Modulation of N-type Calcium Channels in Rat Superior Cervical Ganglion Neurons: A Dissertation

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MODULATION OF N-TYPE CALCIUM CHANNELS
IN RAT SUPERIOR CERVICAL GANGLION NEURONS

A dissertation presented
by
Curtis Frank Barrett

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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(Program in Cellular and Molecular Physiology)

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Modulation of N-Type Calcium Channels in Rat Sympathetic Neurons

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DEDICATION

This thesis is dedicated to my fiancée, Kirsten Canté. Kirsten, you’ve provided support at the times I needed it most, and always stood by my side. Without your encouragement and love, this thesis would not have come to fruition (and I would probably be sitting on a beach in the Bahamas right now – so thank you very much).
COPYRIGHT STATEMENT

Portions of the work contained in this thesis have been, or will be, presented in the following publications:

Liu L, Gonzalez PK, Barrett CF, and AR Rittenhouse. Biophysical characterization of the nondihydropyridine L-type calcium channel agonist FPL 64176. (In Preparation)


In addition, portions of this work have been presented in abstract form in the following publications:


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I am indebted to Ann, who has been, at various times, stimulating, encouraging, supportive, and challenging, but at all times, a friend and colleague; you’ve shown me to always keep my eyes open, and taught me that everything is not always as it seems. I’m grateful to Joshua Singer for introducing me to the captivating world of ion channels, and for teaching me that a successful marriage can exist between biological science and engineering. I am also thankful to the members of the Rittenhouse lab, past and present, who have provided enjoyable company and a stimulating atmosphere in which to work.

I also wish to thank Diane Lipscombe, and the members of her laboratory, for providing me with several lines of HEK cells expressing N-type calcium channels, and for teaching me valuable molecular biology techniques. I’ve enjoyed our collaboration.
ABSTRACT

This thesis details my examination of several mechanisms for modulation of N-type calcium channels in neonatal rat superior cervical ganglion (SCG) neurons. The first part of this work characterizes cross-talk between two distinct mechanisms of modulation: readily-reversible inhibition induced by activation of heterotrimeric G-proteins (termed G-protein-mediated inhibition), and phosphorylation of the channel by protein kinase C (PKC). Data previously presented by other groups suggested that one effect of activating PKC is to prevent G-protein-mediated inhibition. The goal of this project was to confirm this hypothesis by testing functional competition between these two pathways. My findings show that G-protein-mediated inhibition blocks the effects of activating PKC, and that phosphorylation by PKC blocks G-protein-mediated inhibition, confirming that these two mechanisms are mutually exclusive.

In addition, I investigated the effect of activating PKC on whole-cell barium currents in the absence of G-protein-mediated inhibition. When endogenous G-proteins were inactivated by dialyzing the cell with GDP-β-S, a guanine nucleotide that prevents activation of the G-protein’s α subunit, activation of PKC with phorbol esters was without obvious effect on whole-cell current amplitude, fast and holding potential-dependent inactivation, and voltage-dependent activation, suggesting that PKC’s principal role in modulating these currents is to prevent G-protein-mediated inhibition. From these results, I advanced Bean’s 1989 model of reluctant and willing gating (induced by G-protein-mediated inhibition and relief of that inhibition, respectively). In
this expanded model, reluctant channels, inhibited by G-proteins, are resistant to phosphorylation by PKC (reluctant/P-resistant). Unmodulated channels are called willing/available, as they exhibit willing gating, and are available for either binding to a G-protein or phosphorylation by PKC. Finally, phosphorylation of a willing/available channel by PKC drives the channel into the willing/G-resistant state, in which the channel gates willingly, and is resistant to G-protein–mediated inhibition. These results are published in the Journal of General Physiology (2000; 115:277–286), and are presented in this thesis as Chapter II.

In addition to membrane-delimited inhibition, N-type calcium channels are also subject to inhibition via a diffusible second-messenger pathway. In SCG neurons, this inhibition can be observed following stimulation of M₁ muscarinic receptors by the agonist oxotremorine-M. Our lab previously hypothesized that the diffusible messenger involved might be the polyunsaturated fatty acid arachidonic acid (AA). To test this hypothesis, our lab examined the effect of bath-applied AA on whole-cell SCG calcium currents, and demonstrated that AA induces inhibition with similar properties as M₁ muscarinic inhibition. An analysis of AA's effects on unitary N-type calcium currents, published by Liu and Rittenhouse in Journal of Physiology (2000; 525:391–404), revealed that this inhibition is mediated, at least in part, by both a significant increase in the occurrence of null-activity sweeps and a significant decrease in mean closed dwell time.

Based on these results, our lab conducted an examination of AA's effects on whole-cell currents in SCG neurons, and found that AA-induced inhibition is mediated
by an increase in holding potential–dependent inactivation and appears independent of AA metabolism. When I examined AA’s effects in greater detail, I discovered that, in addition to inhibition, AA also appeared to cause significant enhancement of whole-cell currents. The results characterizing AA’s general effects on whole-cell calcium currents in SCG neurons have been published in *American Journal of Physiology – Cell Physiology* (2001; 280:C1293–C1305).

Because my finding that AA enhances whole-cell neuronal calcium currents revealed a novel pathway through which this current can be modulated, I proceeded to characterize this effect. My results showed that enhancement develops significantly faster than inhibition, suggesting different mechanisms or pathways. In addition, dialyzing the cell with BSA, a protein that binds fatty acids, blocked the majority of AA-induced inhibition, but did not reduce enhancement, suggesting that enhancement is independent of inhibition and might be mediated at an extracellular site. Using fatty acid analogs that cannot cross the cell membrane, I confirmed that enhancement occurs extracellularly. My data also indicate that AA-induced enhancement of whole-cell currents does not require metabolism of AA, consistent with enhancement being mediated directly by AA. I also examined the biophysical characteristics of enhancement, and found that both an increase in the voltage sensitivity of activation and an increase in activation kinetics underlie this effect. Finally, using both pharmacological agents and a recombinant cell line, I presented the first demonstration that AA enhances N-type calcium current. These findings are described in detail in a paper recently
published in *American Journal of Physiology – Cell Physiology* (2001; 280:C1306–C1318), and are presented in this thesis as Chapter III.

In our investigation of AA's effects on whole-cell calcium currents, we utilized a voltage protocol, in conjunction with pharmacology, to enhance the level of L-type current in these cells. Since whole-cell calcium currents in SCG neurons are comprised of mostly (80–85%) N-type current, with the remaining current comprised of mostly L-type current, this approach allowed us to examine both N- and L-type currents. When currents are recorded in the presence of 1 μM FPL 64174 (FPL), a benzoyl pyrrole L-type calcium channel agonist first described in 1989, stepping the membrane potential to −40 mV following a test pulse to +10 mV generates a slowly-deactivating ("tail") current. This tail current is made up entirely of L-type current, and allows us to readily investigate the effect of various modulatory mechanisms on this current type. Although FPL has been used for almost a decade to study L-type calcium currents, activity of FPL on N-type calcium currents has not been investigated. Because our lab routinely uses micromolar concentrations of FPL to measure whole-cell and unitary calcium currents in neuronal cells, I tested whether FPL has any effects on N-type calcium current. Therefore, I examined the effect of FPL on whole-cell calcium currents in an HEK 293 cell line that expresses recombinant N-type calcium channels. Application of 1 and 10 μM FPL caused significant, voltage-independent inhibition of currents, demonstrating that FPL inhibits N-type calcium current. Thus, at micromolar concentrations, FPL is not selective for L-type calcium current, and any examination of its effects on whole-cell calcium currents should take this into account. The results describing FPL's effects on L-
and N-type calcium currents are included in a manuscript currently in preparation, and are presented as Chapter IV.
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CHAPTER I

BACKGROUND
Voltage-dependent calcium channels

The lipid bilayer of cell membranes serves as a barrier against diffusion of ions such as calcium. However, cells express integral membrane proteins which allow ions to cross this barrier. These proteins, called ion channels, are tightly regulated, such that, when closed, they form an impermeable barrier to ion flow. But under appropriate conditions, these proteins undergo a change in conformation, allowing select ions to flow through their pores, with net ion movement through the channel dictated by both the ion’s concentration gradient and the voltage potential across the membrane.

Calcium entry plays important roles in triggering many cellular processes, including transmitter release, gene expression, and activation of calcium-dependent enzymes (for review, see Berridge, 1998). Because of the wide range of effects of calcium entry, it is not surprising that calcium influx is tightly regulated. In excitable cells, calcium entry is regulated by voltage-gated calcium channels (VDCCs).

VDCCs are composed of several proteins which assemble to form heteromultimers (for review, see Walker and De Waard, 1998; Randall and Benham, 1999). At the heart of the VDCC is the \( \alpha_1 \) subunit (Fig. 1-1). Composed of four repeating pseudosubunit domains within a single protein ranging from 170–250 kDa, the \( \alpha_1 \) subunit contains the minimum necessary components to form a functional channel. Within each repeating domain are six putative transmembrane-spanning helices, S1 through S6. In each domain, the fourth helix, S4, contains the voltage sensor, in the form of positively-charged amino acids, and a P-loop between S5 and S6 is believed to form the pore of the channel. Found in association with the \( \alpha_1 \) subunit are several auxiliary subunits (the
calcium channel $\beta$, $\alpha_2\delta$ and, in skeletal muscle, $\gamma$ subunits), which have diverse effects on current, such as increasing whole-cell current amplitude by increasing channel expression, altering kinetics of activation and/or inactivation, and increasing ligand binding (Walker and De Waard, 1998).

Consistent with calcium influx triggering diverse cellular processes, several different phenotypes of VDCCs exist, including P/Q, L, N, T and R; these channel types are primarily distinguished by their $\alpha_1$ subunit (Randall and Tsien, 1995; Tottene et al., 2000), and can be identified by their voltage-dependent properties and pharmacological sensitivity (reviewed by Jones, 1998). For example, VDCCs can be classified by their voltage threshold for activation. T-type calcium channels are activated by small changes in membrane potential, activating at voltages as negative as $-70$ mV (Huguenard, 1996). Hence, T-type calcium channels are classified as low-voltage activated (LVA). In contrast, other calcium channels require larger changes in membrane potential, and are considered high-voltage activated (HVA). HVA calcium channels can be further distinguished by their sensitivity to select pharmacological agents; L-type calcium channels are sensitive to the dihydropyridine family of drugs, whereas P/Q-type channels are sensitive to the peptide blocker $\omega$-agatoxin IVA and N-type calcium channels are blocked by the peptide $\omega$-conotoxin GVIA.

Recently, a new scheme for naming VDCCs was proposed (Fig. 1-2), in which the $\alpha_1$ subunits are named by a numerical system, with the subunits divided into 3 gene families, $\text{Ca}_{1.1}$, $\text{Ca}_{1.2}$ and $\text{Ca}_{1.3}$ (Ertel et al., 2000). Subunits within each family are further divided by order of discovery and splice variant. Hence, the N-type calcium...
channel’s $\alpha_1$ subunit (formerly $\alpha_{1B}$) is Ca$_v$2.2. Within this thesis, however, I will refer to channel and current types using the former convention.

$N$-type calcium channels

$N$-type voltage-gated calcium channels were first believed to be expressed solely in neurons. However, $N$-type calcium channel expression has also been demonstrated in non-neuronal secretory cells, including adrenal chromaffin cells (Jan et al., 1990), pancreatic beta cells (Pollo et al., 1993), and anterior pituitary cells (Suzuki and Yoshioka, 1989), to name a few. In neurons, $N$-type calcium channels play an important role in coupling excitation to transmitter release. Hence, they are found mainly in presynaptic membranes (Stanley and Atrakchi, 1990; Reber and Reuter, 1991; Cohen et al., 1991), where an action potential at the nerve terminal will cause the channels to gate, thereby allowing calcium to enter the terminal.

$N$-type voltage-gated calcium channels are also present in the cell membrane of neuronal cell bodies. For example, dissociated frog (Jones and Marks, 1989), chick (Golard and Siegelbaum, 1993) and rat (Plummer et al., 1989) sympathetic neurons express predominantly $N$-type calcium current. However, the role of this current type in cell bodies is not well understood, although it may play a role in mediating release of transmitters from cell bodies (Rittenhouse and Zigmond, 1999). Therefore, dissociated sympathetic neurons are often used as a model system for examining the properties of $N$-type calcium channels, with the caveat that channels recorded from cell bodies may not
necessarily correlate to properties of N-type calcium channels in other regions of the cell, for example at the nerve terminal.

Consistent with their role in mediating excitation-secretion coupling at the nerve terminal, N-type calcium channels associate with several components of the vesicle priming/release machinery. The synaptic proteins syntaxin and SNAP-25 have been shown to interact with the cytoplasmic linker connecting the second and third pseudosubunit domains of the channel’s \( \alpha_{1B} \) subunit (the II-III linker) in a region called the synprint (synaptic protein interaction) site (Rettig et al., 1996; Sheng et al., 1997; Vance et al., 1999; Catterall, 1999). This interaction has been shown to have physiological significance, as synaptic transmission in SCG neurons was disrupted by the introduction of peptides corresponding to the synprint region of \( \alpha_{1B} \) (Mochida et al., 1996). Thus, modulation of N-type calcium channel activity can potentially lead to profound changes in presynaptic transmitter release, thus altering synaptic transmission.

First cloned by Dubel et al. (1992), the \( \alpha_{1B} \) subunit of the N-type calcium channel several distinct sites for interacting with other proteins, including the \( \beta \), \( \gamma \), and \( \alpha_2 \delta \) accessory subunits. One site, located within the I–II linker, is called the AID (for (alpha)I–interaction domain; see Figs. 1-1 and 1-3). This domain serves as the binding site for the channel’s accessory \( \beta \) subunit (Pragnell et al., 1994; De Waard et al., 1996; Zamponi et al., 1997). In addition, the \( \beta \gamma \) subunit of heterotrimeric G-proteins (G\(_{\beta\gamma}\)) has been shown to bind to this domain (De Waard et al., 1997; Qin et al., 1997). Finally, this domain also contains several consensus phosphorylation sites for protein kinase C (PKC; Dubel et al., 1992; Hell et al., 1994).
Additional putative sites of interaction between the channel and G-proteins have been identified at the amino terminus of α1B (Page et al., 1998). In addition, the carboxy-terminal tail of α1B (Fig. 1-3) contains an apparent Gα-binding site (Furukawa et al., 1998), as well as a possible binding site for Gβγ (Zamponi et al., 1997; Qin et al., 1997); also located within this sequence are two putative PKC phosphorylation sites (Dubel et al., 1992).

**G-protein-mediated inhibition of N-type calcium channels**

Over two decades ago, Dunlap and Fischbach (1978) reported that application of transmitters led to a reduction in action potential–activated calcium conductance recorded from embryonic chick dorsal root ganglion neurons. These authors proposed that the decrease in conductance likely resulted from a reduction in the number of available calcium channels (Dunlap and Fischbach, 1981). Subsequently, Holz et al. (1986) reported transmitter-activated inhibition of both action potential duration and calcium current in these neurons. The authors noted that this inhibition was blocked by either pre-treating the cells with pertussis toxin or dialyzing the cell with GDP-β-S, raising the possibility that inhibition is mediated by G-proteins, an hypothesis supported by reports that cell dialysis with GTP-γ-S mimicked the effects of transmitters in both chick (Marchetti and Robello, 1989) and rat (Dolphin and Scott, 1987; Dolphin et al., 1988) dorsal root ganglion neurons.

Following up on these observations, Bean (1989) further investigated this inhibition, and proposed a model in which the voltage dependence of calcium channels...
becomes altered following activation of G-proteins. Bean theorized that receptor activation leads to a reduction in voltage sensitivity by shifting the channels into a “reluctant” gating mode, in which the channels are less responsive to changes in membrane potential. Bean noted that the decrease in current amplitude following receptor activation was not observed at all test potentials, but rather was limited to voltages ranging from $-40 \text{ mV}$ through $+30 \text{ mV}$ (Fig. 1-4 A). At more positive potentials, receptor activation appeared to have little effect on current amplitude, supporting a previous hypothesis that G-protein–induced slowed activation might be relieved with successive membrane depolarizations (Marchetti et al., 1986). Thus, Bean concluded that large positive depolarizations could overcome the inhibitory effect of the transmitters. This conclusion fit well with his observation that “willing” channels reached maximum open probability ($P_o$) at more negative voltages than “reluctant” channels (Fig. 1-4 B).

Central to Bean’s hypothesis was the premise that, at any given time, all of the calcium channels will reside in either a “willing” or “reluctant” gating mode, and that activated G-proteins merely increase the proportion of channels in the reluctant mode (Fig. 1-5). Thus, receptor activation does not decrease the number of channels in the membrane, per se, but rather decreases the number of channels willing to gate.

Since those initial observations, a considerable wealth of data has emerged providing strong evidence that G-proteins mediate voltage-dependent inhibition elicited by receptor activation. Song et al. (1989) demonstrated that application of either ACh or NE led to reversible inhibition of calcium currents in rat sympathetic neurons, but that
this inhibition was rendered irreversible when cells were dialyzed with GTP-γ-S. In 1990, Elmslie and his colleagues (Elmslie et al., 1990) investigated the involvement of G-proteins in modulating calcium currents in frog sympathetic neurons, and discovered that inhibition, induced either by applying the transmitter LHRH, or by dialyzing the cell with GTP-γ-S, could be relieved by application of a strong positive voltage step (Fig. 1-6), establishing a protocol by which levels of G-protein–mediated inhibition can be readily measured. Called facilitation, this relief of inhibition was believed to result from dissociation of the G-protein from the channel (Lopez and Brown, 1991). G-protein–mediated inhibition has been observed in essentially all types of neurons examined, suggesting that it is a major pathway for modulation of N-type calcium current.

Despite vast data supporting the hypothesis that G-proteins mediate voltage-dependent inhibition of N-type calcium current in sympathetic neurons (Elmslie et al., 1990; Lopez and Brown, 1991; Plummer et al., 1991; Elmslie, 1992; Swartz, 1993; Swartz et al., 1993; Zhu and Ikeda, 1994), it was unclear whether the G-protein was the final effector molecule, or whether the G-protein activated a downstream pathway, which then acted on the channel. The first evidence that G-proteins appear to directly bind N-type calcium channels was presented by McEnery et al. (1994), who demonstrated that \(G_\alpha\) and \(G_\beta\gamma\) copurified with \(\omega\)-conotoxin GVIA–labeled membrane proteins (i.e., N-type calcium channels) from rat forebrain, suggesting that \(G_\alpha\), \(G_\beta\gamma\), or both, mediate inhibition. Electrophysiological experiments (Ikeda, 1996) showed that when SCG neurons were injected with RNA encoding the G-protein \(\beta_1\) and \(\gamma_2\) subunits, classic G-protein–mediated inhibition was observed. In contrast, injection of RNA encoding a constitutively active
Gα subunit failed to induce inhibition. Thus, Gβγ was the likely subunit for mediating inhibition, and the Gα subunit could be ruled out as a candidate, although Gα binding to the channel has been confirmed (Furukawa et al., 1998), possibly giving rise to a novel modulatory pathway.

Further biochemical evidence that Gβγ binds N-type calcium channels was presented simultaneously in 1997 by Zamponi et al. and De Waard et al. Using overlay binding assays, Zamponi et al. showed that labeled Gβγ strongly binds to a fusion protein containing the I–II linker of the calcium channel α1B subunit, and weakly binds to a protein containing the C-terminal tail of α1B. In contrast, activated Gαα failed to bind the I–II linker, but, like Gβγ, showed slight binding to a C-terminal tail fusion protein. Zamponi et al. then further defined the interaction between Gβγ and the I–II linker by examining prepulse facilitation in the presence of intracellular recombinant Gβγ and peptides corresponding to various regions of the I–II linker. Their results demonstrated that only certain peptides were capable of blocking Gβγ-induced inhibition. Almost simultaneously, De Waard et al. confirmed that Gβγ binds to two distinct regions of the I–II linker (corresponding to the regions identified by Zamponi et al.), and showed that mutation of a single amino acid within the linker abolishes G-protein–mediated inhibition. Finally, De Waard et al. showed that labelled Gβγ binds to the I–II linker of class A, class B and class E calcium channels, but not to class S or class C channels.

Direct modulation by G-proteins is now accepted as a wide-spread pathway for inhibiting the activity of N-type calcium channels. This inhibition is membrane-
delimited, in that the necessary components (the receptor, the G-protein and the channel) are all localized to the membrane, eliminating the need for diffusion through the cytoplasm. This leads to rapid activation of the pathway following receptor binding (<0.5 seconds). Finally, inhibition through this pathway is characterized by a slowing of whole-cell activation kinetics and a decrease in current amplitude, and can be relieved by either strong positive voltage steps or trains of action potentials.

**PKC phosphorylation modulates N-type calcium channels through cross-talk with G-protein-mediated inhibition**

In addition to inhibition by activated G-proteins, it has also been shown that N-type calcium current can be modulated through phosphorylation by protein kinase C (PKC). Using biochemical methods, Ahlijanian et al. (1991) demonstrated that PKC phosphorylated rabbit brain calcium channels labeled with the N-type calcium channel ligand ω-conotoxin GVIA. Patch-clamp evidence to support modulation of these channels by PKC came from reports that N-type calcium currents in both frog (Yang and Tsien, 1993) and rat (Swartz, 1993; Zhu and Ikeda, 1994) sympathetic neurons are enhanced by phorbol esters.

One mechanism by which PKC may enhance N-type calcium current is by reversing G-protein-mediated inhibition. This hypothesis was first advanced by Swartz and colleagues, who observed that transmitter-activated inhibition in rat hippocampal neurons was attenuated following treatment with phorbol esters (Swartz et al., 1993). Given that one site known to interact with Gβγ subunits, the I–II linker of the α1B subunit,
also contains consensus sites for phosphorylation by PKC (Fig. 1-3; Dubel et al., 1992), this observation was not completely surprising. Evidence to support this interaction was later provided by Zamponi et al. (1997), who demonstrated “cross-talk” between G-proteins and PKC.

Using a recombinant expression system, Zamponi and his colleagues showed that prepulse facilitation (measured as the increase in current amplitude following application of a prepulse, in the presence of intracellular recombinant G_{\beta y}) could be abolished by dialyzing the cell with peptides corresponding to select segments of the I–II linker of the \(\alpha_{1B}\) subunit. The I–II linker of \(\alpha_{1B}\) contains four putative phosphorylation sites for PKC (Fig. 1-3), and Zamponi et al. (1997) showed that a peptide which contains the two N-terminal sites (corresponding to \(\alpha_{1B}\) residues 410–428) abolished facilitation, but that a peptide containing the two C-terminal sites (corresponding to \(\alpha_{1B}\) residues 445–463) was without effect on facilitation, suggesting that only two of the four sites play a potential role in modulating G-protein-mediated inhibition. When the peptide corresponding to residues 410–428 was \textit{in vitro} phosphorylated with PKC, it was no longer able to block G-protein-mediated inhibition (Zamponi et al., 1997). Although there are clearly alternative interpretations, these results strongly suggest that cross-talk between G-protein binding and phosphorylation by PKC does exist for N-type calcium channels, in that phosphorylation of select sites on the I–II linker can prevent interaction with the \(\beta y\) subunit of the G-protein.

The previous findings suggested that one or both of the N-terminal PKC consensus sites within the I–II linker might mediate the interaction between
phosphorylation and G-protein-mediated inhibition of N-type calcium channels. This interaction was investigated further by Hamid et al. (1999). Mutation of the two N-terminal sites (Thr^{422} and Ser^{425}) to alanines resulted in a channel which could still undergo G-protein-mediated inhibition (by application of somatostatin). Unlike the wild-type channel, however, somatostatin-induced inhibition of this mutant channel could not be blocked by activation of PKC, confirming that phosphorylation of either or both of these sites by PKC is critical for preventing G-protein-mediated inhibition. In addition, mutation of Thr^{422} to a glutamate (resulting in a pseudo-phosphorylated residue) significantly reduced somatostatin-induced inhibition. In contrast, mutation of Ser^{425} to either an alanine or glutamate was without obvious effect on G-protein-mediated inhibition or PKC-induced reverse of inhibition, suggesting that phosphorylation of Thr^{422} is both necessary and sufficient to prevent inhibition by G-proteins. Taken together, these results provide evidence that one amino acid in the I–II linker of the α_{1B} subunit (Thr^{422}) serves as the site for cross-talk between G-protein-mediated inhibition and phosphorylation by PKC.

Although the above studies demonstrated that phosphorylation of the I–II linker excludes inhibition by G-proteins, functional evidence that inhibition and phosphorylation are mutually exclusive was lacking. For example, phosphorylation by PKC appears to block the effect of G-proteins, but do G-protein–calcium channel interactions block the effect of PKC? In addition, N-type calcium channels contain PKC consensus sites in addition to those located within the I–II linker (Dubel et al., 1992). For example, several sites exist at the C-terminal tail of the α_{1B} subunit, a region that demonstrates weak
binding to $G_{\alpha}$ and $G_{\beta\gamma}$ proteins (Furukawa et al., 1998; Zamponi et al., 1997). Therefore, in addition to preventing G-protein interactions with the I–II linker, activation of PKC might have other effects on the channel. The experiments detailed in Chapter II of this thesis address these questions. In addition, a preliminary examination of PKC activation on unitary N-type calcium channel activity is presented in Appendix I.

Inhibition of calcium current by a diffusible second-messenger pathway

In addition to modulation by direct interactions with G-proteins, calcium channels in SCG neurons have been shown to exhibit modulation by a diffusible second-messenger pathway (for review, see Hille et al., 1995). This pathway has several characteristics that distinguish it from membrane-delimited inhibition. First, the diffusible second-messenger pathway appears to involve pertussis toxin-insensitive G-proteins (Bernheim et al., 1992). In contrast, membrane-delimited inhibition is mediated by a pertussin toxin-sensitive G-protein, most likely $G_\alpha$ in SCG neurons (Wanke et al., 1987). Second, the diffusible second-messenger pathway develops over seconds, whereas membrane-delimited inhibition is activated in less than a second (Bernheim et al., 1991).

Membrane-delimited inhibition of calcium current induces significant effects on channel gating, leading to slowed activation kinetics and decreased fast inactivation (Chapter II). In contrast, inhibition by the diffusible second-messenger pathways induces no obvious change in activation or inactivation kinetics. In addition, unlike the membrane-delimited pathway, inhibition through the diffusible second-messenger
pathway is calcium-dependent, as it can be blocked by high intracellular concentrations (e.g., 20 mM) of the calcium chelator BAPTA (Beech et al., 1991; Mathie et al., 1992). Finally, in SCG neurons, the two pathways are activated through different G-protein-coupled receptors. The diffusible second-messenger pathway is selectively activated by stimulation of M₁ muscarinic and APY receptors (Wanke et al., 1987; Bernheim et al., 1992; Shapiro et al., 1994), whereas membrane-delimited inhibition is mediated by M₂/M₄ receptors, as well as α₂ adrenergic, somatostatin, and prostaglandin E₂ receptors, to name a few.

*Modulation of N-type calcium channels by arachidonic acid*

Several candidates have been tested as the second messenger involved in mediating M₁ muscarinic inhibition of calcium current. These candidates include cAMP, cGMP, nitric oxide, protein kinases, and others (Wanke et al., 1987; Bley and Tsien, 1990; Beech et al., 1991; Chen and Schofield, 1993; Shapiro et al., 1994). However, the molecule involved has remained unidentified. Using SCG neurons, our lab previously observed that bath application of the fatty acid arachidonic acid (AA) induced inhibition that was highly reminiscent of inhibition by the diffusible second-messenger pathway (L. Liu and A. Rittenhouse, unpublished data). This inhibition induced no obvious slowing of activation or inactivation kinetics, and occluded inhibition by M₁ muscarinic agonists (L. Liu, unpublished data).

In SCG neurons, M₁ muscarinic receptors couple to pertussin toxin-insensitive G₉/₁₁ G-proteins (Caulfield et al., 1994; Delmas et al., 1998; Haley et al., 1998). These
G-proteins activate the enzyme phospholipase A\textsubscript{2} (PLA\textsubscript{2}; Axelrod et al., 1988), which catalyzes the cleavage of the \textit{sn}-2 ester bond in phospholipids to generate free AA and lysophospholipids (Fig. 1-7 A; Balsinde et al., 1999). An alternative pathway for generating AA involves phospholipase C, which liberates diacylglycerol (DAG); AA can then be generated from DAG by the action of the DAG lipase (Fig. 1-7 B). Because the hydrocarbon chain of AA is lipophilic, free AA can readily diffuse across cell membranes; kinetic experiments have estimated that AA can cross lipid bilayers in less than one second (Kamp and Hamilton, 1993). Thus, AA’s effects are not necessarily localized to the cell or cellular compartment from which it is released. In addition, because AA is a major fatty acid in cell membranes, it could represent a readily-available molecule for mediating diffusible modulation of both pre- and postsynaptic neurons.

Because the actions of AA seemed to satisfy the conditions required for mediating diffusible second-messenger–induced inhibition, our lab hypothesized that AA might be involved in mediating M\textsubscript{1} muscarinic inhibition of calcium current in SCG neurons (Liu and Rittenhouse, 1996, 1998). L. Liu in the lab has been testing this hypothesis, using a pharmacological approach to systematically disrupt signaling from the M\textsubscript{1} muscarinic receptor through liberation of AA from the membrane. His results demonstrate that blockade of each step in the pathway, including receptor activation by muscarinic agonists, activation of G\textsubscript{q/11}, activation of phospholipases C and A\textsubscript{2}, and activation of DAG lipase, significantly inhibits M\textsubscript{1}-mediated diffusible second-messenger inhibition of whole-cell currents (Liu and Rittenhouse, 1996, 1998). In contrast, membrane-delimited
inhibition of N-type current was unaffected when these steps were blocked, consistent with this inhibition occurring through a separate pathway.

AA has been shown to modulate calcium currents in a number of cell types, inhibiting current in some cases (Keyser and Alger, 1990; Khurana and Bennett, 1993; Nagano et al., 1995; Schmitt and Meves, 1995) and enhancing current in others (Vacher et al., 1989; Huang et al., 1992; Chesnoy-Marchais and Fritsch, 1994). Previously, our lab examined the effect of exogenous AA on currents recorded from SCG neurons, and found that, at micromolar concentrations, AA inhibits whole-cell currents through both N- and L-type calcium channels (Liu and Rittenhouse, 2000). Moreover, an examination of AA’s effects on the biophysics of these channels revealed that this inhibition is characterized by a dramatic decrease in channel open probability, arising from an increase in mean closed dwell time, first latency and occurrence of null-activity sweeps, suggesting that AA may act by stabilizing the channel in the closed or inactivated state. In contrast, unitary conductance was unaffected by AA, indicating that AA’s inhibitory effects are independent of changes in permeation.

The effects of AA on calcium current are independent of G-proteins. First, the voltage-dependence of AA-induced inhibition is quite different from G-protein–mediated inhibition. When whole-cell currents were measured in the continued presence of the L-type calcium channel agonist (+)-202-791, AA appeared to induce little inhibition at negative voltages, with the greatest inhibition observed positive of 0 mV (Fig. 1-8). In contrast, G-protein–mediated inhibition is greatest at negative voltages, and is undetectable at test potentials more positive than +30 mV (Fig. 1-4). In addition, AA
induces inhibition of whole-cell currents even when GTP is excluded from the pipette recording solution (Liu and Rittenhouse, 2000), suggesting that AA’s effects are mediated downstream of G-protein activation.

Although some fatty acids have been shown to mediate their effects through direct interactions with ion channels (Ordway et al., 1991), it remains unclear if AA or a metabolite inhibits calcium currents in rat SCG neurons through another effector molecule such as an AA-sensitive kinase or phosphatase (Keyser and Alger, 1990; Petit-Jacques and Harzell, 1996). Moreover, the exact mechanism of AA-induced inhibition is unknown. Finally, AA may exert additional, previously unidentified effects on calcium currents in these cells. The experiments outlined in Chapter III and Appendix II were performed to address these questions.
FIGURE 1-1

Voltage-gated calcium channels are composed of several gene products (subunits) which assemble through covalent and noncovalent bonds. At the center of the channel’s structure is the pore-forming $\alpha_1$ subunit, which contains four repeating domains (I–IV), each containing six putative membrane-spanning helices (S1–S6). The $\alpha_1$-interaction domain (AID), residing in the cytoplasmic loop connecting domains I and II, is indicated. Also indicated is one the four voltage sensors, made up of positively-charged amino acids, in S4 of each domain. The synprint site, within the cytoplasmic loop connecting domains II and III, is not visible in this view. Additional subunits are the cytoplasmic $\beta$ subunit (with it’s $\beta$-interaction domain, or BID, indicated), the transmembrane $\gamma$ and $\delta$ subunits, and the extracellular $\alpha_2$ subunit. The two disulfide bonds (S---S) that connect the $\delta$ and $\alpha_2$ subunits are indicated. From Randall and Benham (1999).
FIGURE 1-2

Table depicting the nomenclature of cloned $\alpha_1$ subunits of the various voltage-gated calcium channel subtypes. Proposed names are listed alongside the previous names. Adapted from Ertel et al. (2000).
**FIGURE 1-2**

Proposed Nomenclature for Voltage-Gated Calcium Channels

<table>
<thead>
<tr>
<th>Name</th>
<th>Former Name</th>
<th>Current Type</th>
<th>Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca_{v}1.1 α_{1.1}</td>
<td>α_{1S}</td>
<td>L-type</td>
<td>Skeletal muscle</td>
</tr>
<tr>
<td>Ca_{v}1.2 α_{1.2}</td>
<td>α_{1C}</td>
<td>L-type</td>
<td>Heart, smooth muscle, brain, pituitary, adrenal</td>
</tr>
<tr>
<td>Ca_{v}1.3 α_{1.3}</td>
<td>α_{1D}</td>
<td>L-type</td>
<td>Brain, pancreas, kidney, ovary, cochlea</td>
</tr>
<tr>
<td>Ca_{v}1.4 α_{1.4}</td>
<td>α_{1F}</td>
<td>L-type</td>
<td>Retina</td>
</tr>
<tr>
<td>Ca_{v}2.1 α_{2.1}</td>
<td>α_{1A}</td>
<td>P/Q-type</td>
<td>Brain, cochlea, pituitary</td>
</tr>
<tr>
<td>Ca_{v}2.2 α_{2.2}</td>
<td>α_{1B}</td>
<td>N-type</td>
<td>Brain, nervous system</td>
</tr>
<tr>
<td>Ca_{v}2.3 α_{2.3}</td>
<td>α_{1E}</td>
<td>R-type</td>
<td>Brain, cochlea, retina, heart, pituitary</td>
</tr>
<tr>
<td>Ca_{v}3.1 α_{3.1}</td>
<td>α_{1G}</td>
<td>T-type</td>
<td>Brain, nervous system</td>
</tr>
<tr>
<td>Ca_{v}3.2 α_{3.2}</td>
<td>α_{1H}</td>
<td>T-type</td>
<td>Brain, heart, kidney, liver</td>
</tr>
<tr>
<td>Ca_{v}3.3 α_{3.3}</td>
<td>α_{1I}</td>
<td>T-type</td>
<td>Brain</td>
</tr>
</tbody>
</table>

Adapted from Ertel et al. (2000)
FIGURE 1-3

Schematic of the $\alpha_1$ subunit of the N-type calcium channel, and its sites of interaction with accessory and modulatory proteins. The four repeating domains (I-IV) are indicated. The $\alpha_1$-interaction domain (AID), located within the cytoplasmic linker between the first and second repeats, contains several consensus sites for phosphorylation by protein kinase C (●), as well as binding sites for the calcium channel $\beta$ subunit and the $\beta_y$ subunit of the heterotrimeric G-protein. The carboxy-terminal tail of the $\alpha_1$ subunit, shown at the right, contains an apparent $G_{\alpha}$-binding site within amino acids 1931-1949 of the rabbit $\alpha_{1B}$ subunit. Within this sequence are two putative PKC phosphorylation sites (○). Although not shown in this schematic, the G-protein $\beta_y$ subunit has also been demonstrated to bind to the carboxy-terminal tail of $\alpha_{1B}$. 
FIGURE 1-3

extracellular

S: Serine
T: Threonine

β₃

Gβγ

Gα
FIGURE 1-4

Neurotransmitter application reduces whole-cell calcium currents in sympathetic neurons. Shown are idealized, simulated data adapted from Bean (1989). (A) Idealized data representing the whole-cell current–voltage relationship before (O) and after (●) bath application of neurotransmitter (NT; for example, norepinephrine). Note that, in this example, inhibition by NT is most prominent from −40 mV through +30 mV, and that virtually no inhibition is observed at test potentials more positive than +30 mV. (B) Activation of receptors by neurotransmitter induces a decrease in voltage sensitivity, leading to a positive shift in the activation curve. Simulated, normalized open probability ($P_o$) is plotted against test potential. Shown are data collected in control (—) or following application of neurotransmitter (----).
FIGURE 1-4

A

I (nA)

mV

-100  -50   50   100

-1.2  -0.6   0   0.6  1.2

○ CON

■ NT

B

Normalized P₀

CON  NT

-100  -50   0   50   100

0.0   0.2   0.4   0.6   0.8   1.0
FIGURE 1-5

Model of reluctant/willing gating of voltage-gated N-type calcium channels. A channel not bound by a G-protein resides in the “willing” mode. Upon binding to a G-protein, the channel shifts into the “reluctant” mode, in which transitions from the closed (C) to open (O) state occur significantly more slowly. Note that this model allows for channels to open in the G-protein–bound form (displaying “reluctant” openings).
FIGURE 1-5

"WILLING"

C → → → O

G-Protein

C → → → O

"RELUCTANT"
FIGURE 1-6

Illustration of facilitation, using simulated neuronal whole-cell calcium currents. (A) The voltage protocol used to create the idealized current traces shown in B. The delay between the prepulse to +80 mV and Pulse II is brief (on the order of milliseconds), and the break following Pulse II is on the order of seconds. (B₁) Idealized currents are shown, with a model of the N-type calcium channel shown below. In control solution (in the presumed absence of G-protein activation), application of a test pulse to +10 mV (Pulse I) elicits an inward current, and preceding the test pulse with a prepulse to +80 mV is without effect (Pulse II). (B₂) Activation of G-proteins (either by applying neurotransmitter or dialyzing the cell with GTP-γ-S) leads to G-protein binding to the channel (indicated by the small rectangle associated with the channel). Currents elicited without a prepulse now show G-protein–mediated inhibition, which can be relieved temporarily by applying a prepulse (PP). This relief of inhibition, called facilitation, decays over a course of seconds, during which time the G-protein will re-associate with the channel, leading to recovery of inhibition (Pulse III, lower set of traces).
FIGURE 1-6

A

\[-90 \text{ mV} \quad I \quad +10 \text{ mV} \quad \text{PP} \quad +80 \text{ mV} \quad II \quad \Delta \text{ time} \quad \text{III}\]

B₁  CONTROL

B₂  NT or GTP-γ-S
FIGURE 1-7

Arachidonic acid (cis-5,8,11,14-eicosatetraenoic acid; AA) is a 20-carbon, polyunsaturated fatty acid stored in phospholipid cell membranes. AA can be liberated from membranes by two pathways. (A) Cleavage of the sn-2 ester bond by phospholipase A2 generates free AA and a lysophospholipid. (B) Phospholipase C (PLC) cleaves PI 4,5-bisphosphate (PIP2) into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3). Free AA can then be liberated from DAG by the enzyme DAG lipase.
FIGURE 1-7

A

- membrane phospholipid

  \[
  \text{membrane phospholipid}
  \]

  \[
  \text{phospholipase A}_2
  \]

- free AA extended

  \[
  \text{free AA extended}
  \]

- free AA folded

  \[
  \text{free AA folded}
  \]

B

- PLC

  \[
  \text{PLC}
  \]

- DAG lipase

  \[
  \text{DAG lipase}
  \]
FIGURE 1-8

AA-induced inhibition of whole-cell calcium currents in rat SCG neurons is independent of test potential. Mean whole-cell current–voltage plots (n = 5 cells) were generated before (○) and after (●) bath application of 5 μM AA, in the continued presence of the dihydropyridine L-type calcium channel agonist (+)-202-791 (1 μM). Almost no inhibition occurred at negative voltages, and inhibition was essentially constant from +10 mV through +90 mV. For these experiments, the pipette recording solution contained no GTP analog. Adapted from Liu and Rittenhouse (2000).
FIGURE 1-8

The figure shows a graph plotting pA (picoamperes) on the y-axis against mV (millivolts) on the x-axis. Two conditions are compared: 
- Open circles: (+)-202-791
- Solid circles: (+)-202-791 + AA

The graph demonstrates the modulation of N-type calcium channels in rat SCG neurons under these conditions.
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CHAPTER II

MODULATION OF N-TYPE CALCIUM CHANNEL ACTIVITY BY G-PROTEINS AND PROTEIN KINASE C*

*Adapted from:

ABSTRACT

N-type voltage-gated calcium channel activity in rat superior cervical ganglion (SCG) neurons is modulated by a variety of pathways. Activation of heterotrimeric G-proteins reduces whole-cell current amplitude, whereas phosphorylation by protein kinase C (PKC) leads to an increase in current amplitude. It has been proposed that these two distinct pathways converge on the channel's pore-forming $\alpha_{1B}$ subunit, such that the actions of one pathway can preclude those of the other. In this study we have characterized further the actions of PKC on whole-cell barium currents in neonatal rat SCG neurons. We first examined whether the effects of G-protein-mediated inhibition and phosphorylation by PKC are mutually exclusive. G-proteins were activated by including 0.4 mM GTP or 0.1 mM GTP-γ-S in the pipette and PKC was activated by bath application of 500 nM phorbol 12-myristate 13-acetate (PMA). We found that activated PKC was unable to reverse tonic inhibition unless prepulses were given, indicating that reversal of inhibition by phosphorylation appears to occur only after dissociation of the G-protein from the channel. Once inhibition is relieved, activation of PKC is sufficient to prevent reinhibition of current by G-proteins, indicating that under phosphorylating conditions, channels are resistant to G-protein-mediated modulation. We then examined what effect, if any, phosphorylation by PKC has on N-type barium currents beyond antagonizing G-protein-mediated inhibition. We found that G-protein activation significantly affected peak current amplitude, voltage-dependent inactivation, and voltage-dependent activation. However, when G-protein activation was minimized by dialysis of the cytoplasm with 0.1 mM GDP-β-S, these parameters were not affected by
bath application of PMA. These results indicate that under our recording conditions, phosphorylation by PKC has no effect on whole-cell N-type currents, other than preventing inhibition by G-proteins.

INTRODUCTION

N-type voltage-gated calcium channel activity in rat superior cervical ganglion (SCG) neurons is modulated by a variety of mechanisms. G-protein–coupled, membrane-delimited pathways have been shown to decrease whole-cell barium current amplitude by selective inhibition of N-type current (Plummer et al., 1991; for review, see Hille et al., 1995). There are several features of membrane-delimited inhibition. Current inhibition is mediated by heterotrimeric G-proteins, either activated via G-protein coupled receptors (Wanke et al., 1987; Plummer et al., 1991), or stimulated directly by GTP (Plummer et al., 1989; Ikeda, 1991). G-protein–inhibited whole-cell currents characteristically exhibit slowed or “reluctant” voltage-dependent activation (Bean, 1989). Finally, G-protein–mediated inhibition can be blocked by dialysis of the cell with GDP-β-S, or relieved temporarily by application of a strong positive voltage step (Bean, 1989; Ikeda, 1991).

The rate of reinhibition of currents facilitated by prepulse application is related to the concentration of activated G-proteins. This was demonstrated with increasing concentrations of GTP-γ-S (Lopez and Brown, 1991), neurotransmitter (Erlich and Elmslie, 1995), or free intracellular Gγ (Zamponi and Snutch, 1998). Each of these studies showed that higher levels of G-protein activity resulted in faster rates of
reinhibition following facilitation, thereby suggesting that prepulses lead to dissociation of the G-protein from the channel.

Additional modulation of N-type calcium channel activity exists via phosphorylation by protein kinase C (PKC). Activation of PKC by phorbol esters leads to an enhancement of whole-cell current amplitude in sympathetic neurons, as well as an attenuation of subsequent transmitter-induced, membrane-delimited inhibition (Swartz, 1993; Swartz et al., 1993; Zhu and Ikeda, 1994). Additionally, PKC activation reverses tonic G-protein–mediated inhibition in adult SCG neurons (Swartz, 1993; Zhu and Ikeda, 1994), suggesting that one effect of PKC phosphorylation on whole-cell currents is a relief of G-protein–mediated inhibition. The cytoplasmic linker between the first and second domains of the α1B subunit of the channel has been proposed as one possible site for convergence of these pathways (Zamponi et al., 1997; Hamid et al., 1999).

Previous data (Swartz, 1993; Swartz et al., 1993; Zhu and Ikeda, 1994) have implicated PKC activity in blocking or reversing G-protein–mediated inhibition. Moreover, it has been suggested that G-protein binding to the channel will block the effects of PKC activation (Swartz, 1993). Although these findings suggest mutual exclusivity between these pathways, conclusive evidence to support this hypothesis has not been documented previously. Our results demonstrate that activation of PKC is sufficient to block G-protein–mediated inhibition. In addition, we provide evidence that G-protein binding is sufficient to prevent PKC-induced enhancement of whole-cell current amplitude. Finally, we examined the effect of activating PKC on whole-cell currents in the absence of G-protein–mediated inhibition. Our results show that, when
GDP-β-S is included in the pipette solution, phosphorylation by PKC is without effect on whole-cell current amplitude, voltage-dependent activation, fast inactivation or holding potential-dependent inactivation. Taken together, these data support a model of mutual exclusivity, and suggest that the primary role of phosphorylation by PKC, in this cell type, is to prevent the channel from exhibiting reluctant gating.

METHODS

Cell preparation and culture

Superior cervical ganglia (SCG) were dissected from 1–4 day old Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) and dissociated by trituration (Hawrot and Patterson, 1979). SCG neurons were used for these experiments because approximately 80–90% of the whole-cell barium current is N-type (Plummer et al., 1989). Moreover, this cell type has been shown previously to be sensitive to modulation by G-proteins and phosphorylation by protein kinase C (Swartz, 1993; Zhu and Ikeda, 1994). Cells were maintained in 5% CO₂ at 37°C. The culture medium consisted of DMEM supplemented with 7.5% fetal bovine serum, 7.5% calf serum, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 4 mM L-glutamine (all from Sigma, St. Louis, MO), and 0.2 µg/ml nerve growth factor (Bioproducts for Science, Indianapolis, IN). Following dissociation, cells were plated on poly-L-lysine-coated glass coverslips and incubated for at least four hours prior to recording. Spherical neurons lacking visible processes were selected for recording, and used within 16 hours of preparation.
Electrophysiology

Barium currents were recorded using the whole-cell configuration of an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA). Except where noted, voltage steps to were applied every four seconds from a holding potential of −90 mV. When used, prepulses preceded the test pulse by 5 msec. Currents were recorded at 20–24°C, passed through a 4-pole low-pass Bessel filter at 1 kHz, then digitized at 5 kHz with a 1401plus interface (Cambridge Electronic Design, Cambridge, UK), except the activation data, which were filtered at 5 kHz and digitized at 20 kHz. Data were collected using the Patch software suite, version 6.3 (Cambridge Electronic Design), and stored on a personal computer for offline analysis. Capacitive currents were corrected on-line, and leak currents were subtracted using a scaled-up hyperpolarizing pulse. Pipettes were pulled (model PB-7 puller, Narishige, Tokyo, Japan) from borosilicate capillary tubes (#2-000-210, Drummond Scientific, Broomall, PA) and heat-polished just prior to use (model MF-9 microforge, Narishige), leading to pipette tip resistances ranging from 2–2.5 MΩ. For most recordings, pipette tips were coated with Sylgard (Dow Corning, Midland, MI) to minimize capacitance. Drugs were applied via gravity-driven bath perfusion, with an estimated time to complete bath exchange of 5–10 seconds.

The control bath solution consisted of (in mM): 125 N-methyl-D-glucamine (NMG)–aspartate, 10 HEPES, 20 barium-acetate, 0.0005 tetrodotoxin (TTX), pH 7.5 (296 mOsm). The pipette solution contained (in mM): 122 cesium-aspartate, 10 HEPES,
10 EGTA, 5 MgCl₂, 4 ATP (disodium salt), 0.4 GTP (sodium salt), pH 7.5 (293 mOsm); where indicated, GTP was substituted with 0.1 mM of either GTP-γ-S or GDP-β-S.

Transmitters were excluded from the bath, and G-proteins were directly activated by including GTP or GTP-γ-S in the pipette solution (Ikeda, 1991). This allowed us to avoid receptor desensitization (Huganir and Greengard, 1990), and achieve a reproducible steady-state level of G-protein activation. To minimize modulation of channel activity by a pertussis-toxin-insensitive, calcium-dependent diffusible second-messenger pathway, 10 mM EGTA was included in the pipette solution (Hille et al., 1995).

Pharmacology

Phorbol 12-myristate 13-acetate (PMA) and 4-α-phorbol 12-myristate 13-acetate (4-α-PMA) were obtained from RBI (Natick, MA). GTP-γ-S (tetralithium salt) and GDP-β-S (trilithium salt) were obtained from either RBI or Sigma. The PKC inhibitor bisindolylmaleimide I (BLM) was obtained from Calbiochem (La Jolla, CA), and ω-conotoxin GVIA (CTX) was from Bachem (Torrance, CA). All other chemicals and reagents were obtained from Sigma. Stock solutions of TTX and CTX were prepared in double-distilled water; stock solutions of PMA, 4-α-PMA and BLM were prepared in DMSO. Currents obtained in control bath solution containing the maximal final concentration of DMSO (0.124%) were indistinguishable from solutions lacking DMSO (not shown).
Data analysis

Analysis software included Patch 6.3, Microsoft Excel 97, and Origin 5.0 (Microcal Software, Northampton, MA). Current amplitude was measured isochronically for all recordings. Data are presented as mean ± SEM. Statistical significance was determined using a Student’s two-tailed, paired t-test or a two-way t-test for two means; data were considered significantly different if $P < 0.05$. Sample size is given in parentheses within the figures, unless provided elsewhere. Fraction available was measured as the ratio of current amplitude at the end of the test pulse to the amplitude at the onset of the test pulse (see Fig. 2-3A); this method of measuring fast inactivation has been described previously as residual fraction of peak current (de Leon et al., 1995). The Boltzmann fits presented in Fig. 2-8, and the data shown in Table 1, were calculated using the equation:

$$\frac{I}{I_{\text{max}}} = \frac{I_1 - I_2}{1 + e^{(V-V_h)/k}} + I_2$$

where $I/I_{\text{max}}$ is normalized current; $V$ is voltage in mV; $V_h$ is the voltage at half-maximal $I/I_{\text{max}}$; $k$ is the slope factor of activation at $V_h$, in mV/e-fold change in $I/I_{\text{max}}$; and $I_1$ and $I_2$ are the minimum and maximum values of $I/I_{\text{max}}$, respectively.

RESULTS

Neonatal Rat SCG neurons exhibit tonic G-protein–mediated inhibition

To examine the effects of PKC on whole-cell currents, it was necessary to first confirm that, under our recording conditions, tonic inhibition of whole-cell currents by G-
proteins could be observed, and that activating PKC could block this inhibition. Tonic inhibition, induced by activation of endogenous heterotrimeric G-proteins, was observed in neonatal rat SCG neurons in the absence of G-protein–coupled receptor activation, when GTP was included in the pipette solution. Stepping from −90 mV to +10 mV (Fig. 2-1 A) elicited an inward current whose amplitude was greatly facilitated if this step was preceded by a pulse to +80 mV (Fig. 2-1 B, left panel and Fig 2-2). Four seconds later, current amplitude had returned to baseline, consistent with time-dependent recovery of tonic G-protein–mediated inhibition (Lopez and Brown, 1991; Zamponi and Snutch, 1998).

The increase in current amplitude following a prepulse was voltage-dependent, as shown by the current–voltage analysis (Fig. 2-1 B, right). Maximal facilitation was observed from 0 mV to +10 mV, and dropped to undetectable levels as the test pulse became more positive, consistent with voltage-dependent relief of G-protein–mediated inhibition (Bean, 1989; Ikeda, 1991). Because both maximal inward current and maximal facilitation were observed at a test potential of +10 mV, this voltage was used for subsequent experiments.

Tonic inhibition of whole-cell currents displayed additional characteristics that have been described previously. In addition to decreased current amplitude, G-protein–inhibited whole-cell currents also exhibited slowed activation kinetics and increased facilitation; these effects were more pronounced when GTP-γ-S was substituted for GTP in the pipette solution (Figs. 2-1 C and 2-2). In contrast, G-protein–mediated inhibition
was minimized by dialysis of the cell with GDP-β-S (Fig. 2-1 D). This was reflected by a loss of prepulse-induced facilitation (Fig. 2-2).

In addition to slowing voltage-dependent activation, modulation by G-proteins has also been shown to decrease voltage-dependent fast inactivation (Netzer et al., 1994). To confirm that the currents recorded under our conditions also displayed a decrease in fast inactivation as a result of modulation by G-proteins, we quantified inactivation by measuring the fraction of initial inward current remaining at the end of the test pulse (Fig. 2-3; see also Fig. 2-1). As expected, currents elicited in control bath solution, with GTP in the pipette, showed little inactivation, and application of a prepulse greatly increased fast inactivation. Dialyzing the cell with GDP-β-S had essentially the same effect on fast inactivation as prepulses. In contrast, dialysis with GTP-γ-S greatly decreased inactivation, although application of a prepulse was sufficient to increase fast inactivation to the same level as with GDP-β-S.

Taken together, these results verify that, under the conditions used in this study, inhibition of whole-cell currents by G-proteins is readily observable. Moreover, the effects of this inhibition on current amplitude, facilitation, and kinetics can be completely reversed either by applying prepulses or by including GDP-β-S in the pipette solution.

PMA enhances whole-cell N-type currents and prevents tonic G-protein–mediated inhibition

PKC activation has been shown previously to enhance whole-cell currents and lead to a reduction in G-protein–mediated inhibition in SCG neurons (Swartz, 1993; Zhu
and Ikeda, 1994), thereby minimizing prepulse facilitation. Activating PKC had similar effects under our recording conditions. When the phorbol ester PMA (500 nM) was added to the bath of the cell shown in Fig. 2-1 B, current amplitude significantly increased (Figs. 2-1 E and 2-4). Application of a prepulse following PMA treatment had no effect on amplitude (Figs. 2-1 E and 2-4), consistent with previous findings that PMA reduces G-protein–mediated inhibition and prepulse facilitation. PMA increased current amplitude over a range of test potentials (Fig. 2-1 E, right), consistent with a loss of voltage-dependent inhibition. In addition to modulating current amplitude and facilitation, PMA also affected fast inactivation, significantly decreasing fraction remaining to a level not significantly different than that observed following dialysis with GDP-β-S (Figs. 2-1 and 2-3).

If PKC activation is sufficient to account for the increased current amplitude, decreased facilitation, and altered kinetics observed in cells that show tonic inhibition, then we would predict that these three parameters should change along a similar time course. This was addressed by measuring each of these parameters in a single recording (Fig. 2-5). Indeed, as expected, following application of PMA to the bath, the changes observed in facilitation and fraction remaining closely paralleled the change in unfacilitated current amplitude.

To confirm that the effects observed following PMA application were due to activation of PKC, we conducted two control experiments (Figs. 2-2, 2-3 and 2-4). First, the inactive PMA analog 4-α-PMA (Van Duuren et al., 1979) was applied to the bath to determine whether any of PMA’s effects on currents were due to nonspecific actions of
phorbol esters. 4-α-PMA was without effect on facilitation, current amplitude, or fast inactivation. Second, to determine whether PMA’s effects were due to selective activation of PKC, the PKC-specific inhibitor BLM (Toullec et al., 1991) was applied to the bath prior to application of PMA. BLM effectively blocked PMA’s ability to affect facilitation, current amplitude, and inactivation, indicating that PMA’s effects on whole-cell currents are mediated selectively through PKC activation.

Because neonatal rat SCG neurons contain additional types of calcium current, the changes in whole-cell currents observed following PMA application could be due to actions on currents other than N-type. Therefore, to examine the effects of PMA on non-N-type currents, cells were incubated in a solution containing the N-type calcium channel blocker ω-conotoxin GVIA (ω-CgTX; 1 μM) for ten minutes prior to patch-clamp recording (Figs. 2-2 and 2-4). ω-CgTX was excluded from the recording solutions to allow washout of any reversible block of non–N-type calcium channels (McCleskey et al., 1987; Plummer et al., 1989). Consistent with neonatal SCG neurons having mostly N-type calcium channels, ω-CgTX blocked 80–85% of the whole-cell current (not shown). As previously demonstrated in adult rat SCG neurons (Ikeda, 1991), facilitation of whole-cell currents was lost following treatment with ω-CgTX (Fig. 2-2), consistent with G-protein–mediated inhibition being restricted to N-type currents. Even after N-type channels were blocked, however, bath application of 500 nM PMA caused a slight but significant increase in the amplitude of currents elicited both with and without a prepulse (Fig. 2-4). There was no significant difference between the fold change of unfacilitated and facilitated currents, suggesting that PMA’s effect on non–N-type current
is independent of voltage. These data are consistent with previous studies of rat CA3 hippocampal neurons (Swartz, 1993), frog sympathetic neurons (Yang and Tsien, 1993), and adult rat SCG neurons (Zhu and Ikeda, 1994), in which phorbol esters increased current amplitudes after application of ω-CgTX, suggesting that non–N-type calcium channel activity might also be modulated by protein kinase C. This increase was approximately 15% of currents already inhibited at least 80% by ω-CgTX treatment. Therefore, the contribution of non–N-type calcium current in response to PMA in untreated cells can be considered negligible and is not considered further.

G-protein–mediated inhibition blocks PKC’s effect on whole-cell currents

We observed that PMA only affected whole-cell currents that demonstrated G-protein–mediated inhibition, causing a relief of that inhibition (Figs. 2-1 and 2-4). Moreover, PMA appeared to act faster when prepulses were applied during the recording. This is consistent with the previous observation that relatively long prepulses were required to demonstrate PMA’s effect on G-protein–mediated inhibition (Swartz, 1993). Because prepulses are thought to dissociate the G-protein from the channel (Lopez and Brown, 1991; Zamponi and Snutch, 1998), we examined whether phosphorylation by PKC occurs only when the channel is not associated with a G-protein. To address this, we measured the effect of prepulses on PMA’s ability to enhance maximally inhibited currents; i.e., when GTP-γ-S was included in the pipette solution.

After membrane breakthrough, current amplitude rapidly decreased (Fig. 2-6 A). This decrease was due to influx of GTP-γ-S and subsequent activation of G-proteins, as
application of a prepulse was sufficient to restore current amplitude. Currents were then elicited without prepulses, to minimize dissociation of G-protein–channel interactions. In the absence of prepulses, PMA was essentially without effect on whole-cell current amplitude (Fig. 2-6 C). Even after 8 minutes of stimulation with PMA, considerable G-protein–mediated inhibition remained, as prepulses could still facilitate current amplitude (Fig. 2-6 A).

In separate experiments, currents were recorded in which every other test pulse to +10 mV was preceded by a pulse to +80 mV (Fig. 2-6 B). Under these conditions, application of PMA was sufficient to enhance whole-cell current amplitude. Because GTP-γ-S increases the level of available G\textsubscript{P}{\gamma} subunits in the cytoplasm, we would expect PMA to have a slower effect when recording with GTP-γ-S than with GTP. We therefore measured the time constants of PMA’s effect when prepulses were applied under both conditions. The time constant observed was 3.85 ± 0.69 minutes (n = 13) with GTP, and 5.18 ± 0.68 minutes (n = 3) with GTP-γ-S. In addition, because GTP-γ-S leads to a greater inhibition of current than does GTP (Figs. 2-1 and 2-2), we would expect PMA to cause a greater enhancement of whole-cell currents after dialysis of GTP-γ-S. As predicted, when prepulses were applied throughout the recording, PMA increased current amplitude approximately 2-fold (Fig. 2-6 C), compared to 1.3-fold with GTP (Fig. 2-4). These results indicate that phosphorylation occurred only after G-proteins were displaced from the channel. Together with the finding that phosphorylation by PKC prevents G-protein–mediated inhibition, these findings support mutual exclusivity between G-protein binding and phosphorylation.
GDP-β-S precludes PKC-mediated enhancement of current amplitude

The above data indicate that PKC activation prevents G-protein-mediated inhibition. However, it is unclear whether PKC affects whole-cell currents in additional ways. Therefore, we next examined whether PKC modulates whole-cell currents in the absence of G-protein activity. When GDP-β-S was included in the pipette, we observed an increase in current amplitude following membrane breakthrough (not shown), consistent with previously published results (Netzer et al., 1994). This increase is believed to be the result of a loss of tonic G-protein-mediated inhibition, an hypothesis supported by the complete loss of observable facilitation (Figs. 2-1 D and 2-2). Once this effect reached steady-state (typically within one minute of membrane breakthrough), we studied the effect of activating PKC on whole-cell currents. Unlike the significant increase observed when GTP or GTP-γ-S was included in the pipette, PMA had no significant effect on current amplitude when GDP-β-S was used (Fig. 2-4).

Holding potential–induced inactivation of whole-cell currents is not affected by PKC activation

Although it did not affect current amplitude, PKC activation might affect other properties of the current. Thus, we next examined whether phosphorylation has an effect on holding potential–dependent inactivation. Data were collected from 100-msec test pulses to +10 mV, preceded by 2.2-second prepulses to varying potentials. For these experiments, no attempt was made to isolate fast inactivation from steady-state
inactivation; hence all inactivation measured with this protocol was defined as holding potential-induced inactivation (Fox et al., 1987; Jones and Marks, 1989; Patil et al., 1998). Normalized inactivation curves generated with this protocol are shown in Fig. 2-7 A. When GDP-β-S was included in the pipette, normalized current amplitude decreased as holding potential became less negative, reaching a minimum of ~0.3 at around +20 mV. A slight but statistically significant recovery from inactivation was observed as the holding potential became more positive; this is consistent with previous reports of "U"-shaped inactivation curves recorded under similar conditions (Patil et al., 1998). Bath application of PMA was without effect on holding potential-induced inactivation, suggesting that phosphorylation, in the absence of G-protein activation, has no effect on inactivation. In contrast, including GTP in the pipette led to a lower degree of holding potential-induced inactivation, suggesting a possible role of G-protein modulation in protecting the channels from this form of inactivation.

Changes in holding potential can also affect fast inactivation (Patil et al., 1998). Therefore, analyzing the same recordings presented in Fig. 2-7 A for fraction remaining provided an additional means of examining the effects of phosphorylation on inactivation in the absence of modulation by G-proteins. Fig. 2-7 B shows that, at negative holding potentials, cells containing GTP showed little fast inactivation, whereas cells containing GDP-β-S showed significantly more, consistent with the data presented in Fig. 2-3. At less negative holding potentials, fast inactivation decreased, reaching a fraction remaining of ~1. Applying PMA to cells dialyzed with GDP-β-S was without significant effect on
fraction remaining, demonstrating that phosphorylation by PKC, by itself, does not affect fast inactivation of channels.

*Stimulation of PKC does not affect voltage-dependent activation of whole-cell currents*

Our data suggest that G-protein–mediated inhibition shifts the voltage-dependence of current activation to more positive voltages (Fig. 2-1). Moreover, previous data (Zhu and Ikeda, 1994) demonstrated a voltage-dependent increase in tail current amplitude following application of PMA. Based on these findings, we hypothesized that this increase was due to a relief of G-protein–mediated inhibition. To test this, voltage-dependent activation was examined under various recording conditions. First, activation was measured when endogenous G-proteins were maximally stimulated by dialysis with GTP-γ-S (Fig. 2-8 B). Consistent with relief of G-protein–mediated inhibition, prepulses significantly facilitated voltage-dependent activation, without shifting the threshold of activation. Cells dialyzed with GDP-β-S (Fig. 2-8 C) displayed voltage-dependent activation that was similar to cells recorded with prepulses following GTP-γ-S dialysis (see Table 2-1). This is consistent with a loss of G-protein–mediated inhibition. Subsequent application of PMA had no effect on voltage-dependent activation, supporting the hypothesis of Zhu and Ikeda’s (1994) that the increase in tail current amplitude was due to the loss of G-protein–mediated inhibition. More importantly, these results indicate that activation of PKC, in the absence of G-protein–mediated inhibition, does not affect activation of whole-cell currents.
DISCUSSION

In this study, we sought to determine what effect, if any, activation of PKC by PMA has on whole-cell barium currents in neonatal rat SCG neurons, in the absence of G-protein modulation. Before proceeding with this analysis, however, we established that, under our recording conditions, whole-cell currents can undergo voltage-dependent G-protein–mediated inhibition, and that this inhibition is restricted to N-type currents (Plummer et al., 1991). Moreover, dialysis with GDP-β-S was sufficient to minimize this inhibition. We also confirmed that, in cells dialyzed with GTP, activation of PKC reduced prepulse facilitation, enhanced current amplitude, and increased fast inactivation. In addition, PMA reversed tonic inhibition, as demonstrated by comparing the effects of PMA with the effects of either applying prepulses or dialyzing the cell with GDP-β-S. Finally, as previously demonstrated (Zhu and Ikeda, 1994), we found that activation of PKC enhanced non–N-type currents present in this cell type.

Having confirmed the presence of tonic G-protein–mediated inhibition, and its modulation by PKC, we examined whether these two mechanisms can preclude one another. By examining the effect of PMA on current amplitude in the absence of prepulses, we demonstrated that G-protein–mediated inhibition is sufficient to block the effects of PKC activity. Moreover, our findings are consistent with the hypothesis that activation of PKC is sufficient to block G-protein–mediated inhibition. These results support a model of mutual exclusivity between phosphorylation and G-protein–mediated inhibition, consistent with previously published results (Swartz, 1993; Zhu and Ikeda, 1994; Zamponi et al., 1997; Hamid et al., 1999).
Lastly, we examined whether PKC activation in the absence of G-protein-mediated inhibition causes additional modulation of whole-cell currents. When inhibition was first minimized by including GDP-β-S in the pipette solution, bath application of PMA was without effect on current amplitude, fast and holding potential-dependent inactivation, or voltage-dependent activation, suggesting that PKC’s only role in modulating N-type currents is to block G-protein-mediated inhibition. These results are somewhat surprising, since multiple putative PKC consensus sites are present on the pore-forming α1B subunit (Dubel et al., 1992). However, these findings are consistent with some recombinant studies that indicate that phosphorylation of sites in the I–II linker of the α1B subunit can account for the loss of inhibition by G-proteins (Zamponi et al., 1997; Hamid et al., 1999).

N-type calcium channels inhibited by G-proteins have been termed “reluctant” by Bean (1989), reflecting the channel’s diminished response to changes in membrane potential (Wanke et al., 1987; Bean, 1989; Ikeda, 1991; Patil et al., 1996). Because G-protein binding to the channel is sufficient to block PKC’s effects, “reluctant” can be further defined as “reluctant and P-resistant”, indicating that the channel is not only reluctant to open, but also resistant to phosphorylation by PKC (Fig. 2-9). This G-protein–bound form of the channel may be important in mediating readily reversible modulation of neurotransmitter release (Koh and Hille, 1997).

In contrast, channels not inhibited by G-proteins have been called “willing”, indicating a more rapid response to changes in membrane potential (Bean, 1989). Channel activity can shift from reluctant to willing with the application of a strong
positive voltage pulse (Bean, 1989; Ikeda, 1991), or by a train of action potentials (Williams et al., 1997), leading to facilitated whole-cell currents. Our results indicate the existence of at least two distinct willing forms of the channel, with phosphorylation by protein kinase C serving as the molecular switch between them (Fig. 2-9). Channels not modulated by either G-proteins or phosphorylation constitute one willing form, which we call “willing and available”, as these channels are willing to open, and are available for modulation by either G-protein activation or phosphorylation by PKC. Subsequent phosphorylation by PKC drives the channel into a second willing form, called “willing and G-resistant”, meaning willing to open, but resistant to G-protein modulation. In contrast to the P-resistant form, this resistant form would allow for long-term facilitation of calcium currents in synaptic membranes.

In conclusion, we have confirmed that N-type calcium channel activity in neonatal rat SCG neurons undergoes voltage-dependent G-protein–mediated inhibition. In addition, stimulation of PKC enhances whole-cell barium currents by blocking this inhibition. Moreover, when G-proteins are activated with GTP-γ-S, enhancement by PMA only occurs following prepulses, indicating that G-proteins must dissociate from the channel in order to observe the effects of phosphorylation by PKC. Finally, we have demonstrated that, under our recording conditions, there appears to be no functional effect of phosphorylation by PKC on N-type calcium channel activity beyond causing a long-term block of G-protein–mediated inhibition. Because of the existence of many PKC consensus sites on the N-type calcium channel (Dubel et al., 1992), future studies using other recording conditions might reveal additional effects of phosphorylation.
FIGURE 2-1

Modulation of whole-cell barium currents by G-proteins and PMA. (A) The voltage protocol used to elicit the currents shown in B-E. (Left) whole-cell sweeps; (right) current-voltage plots elicited with (●, n = 6–12) or without (○, n = 7–20) prepulses to +80 mV. In this and subsequent figures, the delay between the prepulse and test pulse is 5 msec, and error bars not visible are contained within the symbols. (B) Whole-cell currents were elicited in control bath solution with GTP in the pipette. (C) GTP-γ-S was substituted for GTP in the pipette solution. (D) GDP-β-S was substituted for GTP in the pipette. (E) The same cell shown in B, 2 minutes after bath application of 500 nM PMA. For all sweeps, the horizontal calibration bars are 100 msec, and the vertical calibration bars are 500 pA.
FIGURE 2-1

A

-90 mV

+10 mV

+80 mV

1 sec

4 sec

+10 mV

IBa (nA)

B

GTP

C

GTP-γ-S

D

GDP-β-S

E

GTP + PMA
FIGURE 2-2

Summary of facilitation, expressed as the ratio of current amplitude following a prepulse to current amplitude before a prepulse. Control facilitation was 1.71 ± 0.06 (n = 34). Application of 500 nM PMA reduced facilitation to 1.01 ± 0.02 (n = 19). In contrast, 500 nM 4-α-PMA, 100 nM BLM, and PMA following BLM were all without significant effect. Substituting 0.1 mM GDP-β-S for GTP in the pipette solution decreased facilitation to 0.91 ± 0.02 (n = 11), whereas substituting 0.1 mM GTP-γ-S for GTP increased facilitation to 2.76 ± 0.14 (n = 9). Following a 10-minute incubation with 1 μM ω-CgTX, control facilitation was lost (0.92 ± 0.03; n = 12), and subsequent application of PMA was without further effect (0.91 ± 0.04; n = 4). *P < 0.001 compared with control.
FIGURE 2-2

Facilitation

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Facilitation</th>
<th>Number of Measurements</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON (34)</td>
<td>*</td>
<td>10</td>
</tr>
<tr>
<td>PMA (19)</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>4-α-PMA (7)</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>BLM (8)</td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>BLM + PMA (8)</td>
<td>*</td>
<td>10</td>
</tr>
<tr>
<td>GDP-β-S (11)</td>
<td>*</td>
<td>10</td>
</tr>
<tr>
<td>GTP-γ-S (9)</td>
<td>*</td>
<td>10</td>
</tr>
<tr>
<td>ω-CgTX (12)</td>
<td>*</td>
<td>10</td>
</tr>
<tr>
<td>ω-CgTX + PMA (4)</td>
<td></td>
<td>10</td>
</tr>
</tbody>
</table>
FIGURE 2-3

Prepulses and PMA both increase voltage-dependent fast inactivation. (A) Fast inactivation is measured as the fraction of the initial inward current (line 1) that remains at the end of a 100-msec test pulse (line 2). (a) Control current elicited by the voltage command shown; (b) current elicited following bath application of 500 nM PMA. (B) Summary of fraction remaining, as defined in A, for unfacilitated (no prepulse, hatched columns) and facilitated (prepulse to +80 mV, solid columns) whole-cell currents. In control solution, application of a prepulse considerably reduced the fraction remaining from 0.91 ± 0.02 to 0.77 ± 0.01 (n = 30). Bath application of 500 nM PMA (n = 15) led to a reduction in unfacilitated fraction remaining (0.74 ± 0.02), whereas facilitated currents in PMA were not significantly different than facilitated control currents (0.80 ± 0.01). 500 nM 4-α-PMA, 100 nM BLM, and PMA following BLM were all without effect. As with PMA, dialysis with GDP-β-S (n = 11) reduced the unfacilitated fraction remaining (0.71 ± 0.01), but was without effect on facilitated currents (0.78 ± 0.01). In contrast, dialysis with GTP-γ-S (n = 7) greatly increased the unfacilitated fraction remaining (1.11 ± 0.04), but, as with all other treatments, was without effect on facilitated currents (0.74 ± 0.02). *P < 0.05 compared with unfacilitated control.
FIGURE 2-3

A

B

Fraction Remaining

CON (30)  PMA (15)  4-α-PMA (7)  BLM (8)  BLM + PMA (8)  GDP-β-S (11)  GTP-γ-S (7)
FIGURE 2-4

Summary of fold change in peak inward current amplitude for unfacilitated (no prepulse, hatched columns) and facilitated (prepulse to +80 mV, solid columns) whole-cell currents elicited at +10 mV. 500 nM PMA ($n = 18$) increased unfacilitated currents by $1.31 \pm 0.05$-fold, but was without effect on facilitated currents ($0.92 \pm 0.04$-fold). 500 nM 4-α-PMA was without effect, and 100 mM BLM blocked PMA’s effects. Application of PMA following dialysis with GDP-β-S ($n = 8$) was without effect ($1.03 \pm 0.09$-fold for unfacilitated, and $0.94 \pm 0.06$-fold for facilitated). Following a ten-minute preincubation in 1μM ω-CgTX ($n = 8$), PMA caused slight but significant increases in both unfacilitated and facilitated currents ($1.14 \pm 0.02$-fold and $1.16 \pm 0.02$-fold, respectively). *$P < 0.005$ compared with control.
FIGURE 2-4

Fold Change in I_{Ba} vs Control

- PMA (18)
- 4-α-PMA (7)
- BLM (8)
- BLM + PMA (8)
- GDP-β-S + PMA (8)
- ω-CgTX + PMA (8)
FIGURE 2-5

The effects of PMA on current amplitude, facilitation, and fast inactivation follow a parallel time course in a single whole-cell recording. (A and C) Open symbols represent currents elicited without a prepulse; closed symbols represent currents elicited following a prepulse to +80 mV. 500 nM PMA was applied to the bath where indicated by the solid bar. (A) Time course of peak inward current. (B) Time course of facilitation as calculated in Fig. 2-2. (C) Fraction remaining was calculated as in Fig. 2-3 and plotted against time.
FIGURE 2-5

A

\[ I_{Ba} \text{ (-pA)} \]

- Prepulse
- + Prepulse

B

Facilitation

C

Fraction Remaining

- Prepulse
- + Prepulse

Time (min)
FIGURE 2-6

G-protein-mediated inhibition blocks PMA’s effects on whole-cell currents. (A–B) Peak inward currents were elicited by stepping to +10 mV and plotted against time (left). GTP-γ-S was included in the pipette solution, and 500 nM PMA was applied to the bath as indicated by the solid bars. At the right are individual sweeps from the times indicated. (A) Currents were elicited without applying prepulses, except where indicated by ○, which were evoked following a prepulse to +80 mV. (B) Currents were elicited using the voltage protocol shown in A; prepulses were applied every other sweep, and currents evoked without a prepulse are plotted. (C) Summary of the fold change in current amplitude following application of PMA. In the absence of prepulses, PMA caused a 1.04 ± 0.13-fold change in current amplitude (n = 4). In contrast, when alternating test pulses were preceded by prepulses, applying PMA increased current amplitude 2.12 ± 0.17-fold (n = 4). *P < 0.005 compared with those without prepulses.
FIGURE 2-6

A

- Prepulses

B

+ Prepulses

C

- Prepulses

+ Prepulses

Fold Change

After PMA

Time (min)
FIGURE 2-7

Activation of PKC does not affect holding potential–dependent inactivation. Currents were elicited by first applying a 2.2-second prepulse of varying voltage, followed 5 msec later by a 100-msec step to +10 mV. ○ were obtained with GTP in the pipette \((n = 6)\), □ were obtained with 0.1 mM GDP-β-S substituted for GTP \((n = 7)\), and ■ were obtained with 0.1 mM GDP-β-S in the pipette and 500 nM PMA in the bath \((n = 6)\). The symbol key pertains to both A and B. (A) Normalized holding potential–induced inactivation plots were generated by measuring the peak inward current during test pulses to +10 mV from each holding potential, then dividing by the maximum current obtained. No statistically significant difference exists between the two sets of GDP-β-S data. (B) The fraction of current remaining was calculated as in Fig. 2-3. No statistically significant difference exists between the two sets of GDP-β-S data. *\(P < 0.05\) versus GDP-β-S and versus GDP-β-S + PMA.
FIGURE 2-7

A

Normalized Current

- GTP
- GDP-β-S
- GDP-β-S + PMA

B

Fraction Remaining

Holding Potential (mV)

-120 -80 -40 0 40 80
FIGURE 2-8

Activation of PKC does not alter the voltage dependence of activation of whole-cell barium currents. (A) Activation plots were generated using a voltage command consisting of 15-msec pulses to incremental test potentials, either with or without a prepulse to +80 mV. Shown are two currents elicited, at the voltage indicated, with GDP-β-S in the pipette solution. Fast tail current amplitudes (A, inset) were measured at the dashed line, normalized, then plotted against test potential (B). Boltzmann fits were applied to the data (solid lines), and all curves were fit with a Chi-square value of <0.002. (B) When 0.1 mM GTP-γ-S was included in the pipette solution, the threshold for activation was approximately −20 mV (−PP, ○). Applying prepulses (+PP, ●) was without effect on the threshold of activation, but the voltage eliciting half-maximal activation was shifted negative. *P < 0.01, versus no prepulse. (C) When the pipette solution contained 0.1 mM GDP-β-S, unfacilitated control (◇) and PMA (500 nM, △) activation plots were virtually indistinguishable. See Table 2-1 for values and sample sizes.
FIGURE 2-8

A

B

C

Normalised Tail Current

-60 -30 0 30 60 90

mV

1.2

0.8

0.4

0.0

GTP-γ-S

+ PP

- PP

GDP-β-S

CON

PMA

mV

-60 -30 0 30 60 90

mV

* * * * *
FIGURE 2-9

Phosphorylation by PKC and modulation by G-proteins are mutually exclusive. Unmodulated N-type calcium channels are willing and available for either G-protein modulation or phosphorylation by PKC (Willing/Available). G-protein binding leads to a reluctant channel that is resistant to phosphorylation (Reluctant/P-Resistant). Alternatively, phosphorylation by PKC leads to a willing channel that is resistant to G-protein interactions (Willing/G-Resistant). Note that shifting from P-reluctant to G-reluctant (and vice versa) requires a transition through the willing and available form of the channel.
FIGURE 2-9

Reluctant/ P-Resistant  Willing/ Available  Willing/ G-Resistant

N-type Calcium Channel

Phosphorylation by Protein Kinase C  G-protein

P
TABLE 2-1

Boltzmann Analysis of Activation Curves

<table>
<thead>
<tr>
<th>Bath Solution Contains:</th>
<th>Pipette Solution Contains:</th>
<th>Prepulse</th>
<th>n</th>
<th>Vh (mV)*</th>
<th>k ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>○ § GTP-γ-S</td>
<td>–</td>
<td>6</td>
<td>36.9</td>
<td>19.8</td>
</tr>
<tr>
<td>Control</td>
<td>● GTP-γ-S</td>
<td>+</td>
<td>6</td>
<td>10.6</td>
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</tr>
<tr>
<td>BLM</td>
<td>GTP-γ-S</td>
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<td>6</td>
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<tr>
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<td>7</td>
<td>1.2</td>
<td>4.1</td>
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</table>

* Voltage eliciting half-maximal activation
‡ Slope constant (mV/e-fold change)
§ Symbols correspond to Fig. 8
REFERENCES


CHAPTER III

ARACHIDONIC ACID REVERSIBLY ENHANCES N-TYPE CALCIUM CURRENT AT AN EXTRACELLULAR SITE*

*Adapted from:

ABSTRACT

We examined the effects of arachidonic acid (AA) on whole-cell calcium channel activity in rat superior cervical ganglion neurons. Our companion paper (see Appendix II) demonstrates that AA induces several effects, including enhancement of current amplitude at negative voltages, and increased activation rate. This study examines the mechanisms underlying these effects. First, enhancement is rapidly reversible by bath application of bovine serum albumin. Second, enhancement appears to occur extracellularly, since intracellular albumin was without effect on enhancement, and bath-applied arachidonoyl coenzyme A, an amphiphilic AA analog that cannot cross the cell membrane, mimicked enhancement. In addition, enhancement is voltage-dependent, in that currents were enhanced to the greatest degree at −10 mV, whereas virtually no enhancement occurred positive of +30 mV. We also demonstrate that the AA-induced increase in activation kinetics is correlated with enhancement of current amplitude. An observed increase in the voltage sensitivity of activation may underlie these effects. Finally, the majority of enhancement is mediated through N-type current, providing the first demonstration that this current type can be enhanced by AA.

INTRODUCTION

In rat superior cervical ganglion (SCG) neurons, the polyunsaturated fatty acid arachidonic acid (AA; 20:4, n-6) is a potent inhibitor of calcium channel activity. When unitary currents were elicited in cell-attached patches, using barium as the charge carrier, applying 5 μM AA led to a significant decrease in the activity of both L- and N-type
calcium channels (Liu and Rittenhouse, 2000). In our companion paper (Liu et al., 2001), we examined the effects of exogeneous AA on whole-cell currents in SCG neurons. In doing so, we discovered that AA induces several effects in addition to inhibiting L- and N-type currents. Consistent with inhibition, AA increased holding potential-dependent inactivation, in agreement with our observation that AA increases the occurrence of null sweeps in cell-attached patch recordings of single L- and N-type calcium channels (Liu and Rittenhouse, 2000). In addition, AA increased the rate of whole-cell current activation. This effect was largely selective for N-type calcium channels, and was independent of G-protein activity.

AA also induced a significant increase in current amplitude at negative voltages (Liu et al., 2001). This enhancement could be separated from inhibition, as dialyzing the cell with bovine serum albumin (BSA), a protein that can bind fatty acids (Spector, 1975), blocked the majority of inhibition, but was without effect on enhancement. Finally, as with both inhibition and faster activation, enhancement is independent of G-protein activity.

Although AA-induced enhancement of calcium currents has been reported in other preparations (Chesnoy-Marchais and Fritsch, 1994; Huang et al., 1992; Vacher et al., 1989), this effect has not been observed previously in neurons. Because calcium entry through neuronal voltage-gated calcium channels can play an important role in coordinating electrical signaling with cellular processes like neurotransmitter release and enzyme activation (Berridge, 1998; Rittenhouse and Zigmond, 1999), a pathway that induces enhanced calcium influx in neurons is of particular interest. Therefore, the
The purpose of this study was to examine the mechanism and properties of AA-induced enhancement of calcium currents in SCG neurons. In this report, we present the first evidence that AA reversibly enhances whole-cell N-type current by a mechanism distinct from its inhibitory effects on L- and N-type currents. This effect is voltage-dependent, with the greatest enhancement observed at −10 mV. In addition, enhancement does not require AA metabolism, and appears to be mediated extracellularly. Finally, our results indicate that the mechanism of AA-induced enhancement involves both an increase in activation rate and an increase in voltage sensitivity. Together with the accompanying paper, our findings add to the growing body of evidence that AA can exert a diverse range of effects on neuronal calcium currents.

METHODS

Preparation and culture of SCG neurons

Following decapitation, the superior cervical ganglia (SCG) were removed from 1–4 d old Sprague-Dawley rats (Charles River Laboratories, Inc., Wilmington, MA), and neurons were dissociated by trituration through a 22-gauge, 1½-inch needle. Following dissociation, cells were plated on poly-L-lysine-coated glass coverslips in 35-mm culture dishes, and incubated at least 4 hours prior to recording (Hawrot and Patterson, 1979). Cells were maintained in 5% CO₂ at 37°C, in DMEM supplemented with 7.5% fetal bovine serum, 7.5% calf serum, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 4 mM L-glutamine (all from Sigma Chemical Co., St. Louis, MO), and 0.2 μg/ml nerve growth
factor (Bioproducts for Science, Indianapolis, IN); cells were used within 12 hours of preparation. Spherical neurons lacking visible processes were selected for recording.

**Preparation and culture of tsA201 cells**

The SV40 large T antigen–transformed HEK 293 subclone tsA201, transfected to stably express the rat N-type calcium channel α1B-a splice variant (ΔA415/ΔSFMG/+ET; see Table 2 in Lin et al., 1997) together with rat neuronal–derived β3 and α2/δ-1 subunits, was a generous gift from Y. Lin and D. Lipscombe (Brown University; Lin and Lipscombe, 2000). Cells were maintained in 5% CO2 at 37°C, in DMEM supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 1X antibiotic-antimycotic (100 IU/ml penicillin, 0.1 mg/ml streptomycin and 0.25 μg/ml amphotericin B; Gibco BRL, Rockville, MD), 25 μg/ml Zeocin (Invitrogen, Carlsbad, CA) and 5 μg/ml Blasticidin (Invitrogen). For recording, semi-confluent cells were trypsinized, plated on poly-L-lysine–coated glass coverslips, and cultured for at least 1 hour prior to recording. Cells were used for recording within 6 hours of preparation.

**Electrophysiology**

Ba2+ currents were recorded at room temperature (20–24°C) using the whole-cell configuration of an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA). Except where noted, voltage steps of 100 ms duration were applied every 4 s from a holding potential of −90 mV. Currents were passed through the amplifier’s 4-pole low-pass Bessel filter set at 2 kHz, then digitized at 20 kHz with a 1401plus interface
(Cambridge Electronic Design, Ltd., Cambridge, England). Data were collected using the Patch software suite, version 6.3 (Cambridge Electronic Design, Ltd.), and stored on a personal computer. Prior to analysis, capacitive and leak currents were subtracted using a scaled-up current elicited with a test pulse to -115 mV. Recording pipettes were pulled from borosilicate capillary tubes (Drummond Scientific Co., Broomall, PA; #2-000-210), and heat-polished just before use; when filled with internal solution, pipette resistance ranged from 2.5–3 MΩ. Drugs were applied via gravity-driven bath perfusion, with an estimated time to complete bath exchange of 5–10 s. For SCG experiments using the irreversible N-type calcium channel blocker ω-conotoxin GVIA (ω-CgTX), cells were placed in Tyrode’s solution (145 mM NaCl, 5.4 mM KCI, 10 mM HEPES, pH 7.5) containing 1 μM ω-CgTX for at least 10 min prior to recording. Recording solutions containing 1,4-dihydropyridines (nimodipine and (+)-202-791) were protected from light until use.

The control bath solution contained, in mM: 125 N-methyl-D-glucamine-aspartate, 10 N-(2-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid) (HEPES), 20 barium-acetate, 0.0005 tetrodotoxin (TTX), pH 7.5; for tsA201 cell recordings, TTX was excluded. The pipette solution contained, in mM: 122 cesium-aspartate, 10 HEPES, 0.1 1,2-bis(2-aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid (BAPTA), 5 MgCl₂, 4 ATP (disodium salt), and 0.1 mM GDP-β-S (trilithium salt), pH 7.5.

For experiments with bovine serum albumin (BSA), 0.5 mg/ml BSA (essentially fatty acid-free; Sigma Chemical Co.) was directly dissolved into the recording solutions.
When internal BSA was used, the pipette tip was first filled with BSA-free pipette solution, then backfilled with BSA solution.

**Pharmacology**

Arachidonic (5,8,11,14-eicosatetraenoic) acid (AA) was obtained from Nu-Check-Prep, Inc. (Minneapolis, MN). GDP-β-S was obtained from either Research Biochemicals, Inc. (Natick, MA) or Sigma Chemical Co. ω-CgTX was from Bachem Bioscience, Inc. (King of Prussia, PA), and (+)-202-791 was from a gift from Sandoz Pharmaceuticals (Sandoz, Switzerland). FPL 64176 and nimodipine were both obtained from Research Biochemicals, Inc. Except where noted, all other chemicals and reagents were obtained from Sigma Chemical Co.

Stock solutions of TTX, FPL 64176, ω-CgTX, arachidonoyl coenzyme A and palmitoyl coenzyme A were prepared in double-distilled water and stored at -25°C. Stock solutions of (+)-202-791 and nimodipine were prepared in 100% ethanol and stored at -25°C. Stock solutions of AA were prepared in 100% ethanol and stored under nitrogen in glass vials at -95°C. Stock solutions of 5,8,11,14-eicosatetraynoic acid (ETYA) were prepared in DMSO and used within 1 day. Neither ethanol nor DMSO, at the maximal concentrations used in these experiments (0.05% and 0.1%, respectively), had a significant effect on whole-cell currents (not shown).
Data analysis

Analysis software included the Patch software suite, version 6.3 (Cambridge Electronic Design, Ltd.), and Origin 5.0 (Microcal Software, Northampton, MA). Data are presented as mean ± SEM, where applicable. Statistical significance was determined using a two-way paired or unpaired t-test, as appropriate. Sample size (n) represents the number of cells recorded under each condition. Percent inhibition and percent enhancement were calculated by the equations \(100 \times \frac{1 - (I_{\text{DRUG}}/I_{\text{CON}})}{1} \) and \(100 \times \frac{(I_{\text{DRUG}}/I_{\text{CON}}) - 1}{1}\) respectively, where \(I_{\text{DRUG}}\) is the current amplitude measured at a given time after drug application, and \(I_{\text{CON}}\) is the current amplitude before drug application. The Boltzmann fits of the activation data presented in Fig. 3-5 D were calculated using the equation \(I/I_{\text{max}} = \frac{[(I_1 - I_2)/(1 + e^{-\frac{(V - V_h)/k}) + I_2)]}{I_1}\), where \(I/I_{\text{max}}\) is normalized tail current amplitude, \(I_1\) and \(I_2\) are the minimum and maximum values of \(I/I_{\text{max}}\), respectively, \(V\) is test potential in mV, \(V_h\) is the voltage at half-maximal \(I/I_{\text{max}}\), and \(k\) is the slope factor of activation in mV/e-fold change in \(I/I_{\text{max}}\) at \(V_h\).

RESULTS

AA-induced enhancement and inhibition of whole-cell \(Ba^{2+}\) currents follow distinctly different time courses

The first step in our analysis was to test whether current enhancement by AA is mediated at a different site than inhibition. If enhancement and inhibition are mediated at the same site, then these effects should develop along a similar time course. Therefore, in the same recording, we applied alternating test pulses to +10 mV and -10 mV, to monitor
inhibition and enhancement, respectively (Fig. 3-1, A and B). Both inhibition and enhancement were then fit to single-exponential time constants (Fig. 3-1 C). Bath application of 5 μM AA led to a rapid increase in current amplitude at both voltages (Fig. 3-1 A); this increase was observed in 6 out of 6 recordings. However, after the initial increase in amplitude, currents elicited at +10 mV then began to decrease (Fig. 3-1 A), with a mean time constant of 4.30 ± 0.51 min (Fig. 3-1 C). Thus, after 5 min, the overall effect of AA on currents elicited at +10 mV was an inhibition of 40.5 ± 9.7% (Figs. 3-1 D and 3-12 A). In contrast, enhancement of currents elicited at −10 mV occurred with a mean time constant of 0.69 ± 0.10 min (Fig. 3-1 C); after 5 min, AA enhanced current amplitude at −10 mV by 155.5 ± 28.1% (Figs. 3-1 D and 3-12 B). These results demonstrate that enhancement develops significantly faster than inhibition, providing evidence that AA may have separate sites of action for these two effects.

Our companion paper shows that dialysis with BSA, a protein which can bind fatty acids (Spector, 1975), blocked the majority of AA-induced inhibition, but did not decrease the magnitude of AA-induced enhancement (Liu et al., 2001). To examine if intracellular BSA affects the time course of AA-induced enhancement, we measured the time constant of enhancement after dialysis with 0.5 mg/ml BSA. Even in the presence of cytoplasmic BSA, AA enhanced currents elicited at −10 mV with an average time constant of 0.82 ± 0.13 min (Fig. 3-1 C). This value was not significantly different from that observed in the absence of BSA (P > 0.1). Thus, intracellular BSA has no effect on the rate of AA-induced enhancement.
Enhancement of whole-cell current amplitude by AA is reversible

AA-induced inhibition was largely reversed by bath application of BSA (Liu and Rittenhouse, 2000; Liu et al., 2001). In this study, we employed a similar approach to determine if enhancement is also reversible. We first examined if enhancement could be reversed by washing control solution (without BSA) into the recording chamber. Washing with control solution for 1 min reversed 24.6 ± 8.2% of AA-induced enhancement (n = 3). Although significant (P < 0.05), this reversal was far from complete. Therefore, 0.5 mg/ml BSA was added to the wash solution. A typical time course is shown in Fig. 3-2 A. After 1 min in AA, introduction of wash solution containing BSA rapidly reduced current amplitude to control levels. AA’s effect on current amplitude at −10 mV, and its reversibility by BSA, are summarized in Fig. 3-2 C. Washing out AA with BSA reversed 97.2 ± 6.1% of AA-induced enhancement (n = 6).

AA-induced enhancement occurs extracellularly

The observation that intracellular BSA was without effect on AA-induced enhancement suggests that this effect may be mediated from the outside of the cell. To test this, we bath-applied the fatty acid analog palmitoyl coenzyme A (PCoA), which contains a negatively-charged moiety that prevents ‘flipping’ across cell membranes (Boylan and Hamilton, 1992). This molecule has been shown previously to mimic the effects of AA in increasing the activity of potassium channels in smooth muscle cells (Petrou et al., 1994). In SCG neurons, bath application of 10 μM PCoA led to a rapid, sustained enhancement of current amplitude (Fig. 3-3 A). This enhancement was over a
narrow range of test potentials, with significant enhancement observed at 0 mV (Fig. 3-3, B and C), providing evidence that fatty acid–induced enhancement is voltage-dependent. In addition, unlike AA, PCoA caused no inhibition of current amplitude at any voltage tested, suggesting that inhibition is likely mediated from the cytoplasmic side, consistent with our intracellular BSA data.

Palmitate (the derivative of PCoA) is a 16-carbon fatty acid, whereas arachidonic acid is a 20-carbon fatty acid. Thus, we also tested arachidonoyl coenzyme A (ACoA), an analog of AA containing a CoA group, for its ability to enhance whole-cell currents. As with PCoA, ACoA caused enhancement, yet no inhibition at any voltage tested (Fig. 3-3, D and E). ACoA-induced enhancement occurred over a similar voltage range as with PCoA, with significance observed at both −10 and 0 mV (Fig. 3-3, E and F). Taken together, these findings provide additional support for our hypothesis that AA enhances currents by acting on the outside of SCG neurons, either within the outer leaflet of the cell membrane, or at the extracellular surface.

**Enhancement does not require AA metabolism**

Modulation of ion currents by AA can occur either by direct interaction with the channels, or indirectly by a downstream pathway, sometimes involving the generation of biologically active AA metabolites (for review, see Ordway et al., 1991, and Meves, 1994). However, there are several arguments against an AA metabolite mediating enhancement of Ba\(^{2+}\) currents in SCG neurons. First, enhancement was observed even in the presence of intracellular BSA, a condition that should prevent AA metabolism.
Moreover, its immediate onset, rapid development and rapid reversibility suggest that enhancement might be mediated directly by AA. Finally, the fatty acid analogs PCoA and ACoA, which are unable to enter the cell, mimicked AA-induced enhancement of current amplitude. Therefore, it is highly unlikely that enhancement of current involves AA metabolism.

Despite these arguments, we could not, based on our experiments thus far, exclude the possibility that enhancement involves an AA metabolite. Therefore, to address this possibility, we examined the effect of ETYA (5,8,11,14-eicosatetraynoic acid), an AA analog which cannot be metabolized by the 3 common pathways (the lipoxygenase, cyclo-oxygenase and the epoxygenase pathways) known to generate biologically active products (Tobias and Hamilton, 1979; Taylor et al., 1985). Bath application of 30 μM ETYA led to a rapid, sustained enhancement of whole-cell current amplitude (Fig. 3-4, A and B). As with PCoA and ACoA, this enhancement was voltage-dependent (Fig. 3-4, C and D). ETYA caused no inhibition at any voltage tested, consistent with the results in our companion paper (Liu et al., 2001). These findings support the hypothesis that AA-induced enhancement of current amplitude does not require metabolism. Moreover, because ETYA mimics enhancement, but not inhibition, these results provide further evidence that these two effects are likely mediated by distinct mechanisms.
AA increases the voltage dependence of activation

In our companion paper, we examined the effect of AA on voltage-dependent activation. Consistent with AA's effects on current amplitude, application of AA for 5 min induced a slight increase in normalized tail current amplitude at negative voltages. However, possibly due to the influence of inhibition in the presence of AA, no significant change in voltage-dependent activation was noted. Therefore, we investigated the effect of AA on activation under recording conditions designed to maximize enhancement and minimize inhibition: 0.5 mg/ml BSA was included in the pipette solution, and the effect of 5 μM AA was examined at 1 min. We first measured the whole-cell current-voltage relationship under these recording conditions before and after application of AA. Currents were elicited by applying 200-ms voltage ramps from -60 mV to +80 mV. Fig. 3-5 A shows that application of AA increased current amplitude between -30 mV and +30 mV, but was without effect on the reversal potential. Moreover, dialyzing the cell with BSA was without effect on the voltage threshold for activation, peak inward current, or reversal potential (compare the control current-voltage plots in Figs. 3-5 A and Fig. 3-1 D).

Having demonstrated that including BSA in the pipette solution was without effect on the current-voltage properties of whole-cell currents, we next examined the effect of AA on the voltage dependence of activation. For these experiments, 15-ms test pulses were applied at incremental voltages to generate current-voltage and activation-voltage plots (Fig. 3-5, B-D). Consistent with previous results, application of AA induced a significant increase in current amplitude at negative voltages, but was without
effect on currents elicited at positive voltages (Fig. 3-5 B). Moreover, in agreement with the results obtained with PCoA, ACoA and ETYA, maximum AA-induced enhancement occurred at -10 mV, and decreased as more positive test pulses were applied (Fig. 3-5 C), confirming that AA-induced enhancement has voltage-dependent properties.

To measure the effect of AA on activation, tail current amplitude from the recordings in Fig. 3-5 B was measured at each voltage, and normalized to maximum amplitude to generate activation–voltage plots (Fig. 3-5 D). Application of AA had no significant effect on the slope of activation, but induced a negative shift of ~30 mV in $V_{b}$ (the voltage which elicits half-maximal activation). These results suggest that AA enhances currents, at least in part, by increasing the channels’ sensitivity to voltage, or possibly by altering surface charge.

AA-induced enhancement is associated with faster activation

In our examination of AA’s effects on whole-cell currents elicited at +10 mV, we observed that, in addition to changing current amplitude, AA appeared to cause an increase in the rate of activation at this voltage (Liu et al., 2001). This increase in activation rate is not likely associated with inhibition, since in cell-attached patch recordings, channels inhibited by AA exhibited increased first latency (Liu and Rittenhouse, 2000). Nevertheless, we explored whether this effect occurs in association with either inhibition or enhancement.

Whole-cell activation was examined by normalizing inward current at each time point to the peak inward current obtained during a 100-ms voltage step to +10 mV.
Normalized data from several experiments were then averaged for each condition (Fig. 3-6). Fig. 3-6 A shows that bath application of 5 μM AA for 1 min led to a significant increase in activation rate. Because AA-induced enhancement of current amplitude appears to reach steady-state within approximately 1 min, whereas inhibition takes several minutes to reach steady-state (see Fig. 3-1, A and C), we tested whether the increase in activation rate also reached steady-state within 1 min. Activation was therefore examined after treatment for 5 min with AA for comparison (Fig. 3-6 B). At 5 min, the rate of activation was not noticeably different from that measured at 1 min, as the 2 curves were similar (compare the AA curves in Fig. 3-6, A and B). Therefore, as with enhancement, AA-induced changes in activation reached steady-state within 1 min, suggesting that increased activation kinetics may be associated with enhancement, rather than inhibition.

We showed that intracellular BSA was sufficient to block the majority of AA-induced inhibition, but did not block enhancement. Therefore, to confirm that the AA-induced increase in activation is not a component of inhibition, we examined whether AA could induce a change in activation kinetics in the presence of intracellular BSA. When cells were dialyzed for at least 1 min with 0.5 mg/ml BSA, bath application of 5 μM AA for 1 min still led to a significant increase in activation (Fig. 3-6 C), confirming that this effect is not associated with AA’s inhibitory effects on whole-cell currents.

We next looked for further evidence that the increase in activation kinetics is associated with enhancement of current amplitude. Because both ACoA and ETYA mimic enhancement of current amplitude, we examined if these compounds could also
induce faster activation kinetics. Bath application of 10 μM ACoA for 1 min induced faster activation (Fig. 3-6 D). In separate experiments, a similar increase in activation rate was observed 1 min after application of 30 μM ETYA (not shown). Taken together, these results suggest that the increased activation rate is associated with AA-induced enhancement of current amplitude.

If faster activation kinetics is associated with enhancement, the changes in activation should be correlated to the increase in amplitude observed following application of AA. Therefore, we compared the change in activation with the change in amplitude upon application of AA. In 4 cell recordings, we examined the first 10 sweeps (applied at 4-sec intervals) following bath application of 5 μM AA. For amplitude, current in each sweep was normalized to the peak current obtained in the 10th sweep. Superimposed, mean normalized currents are shown in Fig. 3-7 A. Activation was then calculated for the same 10 sweeps per experiment by normalizing each sweep to the peak inward current of that sweep, as in Fig. 3-6 (Fig. 3-7 B). We then plotted the mean, normalized values obtained in Fig. 3-7 A against the mean, normalized values obtained in Fig. 3-7 B; this plot is shown in Fig. 3-7 C. A linear regression analysis of these data shows a strong correlation in time between AA-induced enhancement of current amplitude and AA-induced increased kinetics of activation, indicating that these two effects are directly associated.
AA enhances N-type current

Our analysis of AA’s effects revealed that the majority of increased activation kinetics is selective for N-type current, as it was still observed in the presence of the L-type calcium channel antagonist nimodipine (NMN), but was largely lost when cells were pre-treated with the N-type calcium channel blocker ω-conotoxin GVIA (Liu et al., 2001). Given that this effect appears to be associated with AA-induced enhancement, it seems likely that enhancement should be mediated, at least in part, by N-type current. Therefore, if AA enhances N-type current, then application of AA, in the continued presence of NMN, should still lead to enhancement. Bath application of 5 μM AA, in the presence of 1 μM NMN, led to an initial enhancement of current amplitude at both -10 and +10 mV (Fig. 3-8 A; n = 7). As observed in the absence of NMN, currents elicited at +10 mV began to decrease after about one minute, leading to a net result of significant inhibition after treatment for 5 min with AA (Fig. 3-12 A). Also, as seen in the absence of NMN, the enhancement observed at -10 mV was sustained throughout the recording (Figs. 3-8 and 3-12 B). AA-induced enhancement at -10 mV, in the presence of NMN, was 92.9 ± 22.7%, compared to 155.5 ± 28.1% in the absence of NMN (Fig. 3-12 B). This decreased level of enhancement observed in the presence of NMN raises the possibility that NMN-sensitive (e.g., L-type) current may also be enhanced by AA.

If AA-induced enhancement is mediated by N-type current, then blocking this current should preclude at least part of the enhancement. Therefore, cells were incubated with ω-conotoxin GVIA (ω-CgTX, 1 μM) prior to whole-cell recording. Following ω-CgTX treatment, 5 μM AA induced a slight initial enhancement of current at both -10
and +10 mV (Fig. 3-9 A). This enhancement was considerably less than that observed in the absence of ω-CgTX, suggesting that the majority of enhancement is mediated by N-type current. After 5 min in AA, significant inhibition was observed at both positive and negative voltages (Figs. 3-9, A and B, and 3-12), suggesting that inhibition of L-type current is similar at +10 and -10 mV. Similar results were obtained when the L-type channel agonist (+)-202-791 was included in the recording solutions to enhance L-type current (Figs. 3-9, C and D, and 3-12). Taken together, these findings suggest that enhancement is mediated in large part by N-type current. Because treatment with ω-CgTX did not completely abolish AA-induced enhancement, these results also raise the possibility that AA also may cause an enhancement of non-N-type current. This finding was not completely surprising, as Huang et al. 1992 demonstrated that fatty acids enhance nifedipine-sensitive (i.e., L-type) calcium channels in guinea pig ventricular myocytes.

To directly demonstrate that AA induces enhancement of N-type currents, we examined whole-cell currents in tsA201 cells expressing rat calcium channel α1β-α, β3 and α2/δ-1 subunits (Lin and Lipscombe, 2000). α1β-α is the principal splice variant in rat SCG neurons (Lin et al., 1997), allowing a comparison with our SCG results. Expression of N-type channels was confirmed by bath application of 1 μM ω-CgTX, which eliminated greater than 95% of whole-cell Ba2+ currents elicited at both -10 and +10 mV (Fig. 3-10, A and B). In separate experiments, the effect of AA on whole-cell currents was then examined. In 4 out of 4 cells, currents elicited at both voltages were initially enhanced following bath application of 5 μM AA (Fig. 3-10 C). After this initial
enhancement, currents elicited at +10 mV began to decrease in amplitude, resulting in significant inhibition by 5 min (Figs. 3-10 D and 3-12 A). In contrast, currents elicited at -10 mV remained significantly enhanced throughout the recording (Figs. 3-10, C and D, and 3-12 B). These data are in agreement with those collected from SCG neurons, and confirm that N-type current can be enhanced by bath application of AA.

**L-type calcium channel agonists do not preclude AA-induced enhancement**

Our lab previously presented whole-cell current–voltage (I–V) plots generated before and several minutes after bath application of AA, in the continued presence of (+)-202-791 (Liu and Rittenhouse, 2000). Under those conditions, the dominant observed effect of AA was an inhibition of current amplitude; no AA-induced enhancement was observed at any voltage, suggesting that the presence of (+)-202-791 may have precluded AA-induced enhancement. Since the data in the present study indicate that the presence of L-type calcium channel ligands should not affect AA-induced enhancement, we sought to resolve this discrepancy.

The I–V plots presented by Liu and Rittenhouse (2000) represented data collected in control solution, and again after at least 7 min of AA treatment. Thus, if enhancement did initially occur, but was then offset by inhibition, this would not be observed simply by comparing I–V plots. Therefore, in addition to I–V plots, we also examined time courses of current amplitude during application of AA in the continued presence of L-type calcium channel agonists.
When whole-cell currents were elicited in the continued presence of 1 μM (+)-202-791, bath application of 5 μM AA had a similar initial effect as was observed without an agonist: currents elicited at both +10 and −10 mV were initially enhanced by AA (Fig. 3-11 A; n = 6). At +10 mV, inhibition became the dominant effect after several min (Figs. 3-11, A and B, and 3-12 A). At −10 mV, current also decreased, but only to approximately control levels (Figs. 3-11, A and B, and 3-12 B), thereby explaining why I–V plots generated before and several minutes after AA showed no significant enhancement or inhibition at negative voltages. Similar I–V results were obtained with the non-dihydropyridine L-type channel agonist FPL 64176 (1 μM; Figs. 3-11 C and 3-12). These findings suggest that, in recording conditions designed to enhance L-type current, inhibition can be observed at −10 mV, and this inhibition can eventually occlude enhancement.

We next tested whether the presence of L-type calcium channel agonists affects AA-induced enhancement at −10 mV. Under control conditions (with no agonist present), application of AA for 1 min increased current amplitude by 69.7 ± 11.2 pA (n = 6; Fig. 3-11 D). In the presence of 1 μM (+)-202-791, application of AA increased amplitude by 61.3 ± 13.6 pA (n = 6; P > 0.5, compared to without (+)-202-791). Thus, AA is able to induce similar levels of enhancement at −10 mV in the presence or absence of an L-type calcium channel agonist. After 5 min treatment with AA, control currents were still significantly enhanced, whereas currents recorded in the presence of (+)-202-791 had decreased, further supporting our hypothesis that inhibition can be observed under conditions designed to enhance L-type current.
DISCUSSION

In the accompanying paper (Liu et al., 2001), we presented evidence that exogenous AA induces several effects on whole-cell Ba\(^{2+}\) currents in neonatal rat SCG neurons, including inhibition of both L- and N-type currents and increased holding potential–dependent inactivation. AA also induces faster activation kinetics, largely selective for N-type current. Finally, we found that application of AA causes an enhancement of whole-cell current amplitude. This enhancement appeared distinct from inhibition, as including bovine serum albumin (BSA) in the pipette solution largely reduced inhibition, but did not affect enhancement. The purpose of this study was to investigate the properties of this enhancement.

Our results show that AA reversibly enhances N-type current by inducing an increase in voltage-dependent activation, as well as an increase in activation kinetics. Blocking L-type current with NMN did not affect AA’s capacity to enhance current amplitude, suggesting that the remaining current type is enhanced by AA. Moreover, when cells were pretreated with the irreversible N-type calcium channel blocker \(\omega\)-CgTX, most, but not all AA-induced enhancement was abolished, also raising the possibility that AA may enhance non-N-type current. This is consistent with our finding that AA induced a lower level of enhancement in the presence of NMN. Finally, when AA was applied to tsA201 cells expressing recombinant N-type channels, currents elicited at negative voltages increased in amplitude to the same degree as SCG neurons recorded under similar conditions.
Since AA-induced enhancement is mediated, for the most part, through N-type channels, then AA, in the continued presence of L-type channel agonists, should still cause an enhancement at negative voltages; i.e., the agonist's effects and AA-induced enhancement should sum. Our data demonstrate exactly that: AA caused the same increase in current amplitude at -10 mV whether the L-type channel agonist (+)-202-791 was present or not. In addition, the enhanced current elicited in the presence of (+)-202-791 decreased over time, possibly explaining why, in the presence of L-type calcium channel agonists, no apparent change in current amplitude was observed at negative voltages after several minutes in AA (Liu and Rittenhouse, 2000).

Our findings suggest that AA has at least two distinct sites of action, one mediating inhibition, and one mediating enhancement. Dialyzing the cytoplasm with BSA largely blocked AA-induced inhibition, but was without effect on either the magnitude or rate of enhancement. In addition, bath-applied PCoA and ACoA, which cannot cross the cell membrane, each caused enhancement, but not inhibition.

AA-induced enhancement of calcium currents has been observed in GH3/B6 pituitary cells (Vacher et al., 1989), rat osteoblasts (Chesnoy-Marchais and Fritsch, 1994) and guinea pig ventricular myocytes (Huang et al., 1992). In pituitary cells and osteoblasts, this enhancement was voltage-dependent. When we examined enhancement in SCG neurons, we found that enhancement of Ba2+ currents by AA in SCG currents also is voltage-dependent.

Under our recording conditions, AA-induced enhancement of whole-cell currents was most readily observed at negative test potentials. In addition, at voltages more
positive than 0 mV, where neuronal calcium currents are often examined, inhibition became the dominant effect over time, thus occluding enhancement. Finally, our analysis suggests that AA appears to enhance N-type current to the greatest degree. Taken together, these findings may explain why AA-induced enhancement of currents has not been previously identified in other neuronal preparations.

In ventricular myocytes, L-type current appeared to reach peak more rapidly after AA treatment than control (Huang et al., 1992), consistent with AA-induced faster activation. In contrast, no change in activation kinetics was noted in association with AA-induced enhancement of low-voltage-activated calcium currents in GH3/B6 pituitary cells (Vacher et al., 1989) or rat osteoblasts (Chesnoy-Marchais and Fritsch, 1994), although this property was not directly examined. In neurons, changes in activation kinetics could have a profound influence on calcium influx, which could in turn influence excitability. Therefore, it was important to determine if the increased activation kinetics observed in SCG neurons is a component of AA-induced inhibition or enhancement. Our results indicate that increased activation rate is not associated with inhibition, consistent with cell-attached patch recordings of unitary calcium channels, in which AA increased first latency (Liu and Rittenhouse, 2000). However, our findings do support the conclusion that increased activation rate is a component of AA-induced enhancement, as the development of both effects was strongly correlated.

Changes in surface charge can sometimes lead to changes in ion channel permeation and gating (Green and Anderson, 1991; Zhou and Jones, 1995). Therefore, fatty acid–induced enhancement might be the result of altered surface charge. This is
unlikely because the coenzyme A head groups of PCoA and ACoA contain several negative charges, whereas ETYA and AA contain only one negative charge. Although these compounds contain different charges, they induce a similar effect on whole-cell currents. In addition, we would predict that changes in surface charge should occur very rapidly following perfusion of the recording chamber. In contrast, enhancement of current amplitude by AA occurred with a time constant of approximately 41 sec, too slowly to be accounted for by a surface charge effect.

AA can be liberated from cell membrane phospholipids by several pathways (Bazan, 1989). G-protein–coupled receptors can activate phospholipases, which in turn can catalyze the release of AA (Axelrod et al., 1988). A number of neurotransmitters can activate receptor-mediated AA liberation (Axelrod et al., 1988), and SCG neurons are known to express receptors for these agonists, including muscarinic receptors (Lindl et al., 1976; Skok et al., 1999). Therefore, trans-synaptic stimulation of these neurons may lead to diverse modulation of calcium currents by AA.

Together with our accompanying paper, we show that AA can induce several effects on calcium currents in sympathetic neurons. Which effects may be induced may depend on the source of AA and the length of exposure to AA. Our results suggest that a brief external exposure to AA may be sufficient to enhance calcium influx, even with very brief depolarizations, thereby potentiating neuronal excitability. In contrast, exposure to intracellular AA may lead to inactivation of calcium channels, which would inhibit excitability.
FIGURE 3-1

Arachidonic acid (AA)–induced enhancement and inhibition follow distinct time courses. (A) Currents were elicited by applying alternating 100-ms test pulses to +10 mV (□) and −10 mV (○), then peak inward current was plotted against time. 5 μM AA was applied to the bath as indicated by the solid bar. The solid lines represent single-exponential fits of the data. Shown in B are sweeps from the experiment in A, obtained before (open arrowheads) and after (closed arrowheads) bath application of AA. (C) Summary of the time constants of inhibition and enhancement (see A). Bath application of 5 μM AA inhibited current amplitude, measured at +10 mV, with a time constant of 4.30 ± 0.51 min (n = 15). In contrast, AA increased current amplitude, measured at −10 mV, with a time constant of 0.69 ± 0.10 min (n = 5; **P < 0.001 compared with +10 mV). When cells were first dialyzed for ≥ 1 min with 0.5 mg/ml bovine serum albumin (+BSA), bath application of AA increased current amplitude with a time constant of 0.82 ± 0.13 min (n = 7, P > 0.1 compared to without BSA). (D) Current–voltage (I–V) plots were generated by applying 100-ms test pulses at 10-mV increments. Peak inward current was then plotted against voltage for control (○, n = 7) and 5 min following bath application of 5 μM AA (●, n = 7). In this and subsequent figures, error bars not visible are contained within the symbols. *P < 0.05.
FIGURE 3-1

A

5 μM AA

I (pA)

τ = 4.22 min

τ = 0.55 min

- △ +10 mV
- ○ -10 mV

Time (min)

B

+10 mV

-90 mV

200 pA

50 ms

-90 mV

-10 mV

100 pA

50 ms

C

τ (min)

+10 mV

-10 mV

-10 mV

+ BSA

D

I (nA)

-40

-0.2

-0.3

4.0

80

- CON

- AA

***

***

***

***
FIGURE 3-2

External BSA reverses AA-induced enhancement of whole-cell currents. (A) Currents were elicited at -10 mV and plotted against time. 5 μM AA was applied to the bath as indicated by the solid bar, and wash solution containing 0.5 mg/ml BSA was applied to the bath as indicated by the open bar. Shown in B are traces from the experiment in A, taken at the times indicated. (C) Summary of the effect of AA at -10 mV, and its reversibility by BSA (n = 6). In control solution, current amplitude was 35.3 ± 2.8 pA. One min after application of AA, amplitude had increased to 106.4 ± 11.4 pA. Following AA treatment, bath application of BSA for 1 min reduced current amplitude to 38.2 ± 3.1 pA (P > 0.5 versus CON). *P < 0.005 versus CON and versus BSA.
FIGURE 3-2

A

\[ L_{-10\,\text{mV}} (\text{pA}) \]

\[
\begin{array}{c}
0 & 1 & 2 & 3 & 4 \\
0 & 50 & 100 & 150 \\
\end{array}
\]

Time (min)

B

-90 mV  -10 mV

100 pA  50 ms

C

\[ L_{-10\,\text{mV}} (\text{pA}) \]

\[
\begin{array}{ccc}
\text{CON} & \text{AA} & \text{BSA} \\
40 & 120 & 40 \\
\end{array}
\]

*
FIGURE 3-3

Palmitoyl coenzyme A (PCoA) and arachidonoyl coenzyme A (ACoA) mimic AA-induced enhancement of whole-cell currents. (A and D) Peak inward current was elicited at +10 mV and plotted against time; 10 μM PCoA (A) or 10 μM ACoA (D) was applied to the bath as indicated by the solid bars. The extended breaks in the time courses represent time periods during which I–V data were collected. (B and E) I–V plots were generated before (○, n = 4–5) and after (●, n = 4–5) bath application of 10 μM PCoA (B) or 10 μM ACoA (E). *P < 0.05. (C and F) Percent enhancement by PCoA (C) or ACoA (F) was calculated and plotted against voltage (n = 4–5).
FIGURE 3-3

A

B

C

D

E

F

Time (min)

Voltage (mV)

% Enhancement

Voltage (mV)
FIGURE 3-4

AA-induced enhancement does not require AA metabolism. (A) Peak inward current was elicited at +10 mV and plotted against time; 30 μM eicosatetrynoic acid (ETYA) was applied to the bath as indicated by the solid bar. The extended breaks in the time course represent the time periods during which I–V data were collected. Shown in B are sweeps from the experiment in A, taken before (open arrowhead) and after (closed arrowhead) application of ETYA. (C) I–V plots were generated before (○) and after (●) bath application of 30 μM ETYA (n = 7). *P < 0.05. (D) Percent enhancement by ETYA was calculated and plotted against voltage (n = 7).
FIGURE 3-4

A

L_{20 \text{mV}} (\text{pA})

30 \mu M ETYA

200

150

100

50

0

0 2 4 6

Time (min)

B

-90 mV

-10 mV

250 pA

50 ms

C

I (nA)

-40

-20

0

20

40

60

mV

CON

ETYA

D

% Enhancement

-20

-10

0

10

20

30

40

Voltage (mV)
FIGURE 3-5

AA increases the voltage sensitivity of current activation. For these recordings, the pipette solution contained 0.5 mg/ml BSA. (A) Whole-cell currents were elicited by applying a linear ramp pulse from -60 mV through +80 mV (slope = 0.75 mV/ms) in control solution (CON), and approximately 1 min after bath application of AA. Shown are mean ramp currents generated from 4 recordings; error bars were omitted for clarity. (B) I–V curves were generated by applying 15-ms test pulses to incremental voltages at 1-sec intervals. Current amplitude was measured 13.3 ms into the test pulse, and plotted against voltage. ○, currents elicited in control solution (n = 5); ●, currents elicited 1 min after bath application of 5 μM AA (n = 4). *P < 0.05. (C) The percent of AA-induced enhancement in the recordings shown in B was calculated and plotted against voltage. (D) For the recordings shown in B, tail current amplitude, induced by stepping the membrane potential back to -90 mV following the test pulse, was measured, then normalized to maximum tail current amplitude and plotted against test potential to generate activation–voltage curves. The solid lines represent Boltzmann fits to the data. In control solution, $V_h$ was 25.6 ± 9.4 mV and $k$ was 12.2 ± 3.1 mV/e-fold change in activation; following AA application, $V_h$ was -3.53 ± 1.44 mV ($P < 0.05$ versus control) and $k$ was 8.08 ± 1.79 mV/e-fold change ($P > 0.1$ versus control).
FIGURE 3-5

A

B

C

D

Voltage (mV)

Normalized Tail Current

Test Pulse (mv)
FIGURE 3-6

The AA-induced increased rate of activation shares characteristics with AA-induced enhancement of current amplitude. Whole-cell currents were elicited by applying a voltage step to +10 mV, and for each experiment, average current traces were generated before (CON) and after drug application. For each average trace, peak inward current was determined, and currents were normalized to that value. The first ~8.5 ms of mean, normalized currents ± SEM were then plotted against time from the onset of the voltage step. (A) Currents were elicited before, and 1 min after, bath application of 5 μM AA (n = 5). (B) The same recordings shown in A, before, and 5 min after, application of AA. (C) Cells were first dialyzed for >1 min with 0.5 mg/ml BSA, then currents were elicited before, and 1 min after, bath application of 5 μM AA (n = 5). (D) Currents were elicited before, and 1 min after, bath application of 10 μM ACoA (n = 6).
FIGURE 3-6

A

1 min AA

- $I_{\max}$

1 2 3 4 5 6 7 8

Time (ms)

B

5 min AA

- $I_{\max}$

1 2 3 4 5 6 7 8

Time (ms)

C

1 min AA

Internal BSA

- $I_{\max}$

1 2 3 4 5 6 7 8

Time (ms)

D

1 min ACaO

- $I_{\max}$

1 2 3 4 5 6 7 8

Time (ms)
FIGURE 3-7

AA-induced faster activation is correlated with AA-induced enhancement of current amplitude. (A) Whole-cell currents were elicited for 10 consecutive sweeps following application of 5 µM AA; sweeps were applied at 4-sec intervals. The current at each sweep was then normalized to the maximum current elicited by the 10th sweep. Each trace represents the average of 4 normalized cell recordings (error bars were omitted for clarity, and fast tail currents were clipped). Values at 8 ms (dashed line) were used for the x-coordinates in C as a measure of relative current amplitude. (B) Normalized currents were generated for the same sweeps in A, using the method in Fig. 3-6. Error bars were again omitted. Values at 3 ms (dashed line) were used for the y-coordinates in C as a measure of activation rate. (C) Normalized inward current from sweeps 1–10 in A was plotted along the abscissa, and normalized current from sweeps 1–10 in B was plotted along the ordinate. The solid line represents the linear regression of the data points, and the Pearson correlation coefficient (r) is given.
FIGURE 3-7

A

B

C

\( I_{\text{sweep \, n}} / I_{\text{sweep \, 10}} \)

\( -I/I_{\text{max}} \)

\( -I/I_{\text{max}} @ 3 \text{ ms} \)

\( -I/I_{\text{max}} @ 8 \text{ ms} \)

\( r = 0.939 \)
FIGURE 3-8

The majority of AA-induced enhancement is nimodipine-insensitive. (A) Time course of whole-cell Ba^{2+} currents elicited at +10 mV (□) and -10 mV (○). 1 μM nimodipine (NMN) was present throughout the recording (open bar), and 5 μM AA was applied to the bath as indicated by the solid bar. The extended break in the time course represents the time period during which I–V data were collected. (B) I–V curves were generated, in the continued presence of 1 μM NMN, before (○, n = 8) and 5 min after (●, n = 5) bath application of 5 μM AA. *P < 0.05.
FIGURE 3-8

A

![Graph A showing the effect of 1 µM NMN and 5 µM AA on current (I) at different voltages (+10 mV and -10 mV) over time (min)].

B

![Graph B showing the voltage-current (I-V) relationship with NMN and NMN + AA].

mV
FIGURE 3-9

N-type calcium current mediates the majority of AA-induced enhancement. (A–D) Cells were pretreated with 1 μM ω-conotoxin GVIA (ω-CgTX) prior to recording. (A) Whole-cell currents were elicited at +10 mV (□) and −10 mV (○) and plotted against time; 5 μM AA was applied to the bath as indicated by the solid bar. (B) I–V curves were generated before (○, n = 11) and 5 min after (●, n = 9) bath application of 5 μM AA. (C) Whole-cell currents were elicited at +10 mV (□) and −10 mV (○) and plotted against time; 1 μM (+)-202-791 was present throughout the recording, and 5 μM AA was applied to the bath as indicated by the solid bar. The extended breaks in the time course represent the time period during which I–V data were collected. (D) I–V curves were generated, in the continued presence of 1 μM (+)-202-791, before (○, n = 13) and 5 min after (●, n = 6) bath application of 5 μM AA. *P < 0.05.
FIGURE 3-9

A

<table>
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<td>25</td>
<td>4</td>
</tr>
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<td>6</td>
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1 μM ω-CgTX
5 μM AA

B

<table>
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<tr>
<th>I (nA)</th>
<th>mV</th>
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<td>-40</td>
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ω-CgTX
ω-CgTX + AA

C

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<th>Time (min)</th>
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<tr>
<td>200</td>
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</tr>
<tr>
<td>150</td>
<td>2</td>
</tr>
<tr>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>50</td>
<td>6</td>
</tr>
</tbody>
</table>

1 μM ω-CgTX + 1 μM (+)-202
5 μM AA

D

<table>
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<tr>
<th>I (nA)</th>
<th>mV</th>
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<tbody>
<tr>
<td>0.04</td>
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<tr>
<td>0.08</td>
<td>-40</td>
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</tbody>
</table>

ω-CgTX (+)-202
ω-CgTX + (+)-202 + AA
FIGURE 3-10

AA both enhances and inhibits whole-cell recombinant N-type calcium current. (A) Time course of whole-cell Ba\(^{2+}\) currents in tsA201 cells expressing the rat calcium channel \(\alpha_{1B-3}, \beta_3,\) and \(\alpha_\gamma/\delta-1\) subunits. Bath application of 1 \(\mu\text{M}\) \(\omega\)-CgTX (solid bar) virtually eliminated inward current at +10 mV. (B) I–V plots were generated before (\(\bigcirc, n = 5\)) and 5 min after (\(\bullet, n = 5\)) bath application of 1 \(\mu\text{M}\) \(\omega\)-CgTX. (C) Whole-cell currents were elicited at +10 mV (\(\square\)) and −10 mV (\(\bigcirc\)) and plotted against time; 5 \(\mu\text{M}\) AA was applied to the bath as indicated by the solid bar. (D) I–V plots were generated before (\(\bigcirc, n = 4\)) and 5 min after (\(\bullet, n = 4\)) bath application of 5 \(\mu\text{M}\) AA. *\(P < 0.05\). In A and C, the extended breaks in the time courses represent the time periods during which I–V data were collected.
FIGURE 3-10

A

B

C

D

I (nA)

Time (min)

I (nA)

Time (min)

I (nA)

Time (min)

1 μM ω-CgTX

-1.5

0.5

-1.0

-0.5

-40

40

80

-10 mV

+10 mV

-10 mV

+10 mV

CON

ω-CgTX

CON

AA

ω-CgTX

AA

CON

AA
FIGURE 3-11

L-type calcium channel agonists do not preclude AA-induced enhancement. (A) Time course of whole-cell Ba\(^{2+}\) currents elicited at +10 mV (☐) and −10 mV (○). 1 µM (+)-202-791 was present throughout the recording (open bar), and 5 µM AA was applied to the bath as indicated by the solid bar. The extended breaks in the time course represent the time periods over which I–V data were collected. (B–C) I–V curves were generated before (☐, n = 5–11) and 5 min after (●, n = 4–5) bath application of 5 µM AA. Recordings were conducted in the continued presence of either 1 µM (+)-202-791 (B) or 1 µM FPL 64176 (C). *P < 0.05. (D) Summary of the effect of AA on currents elicited at −10 mV, in the absence (open bars) and presence (solid bars) of 1 µM (+)-202-791. Before AA application (−AA), current amplitude in control and (+)-202-791 was 34.4 ± 4.5 (n = 6) and 53.6 ± 5.0 pA (n = 6), respectively; 1 min after application of AA, current amplitude had increased to 104.1 ± 15.2 and 114.9 ± 16.2 pA, respectively. By 5 min, control current was 99.2 ± 14.1 pA, and current in the presence of (+)-202-791 was 78.6 ± 9.4 pA.
FIGURE 3-11

A

![Graph showing the modulation of N-type calcium channels in rat SCG neurons with (+)-202 and 5 μM AA.](image)

Time (min)

B

![Graph showing current (I) in nA as a function of voltage (mV).](image)

C

![Graph showing a comparison between FPL and FPL + AA.](image)

D

![Bar graph showing changes in I-10 mV (-pA) with different treatments.](image)
FIGURE 3-12

Summary of the effects of AA on the amplitude of whole-cell currents elicited at +10 mV (A) and -10 mV (B). Percent inhibition and percent enhancement were calculated by comparing current amplitude 5 min after bath application of AA to amplitude before application of AA. "—" indicates application of AA in the absence of any other drugs. For all experiments, AA concentration was 5 μM; NMN, ω-CgTX, (+)-202-791, and FPL 64176 were each used at 1 μM. *P < 0.05, compared to before AA treatment; †P < 0.01, compared to "—". Sample size (n) is given in parentheses, and refers to both A and B.
FIGURE 3-12

**A**

% Inhibition by AA at +10 mV

**B**

% Enhancement by AA at -10 mV

<table>
<thead>
<tr>
<th>SCG neurons</th>
<th>NMN (7)</th>
<th>ω-CgTX (5)</th>
<th>(+)-202 (8)</th>
<th>(+)-202 (4)</th>
<th>FPL 64176 (5)</th>
<th>tsA201 cells (5)</th>
</tr>
</thead>
<tbody>
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<td>*</td>
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<td>*</td>
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</tbody>
</table>

The figure shows the modulation of N-type calcium channels in rat SCG neurons.
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CHAPTER IV

THE L-TYPE CALCIUM CHANNEL AGONIST FPL 64176 REVERSIBLY INHIBITS N-TYPE CALCIUM CURRENT
ABSTRACT

The benzoyl pyrrole, non-dihydropyridine L-type calcium channel agonist FPL 64176 (FPL) has been used by this lab and others to characterize L-type calcium currents in a variety of cell types. Because FPL's effect on most non-L-type calcium currents has not been investigated previously, we examined the effect of FPL on whole-cell calcium currents in neonatal rat superior cervical ganglion neurons, in which approximately 85% of the whole-cell calcium current is N-type, and the majority of the remaining current is L-type. In these cells, bath application of 1 μM FPL increased both peak inward current amplitude at +10 mV, as well as the amplitude of a slowly-deactivating tail current component. Raising the FPL concentration to 10 μM caused an additional increase in tail current amplitude, but, surprisingly, reduced peak current amplitude to approximately control levels, suggesting that FPL, at this concentration, may be causing inhibition of currents elicited at +10 mV. Thus, in this cell type, one effect of FPL might be an inhibition of N-type current. To test this directly, we examined the effect of FPL on an HEK 293 cell line expressing recombinant N-type calcium channels. We found that bath application of 1 μM FPL inhibited recombinant whole-cell currents elicited at +10 mV by 12.8 ± 4.9%, and application of 10 μM FPL caused 77.3 ± 3.5% inhibition. This inhibition was voltage-insensitive, and largely reversed upon washout of FPL. These results indicate that, in addition to acting as an agonist of L-type calcium channels, FPL is also an inhibitor of N-type calcium channels. Investigators using FPL should therefore take both of these effects into consideration when examining the effect of FPL on whole-cell currents composed of both L- and N-type currents.
INTRODUCTION

Pharmacological agents represent a useful tool for identifying and characterizing ion channels and their currents. Specificity of a drug for its target channel is critical for allowing an accurate assessment of the individual current type(s) that contribute to the whole-cell current. Although a compound may initially appear to show high selectivity for a particular channel type, subsequent investigations may reveal additional actions on other channel types, and allow researchers to interpret their results accordingly.

FPL 64176 (methyl 2,5-dimethyl-4-[2-(phenylmethyl)benzoyl]-H-pyrrole-3-carboxylate; FPL) is a benzoyl pyrrole first reported to function as an activator of voltage-gated calcium channels in rat pituitary GH₃ cells (McKechnie et al., 1989). FPL has been identified as a potent L-type calcium channel agonist, acting at a different site than the 1,4-dihydropyridine (DHP) family of L-type calcium channel ligands, e.g., nifedipine and Bay K 8644 (McKechnie et al., 1989; Zheng et al., 1991; Kunze and Rampe, 1992; Ginap et al., 1993). An examination of FPL’s effects on calcium currents in rat ventricular myocytes showed that FPL increased the amplitude of whole-cell L-type calcium current, and slowed both activation and inactivation kinetics (Rampe and Lacerda, 1991). The increase in whole-cell current amplitude can be explained by an analysis of FPL’s effects at the cell-attached patch recording level, in which FPL increased the channel’s open probability by inducing long-lasting open times (Kunze and Rampe, 1992). In addition, an increase in first latency appears to underlie the observed slowed activation of whole-cell currents (Kunze and Rampe, 1992).
Since its introduction, FPL has served as a valuable tool for identifying and characterizing L-type calcium currents in a variety of cell preparations, including neurons (Jeong and Wurster, 1997a; Jeong and Wurster, 1997b; Shapiro et al., 1999; Carlin et al., 2000; N’Gouemo and Rittenhouse, 2000; Barrett et al., 2001; Liu et al., 2001). It has been reported that recombinant class A (α1A; P/Q-type) calcium channels are largely insensitive to both DHPs and FPL (Sather et al., 1993; Grabner et al., 1996), although sensitivity to these drugs is conferred to class A channels by substituting the same select, minimal domains from the class C (α1C; L-type) calcium channel (Grabner et al., 1996). These studies confirmed that, like DHPs, FPL is a ligand for the L-type calcium channel. Moreover, because FPL has no obvious effect on class A calcium channels, FPL may be selective for L-type calcium channels.

FPL has been used in this lab to measure L-type calcium current (Liu and Rittenhouse, 1996; Gonzalez and Rittenhouse, 1998; N’Gouemo and Rittenhouse, 2000). In order to examine the effect of FPL on another calcium current, the N-type current, we investigated the effect of micromolar concentrations of FPL on whole-cell currents in neonatal rat superior cervical ganglion (SCG) neurons, in which approximately 85% of the calcium current is N-type, and the majority of the remaining current is L-type (Plummer et al., 1989). In addition, we examined the effect of FPL in HEK 293 cells expressing N-type calcium channels. Our results show that FPL, in micromolar concentrations, significantly inhibits N-type calcium current.
Curtis F. Barrett  
*Modulation of N-type Calcium Channels in Rat SCG Neurons*  
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METHODS

*Preparation and culture of SCG neurons*

Following decapitation, the superior cervical ganglia (SCG) were removed from 1–4 d old Sprague-Dawley rats (Charles River Laboratories, Inc., Wilmington, MA), and neurons were dissociated by trituration through a 22-gauge, 1½-inch needle. Following dissociation, cells were plated on poly-L-lysine-coated glass coverslips in 35-mm culture dishes, and incubated at least 4 hours prior to recording. Cells were maintained in 5% CO₂ at 37°C, in DMEM supplemented with 7.5% fetal bovine serum, 7.5% calf serum, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 4 mM L-glutamine (all from Sigma Chemical Co., St. Louis, MO), and 0.2 μg/ml nerve growth factor (Bioproducts for Science, Indianapolis, IN); cells were used within 12 hours of preparation. Spherical neurons lacking visible processes were selected for recording.

*Preparation and culture of tsA201 cells*

The SV40 large T antigen–transformed HEK 293 subclone tsA201, transfected to stably express the rat N-type calcium channel α₁B-a splice variant (ΔA415/ΔSFMG/+ET; see Table 2 in Lin et al., 1997) together with rat neuronal–derived β₃ and α₂δ-1 subunits, was a generous gift from Y. Lin and D. Lipscombe (Brown University; Lin and Lipscombe, 2000). Cells were maintained in 5% CO₂ at 37°C, in DMEM supplemented with 10% fetal bovine serum, 4 mM L-glutamine, 1X antibiotic-antimycotic (100 IU/ml penicillin, 0.1 mg/ml streptomycin and 0.25 μg/ml amphotericin B; Gibco BRL, Rockville, MD), 25 μg/ml Zeocin (Invitrogen, Carlsbad, CA) and 5 μg/ml Blasticidin.
(Invitrogen). For recording, semi-confluent cells were trypsinized, plated on poly-L-lysine-coated glass coverslips, and cultured for at least 1 hour prior to recording. Cells were used for recording within 6 hours of preparation.

**Electrophysiology**

$\text{Ba}^{2+}$ currents were recorded at room temperature (20–24°C) using the whole-cell configuration of an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA). Test pulses were applied every 4 s from a holding potential of $-90$ mV. Currents were passed through the amplifier’s 4-pole low-pass Bessel filter set at 2 kHz, then digitized at 20 kHz with a 1401plus interface (Cambridge Electronic Design, Ltd., Cambridge, England). Data were collected using the Patch software suite, version 6.3 (Cambridge Electronic Design, Ltd.), and stored on a personal computer. Prior to analysis, capacitive and leak currents were subtracted using a scaled-up current elicited with a test pulse to $-115$ mV. Recording pipettes were pulled from borosilicate capillary tubes (Drummond Scientific Co., Broomall, PA; #2-000-210), and heat-polished just before use; when filled with internal solution, pipette resistance ranged from 2–3 MΩ. Drugs were applied via gravity-driven bath perfusion, with an estimated time to complete bath exchange of 5–10 s.

The control bath solution contained, in mM: $125\ \text{N}$-methyl-$\text{D}$-glucamine-aspartate, $10\ \text{N}$-(2-hydroxyethyl)piperazine-$\text{N}''$-(2-ethanesulfonic acid) (HEPES), $20$ barium-acetate, $0.0005$ tetrodotoxin (TTX), pH 7.5; for tsA201 cell recordings, TTX was
excluded. The pipette solution contained (in mM): 122 cesium-aspartate, 10 HEPES, 10 EGTA, 5 MgCl₂, 4 ATP (disodium salt), 0.1 GDP-β-S (trilithium salt), pH 7.5.

Because N-type current in these cells is highly sensitive to inhibition by activated G-proteins, 0.1 mM GDP-β-S was included in the pipette solution to ensure that changes in current amplitude are not due to changes in G-protein–mediated inhibition (Barrett and Rittenhouse, 2000).

FPL 64176 was obtained from Research Biochemicals, Inc. Except where noted, all other chemicals and reagents were obtained from Sigma Chemical Co. Stock solutions of TTX and FPL 64176 were prepared in double-distilled water and stored at −25°C.

In the recombinant cell line, whole-cell barium currents exhibit a high level of steady-state inactivation, which is largely relieved by the application of hyperpolarizing test pulses. To monitor leak and capacitive currents, we typically applied 5 consecutive hyperpolarizing test pulses at periodic intervals throughout the recordings. In this cell line, this often caused a transient increase in current amplitude (e.g., see Fig. 4-4). Therefore, we measured current amplitude once the current had reached steady-state.

**Data analysis**

Analysis software included the Patch software suite, version 6.3 (Cambridge Electronic Design, Ltd.), and Origin 5.0 (Microcal Software, Northampton, MA). Data are presented as mean ± SEM, where applicable. Statistical significance was determined using a two-way paired or unpaired t-test, as appropriate. Sample size (n) represents the number of cells recorded under each condition. Percent inhibition and percent increase
were calculated by the equations \([1 - (I_{\text{DRUG}}/I_{\text{CON}})] \times 100\) and \(([I_{\text{DRUG}}/I_{\text{CON}}] - 1) \times 100\), respectively, where \(I_{\text{DRUG}}\) is the current amplitude measured after drug application, and \(I_{\text{CON}}\) is the current amplitude before drug application.

**RESULTS**

*FPL 64176 induces a slowly-deactivating tail current in rat SCG neurons*

In this study, we utilized a voltage protocol (Fig. 4-1 A) which allowed us to measure both total membrane current (made up of both N- and L-type currents), as well as a current comprised exclusively of L-type current (Plummer et al., 1989). Total membrane ("peak") current was measured during a voltage step to +10 mV from a holding potential of -90 mV. After 20 ms, membrane potential was stepped to -40 mV; at this voltage, both N- and L-type calcium channels rapidly deactivate. However, in the presence of an L-type calcium channel agonist, a slowly-deactivating ("tail") current, comprised solely of L-type current, is observed (Plummer et al., 1989; Plummer et al., 1991; Liu and Rittenhouse, 2000). Thus, when SCG currents were recorded using this voltage protocol, bath application of 1 μM FPL 64176 (FPL) both increased peak current amplitude and induced a slowly-deactivating tail current (Fig. 4-1), consistent with previous reports (Shapiro et al., 1999; Liu et al., 2001). The increase in tail current amplitude was rapidly reversed upon washout of FPL, and reproduced upon reapplication of 1 μM FPL (Fig. 4-1 B). Finally, our lab previously demonstrated that the slowly-deactivating tail current induced by FPL is insensitive to the N-type calcium channel
blocker ω-conotoxin GVIA (Liu et al., 2001), confirming that this tail current is composed of L-type current.

10 μM FPL increases tail current amplitude, but not peak current amplitude

Having confirmed that FPL induces an increase in both peak and tail currents, we next investigated whether 1 μM was a saturating concentration, by examining the effect of bath applying 10 μM FPL (Fig. 4-2). Application of 1 μM FPL increased peak current amplitude by 21.4 ± 8.8% (n = 8; Fig. 4-3 A). We then raised the concentration of FPL to 10 μM. Upon application of 10 μM FPL (n = 8), peak current initially increased in amplitude, then decreased to approximately control levels (Fig. 4-2). Peak current amplitude in 10 μM FPL was 343.9 ± 33.6 pA; this value is not significantly different than in control (348.1 ± 51.1 pA). Thus, it appears that, in the presence of 10 μM FPL, either the enhancement of L-type current at +10 mV is lost or occluded, the recording conditions become disrupted, or another channel type is inhibited. An examination of tail currents suggests that the first possibility is not the case. In the presence of 1 μM FPL, the amplitude of slowly-deactivating tail currents was 171.0 ± 25.3 pA, compared to 5.5 ± 1.6 pA in control (Fig. 4-3 B). Application of 10 μM FPL further increased tail current amplitude to 277.4 ± 36.0 pA, indicating that 1 μM FPL was not a saturating concentration, and that high concentrations of FPL do not occlude its effect on L-type current. In addition, because the increased tail current amplitude in the presence of both 1 and 10 μM FPL was stable over time (Fig. 4-2 B), it is unlikely that the higher FPL
concentration caused a disruption of recording conditions. We therefore explored the possibility that another calcium channel type is inhibited by FPL.

**FPL 64176 inhibits recombinant N-type calcium current**

Whole-cell SCG calcium currents elicited at +10 mV are largely comprised of N-type current (Plummer et al., 1989). Thus, we hypothesized that, at 10 μM, FPL inhibits N-type current while enhancing L-type current, with these effects effectively canceling each other. To test this, we examined whole-cell currents in tsA201 cells expressing rat calcium channel α1B-α133 and α2/δ-1 subunits (Lin and Lipscombe, 2000). Our lab previously demonstrated that the calcium current expressed by these cells is highly sensitive to the N-type calcium channel blocker ω-conotoxin GVIA (Barrett et al., 2001), confirming that this cell line expresses N-type current.

Shown in Fig. 4-4 is an example of the effect of FPL on whole-cell current elicited in this recombinant cell line. In this recording, application of 1 μM FPL appeared to induce a slight decrease in current amplitude, although part of this effect may be due to steady-state inactivation. Application of 10 μM FPL, however, led to a much greater inhibition of current. The effects of 1 and 10 μM FPL on recombinant N-type calcium current at various voltages are summarized in Fig. 4-5 A. Currents elicited at +10 mV were inhibited 12.8 ± 4.9% (n = 9; P < 0.05 versus control) in the presence of 1 μM FPL, while application of 10 μM FPL led to 77.3 ± 3.5% inhibition (n = 10; P < 0.01 versus control). As with FPL’s effect on slowly-deactivating tail currents in SCG neurons, FPL-induced inhibition of peak current in the recombinant cells was largely
reversed upon washout of FPL (Figs. 4-4 and 4-5 A), and reproduced upon reapplication of FPL (not shown).

We next examined whether FPL's inhibition of N-type current is affected by voltage, by measuring the degree of inhibition by 10 μM FPL at various test potentials. Fig. 4-5 B shows that, over the range of test potentials shown to elicit current, inhibition by FPL was essentially unaffected by voltage, indicating that FPL 64176–induced inhibition of N-type calcium current is not voltage-sensitive. Moreover, because treatment with either 1 or 10 μM FPL was without obvious effect on the voltage threshold for activation, the voltage eliciting maximal inward current, or the current’s reversal potential (Fig. 4-5 A), inhibition is not likely the result of a surface charge effect.

**DISCUSSION**

In this study we tested the effect of the benzoyl pyrrole FPL 64176 (FPL) on neuronal L- and N-type calcium currents. In rat SCG neurons, in which ~85% of the whole-cell calcium current is N-type, and the majority of the remaining current is L-type, 1 μM FPL induced a slowly-deactivating tail current, yet only slightly increased peak current amplitude. Ten micromolar FPL further increased tail current amplitude, but, surprisingly, decreased peak current to control levels, raising the possibility that FPL might be inhibiting N-type current while enhancing L-type current. To test this directly, we investigated the effect of FPL on currents elicited in an HEK 293 cell line that expresses N-type calcium channels, and found that FPL significantly inhibited this current.
Given the relatively high proportion of N-type current in SCG neurons, 10 µM FPL seemed to induce a rather modest degree of inhibition in these cells, reducing peak current to approximately control levels. This may be because these cells also express L-type calcium channels, which would lead to an underestimation of FPL’s effects on N-type current. In addition, FPL might have different sensitivities for the endogenous and recombinant N-type calcium channels. Nevertheless, FPL’s effects on recombinant N-type calcium channels are consistent with our results with SCG neurons.

Jones and Jacobs (1990) demonstrated that the 1,4-dihydropyridine (DHP) L-type calcium channel agonists BayK 8644 and (+)-202-791 both inhibit non-L-type calcium current in frog sympathetic neurons, when applied in concentrations in the micromolar range. As with rat sympathetic neurons, whole-cell calcium currents in frog sympathetic neurons are composed of ~85% N-type current, and the majority of the remaining current is L-type (Jones and Marks, 1989). The authors concluded that DHP agonists, while largely selective for L-type calcium current in submicromolar concentrations, exhibit antagonist effects on N-type current at higher concentrations. These results fit well with our finding that FPL 64176, although a non-DHP agonist, also can inhibit N-type calcium current.

From binding studies, FPL and DHPs are believed to bind at separate sites on the L-type calcium channel α1 subunit (Kunze and Rampe, 1992; Ginap et al., 1993). However, the binding sites for FPL and DHPs are believed to reside in close proximity to each other, as α1A, a channel insensitive to both FPL and DHPs, was rendered sensitive to these compounds by substituting minimal domains of the the L-type calcium channel α1
subunit (Grabner et al., 1996). Specifically, transfer of the α1C P-loops from the 3rd and 4th domains (see Fig. 1-1) together with transmembrane helices IIIIS5, IIIIS6 and IVS6 was sufficient to confer both FPL and DHP sensitivity to the α1A subunit. We might therefore speculate that DHPs and FPL might act at these same regions within the α1B subunit of the N-type calcium channel, but with antagonistic effects. This prediction is supported by the previous observation that the N-type calcium channel blocker ω-conotoxin GVIA appears to interact with the IIIIS5–IIIIS6 region of α1B (Ellinor et al., 1994). Thus, chimeric channels in which these regions from α1B are substituted into α1C might yield an L-type calcium channel phenotype that is inhibited by DHPs, FPL and ω-conotoxin GVIA. It would also be interesting to determine the effect of these ligands on a chimeric channel in which α1B contains the minimal substitutions from the L-type calcium channel. We would predict that current through such a channel might be enhanced by these compounds, and that such a channel would be insensitive to ω-conotoxin GVIA.

Our results show that FPL’s inhibitory effect on N-type calcium current is independent of test potential, suggesting that FPL’s affinity for N-type channels is not voltage-sensitive. In contrast, FPL’s effect on L-type calcium current is highly sensitive to test potential. In SCG neurons, 1 μM FPL maximally enhanced peak current at −30 mV, whereas virtually no enhancement was observed at test potentials more positive than 0 mV (see Chapter III). These results are consistent with our lab’s examination of FPL on the current–voltage relationship of undifferentiated PC12 cells, a cell line expressing predominantly L-type calcium current (Plummer et al., 1989; Usowicz et al., 1990; Lievano et al., 1994), in which enhancement was greatest at −20 mV (L. Liu, unpublished
results). The apparent absence of enhancement of L-type calcium current at positive test potentials is probably not due to FPL being displaced from the channel, however, as tail current amplitude in PC12 cells was insensitive to test potential at potentials more positive than +20 mV. Thus, FPL’s mechanism and voltage-dependency of action is quite different for L- and N-type calcium currents.

Because SCG neurons contain mostly N- and L-type calcium channels, we limited our investigation to examining the effect of FPL on these current types. Thus, we cannot exclude the possibility that FPL may also affect other current types, including perhaps potassium and sodium currents, which are inhibited by micromolar concentrations of DHPs (Jones and Jacobs, 1990). Our results should serve as a warning that, at micromolar concentrations, FPL 64176 is not selective for L-type calcium channels, and any examination of it’s effects on whole-cell currents should take into account effects on all current types, particularly in cell types which express N-type calcium channels.
FIGURE 4-1

FPL 64176 (FPL) increases the amplitude of slowly-deactivating tail currents in SCG neurons. (A) Whole-cell currents were elicited by applying a 20-msec test pulse to +10 mV, followed by a step to -40 mV. Sweep a, current elicited in control solution. Sweep b, current elicited after bath application of 1 μM FPL. In this and subsequent figures, “peak” and “tail” currents refer to amplitudes measured at line 1 and line 2, respectively. For clarity, fast tail currents were clipped. (B) Time course of peak and tail current amplitudes from the same recording shown in A; lower-case letters refer to times where individual sweeps were obtained from A. 1 μM FPL was applied to the bath as indicated by the gray bars.
FIGURE 4-1

A

+10 mV
-40 mV
-90 mV

a - CON
b - 1 μM FPL

B

I_{PEAK} (pA)

I_{TAIL} (pA)

Time (min)

1 μM FPL

0 2 4 6 8
FIGURE 4-2

The effect of 10 μM FPL on whole-cell peak and tail currents in SCG neurons. (A) Currents were elicited using the voltage command shown. Sweep a, current elicited in control solution. Sweep b, current elicited after bath application of 1 μM FPL. Sweep c (the middle peak sweep), current elicited after bath application of 10 μM FPL. Fast tail currents were clipped. (B) Time course of peak and tail current amplitudes (measured as shown in Fig. 4-1 A) from the same recording shown in A; lower-case letters refer to times where individual sweeps were obtained from A. Bath application of 1 μM and 10 μM FPL is indicated by the gray and black bars, respectively.
FIGURE 4-2

A

+10 mV

-90 mV

-40 mV

a - CON
b - 1 μM FPL
c - 10 μM FPL

B

Time (min)

I_{PEAK} (pA)

I_{TAIL} (pA)

1 μM FPL
10 μM FPL

0 2 4 6
FIGURE 4-3

Summary of the effects of FPL on whole-cell peak and tail currents in rat SCG neurons (n = 8). (A) Peak inward current, elicited at +10 mV, was measured as shown in Fig. 4-1 A. In control solution, peak current amplitude was 348.1 ± 51.1 pA. After bath application of 1 μM FPL, current amplitude was 406.7 ± 52.0 pA. Bath application of 10 μM FPL decreased peak current amplitude to 343.9 ± 33.6 pA. *P < 0.05, versus 1 μM FPL. (B) Slowly-deactivating tail current amplitude, elicited at -40 mV, was measured as shown in Fig. 4-1 A. In control solution, tail current amplitude was 5.5 ± 1.6 pA. After bath application of 1 μM FPL, current amplitude was increased to 171.0 ± 25.3 pA. Bath application of 10 μM FPL further increased tail current amplitude to 277.4 ± 36.0 pA. †P < 0.0005, versus CON; ‡P < 0.0005, versus 1 μM FPL.
FIGURE 4-3

A

\[ I_{\text{PEAK}} (-\text{pA}) \]

\[ \begin{array}{ccc}
\text{CON} & 1 \mu\text{M FPL} & 10 \mu\text{M FPL} \\
\end{array} \]

B

\[ I_{\text{TALI}} (-\text{pA}) \]

\[ \begin{array}{ccc}
\text{CON} & 1 \mu\text{M FPL} & 10 \mu\text{M FPL} \\
\end{array} \]
FIGURE 4-4

FPL 64176 reversibly inhibits recombinant N-type calcium current. Whole-cell currents were recorded from tsA201 cells expressing the rat calcium channel α1B-a, β3, and β2/δ-1 subunits. (A) Currents were elicited with 100-msec test pulses to +10 mV, and peak amplitude was plotted against time. 1 μM and 10 μM FPL were applied to the bath as indicated by the gray and black bars, respectively. Transient increases in current amplitude resulting from the application of hyperpolarizing test pulses (see METHODS) are indicated by arrows, and breaks in the time course represent the time periods during which current–voltage data were collected. (B) Individual sweeps obtained from the recording shown in A, taken at the times indicated. (C) Whole-cell current–voltage plots from the recording shown in A were generated by applying 100-msec test pulses at 10-mV increments. Data were collected in control solution (○), following application of 1 μM (■) or 10 μM (▲) FPL, and after washout of FPL (▽).
FIGURE 4-4

A

\( I_{\text{PEAK}} (-\text{nA}) \)

\( \text{Time (min)} \)

B

\( +10 \text{ mV} \)

\( -90 \text{ mV} \)

\( 0.5 \text{nA} \)

\( 25 \text{ ms} \)

C

\( \text{nA} \)

\( \text{mV} \)

-60  -30  30  90

-2.0  -1.5  -1.0  -0.5  -0.05  0.05  0.5

-  CON

1 \( \mu \text{M} \) FPL

10 \( \mu \text{M} \) FPL

WASH
FIGURE 4-5

FPL-induced inhibition of recombinant N-type calcium current is not voltage-dependent. (A) Summary of the current–voltage relationship of whole-cell recombinant N-type calcium current, in control solution (○), following application of 1 µM (■) or 10 µM (▲) FPL, and after washout of FPL (▼) (n = 5–10 recordings). (B) Percent inhibition by 10 µM FPL was calculated and plotted against voltage (n = 10). Note that the reversal potential for permeant ions is approximately +60 mV.
FIGURE 4-5

A

B
REFERENCES


Jeong SW, and RD Wurster (1997b) Muscarinic receptor activation modulates Ca\textsuperscript{2+} channels in rat intracardiac neurons via a PTX- and voltage-sensitive pathway. Journal of Neurophysiology. 78:1476-1490.


CHAPTER V

DISCUSSION
In this thesis, I’ve explored several pathways through which the activity of N-type voltage-gated calcium channels can be modulated. One pathway involves membrane-delimited, voltage-dependent inhibition via pertussin toxin-sensitive heterotrimeric G-proteins. This inhibition is believed to arise from a direct interaction between the G-protein βγ subunit and the pore-forming α_{iB} subunit of the calcium channel (Chapter I), and is characterized by a slowing of activation kinetics, decreased fast and holding potential-dependent inactivation, and a decrease in whole-cell calcium current amplitude (Chapter II).

There are two mechanisms for reversing G-protein-mediated inhibition of calcium currents. Because the association of the G-protein with the channel is sensitive to voltage, membrane depolarization can disrupt the interaction and temporarily relieve the inhibition. This relief has been demonstrated both with square voltage waveforms (Elmslie et al., 1990; Ikeda, 1991), as well as with action potential-like waveforms (Brody et al., 1997; Williams et al., 1997; Park and Dunlap, 1998; Tosetti et al., 1999), suggesting that this modulatory pathway plays an important role in regulating readily-reversible inhibition of calcium influx. Once membrane potential has returned to resting levels, the G-protein is free to re-associate with the channel, restoring inhibition. Thus, facilitation represents a short-lived form of relief from inhibition, limited only by the concentration of free G_{βγ} subunits and their diffusion back to the channel.

G-protein-mediated inhibition can also be reversed by activation of PKC. PKC-mediated phosphorylation of the channel renders the channel resistant to inhibition by G-proteins (Swartz, 1993; Zhu and Ikeda, 1994; Hamid, 1999; Chapter II). Unlike
facilitation, which requires time and a return to resting membrane potential for its effects to reverse, PKC’s effects require the action of a phosphatase to remove the phosphate group from the channel in order for the channel to again be available for G-protein-mediated inhibition (Fig. 2-9). Thus, phosphorylation may represent a longer-lived state during which the calcium channel is resistant to inhibition by G-proteins, and is therefore more sensitive to membrane depolarization.

Recently, Jarvis et al. (2000) reported a physical interaction between syntaxin 1A, the synprint region of the calcium channel’s α1B subunit, and the G-protein βγ subunit. The authors proposed a model in which syntaxin “tethers” the G-protein in the vicinity of the calcium channel (Fig. 5-1 A). One consequence of this interaction between syntaxin and Gβγ might be an increase in the rate of re-inhibition following voltage-dependent relief of inhibition, an hypothesis supported by preliminary data (G.W. Zamponi, personal communication). It would be interesting to examine whether syntaxin increases the rate of decay of prepulse facilitation. Since my data suggest that the G-protein needs to be displaced from the channel to allow PKC access to select target phosphorylation sites on the channel, one role of syntaxin might be to minimize the time during which the channel is available for phosphorylation by PKC. I have shown that PKC appears to function as a switch between two distinct willing states of the channel (willing/available and willing/G-resistant; see Fig. 2-9), minimizing the likelihood that the channel would enter the G-protein–bound (reluctant/P-resistant) state. Similarly, syntaxin might act as a switch between the reluctant/P-resistant and willing/available states of the channel, minimizing the likelihood that the channel would enter the willing/G-resistant (PKC
phosphorylated) state (Fig. 5-1 B). Thus, syntaxin might serve to prevent PKC-induced facilitation.

In addition to the actions of G_{βγ}, our lab has preliminary evidence suggesting that the alpha subunit of the heterotrimeric G-protein (G_α) may play a role in modulating N-type calcium channels. Our examination of the effect of G-proteins on holding potential-dependent inactivation revealed that, when G-proteins were activated by intracellular GTP, whole-cell currents exhibited a lower degree of inactivation at positive conditioning potentials than when GTP was substituted with GDP-β-S (Fig. 2-7 A). This effect was observed at potentials at which voltage-sensitive G_{βγ}-channel interactions should be disrupted; therefore, the difference in inactivation might arise from interactions with G_α. This would be consistent with previous evidence that G_α binds to the channel’s α_{1B} subunit (McEnery, 1994; Furukawa et al., 1998). Future experiments may confirm this hypothesis. If true, this finding would alter our understanding of the role that G-proteins play in modulating N-type calcium currents, and reveal that interactions with G-proteins lead to more diverse changes in calcium influx than currently known.

Because of the critical role calcium current through N-type calcium channels plays in mediating excitation–secretion coupling, it is perhaps not surprising that multiple modulatory pathways converge on the channel. In addition to membrane-delimited inhibition by pertussis toxin–sensitive G-proteins, these channels are sensitive to modulation by a diffusible second-messenger pathway. In SCG neurons, this pathway can be activated by M_1 muscarinic receptors coupled to pertussis toxin–insensitive G_{q11}-proteins (Hille et al., 1995). Our lab has proposed that this pathway involves the
arachidonic acid (AA) signaling cascade (Chapter I). This hypothesis is supported by extensive research performed by L. Liu in our lab, who pharmacologically dissected the pathway from the receptor through to AA release from phospholipids, and demonstrated that disruption of any step in this pathway prevents muscarinic diffusible second-messenger inhibition (Liu and Rittenhouse, 1996, 1998). Additional evidence to support AA's role in mediating this inhibition came from our lab's finding that AA itself, when applied to SCG neurons, inhibits N-type calcium currents (Liu and Rittenhouse, 2000), and that this inhibition occludes receptor-activated diffusible second-messenger inhibition (L. Liu, personal communication).

Our data demonstrate that AA's actions on N-type calcium current are more complex than just inhibition. AA has an inhibitory action in the cytoplasm, yet acts to enhance current from the extracellular surface. These opposing effects suggest that free AA can exert a diverse range of effects on calcium channels, depending on the site from which it is liberated. In addition, because AA is lipophilic and can readily diffuse through cell membranes, it has the potential to act as both a retrograde and an anterograde signaling molecule (see below).

Based on the results presented in this thesis, a model can be drawn describing these various pathways for modulating the activity of N-type calcium channels (Fig 5-2). Each of these pathways can be potentially activated by specific ligands for G-protein-coupled, seven-transmembrane spanning receptors, located within either the same membrane as the channel, or in a nearby membrane (as in the case of AA, which can diffuse across synapses). One key factor of this model is that a rich diversity of pathways
can converge on the channel, leading to a wide range of modulation, depending on the state of the channel when the pathways are activated. For example, activating the G-protein pathway in the absence of activated PKC would lead to modulation by both G\textsubscript{βγ} and G\textsubscript{α}, but activating this same pathway when the channel has been phosphorylated by PKC might lead to modulation by only G\textsubscript{α}. Adding to the complexity of this model is the observation that some of these pathways can themselves be modulated by other protein-protein interactions. For example, G\textsubscript{βγ}-mediated inhibition is facilitated by the presence of syntaxin 1A (Jarvis et al., 2000), and seems to be attenuated by the presence of the calcium channel β subunit (Roche et al., 1995; Roche and Triestman, 1998).

What might be the physiological significance of so many modulatory pathways converging on N-type calcium channels? To be sure, the existence of such diverse pathways suggests that excitation-secretion coupling through N-type calcium channels is not an 'all or nothing' event. The effect of an action potential on synaptic transmission would be expected to vary greatly, depending on the modulatory state of the channels. For example, if the channels reside in a PKC-phosphorylated state, an action potential would evoke a larger calcium influx at the nerve terminal, leading to an increased level of transmitter release, which in turn would induce a larger postsynaptic potential. Alternatively, high levels of G-protein activation would lead to a reduction in calcium influx, and hence a reduced level of transmitter release.

The physiological significance of arachidonic acid's diverse range of effects on N-type calcium channels is less clear. One possible role of AA's inhibitory effects might be to limit calcium influx following activation of AA-liberating pathways such as
muscarinic stimulation of Gq11, leading to a slowly-reversible form of depression. On the other hand, AA’s extracellular action of enhancing the voltage-dependence of N-type calcium channels might give rise to a retrograde form of potentiation, where AA liberated from the postsynaptic membrane can act on the presynaptic channels. Because of AA’s ability to readily cross lipid bilayers, AA liberated from either the presynaptic or postsynaptic membrane can act on either or both membranes, leading to enhancement or inhibition, or both.

It should also be noted that presynaptic and postsynaptic membranes contain additional ion channels, including sodium and potassium channels, as well as additional calcium channel types. In this thesis, I focused on modulation of N-type calcium channels. The modulatory pathways examined in this thesis may play roles in modulating additional current types. My results demonstrate that membrane-delimited inhibition by G-proteins is selective for N-type calcium current in neonatal rat SCG neurons, in that the other principal current type, L-type, is unaffected by G-proteins. However, I also confirmed that activating protein kinase C significantly increases current through L-type calcium channels, suggesting that, in this cell type, the PKC modulatory pathway has additional roles beyond preventing G-protein–mediated inhibition of N-type calcium channels; the significance of PKC-induced increases in L-type calcium current is not currently known. In addition, the results in this thesis demonstrate that AA significantly inhibits both N- and L-type calcium currents, yet AA’s enhancement effects are largely selective for only N-type current. Finally, AA may modulate additional ion channels, possibly leading to alterations in action potential or postsynaptic potentials.
The pathways shown in Fig. 5-2 are not meant to serve as an exhaustive list for modulating N-type calcium channels. Indeed, additional pathways may well exist, especially given that calcium channels associate with several accessory proteins, each of which may themselves be subject to modulation. The pathways explored in this thesis may therefore represent only a fraction of those present in a native cell, leaving the field open for future investigation.
FIGURE 5-1

Model of syntaxin's role in maintaining N-type calcium channels in the reluctant state. (A) Schematic of syntaxin's interaction with the channel's $\alpha_{1B}$ subunit (light blue) and the G-protein's $\beta\gamma$ subunit (shown in dark and light green). Also shown is the channel's $\beta$ accessory subunit (dark blue). Syntaxin's association with both the calcium channel and the G-protein suggest that syntaxin might serve as a tether to keep the G-protein in the vicinity of the channel. Figure from Jarvis et al. (2000). (B) Model of state-driven modulation of N-type calcium channel gating. Phosphorylation of the channel by PKC drives the willing/available channel into the willing/G-resistant state, in which the channel is resistant to modulation by G-proteins. Alternatively, $G_{\beta\gamma}$ can bind to the willing/available channel, driving it into the reluctant/P-resistant state. Syntaxin may then serve to stabilize this state.
FIGURE 5-1

A

B

Reluctant/ P-Resistant   Willing/ Available   Willing/ G-Resistant

Syntaxin   PKC

G

α₁β

PKC phosphorylation
FIGURE 5-2

Schematic of N-type calcium channel modulatory pathways in rat SCG neurons. The transmitters/receptors indicated (shown as 7-transmembrane G-protein-coupled receptors) have been shown by biochemical and electrophysiological methods to associate with one or more of the signaling pathways detailed in this thesis. Note that overlap can occur, in that muscarinic receptors can potentially lead to activation of several pathways, which, depending on the current state of the channel, could lead to a diverse range of responses.
FIGURE 5-2

Enhancement/Facilitation
Inhibition

Muscarinic

AA

PKC

Vasopressin
Angiotensin II
Bradykinin
Muscarinic

NPY
Muscarinic
VIP
α²-adrenergic

OUT

IN

Gα

Gβγ

COOH
REFERENCES


Williams S, M Serafin, M Mühlethaler, and L Bernheim (1997) Facilitation of N-type calcium current is dependent on the frequency of action potential–like depolarizations in dissociated cholinergic basal forebrain neurons of the guinea pig. *Journal of Neuroscience. 17:1625-1632.*