptors while the remaining receptors exhibited a binding profile that resembled $M_2$ receptors. However, the absolute $K_D$ values for binding
to AF-DX 116 (10 nM) and 4-DAMP (700 nM) for the latter pool were quite different from the affinities expected for classical M₂ receptors (100 nM for AF-DX 116, 20 nM for DAMP). These findings suggest that the "M₂" receptors in toad gastric smooth muscle may be subtly different from mammalian M₂ receptors, or may be a completely different subtype of muscarinic receptor. To obtain a more rigorous identification of this subtype would require analysis of the mRNAs encoding for muscarinic receptors in toad gastric smooth muscle with subtype-specific mRNA probes.

Studies of toad gastric and bovine tracheal smooth muscle also revealed a heterogeneity in the binding of agonist to membrane fractions. However, this heterogeneity in agonist binding reflects heterogeneity in coupling of all receptors to G proteins (see figure 24). It is generally considered that receptors can exist in two states: free receptors with low affinity for agonist and receptors coupled to G proteins with high affinity for agonist; see Fig. 24a). Agonist binding to a receptor induces a conformational change that increases the affinity of the receptor for G protein bound to GDP and causes the formation of a ternary complex consisting of agonist-receptor-G protein. The agonist-receptor complex activates the G protein by promoting the exchange of GDP for GTP. Binding of GTP in turn promotes the dissociation of the ternary complex and causes the dissociation of the G protein into its βγ and α subunits (Fig. 24b). Both the α subunit bound to GTP and βγ subunits may then interact with target sites.

The conclusion that muscarinic receptors were coupled to G proteins
Figure 24. Scheme for receptor-G protein signal transduction. From the unliganded state (a) receptor binds agonist (A) which produces a change (b) in receptor-G protein interaction, allowing GTP, in the presence of Mg$^{2+}$, to replace GDP on the $\alpha$ subunit. The activated $\alpha$-GTP subunit and the $\beta\gamma$ subunits dissociate and one or both interact with effectors. Alternatively, free $\beta\gamma$ may bind other $\alpha$ subunits. The intrinsic GTPase activity of the $\alpha$ subunit hydrolyses GTP to GDP, and $\alpha$-GDP recombines with $\beta\gamma$ (c), ending the activation cycle. (from Neer and Clapham, 1988).

in both airway and gastric smooth muscle was based on two lines of evidence. The first is that in the absence of guanine nucleotides, the agonist oxotremorine interacted with both high and low affinity states of the receptors, with 24% of the gastric and 35% of the tracheal receptors displaying high agonist affinity characteristic of G protein-coupled receptors (see Figs. 16 and 22). The second is that the addition of GTP$\gamma$S, a nonhydrolyzable analog of GTP, completely abolished all high affinity agonist binding without affecting the total
number of receptors. The GTP-dependent shift in agonist binding is characteristic of all receptors coupled to G proteins.

Although we did not obtain direct evidence that both \( M_2 \) and \( M_3 \) receptors were coupled to G proteins, the data were consistent with this possibility. The number of high affinity agonist binding sites in the trachea (35% of the total receptor pool) was too large to be accounted for by the small (10%) population of \( M_3 \) receptors. Moreover, there is reason to expect that high affinity agonist binding was not due solely to the coupling of \( M_2 \) receptors to G proteins. Data from transfection studies (Peralta et al., 1987) demonstrating that both cloned \( M_2 \) and \( M_3 \) receptors were capable of interacting with G proteins, as well as our own data in toad gastric muscle, support this contention.

Moreover, studies using pertussis toxin provided unanticipated results. In other systems, pertussis toxin has been shown to ADP-ribosylate the \( \alpha \) subunits of \( G_1 \) and \( G_6 \), preventing their coupling to receptors and abolishing high affinity agonist binding. In our studies, incubation of both toad gastric and bovine tracheal smooth muscle membranes with toxin and \([^{32}P]NAD \) led to the ribosylation of a single protein with a molecular weight of \( \sim 41 \) kD on SDS-PAGE, a finding consistent with the ribosylation of both of the G proteins mentioned above. However, pretreatment of membranes with pertussis toxin under conditions that caused a nearly complete ribosylation of all of the available \( \sim 41 \) kD substrate (see Fig. 23), abolished high affinity agonist binding in toad gastric smooth muscle membranes but produced only a slight reduction in high affinity agonist binding in trachea.
These results were surprising in several respects. First, in other tissues, most notably the heart, M₂ receptors are coupled to the pertussis-sensitive G protein G₁. However, even though the majority of receptors in tracheal smooth muscle are M₂ and a pertussis-sensitive substrate is present, there was little or no effect of pertussis toxin on agonist binding. Therefore in trachea, most if not all of the M₂ receptors are coupled to a pertussis-insensitive G protein. In this regard, the results of transfection studies using cloned M₂ receptors are of interest (Ashkenazi et al., 1987). When M₂ receptors were expressed in CHO cells at low density they coupled exclusively to pertussis-sensitive G proteins. However, when expressed at a higher receptor density (~ 1.5 x 10⁶ sites/cell) the receptors appeared to be coupled to pertussis-insensitive G proteins. Thus the high receptor density in tracheal smooth muscle may somehow facilitate the interaction of M₂ receptors with toxin-insensitive G proteins.

The finding that the M₃ receptors in toad gastric smooth muscle are coupled to a pertussis substrate was also unexpected, since the coupling of transfected M₃ receptors to G proteins in both NG108-15 cells and RAT 1 cells were insensitive to toxin treatment (Pinkas-Kramarski et al., 1988). The physiologic relevance of the results from transfection experiments may be questionable, however, in that the cell lines used may have differing amounts and/or types of G proteins that could influence the nature of the coupling of the expressed muscarinic receptors. For example, although M₂ receptors were coupled to a pertussis-insensitive G protein in CHO cells, the receptors, when expressed at an identical density in a human embryonic
cell line were coupled to pertussis-sensitive G proteins (Ashkenazi et al., 1987; Peralta et al., 1989).

Our results in both smooth muscle types support the notion originally set forth by Ashkenazi et al. (1987) that the ability of an individual receptor subtype to recognize and interact with various G proteins is differential rather than exclusive and may be determined by the cellular context in which it is evoked. Thus, the nature of coupling of muscarinic receptors to G proteins in a given tissue may depend on such factors as receptor density, the type and amount of G proteins, the affinity of these two components for one another, and their physical arrangement within the cell membrane (Neer and Clapham, 1988).

Comparison of the results obtained in toad gastric smooth muscle with those in bovine tracheal smooth muscle indicate that their common biological responses upon cholinergic activation may be mediated by very different receptor-G protein interactions. Based on our own results, as well as on evidence obtained in other systems, we propose the following models to explain the mechanisms that control the diverse effects of cholinergic agents in each smooth muscle type. In toad gastric smooth muscle (Fig. 25) we hypothesize that M₃ receptors are coupled to a pertussis-sensitive G protein that in turn stimulates phosphoinositide hydrolysis via activation of phospholipase C. The breakdown products of PIP₂, IP₃ and diacylglycerol, in turn act at several effector sites to promote the increase in intracellular Ca²⁺ necessary to cause contraction. IP₃ stimulates Ca²⁺ release from intracellular stores within the sarcoplasmic reticulum (Carsten, et al., 1985; Suematsu et
Figure 25. Proposed mechanisms for muscarinic cholinergic actions on toad gastric smooth muscle.

- **MLCK**: myosin light chain kinase
- **Ca**^2+^: calcium ion
- **Ca pump**: calcium pump
- **DAG**: diacylglycerol
- **IP_3**: inositol1,4,5-trisphosphate
- **PIP2**: phosphatidylinositol 4,5-bisphosphate
- **cAMP**: cyclic AMP
- **cAMP kinase**: cyclic AMP protein kinase
- **K^+ channel**: potassium channel
- **M_3 Subtype**: M_3 muscarinic receptor
- **M_2 Subtype**: M_2 muscarinic receptor
- **Rho**: rho protein
- **Regulator**: regulatory subunit of phospholipase C
- **G alpha G**: G alpha protein
- **G alpha i**: G alpha protein
- **IP_3 receptors**: inositol1,4,5-trisphosphate receptors
- **Ca_2+ channel**: calcium channel
- **Ca pump**: calcium pump
- **MLCK**: myosin light chain kinase
- **Ca^2+ pump**: calcium pump
- **Ca^2+ channel**: calcium channel
- **IP_3 receptors**: inositol1,4,5-trisphosphate receptors
- **Ca^2+**: calcium ion
al., 1985). Diacylglycerol directly activates voltage-sensitive Ca\(^{2+}\) channels (Vivaudou et al., 1988) and suppresses a potassium current termed the M current, (an effect which causes membrane depolarization and consequent opening of voltage sensitive Ca\(^{2+}\) channels). This latter portion of this proposed pathway has been demonstrated for M\(_{3}\) receptors expressed in NG108-15 neuroblastoma x glioma cells (Fukuda et al., 1988) except that in this system the M\(_{3}\) receptors were coupled to pertussis-sensitive G proteins.

The M\(_{2}\) receptors in toad gastric smooth muscle could be coupled through the pertussis-sensitive G\(_{i}\) to an inhibition of adenylyl cyclase. It is generally considered that the \(\beta Y\) subunits from G\(_{i}\) mediate the inhibition of adenylyl cyclase by binding to free \(\alpha_s\) subunits from G\(_s\) (Weiss, et al., 1988; Gilman, 1987). Alternatively, the \(\alpha_i\) subunit from G\(_i\) may directly inhibit the cyclase enzyme. The function of this muscarinic pathway may be to inhibit cAMP-dependent processes that normally produce smooth muscle relaxation such as the lowering of cytosolic Ca\(^{2+}\) levels through the activation of Na\(^+\)/Ca\(^{2+}\) exchange (an effect that is secondary to an increase in Na\(^+\), K\(^+\) - ATPase activity, Scheid et al., 1979; Scheid and Fay, 1984) or the Ca\(^{2+}\) ATPase (Kattenburg and Daniel, 1984) or by changes in myosin-ATPase activity secondary to a decrease in myosin light chain kinase activity (Conti and Adelstein, 1981; Aksoy et al., 1983).

The preferential coupling of M\(_2\) receptors to G\(_{i}\) and the inhibition of adenylyl cyclase has been well established in a number of systems. Studies in cardiac tissue using pirenzepine and AF-DX 116 indicated that AF-DX 116 was much more potent in blocking the muscarinic
agonist-induced inhibition of adenylyl cyclase, suggesting that $M_2$ receptors in the heart are coupled to this enzyme. Moreover, Capon's laboratory demonstrated that $M_2$ receptors cloned into CHO cells (Ashkenazi et al., 1987) or into human embryonic kidney cells (Peralta et al., 1988) were efficiently coupled to adenylyl cyclase inhibition by a pertussis-sensitive G protein.

The results observed in tracheal smooth muscle have led us to suggest that the cholinergic control of the contractile state of this tissue occurs by slightly different mechanisms than those that may occur in toad stomach (Figure 26). As in gastric muscle, we assume that muscarinic receptors modulate both the phosphoinositide and the cAMP second messenger pathways in airway smooth muscle. This assumption is supported by studies using intact tracheal tissue, in which muscarinic agonists were shown to inhibit cAMP production (Madison et al., 1987) and stimulate phosphoinositide metabolism (Takuwa et al., 1986). However, we are postulating that different G proteins are involved in mediating each signalling pathway in the trachea. Specifically, we propose that the high density of muscarinic receptors allows the tracheal $M_2$ receptors to couple to $\text{PIP}_2$ breakdown through a pertussis-insensitive G protein, $G_p$. The $M_2$ receptors may also inhibit adenylyl cyclase activity stimulated by either β-adrenergic, histamine $H_2$ or VIP receptor that are coupled to $G_s$. This can occur in two ways: the $\beta Y$ subunits could be donated from the pertussis-insensitive $G_p$ to bind $\alpha_s$ thereby removing its stimulatory effect on cyclase; alternatively, a small percentage of the $M_2$ receptors could be coupled to the pertussis-sensitive $G_1$ to
Figure 26. Proposed mechanisms for muscarinic cholinergic actions on bovine trachealis smooth muscle.

- **M2 Subtype**
- **M3 Subtype**
- **GL - G protein-like**
- **MLCK - Myosin light chain kinase**
- **PIP2 - Phosphatidylinositol 4,5 bisphosphate**
- **PLC - Phospholipase C**
- **IP3 - Inositol 1,4,5 triphosphate**
- **DAG - Diacylglycerol**
- **Ca pump**
- **Ca channel**
- **cAMP - Cyclic Adenosine Monophosphate**
- **MLCK - Myosin light chain kinase**
- **GI - G protein-like**
- **M2 Subtype**
- **M3 Subtype**
inhibit cyclase by a manner analogous to that described for toad gastric smooth muscle. The fact that we were occasionally able to observe pertussis-toxin effects on agonist binding lends support to this latter possibility. We are also postulating that the small percentage of 
 receptors are coupled to phosphoinositide metabolism by a pertussis-
 insensitive G protein. Such a coupling has been proposed for pancreatic acinar cells in which the muscarinic-induced increases in PIP$_2$
 hydrolysis were found to be sensitive to GTP but insensitive to pertussis toxin. Again this coupling may be physiologically important since the number of M$_3$
 receptors in trachea (∼ 200,000/cell) is identical to the total number of muscarinic receptors on other smooth muscle types (Collins and Crankshaw, 1986; Chapter VI.). Once the effector pathways have been activated, we assume that the second messengers elicit similar changes in Ca$^{2+}$ handling in the trachea as were described for the toad stomach muscle.

To test the models described above, it would first be necessary to demonstrate that each subtype of receptor was capable of interacting with a G protein. This could be achieved by repeating the agonist displacement experiments in each tissue using AF-DX 116 or 4-DAMP as the radioligand. Then using our estimates of the binding constants for antagonists and agonists and the proportions of each receptor species, we could define conditions for the selective activation of a given receptor species. This would allow us to correlate the activation of a specific receptor species with a particular biochemical response. Thus, for example, in toad gastric smooth muscle, in order to show that M$_3$
 receptors are coupled to pertussis-sensitive G proteins and to
phosphoinositide metabolism we would first need to demonstrate that phosphoinositide hydrolysis can be activated by muscarinic agonists at a concentration near the $K_D$ value for G protein coupled receptors. To show that $M_3$ receptors mediate this response we would need to demonstrate that this activation could be blocked by concentrations of 4-DAMP near its $K_D$ value for $M_3$ receptors, but be unaffected by concentrations of AF-DX 116 that selectively interact with $M_2$ receptors. The participation of a pertussis-sensitive G protein could then be determined from the effects of toxin treatment on both the activation of phospholipase C and the suppression of the M current induced by muscarinic agonists. Lastly, we would need to test whether either of the breakdown products of PIP$_2$ (IP$_3$ and diacylglycerol) can mimic the effects of muscarinic agonists on the M current. Similar strategies could be employed to test each of the other proposed pathways for regulating bovine tracheal and gastric smooth muscle contractility.

The models proposed above also predict that each subtype of receptor regulates different transduction pathways within the cell. This may allow for an amplification of the response to acetylcholine, i.e., maximal changes in intracellular Ca$^{2+}$ could be obtained if both second messenger pathways were modulated simultaneously. Alternatively, each signalling pathway could be used separately to respond to different extracellular signals. For example, muscarinic regulation of the adenylyl cyclase system could be used to modulate responses to β adrenergic stimuli. Conversely, the muscarinic activation of the phosphoinositide system may serve as a direct excitatory pathway in response to acetylcholine release from parasympathetic nerve endings.
Thus, the $M_3$ receptor-mediated phospholipid pathway in toad gastric smooth muscle could be used as a direct excitatory pathway whereas the adenylyl cyclase pathway could be used to integrate incoming signals from the parasympathetic and sympathetic nervous systems. In this regard, the adenylyl cyclase signalling system would represent a balance point between inputs from the autonomic nervous system.

These studies allowed us to compare the subtypes and patterns of G protein coupling between gastric and airway smooth muscle. Since data from the literature have demonstrated muscarinic receptor mediated phospholipid metabolism in both tissues, our data suggest that the muscarinic receptors can be coupled to this pathway by different G proteins: a pertussis-insensitive G protein in trachea and a pertussis-sensitive G protein in toad gastric smooth muscle. It may be that the apparent differences in coupling of muscarinic receptors to phosphoinositide hydrolysis within the different tissues may depend on the different stoichiometric relationships between the receptors, G proteins and phospholipase C. An alternative possibility is that muscarinic receptors in different smooth muscle tissues may be linked to different routes of metabolism of phosphatidylinositol (phospholipase C versus phospholipase A$_2$, Peralta et al., 1989).

In summary, the results of these studies have provided an initial identification of the receptor subtypes and G proteins that may mediate the diverse responses of smooth muscle to cholinergic stimuli. Perhaps even more significant are our findings that different smooth muscle types use different muscarinic receptor subtypes and G proteins to regulate the same biochemical signalling systems. Thus, it may be that
the nature of a smooth muscle cell's response to acetylcholine will be dictated by its endowment of receptors, G proteins, and effectors, as well as by the relative concentrations and affinities of all of these compounds.
Chapter IX
ANALYSIS OF DISPLACEMENT CURVES

The general approach to the identification of muscarinic receptor subtypes present in toad gastric and bovine tracheal smooth muscle was to evaluate the ability of unlabeled antagonists, which exhibit varying degrees of selectivity for muscarinic receptor subtypes, to displace the binding of the nonselective antagonist [³H]QNB. Similar displacement experiments for the agonist oxotremorine were used to demonstrate the presence of multiple agonist affinity states.

The mathematical models that describe these interactions is based on the law of mass action which dictates that drugs combine with their receptors by a simple bimolecular reaction at a rate dependent upon the concentration of drug (D) and receptor (R) and that the resulting drug receptor (DR) complex breaks down at a rate proportional to the number of complexes formed:

\[
D + R \xrightarrow{k_1} R\cdot D \quad \text{and} \quad D + R \xrightarrow{k_2} R\cdot D
\]  

the equilibrium dissociation constant \(K_D\) equals

\[
K_D = \frac{\left[D\right]\left[R\right]}{\left[R\cdot D\right]} = \frac{k_2}{k_1}
\]  

Since the total receptor concentrations must be a sum of the free and bound concentrations:

\[
\left[R_{TOT}\right] = \left[R\right] + \left[R\cdot D\right]
\]

combining the last two equations to solve for \(D\cdot R\) gives
\[ [D \cdot R] = \frac{[D][R_{TOT}]}{K_D + [D]} \] (9.4)

which describes the law of mass action and which yields a hyperbolic displacement curve. The mass action model also dictates that for more than one population of receptor sites, the observed binding curve would reflect the "sum of hyperbolas" for each drug-receptor interaction. This assumes that the ligand interacts independently with each receptor class and that the affinity remains unchanged with increasing occupancy.

Whether or not a displacing ligand interacts with the receptors via a simple mass action law (reflecting one hyperbola) or via greater complexity (reflective of multiple hyperbolas) can be determined by examining the overall "shape" of the displacement curve in a plot of % Bound versus the log of the concentration of the displacing ligand. When the radioligand and the displacing compound interact reversibly with the receptor via a simple bimolecular reaction, the competition curve for the displacing compound will proceed for 10% to 90% over an 81-fold concentration of the displacing compound. This generalization was first made by Koshland (1970) for enzymes displaying Michaelis-Menton kinetics. Curves that exhibit this displacement are of "normal steepness" and have a characteristic slope or pseudo-Hill coefficient equal to 1.0. Curves proceeding from 10% to 90% displacement over a greater than 81-fold range of displacing ligand concentration are frequently referred to as "shallow" and possess a pseudo-Hill coefficient less than 1.0. A Hill coefficient < 1.0 for such curves is generally assumed to reflect the sum of binding to two sites, but may also reflect negative cooperativity between binding sites.
such that the overall affinity of the receptor population decreases as its fractional occupancy by the ligand increases.

The analysis of displacement curves can be modeled by several equations and most curve fitting programs fit the data nonlinear least squares regression. Linear regression is unsuitable for fitting displacement curves since the curves are logarithmic functions. Nonlinear regression determines values of parameters ($K_d$, relative number and proportion of binding sites, etc.) that minimize the sum of the squares of the distances of the data points from the curve generated by the equations used to describe the binding reactions (Motulsky, 1987). Two nonlinear least squares regression programs were used for curve fitting in our studies: ALLFIT developed by De Lean (1978) and LIGAND developed by Munson and Rodbard (1980).

The ALLFIT program is a four parameter logistics equation that is mathematically analogous to the Hill equation:

$$ Y = \frac{a - d}{1 + \frac{X}{C}} + d \quad (9.5) $$

where

$Y =$ concentration of the radioligand bound

$X =$ concentration of displacing ligand bound

$C =$ $IC_{50}$ of displacing ligand

$b =$ steepness factor or pseudo–Hill coefficient

$a =$ extrapolated upper limit for $Y$ (total binding)

$d =$ extrapolated lower limits for $Y$ (nonspecific binding)

The primary advantage of the ALLFIT program is that it allows for a
constrained, simultaneous fit to several curves, forcing them to share certain parameters in common. In our case, the constrained fit to the curves forced them to share either C (IC\textsubscript{50}) or B (the slope factor or pseudo-Hill coefficient). For analysis of individual curves, the standard errors and the confidence limits of the parameter estimates in nonlinear regression are only approximate since they are logarithmically distributed rather than normally distributed; therefore, any conclusions regarding the equality of corresponding parameters are also approximate (De Lean, 1978). In contrast, simultaneous constrained curve fitting permits testing the equality of parameters by inspecting the consequences of forcing them to be equal.

The ALLFIT program was thus used to provide the value of the pseudo-Hill coefficient for the agonist and antagonist displacement experiments. This program, however, is unable to resolve the complex binding curves further. Therefore, if the pseudo-Hill coefficient was significantly different from 1.0, the data were then analyzed with the LIGAND program to obtain estimates of the $K_D$ values and proportion of the different binding sites. Implicit in this statement is the assumption that the binding reactions between $[^3H]QNB$, the displacing ligands and the receptors follow the mass action model assumed for the LIGAND program (as opposed to reflecting negative cooperativity between binding sites, Limbird, 1986).

The mathematical model of the LIGAND program is analogous to a "sum of hyperbolas" description for complex binding similar to equation 9.4. The algebraic descriptions below are from [1983].

1) For a single ligand binding to a single class of binding sites:
\[ B = \frac{KR}{(1 + KF) + N}F \] (9.6)

\[ T = B + F \]

where:
- \( T \) = concentration of total ligand added
- \( B \) = concentration of bound ligand
- \( F \) = concentration of free ligand
- \( R \) = receptor density
- \( N \) = ratio of nonspecifically bound ligand to free ligand
- \( K \) = equilibrium association constant in units \( M^{-1} \)

2) For two ligands binding to a single class of receptors as occur in displacement experiments, the mathematical model becomes

\[ B_1 = \frac{K_1R}{1 + K_{1F_1} + K_{2F_2}} + \frac{N_1}{F_1} \] (9.7)

\[ B_2 = \frac{K_2R}{1 + K_{1F_1} + K_{2F_2}} + \frac{N_2}{F_2} \] (9.8)

\[ T_1 = B_1 + F_1 \]

\[ T_2 = B_2 + F_2 \]

Where the subscripts refer to ligands \( L_1 \) or \( L_2 \). The value \( K_1 \) for the radioligand, \(^3\text{H}QNB\), is determined in independent experiments by Scatchard analyses of QNB binding data. These equations take into account the fraction of the receptor population that will be occupied by each ligand.

3) For a ligand binding to 2 classes of receptors, a double subscript is used in the LIGAND program to describe the affinity constant \( K_{xy} \),

\[ B_x = \frac{K_{11}R_1}{1 + K_{11}F_1 + K_{21}F_2} + \frac{K_{12}R_2}{1 + K_{12F_1} + K_{22}F_2} + \frac{N_1}{F_1} \]
\[ B_x = \frac{K_{21}R_{x1}}{1 + K_{11}F_1 + K_{21}F_2} + \]
\[ K_{22}R_{x2}/(1 + K_{12}F_1 + K_{22}F_2) + N_a F_2 \]

where \( x = \text{Ligand} \) 1 or 2 and \( y = \text{receptor} \) 1 or 2. The program also introduces a correction factor that adjusts for varying receptor concentrations between experiments and thus permits simultaneous analysis and comparison of the data from several experiments.

The LIGAND program has several features that demonstrate the advantages of computer-assisted analysis for the estimation of binding parameters. First, the computations are done using the concentration of the ligand added to the reaction, which can be precisely determined. Therefore, most of the measurement error is confined to a single variable, the concentration of bound radioligand. In contrast, Scatchard analysis introduces error in both the Bound and the Bound/Free axes. Secondly, the program weights the data based on the reciprocal of their variance so that the analysis is most significantly influenced by the most reliable data. Without this procedure the nonlinear regression program would tend to emphasize points with a large variance from the predicted curve. Third, the curve-fitting program provides a variety of statistical methods to determine "the goodness of fit" for a given model (one-site versus a two-site model, etc., see below).

To analyze the results from the displacement experiments, the following data are entered in the ligand program: the specific activity of \(^{3}H\)QNB, the total cpm added to each tube, and the cpm bound at each concentration of displacing ligand. The data is first fit to the simpler one site model; since a nonlinear equation cannot be resolved in
a single step, initial estimates of each parameter are supplied. The nonlinear regression procedure then adjusts these values to improve the fit of the curve to the data. The initial values of the affinity for [\(^3\text{H}\)]QNB are obtained from previous saturation binding experiments, the estimates of \(R\) and \(K_{21}\) (affinity for the displacing ligand) are based on the raw data. For example, a suitable initial estimate for the affinity of the displacer would be the concentration of the drug that caused a 50% reduction in total cpm bound.

The data are then fit to the more complex two-site model. Initial estimates are also provided for this fit. The program then compares the two models to determine if the more complex model resulted in a better fit to the data. The comparison of two models with a different number of parameters is not straightforward because an increased number of parameters gives more flexibility to the curve fitting procedure and almost always leads to a curve that is close to the points (remember that a nonlinear least squares program iterates until the average sums of squares of the distance between the data points and the curve are minimized). The question is whether the improved fit is worth the loss of degrees of freedom (number of data points minus the number of parameters). This question can be answered statistically by performing the \(F\) test.

\[
F = \frac{(SS_1 - SS_2)/(df_1 - df_2)}{SS_2/df_2}
\]

where \(SS_1\) and \(SS_2\) equal the total sums of squares for the one site and two site models, respectively, and \(df\) refers to the number of
degrees of freedom. A P value is then obtained by the program by comparing this calculated F value with tabulated values for (df$_1$ - df$_2$) and df$_2$ degrees of freedom. A P value < 0.05 indicates that the more complex model fits the data significantly better than a one-site model.

A problem with LIGAND program that was identified in these studies is the manner in which it defines nonspecific binding (N). In analysis of individual curves, N is defined as a function of the total counts added (T) (i.e. nonspecific binding is "the limiting ratio of bound/total as T approaches infinity"). However, in composite analysis, as described above, a correction factor (C) is introduced to account for differences in receptor concentrations between experiments; this factor is also used to scale the nonspecific binding value. Therefore, in composite analysis, N is defined as a function of receptor concentration (i.e. "amount bound"). The scaling of N may lead to an overestimate of the contribution of nonspecific binding to the composite curves and may obscure the detection of a small population of receptors with low affinity for the ligand. This problem was recognized in the composite analysis of AF-DX 116 displacement curves in tracheal smooth muscle in which the low affinity binding site represented ~10% of the total number of sites. To adjust for this problem, we corrected each individual curve for non-specific binding and held N constant at zero for the composite analysis. After this correction was performed, the composite analysis was able to resolve the second low affinity site for AF-DX binding.
DeLean and associates (1981) provided an empirical test of this computer modeling method by analyzing radioligand binding data for mixtures of receptor subtypes and helped determine the limits of the analysis. They utilized LIGAND to analyze complex binding data which resulted from mixing known proportions of β adrenergic receptor subtypes in radioligand binding reactions. Such mixtures of receptor subtypes were created by combining various proportions of previously characterized pure β₁ and β₂ receptors populations from turkey and frog erythrocyte membranes (DeLean et al., 1981). It was determined that accurate resolution of the two subtypes could be obtained using an agent with only a 5 to 8 fold selectivity for a particular receptor subtypes when the receptors were present in a 50/50 mix. However, the practical limit of the program's ability to statistically resolve two receptor populations from one another was reached with a 90%/10% mix of receptors. In this case, the displacing ligand needed at least a 30 to 100-fold selectivity for one receptor subtype in order for the statistics in the computer modeling program to favor a two-site model over a one-site model.

These results may explain our inability to discriminate 4-DAMP binding to the ~ 85% M₂ / 15% M₃ receptor mixture in tracheal smooth muscle. 4-DAMP, based on the ratio of its affinity for M₃ receptors in exocrine glands and M₂ receptors in cardiac membranes, has only a 10-fold selectivity towards M₃ receptors. Therefore, given the mixture of receptor subtypes in trachea, it is unlikely that both sites would be resolved. On the other hand, it was curious that both M₂ and M₃ receptors were resolved in toad gastric membranes given
the 87% M3/13% M2 mixture of receptors. Although the "M2" receptors in this tissue shared the same relative binding to pirenzepine, AF-DX 116 and 4-DAMP as classical M2 receptors, the absolute binding constant for AF-DX 116 and 4-DAMP were different than those expected for classical M2 receptors (i.e. 100 nM for AF-DX 116, 20 nM for 4-DAMP). Analysis of the data indicated that this M2 receptor exhibited 10 nM affinity for AF-DX 116 and ~700 nM affinity for 4-DAMP. Therefore, the apparent selectivity of 4-DAMP was 100-fold for the M3 receptor, based on the ratio of the Kd values for the 2 receptor subtypes. These results suggest that the class of receptors defined as M2 in the toad stomach muscle may be subtly different than mammalian M2 receptors or may actually represent a novel receptor subtype. Alternatively, the phospholipid environment of the membrane surrounding the receptor may be somewhat different in the amphibian tissue, forcing the M2 receptor into a slightly altered conformation that would affect binding to AF-DX 116 and 4-DAMP.
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