# ACTIVATION OF VOLTAGE-GATED CALCIUM CURRENT BY ACTION POTENTIALS AND MODULATION BY G PROTEINS

by

Debra Elaine Artim

BS, Chapman University, 1996

MS, University of Pittsburgh, 2000

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This dissertation was presented

by

Debra Elaine Artim

It was defended on

February 23, 2005

and approved by

Stephen Dr. Meriney, Ph.D.

Jon Johnson, Ph.D.

Alison Barth, Ph.D.

Guogiang Bi, Ph.D.

Stephen Jones, Ph.D.

Committee chairperson: David Wood, Ph.D.

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Voltage-gated calcium channels are ubiquitously expressed in neurons and are of vital importance to proper cellular functioning. Calcium entry into cells via activation of voltage-gated calcium channels controls a wide variety of cellular functions including neurotransmitter release, muscle excitation-contraction coupling, gene regulation and activation of signaling cascades. Regulation of calcium channels via activation of G protein-coupled receptors is a prominent mechanism of calcium current inhibition and neurotransmitter release. An intriguing characteristic of this modulatory pathway is its voltage dependence whereby the degree of calcium current inhibition varies depending on the membrane voltage of the cell, and is susceptible to activity-dependent relief by trains of action potentials. Previously, it has been suggested that in chick ciliary ganglion neurons, somatostatin inhibits calcium current in a voltage dependent manner. Interestingly, the specific characteristics of the inhibition varied depending on the recording configuration used to collect data. Thus, I measured the voltage-dependence of somatostatin-mediated calcium current inhibition in individual ciliary ganglion neurons using the whole-cell and perforated patch configuration of voltage clamp recordings. The results indicate that the cytoplasmic dialysis that occurs during whole-cell recordings enhances the voltage dependence of calcium current inhibition and suggests that there is a greater concentration of activated G protein subunits in this configuration. While much is known about step depolarization-evoked calcium current and the kinetic changes that accompany G protein-mediated inhibition, relatively little is known about the effects of kinetic slowing on AP-evoked calcium current. Therefore, I used a modification of action potential waveforms was used to determine the effect of G protein activation on the kinetics of single action potential-evoked calcium current. The results demonstrate that kinetic slowing does not alter the time course of action potential-evoked calcium current and suggests that modulated channels may not contribute to AP-evoked calcium current.

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# I. INTRODUCTION

## A. OVERVIEW

Voltage-gated calcium channels are of vital importance to cellular function by opening in response to depolarization and allowing calcium entry into cells. Calcium ions are important signaling molecules for a variety of cellular functions including gene transcription, activating second messenger cascades, muscle contraction and neurotransmitter release. Therefore calcium channels serve as transducers linking membrane depolarization (neuronal activity) to cellular signaling events. Since their discovery, voltage-gated calcium channels in neurons have been the focus of numerous studies and much has been learned about their molecular structure, biophysical properties and functions. One area of particular interest is the role of calcium channels in neurotransmitter release. Action potential invasion of the nerve terminal depolarizes the membrane, resulting in the opening of presynaptic voltage-gated calcium channels. The subsequent influx of calcium ions triggers the release of chemical neurotransmitters from synaptic vesicles. Release of neurotransmitter is tightly regulated by the influx of calcium into neurons and this process is in turn highly regulated by a number of protein - protein interactions and second messenger pathways. Due to their importance in all excitable cells, the study of calcium channels remains an important area of research. The experiments described in this dissertation were designed to further elucidate mechanisms underlying calcium channel activation and modulation.

## B. VOLTAGE-GATED CALCIUM CHANNELS

### 1. Classification

Voltage-gated calcium channels are a class of membrane spanning ion channels that allow the influx of calcium ions into cells in response to membrane depolarization. There are two main categories of voltage-gated calcium channels: low voltage-activated and high voltage-activated. The high voltage-activated calcium channels exhibit marked biophysical diversity. Numerous functional criteria have been used to discriminate among voltage-dependent calcium channels. Properties such as single-channel conductance, time- and voltage- dependence of activation and inactivation, the relative permeability to several divalent cations, the distribution of open- and close-time durations, gating modes, and blockade by various toxins and peptides have been used to identify various channel subtypes. Much has been learned from the correlation of observations of calcium channel function at the cellular level with the function of cloned calcium channels in expression systems. Consequently, channels have been classified according to the specific gene produce that forms the pore-forming $\alpha_1$ subunit. Recently, a new system of nomenclature has been proposed (Ertel et al. 2000), that organizes calcium channels into 3 families based on their structural and functional relationships (Reviewed by Catterall, 2000; see Table 1). The $Ca_v1$ family consists of the L-type, dihydropyridine-sensitive calcium channels and includes the $\alpha_{1s}$ channel found in skeletal muscle and the $\alpha_{1C}$ cardiac calcium channel. The $Ca_v2$ family is prominently expressed in neurons and contains the calcium channels underlying neurotransmitter release. This family consists of the N-, P/Q – and R- type calcium channels which can be regulated by direct binding of G proteins, synaptic proteins and protein phosphorylation. This dissertation focuses on the $Ca_V2.2$ calcium channel, which is the N-type, or $\alpha_{1B}$ containing calcium channel, and is pharmacologically identified by its sensitivity to blockade by ω-conotoxin GVIA (Nowycky et al. 1985). Finally, the $Ca_V3$ family contains the low

**Table 1. High voltage-activated calcium channels: subunit composition, pharmacology and functions.**

| Calcium channel | Calcium current type | Name of α1 subunit | Specific blocker | Primary Localizations | Functions |
|---|---|---|---|---|---|
| $Ca_V1.1$ | L | $\alpha_{1S}$ | Dihydropyridines | Skeletal muscle | Excitation-contraction coupling, Calcium homeostasis, gene regulation |
| $Ca_V1.2$ | L | $\alpha_{1C}$ | Dihydropyridines | Cardiac Muscle, Endocrine cells | Excitation-contraction coupling, hormone secretion, gene regulation |
| $Ca_V1.3$ | L | $\alpha_{1D}$ | Dihydropyridines | Endocrine cells, neurons | Hormone secretion, gene regulation |
| $Ca_V1.4$ | L | $\alpha_{1F}$ | | Retina | Tonic neurotransmitter release |
| $Ca_V2.1$ | P/Q | $\alpha_{1A}$ | ω-Agatoxin | Nerve terminals, dendrites | Neurotransmitter release, dendritic calcium transients |
| $Ca_V2.2$ | N | $\alpha_{1B}$ | ω-Conotoxin-GVIA | Nerve terminals, dendrites | Neurotransmitter release, dendritic calcium transients |
| $Ca_V2.3$ | R | $\alpha_{1E}$ | None | Cell bodies, dendrites, nerve terminals | Neurotransmitter release, calcium-dependent action potentials |

Adapted from Catterall, W.A., 2000.

voltage-activated (T-type) calcium channels which have a prominent role in repetitive neuronal firing and setting neuronal excitability. Calcium channels in the central nervous system exist as heteromeric structures consisting of $\alpha_1$, β, and $\alpha_2/\delta$ subunits (Figure 1). The $\alpha_1$ subunit is the primary determinant of calcium channel biophysics and pharmacology while the other two auxiliary subunits function to modify channel expression and gating properties. Each subunit will be discussed in further detail below.

2. Subunit structure and function.

The $\alpha_1$ subunit is the pore-forming subunit of the calcium channel and is sufficient to produce a functional channel when expressed. This subunit confers the major biophysical properties (including ion permeation and channel gating) and pharmacological properties of the calcium channel. To date, ten genes encoding $\alpha_1$ subunits have been identified and cloned. Each subunit possesses four successive domains of very similar structure termed I-IV. Each repeated domain contains six putative transmembrane segments (S1-S6). These 24 segments are arranged in an orderly manner to define a central ionic pore. The transmembrane segments are highly conserved among channel subtypes and across species. The variable regions include the intracellular loops connecting the segments and the carboxyl terminus. The fourth segment (S4) in each repeat is 20 amino acids in length and contains repeated motifs of one positively charged amino acid at every third or fourth position followed by several hydrophobic amino acids (DeWaard et al. 1996). Studies of single point mutations of the positively charged amino acids of the S4 segment suggest that these segments represent the voltage sensors that may initiate channel opening during channel activation. This hypothesis is supported by data that demonstrate changes in S4 segment conformation are responsible for part of the gating current triggered by membrane depolarization (Guy and Conti, 1990). Two short segments located between S5 and S6 in each of the four repeated domains, along with the α helical

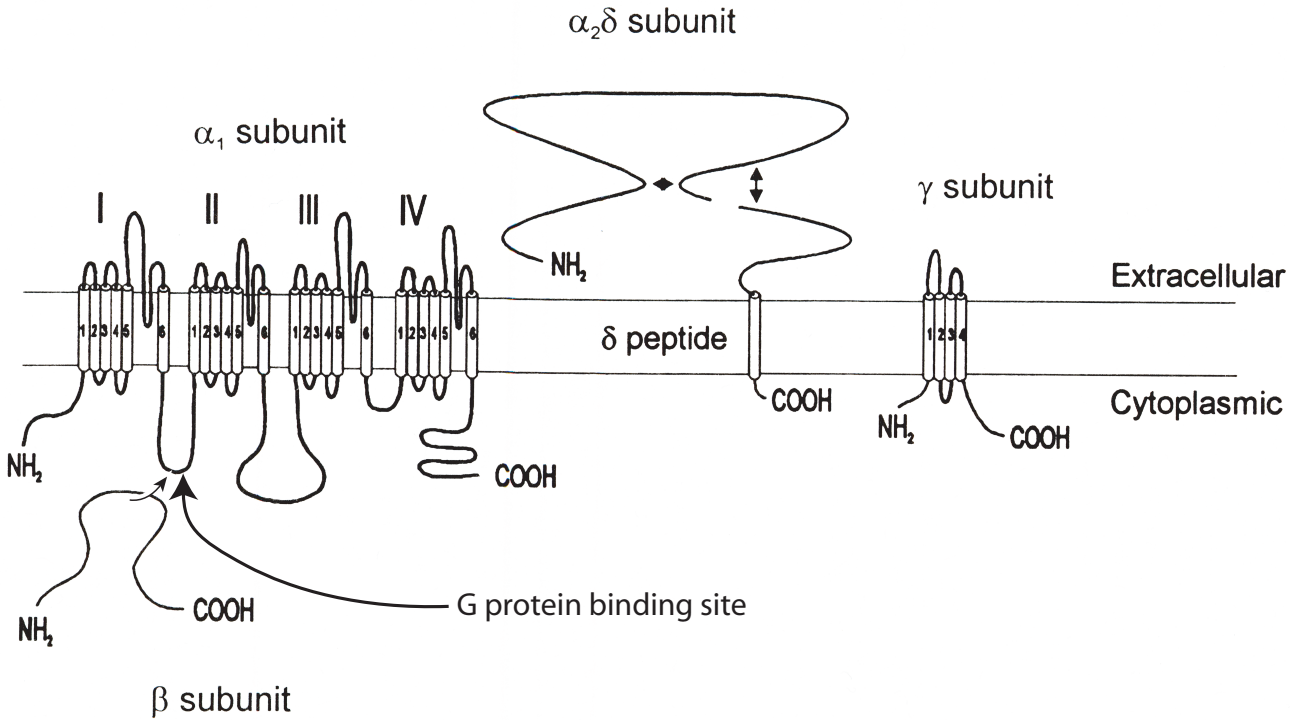


**Figure 1. Schematic of the voltage-gated calcium channel**. Diagram of the calcium channel α1, β, α2/δ, and γ subunits. Arrows indicate the I-II linker of the α1 subunit, which contains the binding sites for the calcium channel β subunit and G protein βγ subunits. Diagram is adapted from DeWaard et al., 1996.

portions of S5 and S6 are proposed to form the permeation pore. This region is thought to be responsible for conferring the ion selectivity of the channel (Heinemann et al. 1992).

The β subunit of voltage-gated calcium channels does not contain any putative transmembrane domain and thus is a completely intracellular protein. Four β subunit genes have been identified, each of which is subject to alternative splicing to yield a variety of gene products. Beta subunits contain 7 α-helical domains and four heptad repeats in which most of the first and fourth residues of every seven are hydrophobic (DeWaard et al. 1996). These repeats are thought to be involved in interactions with cytoskeletal proteins (Fuchs & Hanukoglu, 1983). Coexpression of β subunits with $\alpha_1$ subunits increases the functional expression of calcium channels and modifies activation and inactivation properties. Different β subunits can affect the biophysical properties of calcium channels in different ways and to varying extents (reviewed by Dolphin, 2003). For example, coexpression of β subunits with any $\alpha_1$ subunit increases calcium current amplitude but to varying extents depending on which α1 subunit is expressed. Coexpression of $\beta_{1b}$ with $\alpha_{1A}$, $\alpha_{1B}$, or $\alpha_{1E}$ produces an 18-fold, a 4-fold and a 3-fold increase in peak current amplitude respectively (DeWaard et al. 1994; Stea et al. 1993; Wakamori et al. 1994). The magnitude of current amplitude increase may also depend upon the specific β subunit expressed Hofmann et al. 1994; Hosey et al. 1996). For example, coexpression of the various β subunits with the $\alpha_{1C}$ subunit results in between a 2-fold and a 19-fold increase in current magnitude. Additionally, biophysical properties of the calcium channel can be differentially affected by various β subunits (Meir & Dolphin, 2002; Olcese et al. 1994). For example, the $\beta_{1b}$, $\beta_{2a}$ and $\beta_4$ subunits decrease channel inactivation rate while the $\beta_3$ subunit enhances channel inactivation rate. Thus, expression of an $\alpha_1$ subunit with various β subunits gives the potential for remarkable physiological diversity. Since the β subunit is entirely cytoplasmic, interactions of the β subunit with the α subunit are confined to intracellular sites. Two domains involved in β subunit binding to the $\alpha_1$ subunit have been identified (Dolphin et al. 1999). The $\alpha_1$ interaction domain (AID) binds β subunits and is located in the I-II cytoplasmic

linker of all α1 subunits. This domain is 18 amino acids in length, of which nine are highly conserved and thought to participate in β subunit binding. The β interaction domain (BID) interacts with AID, and consists of a sequence of 30 amino acids located at the N-terminus of the β subunit. The BID is thought to be a potential regulatory target for protein kinase C.

The $\alpha_2/\delta$ subunit consists of the δ subunit which has a single transmembrane segment and is linked by disulfide bond to the $\alpha_2$ subunit, which is entirely extracellular. Thus, only 5 amino acids in the δ subunit are located intracellularly (Randall & Benham, 1999). There are currently 4 known $\alpha_2/\delta$ subunits and they have been shown to increase expression of calcium channels in the cell membrane, prevent prepulse facilitation of calcium current and modify gating properties (Klugbauer et al. 1999). However, the effects of the $\alpha_2/\delta$ subunits are generally smaller than those of the β subunits and are limited primarily to current amplitude and inactivation kinetics (DeWaard et al. 1996). As with the β subunits, the magnitude of current amplitude increase varies with different subunit combinations. For example, $\alpha_2/\delta 1$ induces a 1.5-fold increase in current amplitude when expressed with $\alpha_{1D}$ and a 17-fold increase when expressed with $\alpha_{1C}$. Additionally, it has been demonstrated that the coexpression of the β subunit may be required to allow the maximum effect of the $\alpha_2/\delta$ subunit to be exhibited (DeWaard et al., 1995).

The gamma (γ) subunit was originally found to be colocalized with L-type calcium channels in skeletal muscle (Takahashi et al. 1987). This subunit contains four putative transmembrane domains with intracellular amino and carboxy terminals and is thought to modify the inactivation of skeletal muscle calcium channels. Recently, a novel γ subunit has been identified that can modulate the voltage dependence of $\alpha 1_A$-containing calcium channels (Letts et al, 1998). It remains to be determined if all neuronal calcium channels are associated with a γ subunit.

3. Role in disease.

Channelopathies are a class of inherited diseases characterized by mutations in genes for ion channels. Several spontaneously occurring mutations in calcium channel subunits have been found and linked to various diseases. Investigation of calcium channel mutations and their link to channelopathies is fast becoming a productive area of research. Mutations in calcium channel genes can alter the expression, development and function of the channel. Mutations in the $\alpha_{1A}$ subunit have been linked to absence epilepsy and cerebellar ataxia (Fletcher et al. 1996). Mouse models of these mutations (*tottering* and *leaner*) have been instrumental in identifying biophysical properties of the channel that are altered, including a decrease in mean open time and smaller single channel conductance. A study of transmitter release at the parallel fiber synapse of *tottering* mice has shown that release is mediated primarily by N-type calcium channels, as opposed to the P/Q-type channels that control release in wild-type mice (Zhou et al., 2003). Furthermore, since N-type calcium channels are more susceptible to G protein-mediated inhibition than P/Q-type channels, activation of $GABA_B$ receptors results in a greater amount of transmitter release inhibition in *tottering* parallel fiber synapses than in those of wild-type mice. Thus, mutations affect not only calcium current amplitude, but transmitter release properties that may have functional effects on the output of neuronal systems.

Mutations in calcium channels underlying channelopathies are not limited to the $\alpha_1$ subunit. A mutation in the $\beta_4$ subunit is linked to idiopathic generalized epilepsy and episodic ataxia (Escayg et al. 2000). The *lethargic* mutation results in a truncated $\beta_4$ mRNA, and no expression of $\beta_4$ protein (Burgess et al. 1997). As a result, there is a compensatory overexpression of $\beta_{1b}$ subunits (McEnery et al. 1998) that causes alterations in thalamic excitatory synaptic currents that are hypothesized to contribute to the generation of seizures (Caddick et al. 1999). Clearly, proper expression of $\alpha_1$ and $\beta$ subunits is crucial for proper functioning of the synapse and of neuronal circuitry.

## C. G PROTEIN MODULATION OF CALCIUM CURRENT

### 1. Mechanism

Calcium channels of the $Ca_V2$ family (which includes the N-type calcium channel, $Ca_V2.2$, that is the focus of the studies described here) are common targets of modulation by heterotrimeric G proteins. Many studies have investigated this process, first described by Dunlap & Fishbach (1981) who found that NE inhibited calcium current in dorsal root ganglion neurons. The most extensively studied mechanism underlying calcium channel modulation is a membrane-delimited pathway in which activated G protein βγ-subunits (G-βγ) interact directly with the $\alpha_1$ subunit of the calcium channel (Herlitze et al. 1996; Ikeda 1996). This pathway is limited spatially so that all of the elements required for modulation are in close proximity with each other. This was demonstrated by Forscher and colleagues (1986) who studied calcium current inhibition using cell-attached recordings of calcium current They found that modulation occurred only when the agonist was included in the patch pipette, but not in the extracellular solution, thus ruling out a role for soluble second messengers in the pathway mediating calcium current inhibition.

Several portions of the calcium channel α subunit have been implicated in G-βγ binding. A primary binding site for G-βγ is on the I-II linker of the calcium channel $\alpha_1$ and has been shown to be essential for G protein inhibition. It has been suggested that this site may determine G protein specificity because the efficacy of various subtypes of G protein β subunit binding to this site is correlated with the efficacy of β subunit-mediated modulation of N-type calcium channels (Garcia et al. 1998). Additional G-βγ binding sites have been identified using G protein binding experiments, site-directed mutagenesis, and construction of calcium channel chimeras. Researchers have taken advantage of the fact that $Ca_V2.1$ and $Ca_V2.3$ channels are modulated to a lesser degree than are $Ca_V2.2$ channels. Therefore, by constructing channel chimeras, the

contribution of various binding sites to G protein modulation can be determined. These experiments have demonstrated that the I-II linker and the N-terminus of the calcium channel contain binding sites important for G protein modulation (Canti et al. 1999; Page et al. 1998; Qin et al 1997; Simen & Miller 1998). The C–terminus of the calcium channel has also been implicated, and was shown to be required for complete G protein regulation of $Ca_V$ 2.1 (Zhang et al. 1996). Although there are multiple sections of the calcium channel that have been shown to be important for G protein binding to cause maximal inhibition of calcium current, physiological data suggest that calcium current inhibition may be mediated by binding of a single G-βγ subunit to the channel (Zamponi & Snutch, 1998). Thus, it has been suggested that the multiple binding sites cooperate to form a single high affinity G-βγ binding site.

2. Voltage dependence of calcium current inhibition.

A defining characteristic of membrane-delimited, G-βγ-mediated calcium current inhibition is that it is voltage-dependent, typically demonstrated by relief of inhibition by a large step depolarization (reviewed by Jones & Elmslie, 1997; Elmslie, 2003). This form of G protein modulation results in a change in the voltage-dependence of calcium channel gating such that modulated channels require a stronger or longer depolarization to open (Bean, 1989). Modulated channels are hypothesized to be in a reluctant gating mode and a large depolarization is required to shift them back into the willing, unmodulated gating mode. Prepulse-induced relief of inhibition is hypothesized to be due to a voltage-dependent conformational change in the calcium channel that results in decreased affinity of the calcium channel for G proteins, thus G-βγ subunits unbind from the channel. A direct interaction of G-βγ subunits with the calcium channel is suggested by evidence that reinhibition of calcium current following a prepulse (due to rebinding of G-βγ to the calcium channel) depends on the concentration of activated G proteins (Lopez & Brown, 1991; Elmslie & Jones, 1994).

Furthermore, the time course of reinhibition following a prepulse is consistent with inhibition being caused by binding of a single G-βγ subunit to the calcium channel(Zamponi & Snutch, 1998).

Voltage-dependent calcium current inhibition is characterized by a slowing of current activation kinetics, termed kinetic slowing (Bean, 1989), which reflects the gradual shift of channels from the reluctant to willing gating mode during prolonged depolarization. Thus, kinetic slowing is an example of voltage-dependent inhibition relief; modulated channels open more slowly resulting in the appearance of slowed calcium current activation. Single-channel studies of calcium current inhibition have supported this hypothesis, showing that G protein-mediated inhibition results in an increase in the latency to first channel opening (Carabelli et al. 1996; Patil et al. 1996). This increase is correlated with the presence of kinetic slowing in ensemble currents and is relieved by a strong prepulse depolarization. There is also evidence that modulated channels can open directly from the reluctant gating mode, suggesting that G protein unbinding is not necessarily required for channel activation (Colecraft et al. 2000; Lee & Elmslie 2000). Reluctant openings occurred without an increase in first latency and with lower open probability and shorter mean open times than channel openings from the normal (non-modulated) gating mode. It has previously been reported that in whole-cell recordings of calcium current, reluctant openings contributed to action potential-evoked calcium current, and caused calcium current to peak earlier (Colecraft et al., 2000). Analysis of gating currents, which are due to movement of the voltage sensor in the S4 region of the calcium channels, show that they are also slowed by G protein modulation and accelerated by a depolarizing prepulse (Jones et al. 1997). G protein modulation also reduces the ability of a gating charge to result in channel opening. These observations are consistent with a willing / reluctant model of modulated calcium channel gating.

The sensitivity of voltage-dependent calcium current inhibition to relief by a prepulse led investigators to speculate that it may also be sensitive to physiologically relevant stimuli.

Inhibition can be partially relieved by short trains of 1- to 2- ms step depolarizations that were designed to mimic action potential stimulation (Williams et al. 1997; Womack & McCleskey 1995). Since then, it has been shown that trains of action potential waveforms can relieve G protein inhibition of N-type calcium channels in chick dorsal root ganglion neurons (Park & Dunlap, 1998) and of P/Q-type calcium channels expressed in HEK 293 cells (Brody et al. 1997). Furthermore, the amount of inhibition relieved depended on the duration of the action potentials used and the frequency of action potential stimulation (Park & Dunlap, 1998). These data suggest that action potential-induced relief of voltage-dependent inhibition may be relevant under physiological conditions. In support of this hypothesis, Brody & Yue (2000), have shown that relief of G protein-mediated inhibition of calcium current contributes to short-term synaptic facilitation in hippocampal autapses.

3. Interactions of the G protein pathway with other proteins and second messenger systems.

Many proteins can interact with the calcium channel, with G proteins or with both to influence the effectiveness of G protein-mediated calcium current inhibition.

a. Protein kinase C. Protein kinase C (PKC) has effects on calcium current in marked contrast to the effects of G proteins. Phosphorylation of the calcium channel by PKC can enhance calcium current in many preparations. Additionally, PKC phosphorylation can reverse G protein-mediated inhibition of N- and P/Q-type calcium current both in dissociated neurons and in channels expressed in *Xenopus* oocytes. PKC phosphorylation also prevents future G protein modulation of calcium current upon activation of G protein coupled receptors but only when $G\beta_1$ is the activated subunit . Recently, a single site on the G protein $\beta_1$ subunit has been identified that can detect PKC-mediated phosphorylation of the $\alpha 1_B$ subunit (Doering et al. 2004). The site of PKC phosphorylation has been identified as threonine residue 422 on the I-II linker of the

$\alpha_1$ subunit, just downstream of the principle interaction site of G-βγ. Phosphorylation of this site blocks G-βγ binding to this region (Zamponi et al. 1997). Thus the actions of PKC can alter the effectiveness of G protein activation to inhibit calcium current.

b. Calcium channel beta subunit. The interaction of calcium channel β subunits with G protein inhibition of calcium current has been extensively studied and the results have not always been easily interpreted (reviewed by Dolphin, 2003). Several studies have produced data that seem to be in contrast with each other. Early studies provided evidence that the calcium channel β subunit competed with G protein βγ subunits for binding on the calcium channel α1 subunit (Bourinet et al. 1996; Qin et al. 1997). Therefore, it was thought that effective G protein inhibition would only occur in the absence of the β subunit. However, recent studies have provided greater insight into the interaction of calcium channel β subunits and G-βγ with the calcium channel $\alpha_1$ subunit. In contrast to the previous hypothesis, it is now postulated that the calcium channel β subunit is essential for voltage-dependent calcium current inhibition. Coexpression of calcium channel β subunits resulted in more pronounced voltage-dependent calcium current inhibition, which was reflected in an increased rate of facilitation due to G-βγ unbinding (Canti et al. 2000; Roche & Treistman, 1998) These studies showed that in the absence of β subunits, G-βγ can induce a small voltage-independent calcium current inhibition, but robust voltage-dependent inhibition (as measured by relief of inhibition by a prepulse) required the presence of β subunits. It is hypothesized that during voltage-dependent relief of inhibition, the depolarization changes the conformation of α1 / β subunit binding thereby decreasing the stability of G-βγ binding to the calcium channel.

c. Synaptic proteins. The presence of synaptic proteins has been found to influence calcium current attributes. Coexpression of syntaxin 1A, a presynaptic membrane protein which is part of the SNARE complex, with $\alpha_{1B}$ calcium channels results in a 15-20 mV negative shift in the

half-inactivation potential (Bezprozvanny et al. 1995). Furthermore, calcium current has enhanced slow inactivation when coexpressed with syntaxin (Degtiar et al. 2000). There is also evidence that syntaxin can alter G protein-mediated modulation of calcium current. Stanley & Mirotznik (1997) found that botulinum toxin cleavage of syntaxin prevents G protein modulation of N-type calcium channels in chick ciliary ganglion calyx terminals. This led to the hypothesis that the presence of syntaxin was required for G protein activation to effectively inhibit calcium current. To investigate the interaction of syntaxin with G protein-mediated modulation of calcium current, Jarvis et al. (2000) coexpressed syntaxin 1A with $\alpha_{1B}$ subunits in tsA-201 cells. They found that coexpression of syntaxin 1A resulted in a pronounced tonic G protein inhibition of the N-type calcium current that was voltage-dependent, as measured by prepulse relief of inhibition. This effect was attenuated by botulinum toxin C further supporting the role of syntaxin. However, when G βγ subunits were also coexpressed, botulinum toxin was unable to prevent the tonic inhibition. Thus, they concluded that syntaxin optimized G protein modulation, but was not required for it to occur. Overexpression of syntaxin 1A also promoted calcium current inhibition in chick dorsal root ganglion neurons, and shifted the inhibition towards greater voltage-dependence (Lu et al. 2001). It has been demonstrated that syntaxin can bind G protein βγ subunits and the II-III linker of the calcium channel simultaneously (Jarvis et al. 2000). Therefore, it is hypothesized that syntaxin acts to colocalize the calcium channel with G-βγ, thus optimizing conditions for G protein modulation.

In contrast to the effects of syntaxin on calcium current, cysteine string protein (CSP) has been shown to enhance N-type calcium current. At the chick ciliary neuron calyx synapse, injection of recombinant CSP into the terminal resulted in increased calcium current amplitude which was due to recruitment of dormant channels (Chen et al. 2002). However, coexpression of CSP with the N-type calcium channel also resulted in robust tonic inhibition of calcium current, in a G protein and voltage-dependent manner (Magga et al, 2000). As with syntaxin, the C terminal of CSP can bind to the II-III linker of the calcium channel and to G-βγ subunits.

Therefore, it has been proposed that CSP acts by targeting G protein subunits to their binding site on the calcium channel. It addition to binding G-βγ, the J domain of CSP can bind to G protein α subunits and prevent assembly of the G protein heterotrimer, resulting in a greater concentration of free G-βγ subunits (Miller et al. 2003). Thus, the extent of G protein inhibition of calcium current and the degree of voltage-dependence can be influenced by coexpression of certain synaptic proteins, further regulating calcium influx at the synapse.

d. Phosphatidylinositol 4,5-biphosphate ($PIP_2$). $PIP_2$ is a membrane associated phospholipid that has been implicated in a number of cellular functions including endocytosis and regulated exocytosis of peptide in neuroendocrine cells (Czech, 2000). There are two actions of $PIP_2$ on calcium channel activity (Wu et al. 2002). The first is to produce stabilization of P/Q-channel activity. This was demonstrated by the ability of $PIP_2$ to prevent channel rundown during the course of a recording when included in the patch pipette. Secondly, $PIP_2$ can produce a small degree of voltage-dependent inhibition of calcium current which can be prevented and reversed by phosphorylation by PKA. It has been suggested that $PIP_2$ stabilizes a G βγ-sensitive state of the calcium channel, an effect that depends on cytosolic calcium (Rousset et al. 2004). The hypothesis is that stable interactions between the membrane and the calcium channel require both $PIP_2$ and cytosolic calcium and that these interactions are necessary for normal calcium channel G-βγ interactions.

e. Cytoskeleton. Components of the cytoskeleton can interact with calcium channels and alter the characteristics of calcium entry. For example, inactivation of L-type calcium channels has been shown to depend on the cytoskeleton. In macropatch recordings of L-type calcium current, disruption of the cytoskeleton by colchicine or cytochalasin B increased calcium current inactivation and the cytoskeleton stabilizers taxol and phalloidin decreased inactivation (Johnson & Byerly, 1993). A subsequent study investigated the mechanism underlying this

effect and found that microtubule disruption increased channel open probability while microtubule stabilization decreased channel open probability (Galli & DeFelice, 1994). Additionally, it has been demonstrated that cytoskeleton integrity is important for G protein mediated signaling cascades. G protein βγ subunits can bind directly to microtubules (Gundersen & Cook, 1999) and the efficacy of G protein actions can be modified by changes in the structure and stability of the cytoskeleton. In β1-integrin knock-out mice, muscarinic inhibition of L-type current in cardiac myocytes was absent (Bloch et al. 2001). This modulation was also impaired by myocyte treatment with cytochalasin D. Immunostaining revealed that the disruption was due to displacement of G proteins, suggesting that cytoskeletal integrity is required for proper localization and function of G protein signaling. Michel et al (1996) showed that disruption of microfilaments or of microtubules in human erythrocytes inhibited $G_i$ protein signaling by neuropeptide Y and muscarinic receptors at the level of the receptor - G protein interaction without effecting agonist binding to the receptor. Thus, cytoskeleton integrity is vital for effective G protein signaling.

Recently, a direct interaction between cytoskeleton and N-type calcium channels has been demonstrated (Leenders & Sheng, 2004). The yeast two-hybrid system was used to demonstrating binding of the N-channel alpha subunit ($\alpha_{1B}$) to MAP 1 A/B light chains, stabilizing proteins known to interact with microtubules. Interestingly, the MAP proteins bind to the $\alpha_{1B}$ subunit at a sequence in the C-terminus known to also bind G protein $\beta\gamma$-subunits (Leenders & Sheng, 2004). Thus, it is possible that alterations in cytoskeleton can influence calcium channel activity and interactions with other proteins.

3. Desensitization

G protein signaling loses effectiveness during a prolonged exposure to agonist, a process termed desensitization. Desensitization of G protein-mediated signaling can arise from a variety of different mechanisms that vary depending on cell type and on the specific receptor studied (for review see Ferguson, 2001). Rapid desensitization (within seconds to minutes of agonist exposure) can be due to phosphorylation by specific G protein-coupled receptor kinases (GRKs), phosphorylation by second messenger kinases, or receptor internalization with two or more mechanisms often combining to produce desensitization. GRK-induced desensitization results from G protein uncoupling from the receptor in response to receptor phosphorylation. GRKs phosphorylate specifically agonist bound receptors, promoting the binding of β-arrestin. This step leads to receptor uncoupling from G proteins and also targets many G protein-coupled receptors (GPCRs) for internalization in clathrin-coated vesicles.

GRKs are a family of serine / threonine protein kinases that mediate agonist-dependent phosphorlyation of GPCRs and initiate homologous desensitization (Premont et al., 1995; Stoffel et al., 1997). The GRK family includes seven members with marked sequence similarity and three functional domains. The N-terminal domain is thought to be involved with substrate specificity. The central, catalytic domain consists of approximately 240 residues with high sequence identity among the different GRKs and includes the ATP binding domain. The C-terminus is involved in targeting of cytoplasmic GRKs to the plasma membrane. GRK 2 and 3 (βARK 1 and 2) require translocation from the cytosol to the membrane for activation upon agonist stimulation. These GRKs have a 125-residue extension at the C-terminus that interacts with free G protein βγ-subunits. Since free βγ-subunits are found only at sites of activated receptors, this allows for precise localization of GRK to activated receptors. Also, the degree of GRK 2/3 activation depends on the concentration of activated βγ-subunits.

Receptor internalization also contributes to rapid desensitization of GPCR-signaling (Beaumont et al., 1998). Agonist-activated receptors are endocytosed, usually through clathrin-mediated mechanisms, and sequestered into the intracellular membrane compartments of the cell. This process of receptor internalization is thought to be required for dephosphorylation of the GPCR and subsequent recycling of the receptor back to the cell surface. Although the rate of GPCR recycling varies among receptor types (Ferguson et al., 2000), increases in GRK activation increase the rate of receptor internalization (Tsuga et al., 1994).

In addition to the mechanisms described above, G protein signaling is also terminated by a family of proteins called regulators of G protein signaling (RGS proteins). These proteins increase the rate of Gα – bound GTP hydrolysis, thereby promoting formation of the G protein heterotrimer and terminating G protein signaling. Recently, Tosetti et al. (2003) demonstrated a calcium-dependent mechanism for RGS3 activity in chick dorsal root ganglion neurons. There were two components of RGS-mediated desensitization of G protein activity. The first, fast component was calcium-dependent and required direct binding of calcium to the RGS protein while the second, slower component was calcium and calmodulin-dependent. Both components acted to terminate G protein-mediated inhibition of calcium current.

## D. CHICK CILIARY GANGLION

The chick ciliary ganglion has two homogeneous populations of parasympathetic motoneurons: ciliary neurons, which innervate the striated muscle of the ciliary body and iris, and choroid neurons, which innervate the smooth muscle of the choroid coat in the eye (Marwitt et al. 1971, Figure 2). Using immunohistochemical methods, somatostatin has been shown to be expressed in choroid cell somata and nerve terminals innervating the smooth muscle of the

# Chick Ciliary Ganglion

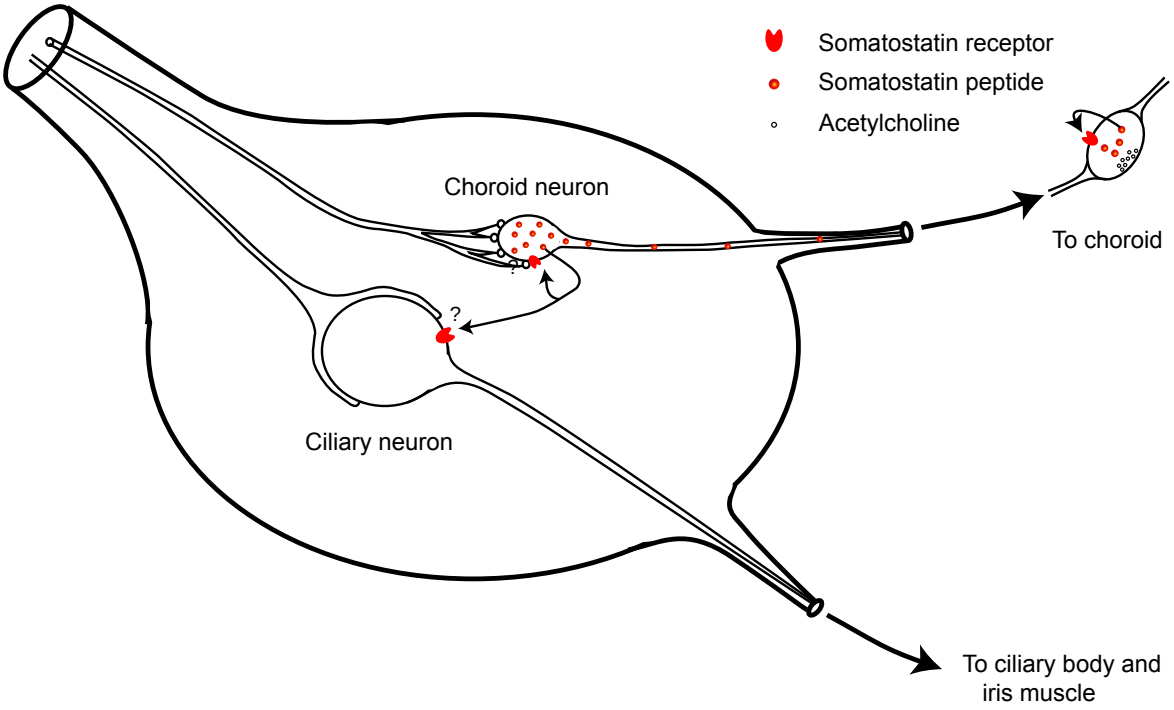


**Figure 2. Diagram of the chick ciliary ganglion.** The chick ciliary ganglion contains two populations of neurons: ciliary neurons, that innervate the striated muscle of the ciliary body and iris, and choroid neurons, which innervate the smooth muscle of the choroid coat in the eye. Both ciliary and choroid neuron cell bodies express functional somatostatin receptors. Somatostatin is expressed in choroid cell somata and nerve terminals innervating the smooth muscle of the choroid coat.

choroid coat (Epstein et al. 1988; De Stefano et al. 1993). In contrast, ciliary neurons and preganglionic Edinger Westfal nerve terminals onto both ciliary and choroid cell bodies do not appear to express somatostatin. Despite this apparently specific localization of somatostatin peptide expression only to choroid neurons, both ciliary and choroid neuron cell bodies clearly express functional somatostatin receptors (Meriney et al. 1994; White et al. 1997). Dissociated ciliary ganglion neurons are a good model system with which to investigate calcium current modulation because they express only 2 types of calcium current (N-type and L-type) and somatostatin produces reliable, robust inhibition of exclusively N-type current (White et al. 1997). Furthermore, a high quality voltage-clamp can be achieved allowing the study of calcium currents with a high degree of biophysical rigor.

## E. SOMATOSTATIN

Somatostatin is a peptide originally identified in the rat hypothalamus as a factor that reduced the release of growth hormone from rat pituitaries. Since its discovery, somatostatin has been implicated in numerous physiological actions including endocrine, neurophysiological and neurotransmission and somatostatin receptors have been found widely distributed in the periphery and in the central nervous system (Schindler et al. 1996). In addition to somatostatin's effect on growth hormone secretion, it has other neuroendocrine functions including in the pancreas where it lowers plasma levels of insulin and glucagon, and in the gastrointestinal tract where it produces potent inhibition of a variety of peptides. Somatostatin has been shown to inhibit high voltage-gated calcium current via activation of pertussis toxin sensitive G proteins in a number of neuronal preparations including CA1 pyramidal neurons (Ishibashi & Akaike, 1995) and the rat amygdala (Viana & Hille, 1996). Additionally,

somatostatin can modulate the activity of inward rectifying potassium channels. In rat locus coeruleus neurons, somatostatin decreases spontaneous firing by opening inward rectifying potassium channels (Chiu et al. 1994). Somatostatin can also activate the "M-type" potassium current and an additional voltage-insensitive potassium conductance (Schweitzer et al. 1998). Modulation of transmitter release has also been attributed to somatostatin actions. In CA1 and CA3 hippocampal neurons, somatostatin application depressed glutamatergic EPSCs. The overall effects of somatostatin tend to be inhibitory and it is hypothesized to act broadly to reduce the spread of epileptiform events through the hippocampus (Tallent & Siggins, 1997).

In the ciliary ganglion, somatostatin has been shown to modulate calcium current and neurotransmitter release. Somatostatin inhibits calcium current in the chick ciliary ganglion (Dryer et al. 1991) and in acutely dissociated chick ciliary ganglion neurons (Meriney et al. 1994, White et al. 1997). The inhibition targets exclusively N-type calcium channels (White et al. 1997), is mediated by activation of pertussis toxin-sensitive G proteins and is voltage-dependent. Furthermore, the characteristics of somatostatin-mediated calcium current inhibition have been shown to vary depending on the recording configuration used to record calcium currents (Meriney et al. 1994). Somatostatin has also been shown to produce robust inhibition of high potassium-evoked acetylcholine (ACh) release from the intact ciliary ganglion and from dissociated ciliary ganglion neurons (Pilar et al. 1996, Gray et al. 1999). Somatostatin is colocalized with ACh in choroid nerve terminals (Epstein et al. 1988; Gray et al. 1989), and is thought to act in a negative feedback role on ACh release (Gray et al. 1989, 1999). Interestingly, somatostatin can also be released from ciliary ganglion neuronal somata (Johnson & Pilar, 1980). Since all ciliary ganglion neurons express somatostatin receptors (Meriney et al. 1994; White et al. 1997), somatostatin may act globally in the ciliary ganglion *in vivo*. Manipulation of a nitric oxide / cGMP-dependent protein kinase (NO/PKG) pathway was demonstrated to alter the ability of somatostatin to inhibit transmitter release from choroid nerve terminals and isolated cell bodies. Blockade of NO synthase or cGMP-dependent protein kinase attenuated

somatostatin-mediated inhibition of ACh release (Gray et al. 1999). This work led to the hypothesis that a cytoplasmic NO/PKG pathway alters the calcium sensitivity of the release apparatus. These alterations in calcium sensitivity are thought to regulate the functional significance of somatostatin-mediated inhibition of calcium current in the ciliary ganglion.

## F. CALCIUM CURRENT AND TRANSMITTER RELEASE

### 1. Presynaptic inhibition

Presynaptic inhibition of transmitter release refers to activation of receptors on the presynaptic membrane that results in subsequent decrease in the amount of transmitter released from the terminal. This is thought to be a mechanism that prevents too much transmitter from being released, especially during periods of high neuronal activity. One well-documented mechanism underlying presynaptic inhibition is the reduction of presynaptic calcium influx via activation of G protein-coupled receptors (reviewed by Wu & Saggau, 1997). For example, activation of adenosine A receptors inhibits presynaptic calcium influx and transmitter release from the calyx of the chick ciliary ganglion (Yawo & Chuhma, 1993). Transmitter release was measured from synapses between co-cultured sympathetic neurons, where activation of neuropeptide Y receptors inhibited transmitter release via reduction in presynaptic calcium entry (Toth et al. 1993). In cerebellar slices, activation of $GABA_B$ receptors resulted in reduced presynaptic calcium signal and inhibition of transmitter release at parallel fiber synapses (Dittman & Regehr, 1996). These are but a few examples of studies that have demonstrated a relationship between G protein-mediated inhibition of calcium current and a reduction in transmitter release. In addition to the G protein effect on presynaptic calcium current, activation of G protein-coupled

receptors can also modulate transmitter release downstream of calcium entry. G protein activation has been shown to decrease the frequency of miniature EPSCs at the neuromuscular junction and at hippocampal and cerebellar synapses. This effect is not mimicked by decreasing extracellular calcium and therefore is likely due to a mechanism that is downstream from calcium entry. Transmitter release is inhibited by serotonin at the lamprey reticulospinal motoneuron synapse, independent of calcium influx (Blackmer et al. 2001). Injection of G protein βγ subunits directly into the nerve terminal also inhibited transmitter release downstream of calcium entry. These authors propose a role for G proteins at the level of the exocytotic fusion machinery. These studies suggest a role for G protein-mediated presynaptic inhibition in feedback regulation of transmitter release, synaptic depression and perhaps in setting synaptic strength.

2. AP broadening

The shape of an action potential waveform is important in determining the precise time course and amplitude of calcium entry into the nerve terminal. These changes in calcium entry in turn regulate the magnitude of transmitter released. Blocking potassium channels that contribute to action potential repolarization will slow the repolarization rate, thus increasing action potential duration and prolonging the stimulus for calcium entry. Therefore changes in ionic conductances can modulate transmitter release indirectly via changes in calcium entry. For example, lithium-induced action potential broadening enhances glutamatergic transmission in CA1 pyramidal cells by a presynaptic mechanism consistent with increased calcium influx (Colino et al. 1998). In slices of rat pituitary terminals Jackson et al. (1991) used simultaneous patch-clamp recordings and fluorimetric calcium measurements to show that action potential broadening induced by application of a potassium channel blocker increased the calcium signal and enhanced transmitter release. However, action potential broadening does not always result

in increased transmitter release. In *Xenopus* nerve-muscle co-cultures, blockade of calcium-activated potassium (BK) channels with iberiotoxin decreased transmitter release (Pattillo et al. 2001). These authors hypothesized that the reduction in transmitter release may be due to a decreased driving force on calcium caused by blocking the BK channels that contribute to the late repolarization phases of the action potential. Thus, the precise time course of action potential shape and repolarization will determine the calcium entry profile and subsequent transmitter release.

## G. GOALS OF THE DISSERTATION

A thorough understanding of the function and modulation of voltage-gated calcium channels is crucial to our understanding of the function of synapses and of the nervous system. The purpose of this dissertation is to gain insight into calcium current activation and modulation by G proteins. Specifically, I was interested in the voltage-dependence of G protein-mediated inhibition. Furthermore, while much is known about modulation of calcium current evoked by step depolarization, I was interested in how action potential-evoked calcium currents are effected by G protein modulation. These goals were pursued by experiments outlined in the following sections.

**1. Voltage-dependence of somatostatin-mediated inhibition in chick ciliary ganglion neurons.** The ciliary ganglion presents a unique opportunity to study voltage-dependent calcium current inhibition. A previous study suggested that the degree of kinetic slowing of modulated calcium current varies depending on the recording configuration used in the experiment (Meriney et al. 1994). I wanted to take advantage of this feature of the preparation

to investigate the effects of voltage-dependent inhibition on relief of inhibition during an action potential train and other characteristics of voltage-dependent inhibition. Therefore, I studied somatostatin-mediated calcium current inhibition in chick ciliary ganglion neurons using the whole-cell and perforated patch recording configurations. These results are presented in Chapter 2.

**2. The effect of action potential broadening on calcium current activation.** This section of the research was designed to evaluate the effects of action potential broadening on calcium current activation. Action potential broadening typically results in increased calcium current and transmitter release. However, in some instances, action potential broadening can result in decreases in transmitter release (Pattillo et al. 2001). This is hypothesized to be due to differential effects of action potential broadening on calcium current. Thus, the experiments presented in Chapter 3 were designed to test the hypothesis that action potential broadening may have differential effects on calcium current depending on the precise characteristics of the broadening.

**3. G protein-mediated modulation of action potential-evoked calcium current.** Many studies have investigated G protein-mediated calcium current inhibition using step depolarizations to evoke calcium current. While this method allows an accurate and in-depth biophysical examination of calcium current inhibition, the physiological relevance of inhibition remains to be determined. The kinetic slowing associated with calcium current inhibition suggests that the time course of action potential-evoked calcium current might also be altered by G protein modulation. The experiments presented in Chapter 4 investigated the effects of G protein modulation on action potential-evoked calcium current.

# II. SOMATOSTATIN-MEDIATED INHIBITION OF CALCIUM CURRENT IN CHICK CILIARY GANGLION NEURONS

## A. SUMMARY

Voltage-dependent relief of somatostatin-mediated inhibition of calcium currents was studied in chick ciliary ganglion neurons. Prompted by a previously reported difference in activation kinetics of G protein-modulated current when recorded with whole-cell versus perforated patch recording techniques, I have examined the differences in the voltage dependence of inhibition that may occur as a result of cytoplasmic dialysis. Trains of action potentials were used to evaluate relief of G protein-mediated modulation of calcium currents. These data revealed that action potential trains could relieve a significantly greater proportion of somatostatin-mediated inhibition under whole-cell than in perforated patch recording conditions. To further investigate differences in the voltage dependence of inhibition between recording conditions, the voltage-dependence of inhibition relief was measured by varying the amplitude of the prepulse used to relieve inhibition. Additionally, reinhibition following a prepulse and the somatostatin concentration – response relationship were measured using whole-cell and perforated patch recording configurations. The results indicate that somatotstatin-mediated calcium current inhibition is more susceptible to relief by prepulses of moderate amplitude and that the rate of reinhibition following a prepulse is faster when measured in whole-cell than in perforated patch recordings. Furthermore, the $IC_{50}$ of somatostatin inhibition of calcium current is slightly lower in

whole-cell than in perforated patch recordings. Taken together, these data suggest that there is a greater efficacy of activated G protein subunits in whole-cell than in perforated patch recordings. Thus, a cytoplasmic component appears to alter the voltage dependence of somatostatin-mediated calcium current inhibition in chick ciliary ganglion neurons.

## B. INTRODUCTION

Voltage-gated calcium channels are ubiquitously expressed in neurons and serve to link neuronal excitability to cellular functions. Specifically, N- and P/Q-type calcium channels are critically important in the regulation of transmitter release (Dunlap et al. 1995), and neuromodulator-mediated inhibition of presynaptic calcium channels is a major means of regulation of synaptic communication between neurons (Wu and Saggau 1997). A well-documented mechanism underlying calcium channel regulation is a direct, membrane-delimited pathway (Hille 1992, 1994; Dolphin 1996) in which G protein βγ-subunits bind directly to the calcium channel (Ikeda 1996; Herlitze et al. 1996). This binding results in altered calcium channel gating so that modulated channels are moved into a 'reluctant' state in which they require longer or stronger depolarization to open (Bean 1989; Elmslie et al. 1990). One prominent characteristic of this form of calcium channel inhibition is its voltage dependence. When studied using long step depolarizations, calcium current inhibition exhibits both a reduction in peak calcium current and a slowing of current activation kinetics, commonly referred to as kinetic slowing, which is thought to be indicative of voltage-dependent inhibition (Elmslie et al. 1990; Golard and Siegelbaum 1993; Elmslie and Jones 1994). In these cases a portion of the inhibition can be relieved transiently by a strong conditioning step depolarization or prepulse (Jones and Elmslie 1997). This is hypothesized to result from a voltage-induced

conformational change in the calcium channel that causes a dissociation of the G protein βγ-subunit from the calcium channel, during which time the channel is shifted back into the 'willing', non-modulated gating mode (Dolphin 1998; Zamponi and Snutch 1998).

An intriguing aspect of voltage-dependent calcium current inhibition is its potential physiological relevance. The voltage dependence of inhibition implies that it may be sensitive to neuronal activity and several groups have investigated the ability of more physiologically relevant stimuli to relieve G protein-mediated inhibition. Relief of inhibition of N-type calcium currents has been demonstrated using trains of 1-2 ms step depolarizations to +30 or +40 mV in dorsal root ganglion neurons (Womack and McClesky 1995) and in cholinergic basal forebrain neurons (Williams et al. 1997). Trains of action potential (AP) waveforms as voltage commands in place of standard step depolarizations failed to relieve inhibition of N-type calcium current in rat sympathetic neurons (Toth and Miller 1995), but markedly relieved inhibition of N-type calcium current in chick dorsal root ganglion neurons (Park and Dunlap 1998) and of recombinant P/Q-type calcium current expressed in HEK-293 cells (Brody et al. 1997). Additionally, it has been demonstrated that relief of G protein-mediated calcium current inhibition may be a form of short-term synaptic facilitation (Brody and Yue, 2000). These studies suggest that voltage-dependent calcium current inhibition may be an important mechanism underlying the precise regulation of neuronal activity.

I have studied the modulation of calcium currents in chick ciliary ganglion neurons. The ciliary ganglion is a parasympathetic ganglion that contains two populations of neurons, ciliary and choroid. Choroid neurons tend to be smaller than ciliary neurons and fire slower APs (Dryer 1994; Smith and O'Dowd, 1994). Both choroid and ciliary neurons express somatostatin receptors (Dryer et al 1991), and calcium current can be modulated by somatostatin in all ganglionic neurons (White et al. 1997). In contrast, the somatostatin peptide has been reported to be expressed only by the smaller choroid neurons (Epstein et al. 1988; De Stefano et al. 1993) that regulate vascular contractility in the choroid coat. Somatostatin is thought to be

relevant to choroid nerve terminal function *in vivo*, by negative feedback of ACh release from ganglionic neurons (Gray et al. 1989; Gray et al. 1990). Ciliary ganglion neurons express approximately 75% N-type calcium current at stage 40, and the neuropeptide somatostatin inhibits exclusively N-type calcium current at this stage (White et al. 1997). Previously, it has been reported that cytoplasmic dialysis during whole-cell patch clamp recordings may alter some of the characteristics of somatostatin-mediated inhibition of calcium current in ciliary ganglion neurons (Meriney et al. 1994). This work suggested that an intracellular mechanism altered the degree to which somatostatin-mediated modulation slows the activation kinetics of calcium current.

The present study was designed to investigate the possibility that the voltage dependence of inhibition may be influenced by cytoplasmic dialysis of ciliary ganglion neurons. This may provide insight into the cellular mechanisms that control the degree to which calcium current inhibition is voltage-dependent. Using trains of action potential waveforms to evoke calcium current, I have shown that somatostatin-mediated modulation of calcium current is more sensitive to activity-induced relief when studied by whole-cell methods that allow cytoplasmic dialysis versus perforated patch methods that leave the cytoplasm relatively intact. Additionally, I have designed protocols to investigate which characteristics of somatostatin-mediated inhibition may be altered by cytoplasmic dialysis. The chick ciliary ganglion may provide a unique opportunity to study G protein modulation as it allows for the manipulation of the degree of voltage dependence of calcium current inhibition. Thus, this preparation is particularly well suited for the described experiments that will further our understanding of G protein modulation of calcium current.

## C. METHODS

1. Cell culture

Ciliary ganglia were dissected from stage 40 (as determined by Hamburger and Hamilton, 1951) White Leghorn chicken embryos (following rapid decapitation) in oxygenated Tyrode solution containing (in mM): 134 NaCl, 3 KCl, 3 $CaCl_2$, 1 $MgCl_2$, 12 glucose and 20 $NaH_2CO_3$, pH = 7.3 adjusted with NaOH. Enzymatic digestion of the ganglion sheath was done using one of two protocols. In some cases the ganglia were incubated sequentially in collagenase type II (0.5 mg/mL) and trypsin-EDTA (0.08%) in $Ca^{++}$ - and $Mg^{++}$-free Tyrode at 37 ° C for 20 minutes each. Alternatively, the ganglia were incubated for 45-60 minutes at 37 ° C in an enzyme cocktail adapted from Stanley (1999) which contained: hyalauronidase (700 U/mL), collagenase type IV (0.6 mg/mL), dispase (12 mg / mL) and soybean trypsin inhibitor (74 µg / mL). After incubation the ganglia were removed from the enzyme by three washes in minimal essential media (MEM) plus 10% heat-inactivated horse serum. Ganglia were dissociated mechanically by gentle trituration through a polished Pasteur pipette. The final suspension of cells was centrifuged at 100 X *g* for 5-8 minutes. The pellet was resuspended in MEM with 10% chick embryo extract and 10% heat-inactivated horse serum, plated onto poly-D-lysine coated 35mm plastic dishes and incubated in 5% $CO_2$ at 37 ° C. Cells were used for experiments 1-6 hours after plating at which time there were no neurites elaborated.

2. Electrophysiology

a. Perforated patch-clamp recordings. In some recordings, the perforated patch technique was used to gain electrical access to the cell interior while allowing only the exchange of small monovalent ions between the pipette and the cell cytoplasm (Horn and Marty, 1988). To isolate calcium current, the cells were bathed in an external solution composed of (in mM): 100 NaCl, 50 TEA-Cl, 10 HEPES, 5 glucose, 5 KCl, 5 $CaCl_2$, 2 $MgCl_2$, 5 3, 4-diaminopyridine and 1 µM tetrodotoxin, pH = 7.3 adjusted with NaOH. Pipettes were pulled on a Flaming / Brown Micropipette Puller (Sutter Instruments Co.; Model P-97), coated with SYLGARD (Dow Corning, Midland, MI), and heat-polished. Electrode resistances ranged from 0.5 to 2 MΩ. The pipettes were filled in a two-step manner. The tip was filled by capillary action during a 5-10 second immersion in an internal solution of (in mM): 75 $Cs_2SO_4$, 55 CsCl, 7 $CaCl_2$, 1 $MgCl_2$ and 10 HEPES, pH = 7.3 adjusted with CsOH. The remainder of the pipette was back-filled with the above solution plus 400 µg/mL of amphotericin B (see Rae et al., 1991). Following the acquisition of a tight seal against the neuron, amphotericin B gradually induced a low-resistance access to the cell that reached equilibrium within 5-15 minutes after seal formation. Access resistance was measured by changes in the area and decay time constant of the capacitive transient evoked by a 10 mV hyperpolarizing voltage step. Series resistance was 14.1 ± 5.3 MΩ (mean ± S.D) and was compensated by at least 80%. High calcium (7 mM) was included in the internal solution to detect spontaneous rupture of the perforated patch, at which point calcium current was rapidly attenuated and the recording was discontinued.

b. Whole-cell patch clamp recordings. In some recordings, traditional whole-cell patch-clamp methods were used (Hamill et al. 1981). The pipettes were filled with the following internal solution (in mM): 120 CsCl, 10 HEPES, 11 EGTA, 5 TEA-Cl, 1 $CaCl_2$, and 4 $MgCl_2$, pH = 7.3 adjusted with CsOH, with 4 ATP-Mg, 0.3 GTP-Na and 0.1 leupeptin added fresh daily to retard

run-down of calcium currents. The bath solution and pipettes were as described above. Series resistance for whole-cell recordings was 7.2 ± 5.1 MΩ (mean ± S.D.) and was compensated by at least 80%

In both recording configurations currents were activated, acquired and leak-subtracted using a hyperpolarizing P/4 protocol by the software package pClamp 6 or pClamp 8 (Axon Instruments, Foster City, CA) running on a Pentium processor-based microcomputer in concert with an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA). The currents were four-pole Bessel-filtered at 5 kHz and digitized at 20 kHz. Before all recordings, I corrected for the liquid junction potential, which was -4.3 mV in perforated patch solutions and in whole-cell solutions was -5.8 mV.

c. Action potential waveform. The perforated patch technique was used as described above except that $K_2SO_4$ and KCl replaced $Cs_2SO_4$ and CsCl in the pipette solution. Tyrode was used as the external solution. The fast current-clamp mode of the Axopatch 200A (Magistretti et al. 1998) was used to evoke and record an action potential at room temperature from small neurons (capacitance = 6-7 pF) presumed to be choroid neurons in the ciliary ganglion. One of these APs was used as a command for voltage-clamp experiments and had the following characteristics: resting potential = -60 mV, peak = +30 mV, 2.2 ms duration at half amplitude. AP trains consisted of 8 identical action potentials delivered at 100 Hz, followed by a 30 ms prepulse to +70 mV and another choroid action potential.

3. Data analysis

The percentage relief of somatostatin-mediated inhibition during AP trains was calculated as the ratio of inhibition recorded during the first AP in a train to inhibition of current evoked by each successive AP in the train. In all cases, the control current from each AP was used to calculate

the inhibition percentage following somatostatin application. This approach corrected for any effects of inactivation during the train of APs. In other experiments, currents were evoked using traditional step depolarization in a 3-step (prepulse) protocol, consisting of a 100-ms step depolarization from -80 mV to +10 mV ($1^{st}$ test pulse), a 30-ms step from -80 mV to +100 mV (prepulse), and another 100-ms step from -80 mV to + 10 mV ($2^{nd}$ test pulse). There was a 500 ms interval between the first test pulse and the prepulse, to ensure it had no effect on the magnitude of inhibition of the second test pulse. For all experiments, the percent somatostatin-mediated inhibition was measured at the time of peak control calcium current. In prepulse experiments, inhibition of calcium current evoked by the first test pulse (step from -80 mV to +10 mV) was calculated according to the following equation; $Inh_1 = (1\text{-}SOM_1/control_1) *100$, where $control_1$ is the amplitude of peak control calcium current and $SOM_1$ is the amplitude of somatostatin inhibited calcium current at the isochronal time point. Similarly, inhibition of calcium current evoked by the second test pulse was calculated according to the equation; $Inh_2 = (1\text{-}SOM_2/control_2) * 100$, where $control_2$ is the amplitude of peak control calcium current and $SOM_2$ is the amplitude of somatostatin inhibited calcium current at the isochronal time point. The percent prepulse-induced relief of inhibition was calculated as follows: Relief = $(1- Inh_1 / Inh_2) * 100$, where $Inh_1$ and $Inh_2$ were defined as described above. All data are reported as mean ± SEM. All statistical analyses were performed using Student's t-test with Layered Bonferroni correction for multiple comparisons when necessary. Significance was defined as $p < 0.05$.

4. Pharmacologic agents.

Somatostatin (Sigma, St. Louis, MO) was dissolved in distilled water to make a 100X stock, stored at – 20 ° C, and diluted into bath saline to a final concentration (1 nM – 200 nM) on the day of the experiment. All other agents were obtained from Sigma and dissolved in saline.

Pharmacologic agents were pressure applied locally through a pipette (20-30 μm tip diameter). Calcium current inhibition is more voltage-dependent with increasing concentrations of activated G proteins (Lu et al. 2001). Therefore to accurately measure the voltage dependence of inhibition, it was important to ensure that all cells were exposed to a saturating concentration of somatostatin. In all experiments where a maximal dose of somatostatin was delivered, somatostatin was determined to have a significant effect when calcium current was inhibited by at least 35%. This ensured that somatostatin was effectively delivered and variations in inhibition relief were not due to incomplete somatostatin-mediated inhibition (see Figure 3). Due to the relatively rapid desensitization of somatostatin effect on calcium current, each cell was used for only one waveform and one somatostatin application.

## D. RESULTS

1. Somatostatin-induced inhibition of calcium current is voltage-dependent.

In chick ciliary ganglion neurons, somatostatin-induced inhibition targets N-type calcium channels exclusively (White et al. 1997) and is partially voltage-dependent. The inhibition exhibits characteristics typically associated with voltage-dependent inhibition, namely robust inhibition of calcium current at moderate depolarizations, slowing of activation kinetics and relief of inhibition by a strong depolarizing prepulse. Application of 200 nM somatostatin inhibited calcium current evoked by 100 ms voltage steps from -80 mV to +10 mV every 10-15 seconds. Figure 4A shows a representative current trace before and after somatostatin application. The modulated current has reduced amplitude and clear slowing of activation kinetics. The current-voltage relationship of calcium current before and after somatostatin application demonstrates

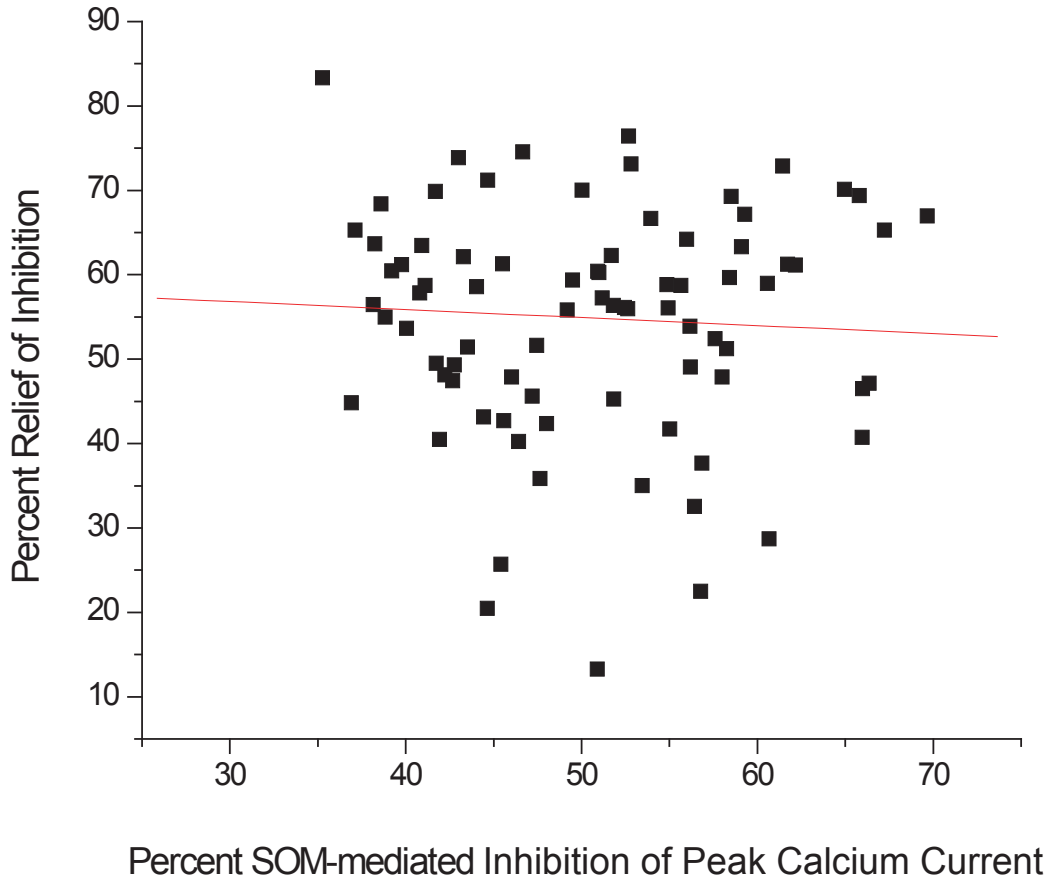


**Figure 3. Relationship between percent somatostatin-mediated inhibition and prepulse relief of inhibition.** Calcium current was evoked by the standard prepulse protocol using whole-cell and perforated patch recording configurations. Somatostatin (200 nM) was applied to inhibit calcium current. For each cell, inhibition of peak calcium current during the first test pulse, and percent prepulse-induced relief of inhibition was measured. The plot includes data from cells with at least 35% inhibition of peak calcium current. Red line is a linear fit of the data. There was no significant correlation between the two measurements ($p > 0.05$).

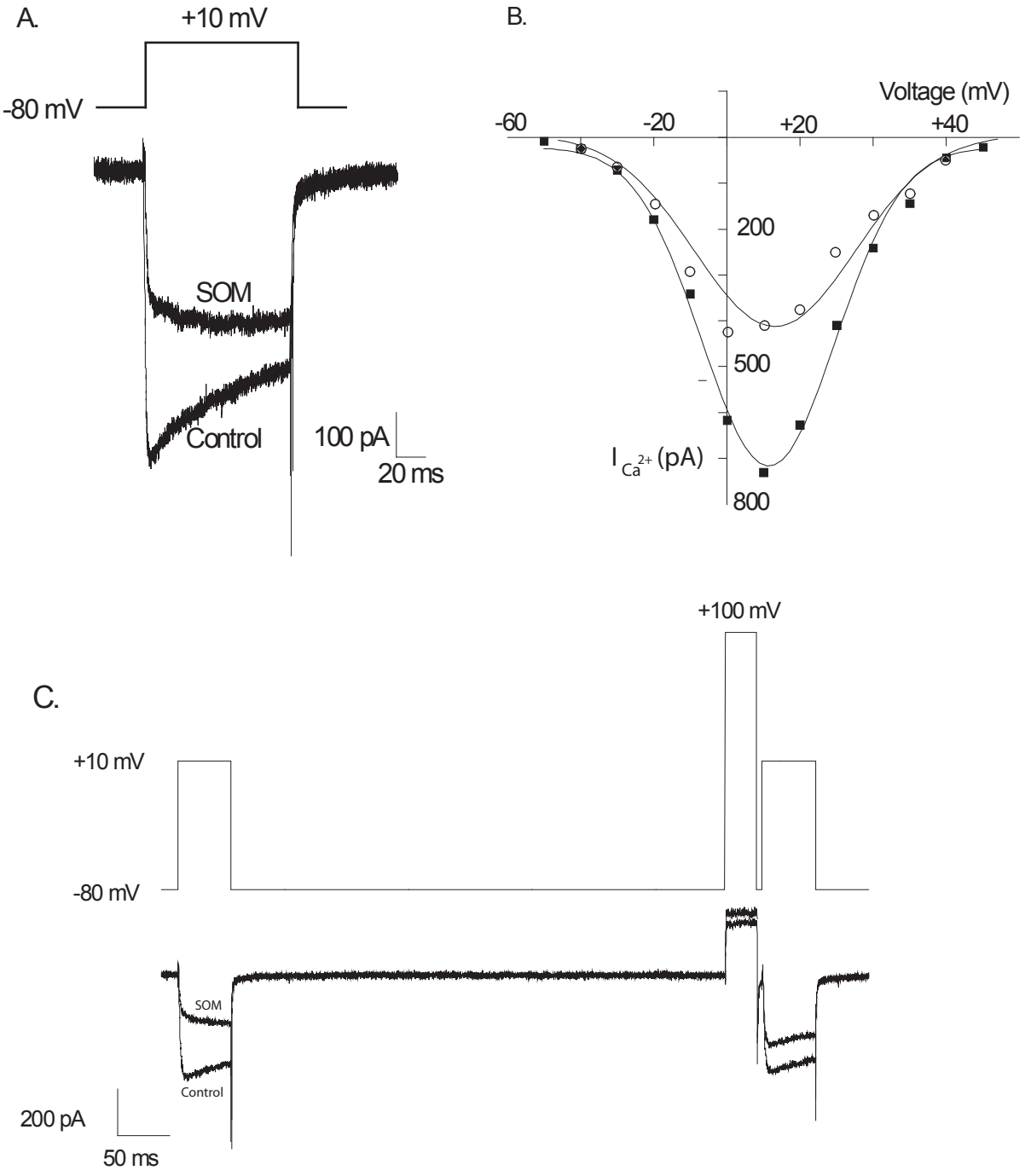


**Figure 4. Somatostatin-mediated inhibition of calcium current is voltage-dependent.** A. Representative current trace evoked by step depolarization from -80 mV to +10 mV before (control) and after (SOM) application of 200 nM somatostatin using standard whole-cell recording configuration. B. Current-voltage relationship from one cell before (closed squares) and after (open circles) somatostatin application demonstrates the voltage-dependence of calcium current inhibition. C. Typical prepulse protocol used to measure relief of inhibition (top). Representative current trace before (control) and after (SOM) somatostatin application. In the presence of somatostatin, current evoked by the first step to +10 mV (first test pulse) is inhibited and has slowed activation kinetics. Current evoked by the second step to +10 mV (second test pulse) shows some relief of inhibition and normal activation kinetics.

the voltage dependence of inhibition. There is robust inhibition of peak calcium current (activated by step depolarization to +10 mV), but little to no inhibition of current by steps to more depolarized potentials (Figure 4B). A typical prepulse protocol used to measure the voltage dependence of calcium current inhibition is illustrated in Figure 4C. Current evoked by the first test pulse to +10 mV is inhibited by somatostatin application and is characterized by kinetic slowing. The prepulse (30-ms step to +100 mV), which precedes the second test pulse by 5 ms, relieves a portion of the inhibition and restores normal activation kinetics of the calcium current (Figure 4C, bottom).

A previous study of somatostatin-inhibition of calcium current in chick ciliary ganglion neurons had suggested that some characteristics of somatostatin-mediated inhibition may vary depending on whether the calcium currents were recording using whole-cell or perforated patch techniques (Meriney et al. 1994). Therefore, I characterized somatostatin-mediated calcium current inhibition using both recording configurations. The mean inhibition of peak calcium current measured in the two configurations was not significantly different (whole-cell = 45 ± 0.82%, perforated patch = 46 ± 1.04%, n = 179 for each condition, $p > 0.05$).

2. Degree of kinetic slowing varies in two different recording configurations

One of the characteristics previously reported to differ between whole-cell and perforated patch recordings of calcium current is the degree to which somatostatin application alters activation kinetics (Meriney et al. 1994). Examination of sample traces from that study suggests that there is a greater degree of kinetic slowing of modulated calcium current in whole-cell recordings. I systematically tested this finding to determine if cytoplasmic dialysis increases somatostatin-induced kinetic slowing. First, a method of quantifying kinetic slowing had to be developed. In order to quantify kinetic slowing in a way that was independent of the kinetics of the control calcium current, therefore not affected by inactivation, kinetic slowing was defined as the ratio of

percent inhibition of peak calcium current to percent inhibition of current after 95 ms of depolarization to +10 mV (see Figure 5A). This quantification allowed a measurement of kinetic slowing in each cell and provided a means of comparison among cells. Figure 5B shows that on average, there was a greater degree of kinetic slowing of modulated calcium currents in whole-cell recordings than in perforated patch recordings (whole-cell kinetic slowing ratio = 1.48 ± 0.04, n=65; perforated patch k.s. ratio = 1.36 ± 0.02, n=71, p = 0.01). These results confirm the previous observation that whole-cell recordings show greater kinetic slowing and suggest that cytoplasmic dialysis may enhance the voltage dependence of somatostatin-mediated calcium current inhibition.

3. Action potential-train induced relief of calcium current inhibition

Since kinetic slowing is indicative of voltage-dependent calcium current inhibition (Boland & Bean, 1993; Artim & Meriney, 2000), the data presented above suggest that the recording configuration may influence the voltage dependence of somatostatin-mediated inhibition. Therefore, I next tested the functional impact of this difference by measuring relief of somatostatin-mediated inhibition. Previous studies have shown that action potential trains can relieve a portion of voltage-dependent inhibition (Park and Dunlap, 1998; Brody et al. 1997). Since this is a more physiologically relevant stimulus than the large depolarizing prepulse often used to measure voltage-dependence, I used a train of action potentials (APs) to measure relief of inhibition as an indicator of voltage-dependent inhibition (Figure 6). The AP train consisted of 8 choroid action potentials (-60 mV resting potential, +30 mV peak, 2.2 ms duration at half-amplitude; see methods) delivered at 100 Hz. This stimulus was used as a voltage clamp command to evoke calcium current in both the whole-cell and perforated patch recording configurations before and after application of 200 nM somatostatin. Inhibition of the current evoked by the 1$^{st}$ AP in the train was not significantly different in the two configurations (45.6

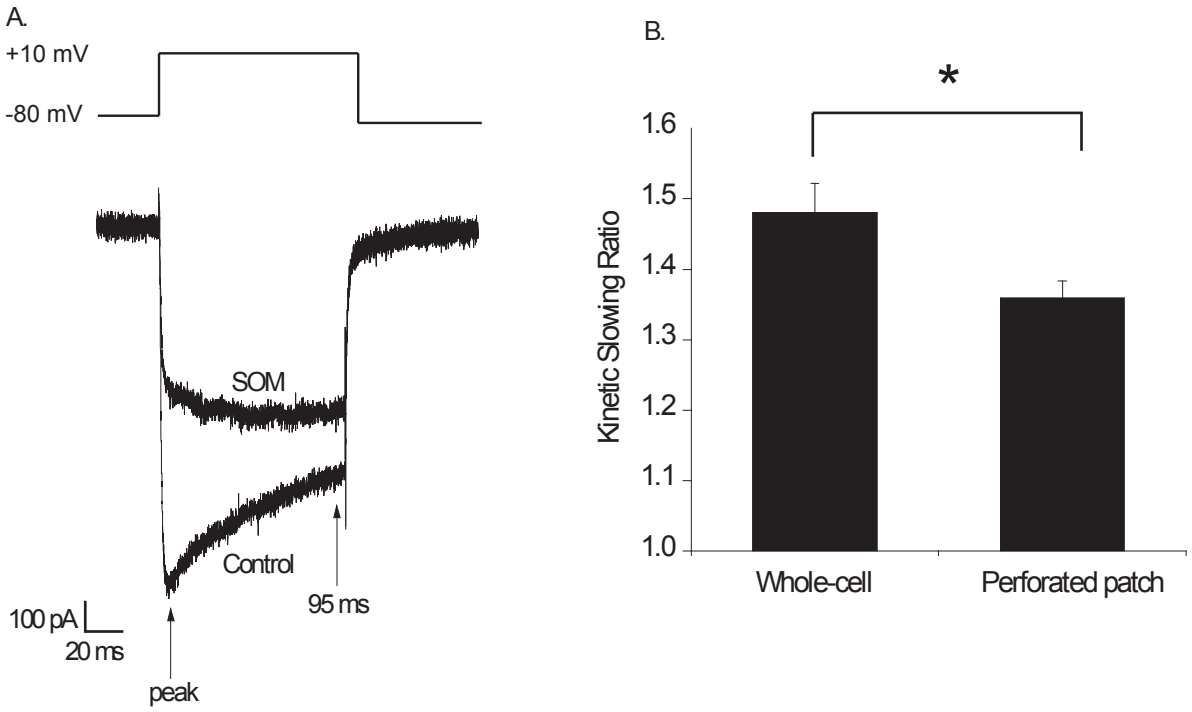


**Figure 5. Measurement of kinetic slowing.** A. Representative current trace, as in Figure 4A, evoked by a step depolarization before (control) and after (SOM) application of 200 nM somatostatin. Arrows point to times at which percent inhibition was measured. Kinetic slowing was defined as the ratio of percent inhibition of peak calcium current to percent inhibition of current after 95 ms of depolarization. B. Mean data show significantly greater kinetic slowing in whole-cell than in perforated patch recordings ($p < 0.05$).

±1.6%, n=10 for whole-cell and 52.1± 3.1%, n=8, for perforated patch, $p > 0.05$) and in both configurations, the AP train relieved a portion of the inhibition. However, there was significantly greater relief of inhibition measured in whole-cell recordings than in perforated patch recordings (Figure 6B). This difference was significant from the 4$^{th}$ through the 8$^{th}$ AP in the train, and although there was only a total of ~6% relief of inhibition by the AP train in perforated patch recordings, there was appreciable relief (~15%) in whole-cell recordings. These data are consistent with the hypothesis that somatostatin-induced calcium current inhibition exhibits greater voltage dependence when measured using traditional whole-cell patch clamp techniques. In some experiments, the AP train was followed by a prepulse (30 ms to +70 mV) and another choroid action potential (Figure 6A). This allowed a measurement of the total amount of voltage-dependent inhibition in the cell. This protocol was used to determine if the AP train was more effective at inhibition relief in whole-cell recordings, or if the total voltage-dependent inhibition was greater in whole-cell recordings. Percent inhibition of the calcium current evoked by the post prepulse AP in the train (following the prepulse) was compared to percent inhibition of the 1$^{st}$ AP in the train to measure relief of inhibition produced by the combination of the AP train and the prepulse (Figure 6C). There was no significant difference in the total relief of inhibition between whole-cell and perforated patch recordings (whole-cell = 57.5 ±1.4%, perforated patch = 55.7 ± 2.9%, $p > 0.05$;). This suggests that the overall voltage-dependent inhibition does not vary between the two recording configurations, but that the AP train alone is more effective at inhibition relief when measured using whole-cell recording conditions.

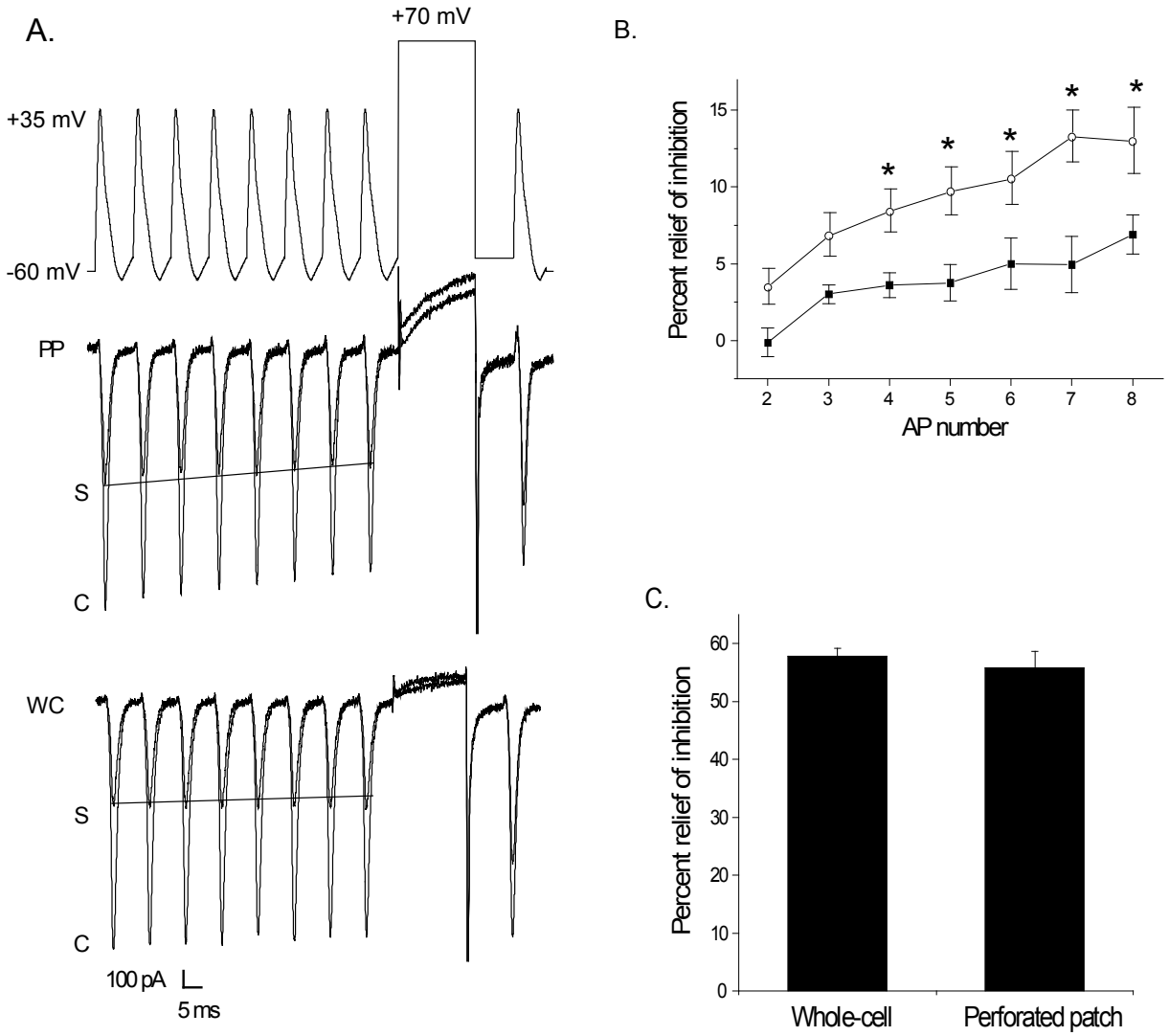


**Figure 6. Relief of inhibition by an action potential train.** A. Choroid action potential train (top) with representative current traces before (C) and after (S) 200 nM somatostatin application in whole-cell (middle) and perforated patch (bottom) recording configurations. Dashed lines represent current amplitude expected if there was no relief of inhibition. B. Summary data plotting percent relief of inhibition in whole-cell (open circles) and perforated patch (closed squares) recordings as a function of action potential number in the train. * is significantly different by Student's t-test, $p < 0.05$ C. Summary data showing relief of inhibition measured at the post prepulse action potential.

4. Characteristics of somatostatin-induced voltage-dependent inhibition in whole-cell and perforated patch recordings.

I next investigated several aspects of somatostatin-induced calcium current inhibition in an attempt to identify which characteristics differed between whole-cell and perforated patch recordings. Specifically, I measured the voltage dependence of somatostatin-mediated inhibition relief, the time course of re-inhibition following a prepulse and the somatostatin concentration / inhibition relationship using both whole-cell and perforated patch techniques. To measure the voltage-dependence of inhibition (hypothesized to be due to unbinding of G protein subunits from the calcium channel) a prepulse protocol was used, and the amplitude of the prepulse varied. Based on the results from action potential trains, where action potentials were more effective at relieving inhibition in whole-cell recordings, I predicted that relief would develop at lower voltages in whole-cell as compared to perforated patch. Thus, prepulses of moderate amplitude, similar to the voltages reached by action potentials, would be more effective at relieving calcium current inhibition in whole-cell recordings. Calcium current was evoked by 100 ms voltage steps from -80 mV to +10 mV before and after a prepulse in the presence and absence of somatostatin. In all experiments in which a prepulse protocol was used, there was a 500 ms interval between the first 'test pulse' and the prepulse to ensure that the first depolarization to +10 mV did not have any effect on the magnitude of inhibition of the current evoked by the second test pulse (see Figure 7). Whole-cell and perforated patch recordings were made of calcium current evoked with the prepulse protocol and the amplitude of the prepulse was varied from -50 mV to +100 mV (Figure 8 A-C). Relief of inhibition produced by the prepulse was calculated for each cell and average relief plotted as a function of prepulse amplitude (Figure 8D). There was greater relief of inhibition produced by prepulses of moderate amplitude (+25 mV) in whole-cell recordings as compared to perforated patch recordings and the $EC_{50}$ for voltage-dependence of inhibition was less depolarized for whole-cell

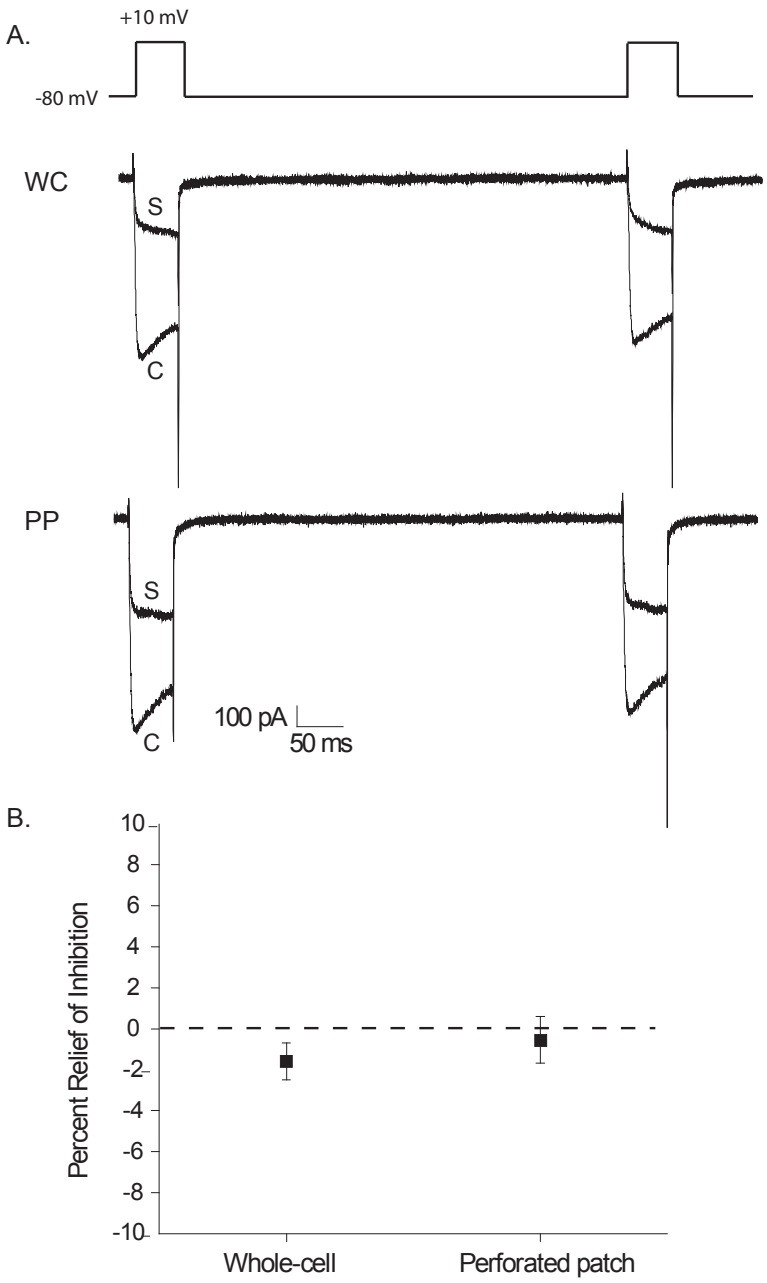


**Figure 7. Effect of two test pulses on percent somatostatin-mediated inhibition.** A. Calcium current was evoked by two step depolarizations from -80 mV to +10 mV separated by 500 ms (top). Representative current traces before (C) and after (S) application of 200 nM somatostatin in whole-cell (middle) and perforated patch (bottom) recording configurations. B. Summary data show there is no significant relief of inhibition caused by the first step depolarization to +10 mV. Each data set not significantly different from 0 by one-population t-test, $p > 0.05$.

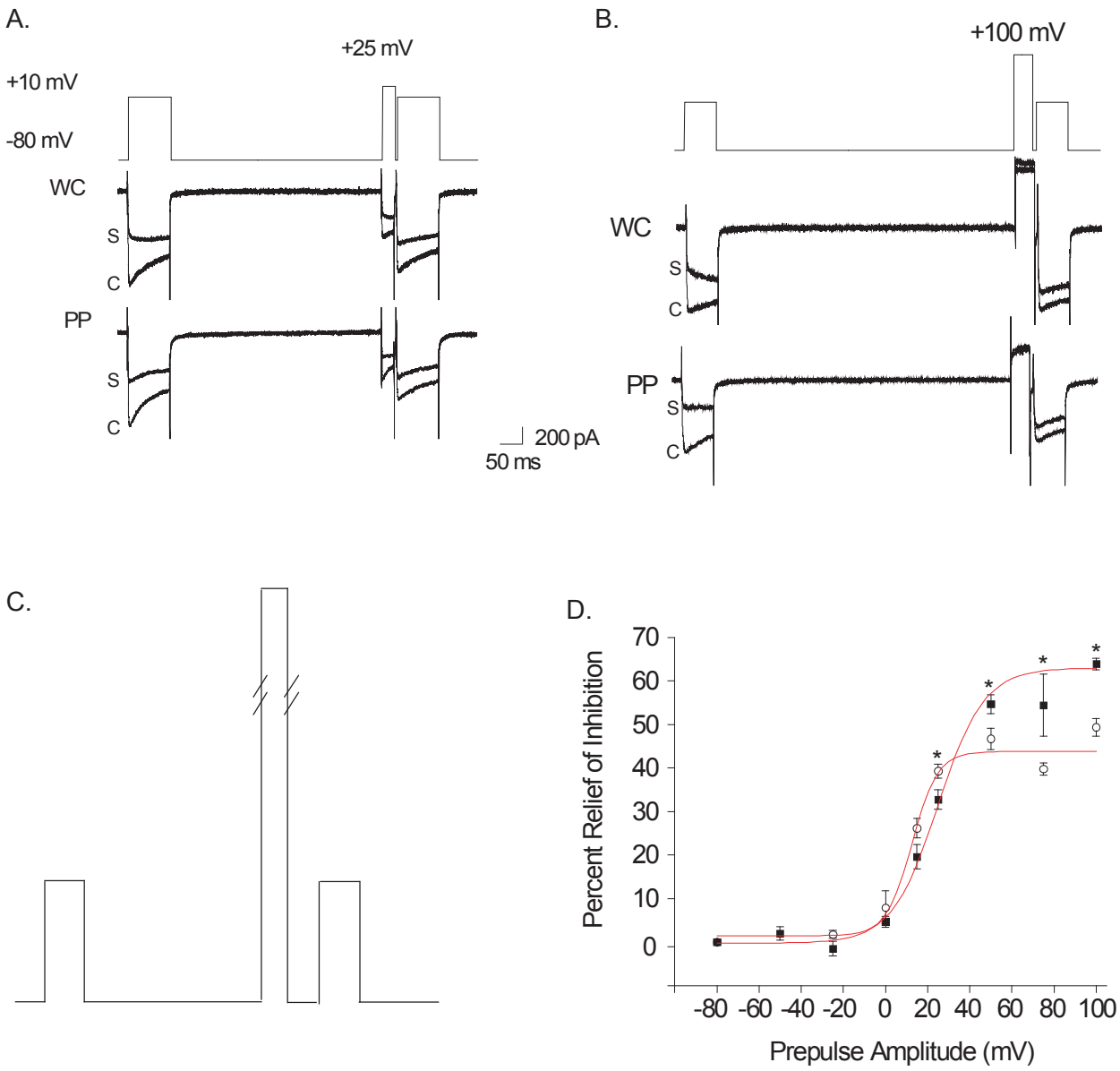


**Figure 8. Voltage dependence of prepulse relief of inhibition.** A. Prepulse protocol with a prepulse to +25 mV (top). Representative current traces in the presence (S) and absence (C) of 200 nM somatostatin in whole-cell (middle) and perforated patch (bottom) recording configurations. B. Same as in A but with a prepulse to +100 mV. C. Schematic of voltage protocol used to measure voltage-dependence of inhibition relief. D. Summary data plot of the percent relief of inhibition as a function of prepulse amplitude for whole-cell (open circles) and perforated patch (filled squares) recordings. Solid lines are Boltzmann fits of the data; * is significantly different by student's t-test, $p < 0.05$.

recordings (whole-cell $EC_{50}$ = 11.9, perforated patch $EC_{50}$ = 21.8). Thus, a difference in the voltage dependence of inhibition relief can at least partially account for the differences in percent relief of inhibition by AP trains between whole-cell and perforated patch recordings, Interestingly, when tested using prepulses to +100 mV, which produce maximum relief of inhibition, there was significantly greater relief of inhibition in perforated patch recordings (whole-cell = 49.3 ± 1.9%, n=49; perforated patch = 63.9 ± 1.4%, n = 41; $p < 0.001$) which, in contrast to the previous data using an action potential train plus a prepulse to +70 mV, suggests that the total degree of voltage-dependent inhibition is greater in intact cells.

It is known that the voltage dependence of calcium current inhibition depends on the concentration of activated G proteins (Elmslie and Jones 1994; Lu et al. 2001). Thus, I hypothesized that the higher degree of voltage dependence seen in whole-cell recordings may reflect a higher effective concentration of G proteins in those recordings. To test this hypothesis, a concentration – response relationship was measured using both whole-cell and perforated patch recordings (Figure 9). Calcium current was evoked by 100 ms step depolarization from -80 mV to +10 mV and somatostatin was applied in concentrations ranging from 1nM to 200 nM. Due to desensitization of somatostatin effect on calcium current, each cell was exposed to only one concentration of somatostatin. Although there was a small trend for increased effectiveness of somatostatin in whole-cell recordings ($EC_{50}$ = 2.7 nM in whole-cell versus 4.5 nM in perforated patch) there was no significant difference in percent inhibition at any concentration tested (figure 9). The biggest difference in percent somatostatin-induced inhibition occurred with 2 nM somatostatin application (whole-cell = 23.8 ± 3.6%, n = 5; perforated patch = 15.3 ± 2.7%, n = 6) however this was not a significant difference ($p = 0.08$). This trend, though not significant, could reflect a greater effectiveness of G protein subunits in whole-cell recordings and suggests that cytoplasmic dialysis may increase the calcium channels' ability to sense activated G protein. This is an avenue worthy of pursuit.

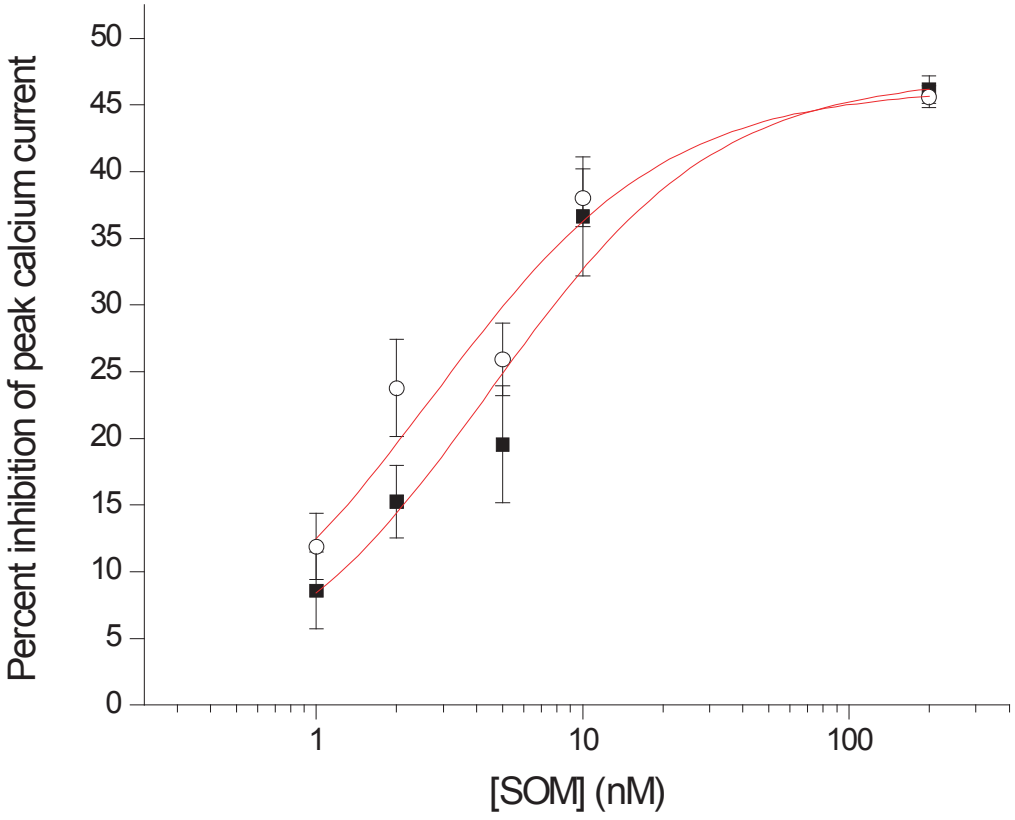


**Figure 9. Concentration – effect relationship of somatostatin inhibition of calcium current.** Calcium currents were evoked by step depolarizaztion from -80 mV to +10 mV before and after application of somatostatin using the whole-cell (open circles) and perforated patch (filled squares) recording configurations. Inhibition of peak calcium current is plotted as a function of somatostatin concentration. Solid lines are Boltzmann fits of the data.

Next, I studied the time course of G protein rebinding to the calcium channel by measuring re-inhibition of calcium current following a 30 ms prepulse to +100 mV. If there is a greater concentration of activated G protein subunits in whole-cell recordings, this should be reflected in a higher rate of reinhibition as this process is also dependent on the concentration of activated G protein βγ-subunits (Elmslie and Jones 1994; Lopez and Brown 1991; Zamponi and Snutch 1998). Calcium currents were collected in whole-cell and perforated patch recording configurations in the presence and absence of 200 nM somatostatin. Currents were evoked using the 3-step prepulse protocol in which the interval between the prepulse and the second test pulse was varied (Figure 10A). As described above, relief of inhibition was calculated for each cell, and the averages plotted as a function of the interval following the prepulse (Figure 10B). The data were fit with a single exponential decay and the tau of the whole-cell data was less then for perforated patch data (whole cell $\tau$= 45.8 ms, perforated patch $\tau$ = 65.3 ms) supporting the hypothesis that G protein rebinding following the prepulse is faster in whole-cell recordings. The individual time points were significantly different at the 10 ms and the 50 ms intervals. Since the total amount of relief with the shortest interval (5 ms) was greater in perforated patch recordings, the data were normalized in order to compare reinhibition relative to the maximum amount of relief (Figure 10C). Again, the rates of reinhibition were different, with whole-cell recordings showing faster reinhibition than perforated patch recordings (whole-cell $\tau$ = 45.1 ms, perforated patch $\tau$ = 57.0 ms). Taken together, these data suggest that the rate of reinhibition following a prepulse is faster in whole-cell recordings and are consistent with the hypothesis that there is a greater effective concentration of activated G protein subunits in whole-cell recordings.

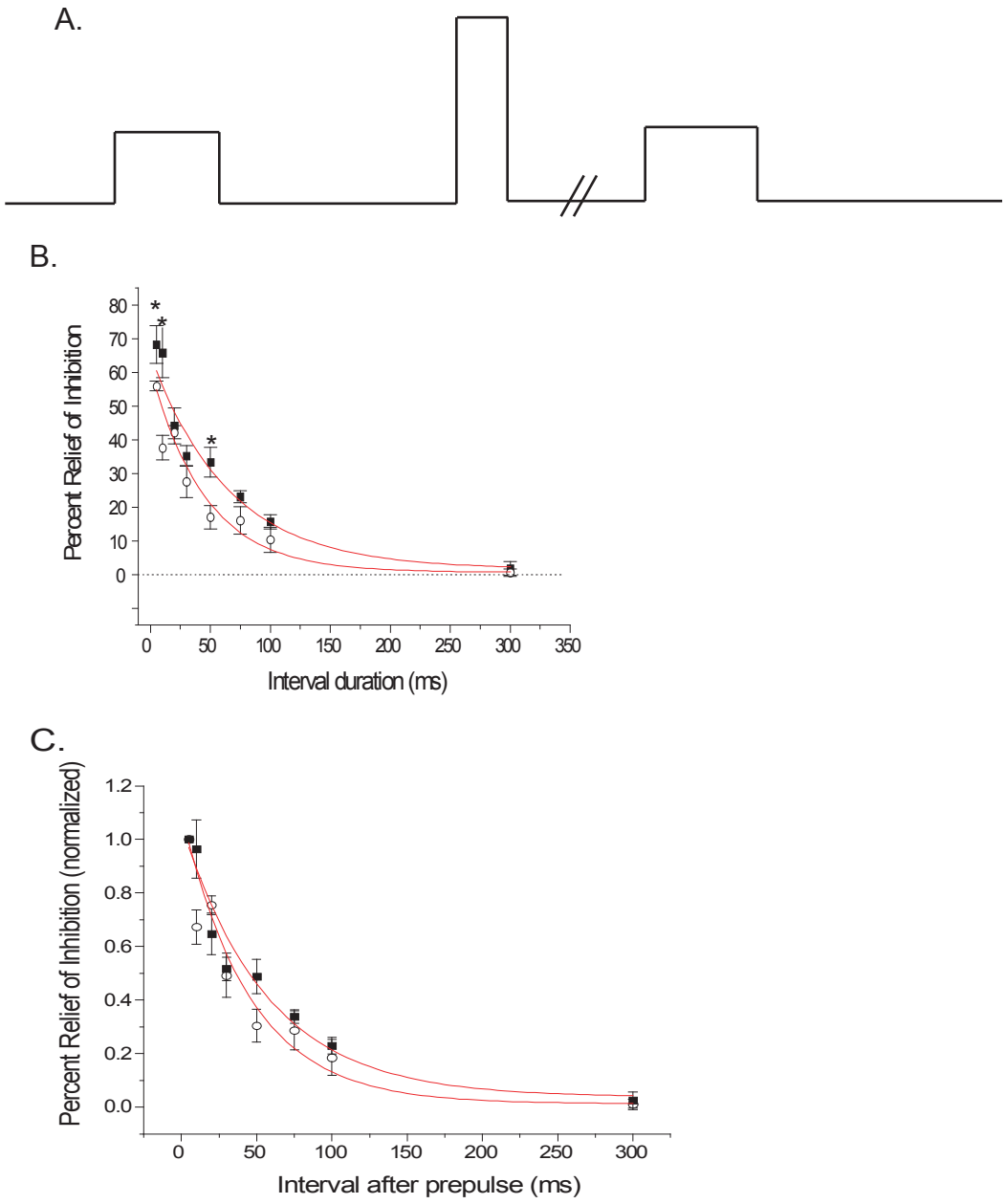


**Figure 10. Reinhibition of calcium current following a prepulse.** A. Schematic of the voltage protocol used to measure the time course of reinhibition of calcium current after a prepulse. B. Summary data from whole-cell (open circles) and perforated patch (filled squares) with percent relief of inhibition plotted as a function of the interval duration. Solid lines are exponential fits of the data. C. Same data as in B, normalized to relief of inhibition with a 5 ms interval. Solid lines are exponential fits of the data. * is significantly different by student's t-test, $p < 0.05$.

5. Role of cytoskeleton disruption in altering voltage-dependence of inhibition

I next tested the hypothesis that alterations in the cytoskeleton underlie the increased voltage dependence of calcium current inhibition seen in whole-cell recordings. It is known that microtubules interact directly with G protein βγ-subunits (Gunderson and Cook 1999) and that disruption of microtubule polymerization can alter the magnitude of G protein-mediated inhibition (Unno et al. 1999). Therefore, if whole-cell methods disrupt microtubule structure this may alter association of G protein subunits with the calcium channel. Cultures were pretreated with colchicine (100 μM) for 1-4 hours to disrupt microtubule polymerization. Calcium current was recorded with the perforated patch method and evoked by choroid AP trains (8 APs delivered at 100 Hz) in the presence and absence of 200 nM somatostatin. Relief of inhibition produced by the action potential train was measured as described above and plotted as a function of the AP number in the train (Figure 11). There was no significant difference in the percent relief of inhibition during the AP train in perforated patch recordings of control versus colchicine treated cultures, suggesting that colchicines treatment did not mimic the whole-cell recording configuration.

6. Investigation of differences in recording conditions

I investigated two additional characteristics that could have accounted for differences between whole-cell and perforated patch recordings of calcium current. First, the inactivation of calcium current, during a step depolarization and during an AP train, was compared between whole-cell and perforated patch recordings (Figure 12). Calcium current was evoked by 100 ms step depolarization from -80 mV to +10 mV and inactivation was calculated as the percent decrease in current measured at the peak and after 95 ms of depolarization. In other cells, calcium current was evoked by trains of 8 choroid APs (100 Hz), and inactivation was calculated as the

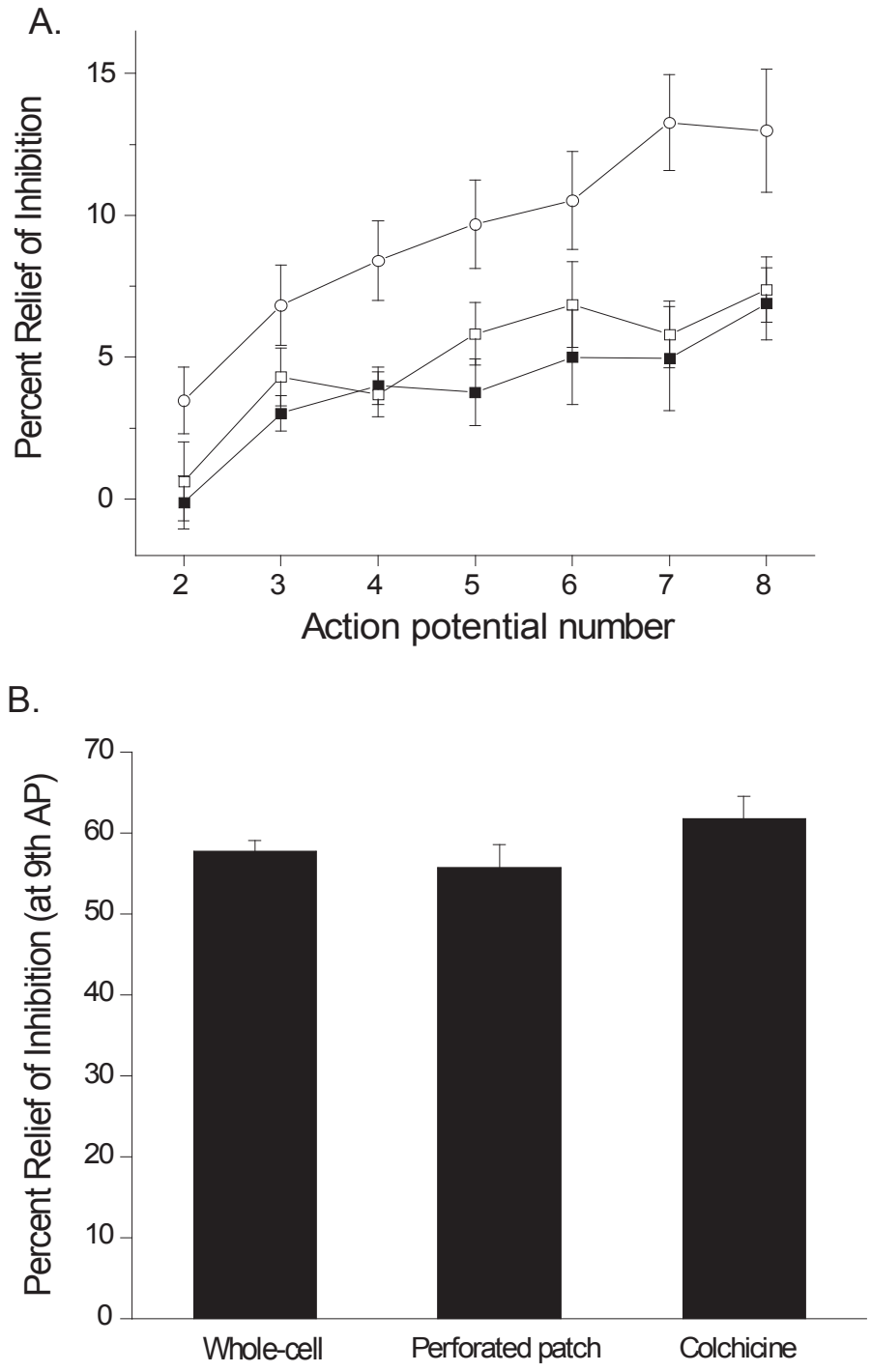


**Figure 11. Effect of colchicine on relief of inhibition.** A. Summary graph plots percent relief of inhibition as a function of action potential number in the train for whole-cell (open circles), perforated patch (solid square), and colchicine treated-perforated patch (open square) recordings. B. Summary data of percent relief of inhibition measured at the post prepulse action potential.

percent decrease of calcium current from the 1st to the 8th AP in the train. For both step depolarization and AP trains, there were no significant differences between the percent inactivation of calcium currents recorded using whole-cell versus perforated patch configurations (Figure 12 B & D).

Another difference between the two recording configurations is the concentration of chloride ions in the internal pipette solution ($[Cl^-]_i$). To assess the effect of $[Cl^-]_i$ on voltage-dependent inhibition, a whole-cell pipette solution with the same $[Cl^-]_i$ as perforated patch solution was used (see Methods). Using this low chloride whole-cell solution, there was a slight effect on the current – voltage relationship and the voltage-dependence of activation, such that currents recorded using the low chloride whole-cell solution were more similar to those recorded using perforated patch techniques (Figure 13 A & B). However, when the voltage dependence of inhibition was measured using the choroid AP train, there was no significant difference in the percent relief of inhibition in control whole-cell versus low chloride whole-cell recordings (Figure 13C). Therefore, the different chloride ion concentrations of the internal solutions cannot account for the difference in the voltage dependence of inhibition seen between whole-cell and perforated patch recordings.

7. Desensitization of somatostatin-induced calcium current inhibition.

Another characteristic of somatostatin-mediated inhibition that may be susceptible to cytoplasmic dialysis is the desensitization of somatostatin effect. Meriney et al. (1994) showed that the magnitude of somatostatin-induced inhibition is diminished with repeated applications of the neuropeptide only when measured in whole-cell recordings. Additionally, the effect of somatostatin during one 3-minute application appeared to desensitize to a greater degree in whole-cell than in perforated patch recordings. However, as this observation was not the focus of the study, it was limited to one example and was not quantified. Therefore, I investigated

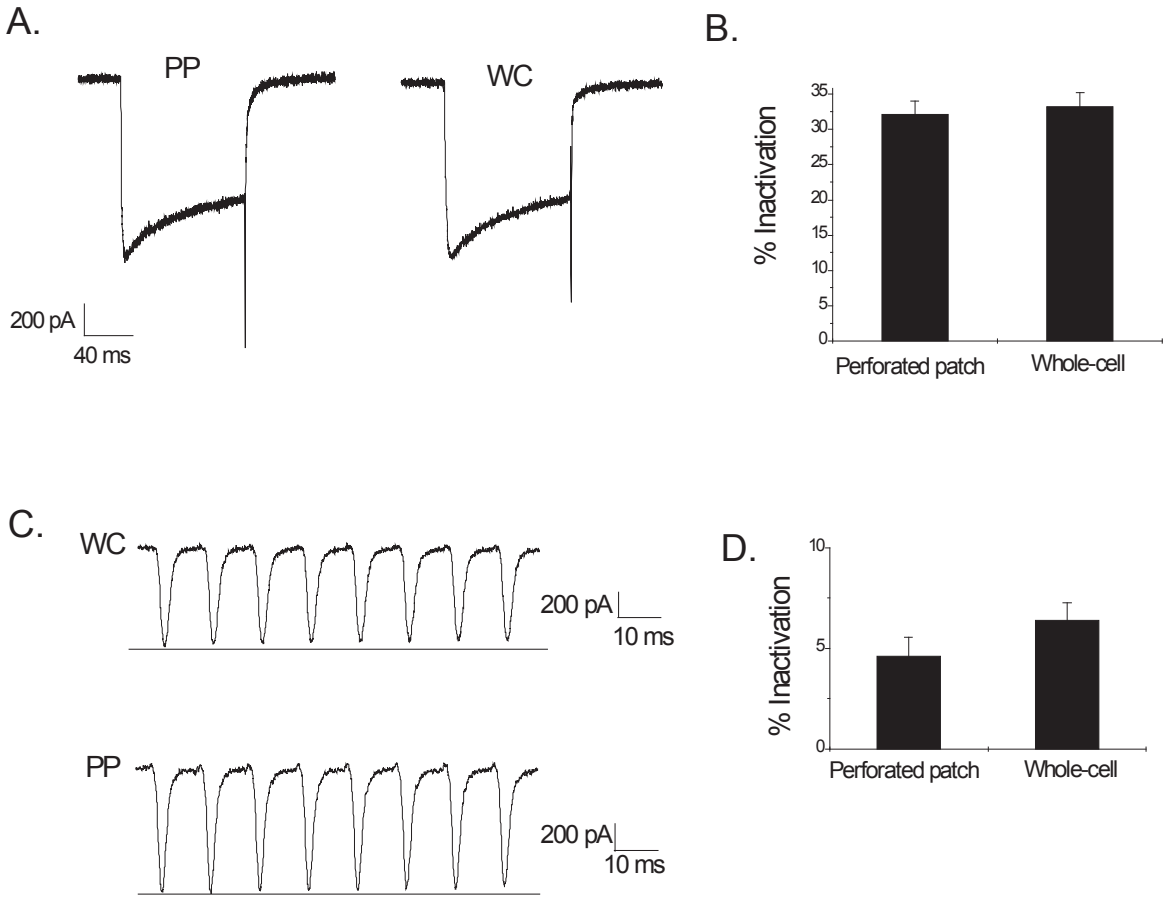


**Figure 12. Inactivation of calcium current**. A. Representative calcium currents activated by steps to +10 mV in whole-cell and perforated patch recording configurations. B. Graph comparing mean percent inactivation of whole-cell and perforated patch recordings during 100 ms step depolarizations. C. Representative calcium currents evoked by a 100 Hz train of 8 choroid action potential waveforms in whole-cell and perforated patch recording configurations. Solid lines indicate peak current amplitude predicted if there was no inactivation during the AP train. D. Graph comparing mean percent inactivation in whole-cell and perforated patch recordings during trains of choroid action potentials. B & D. No significant differences between calcium current inactivation in whole-cell and perforated patch recordings, $p > 0.05$.

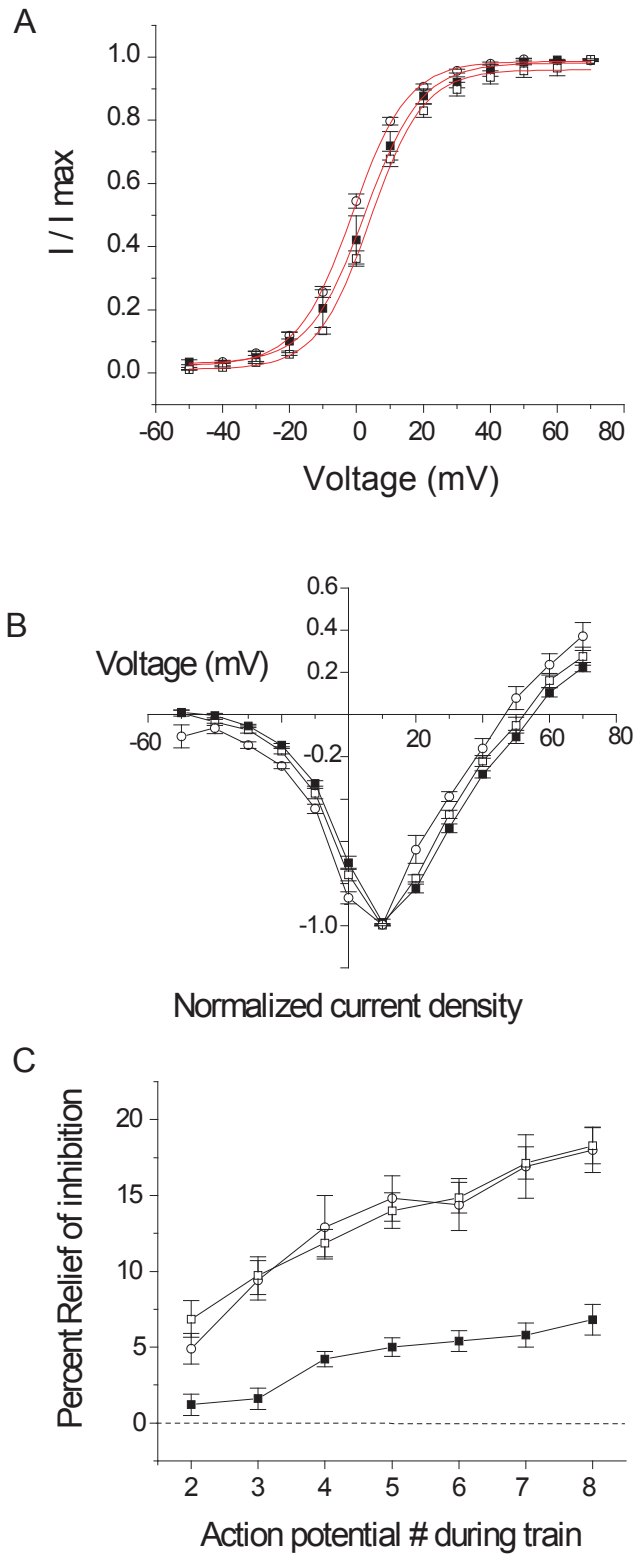


**Figure 13. Calcium current recorded with low-chloride internal solution.**

**Figure 13. Calcium current recorded with low-chloride internal solution**. A. Voltage-dependent activation of calcium currents recorded in the whole-cell recording configuration (open circles), perforated patch recording configuration (filled squares) and low-chloride whole-cell recordings (open squares). Voltage dependence of activation was obtained by plotting normalized tail current amplitude against the voltage of the step used to activate calcium current. Solid lines are Boltzmann fits to the data. B. Current-voltage relationship for calcium currents recording in whole-cell, perforated patch, and low-chloride whole-cell recording configurations. C. Bursts of choroid AP waveforms delivered at 100 Hz relieved SOM-mediated inhibition of calcium currents recorded in the whole-cell configuration with low-chloride internal solution to the same degree as in control whole-cell recordings. Thus, subtle differences in charge screening between whole-cell and perforated patch recordings did not account for the observed differences in AP waveform-induced relief of inhibition between whole-cell and perforated patch recordings.

desensitization of somatostatin effect on calcium current and compared the rate of desensitization between whole-cell and perforated patch recordings. Due to complications arising from run-down of calcium current in whole-cell recordings, measurements of desensitization of peak calcium current inhibition were inconclusive. For that reason, I quantified desensitization of kinetic slowing, which has also been shown to desensitize during prolonged application of neuropeptide (Diverse-Pierluissi et al. 1996). Since the degree of G protein-mediated kinetic slowing is independent of the magnitude of calcium current, this measurement is not affected by calcium current run-down during the recording. Kinetic slowing was measured at the time of maximal somatostatin effect on calcium current, and at 5 second intervals thereafter (Figure 14A). The percent desensitization of kinetic slowing was compared for whole-cell and perforated patch recordings. There was no significant difference in the percent desensitization of kinetic slowing at any time point studied (Figure 14B). Thus, cytoplasmic dialysis does not appear to alter the rate of desensitization of somatostatin-induced kinetic slowing.

## E. DISCUSSION

G protein-mediated inhibition of calcium current is often associated with slowed activation kinetics and strong voltage dependence. I have studied G protein-mediated inhibition of calcium current in parasympathetic chick ciliary ganglion neurons where it has been shown previously that the slowed activation kinetics of modulated current is altered by cytoplasmic dialysis. This system may represent a neuronal model in which the characteristics of G protein-mediated calcium current inhibition are altered by a parallel cytoplasmic signal transduction cascade that is activated independently of the G protein-coupled receptor (Gray et al. 1999). This pathway

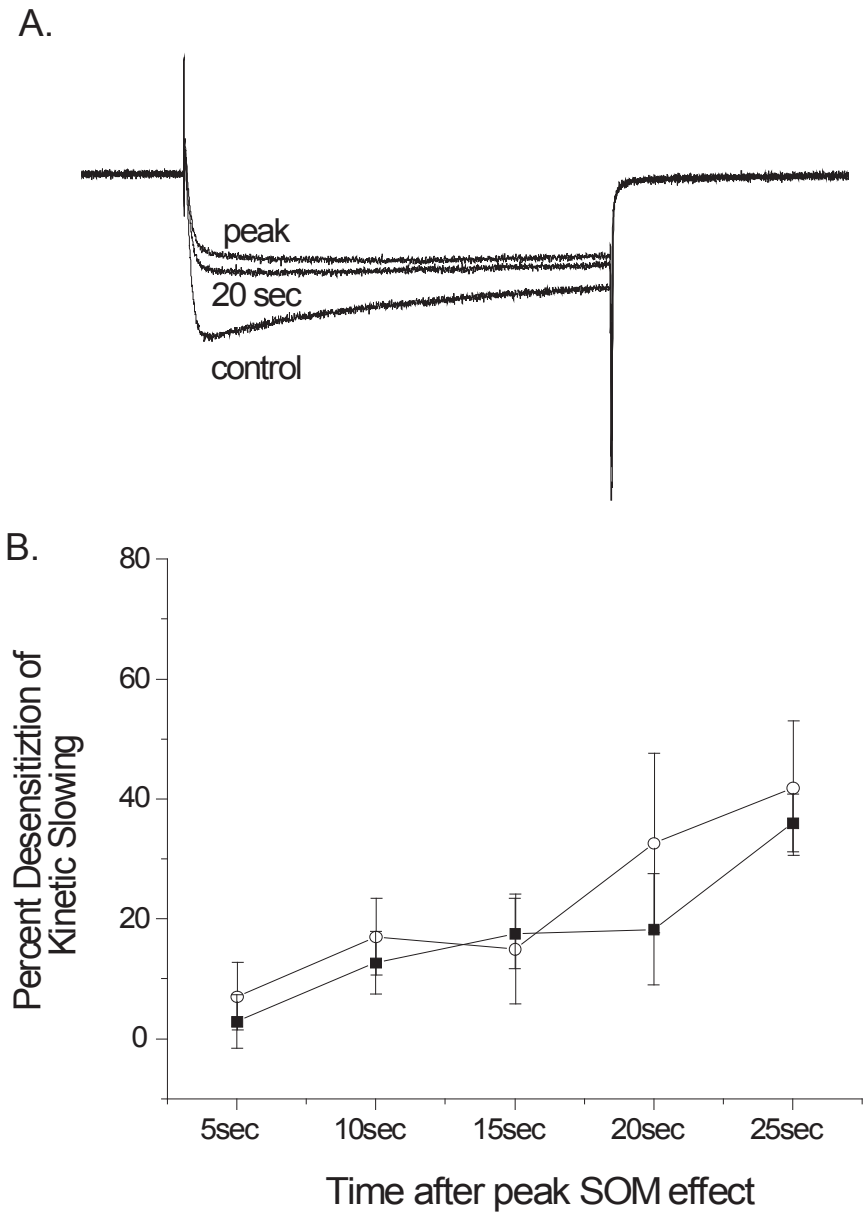


**Figure 14. Desensitization of kinetic slowing.** A. Representative current trace before (control) somatostatin application, during peak somatostatin effect (peak) and after 20 seconds of somatostatin presence. B. Summary graph plots mean percent desensitization of kinetic slowing in whole-cell (open circles) and perforated patch (closed squares) recordings.

may interact with G protein modulation of calcium current to alter calcium influx and transmitter release. Based on the previously reported observation that the slowed activation kinetics of modulated current is greater with cytoplasmic dialysis, I hypothesized that the voltage dependence of somatostatin-mediated inhibition would also be enhanced. To test this hypothesis, calcium current modulation was compared between the whole-cell configuration, in which the cytoplasm is altered by dialysis with the internal pipette solution, and the perforated patch configuration, in which the cytoplasm remains relatively intact. This experimental manipulation was used to mimic the absence and presence of a hypothesized endogenous cytoplasmic modulatory pathway.

Comparison of somatostatin-mediated calcium current inhibition in whole-cell versus perforated patch recordings revealed two results consistent with previous observation of the effect of cytoplasmic dialysis on somatostatin effect. Kinetic slowing was quantified as the ratio of percent inhibition of peak calcium current to the percent inhibition of current after 95 ms of depolarization. Somatostatin caused a significantly greater degree of kinetic slowing when cytoplasm was dialyzed in whole-cell recordings. This result suggested that the degree of voltage dependence would be greater in whole-cell recordings also. To test this hypothesis, calcium current was evoked with a train of choroid action potentials, and relief of inhibition during the train was compared in whole-cell and perforated patch recordings. As predicted, there was significantly greater relief of inhibition in whole-cell recording, suggesting there is a greater degree of voltage-dependence in these recordings. However, relief of inhibition produced by a standard prepulse following the AP train is the same whether measured in whole-cell or perforated patch recordings. Taken together, these results indicate that, while the total voltage dependence of somatostatin-mediated inhibition is the same in whole-cell and perforated patch recordings, AP trains are more effective at relieving inhibition in the whole-cell configuration. Therefore, bursts of AP waveforms might relieve significant calcium current

inhibition in ciliary ganglion neurons, especially when cytoplasmic components are lost or compromised during whole-cell recordings.

Choroid neurons of the ciliary ganglion innervate vascular smooth muscle in the choroid coat at the back of the eye. It is thought that endogenous somatostatin release provides feedback inhibition to these parasympathetic nerve terminals (Gray et al. 1989, 1990). Furthermore, choroid neurons initiate smooth muscle contraction using bursts of APs (Meriney and Pilar, 1987). If the cytoplasmic dialysis-sensitive alteration in the voltage dependence of G protein modulation reported here is a property that is modifiable under physiological conditions, and natural AP bursts can relieve significant somatostatin-mediated inhibition *in vivo*, this might be a mechanism underlying the fine tuning of synaptic transmission in this system. Since choroid neurons fire APs that are of relatively long duration (2.2 ms at half amplitude), bursts of choroid APs under appropriate conditions could be particularly well suited for the relief of G protein-mediated inhibition of calcium current in this system. Given that the relationship between calcium influx and transmitter release is known to be non-linear (Dodge and Rahaminoff 1967; Augustine and Charlton 1986; Wu and Saggau 1997), this relief of calcium current inhibition would be expected to have a significant impact on the magnitude of transmitter released. There is evidence for a similar effect of cytoplasmic dialysis on prepulse relief of peptide-mediated inhibition in mudpuppy cardiac ganglia (Merriam and Parsons 1995). This suggests that the added degree of sensitivity to cytoplasmic components may be representative of signal transduction pathways that control transmitter release within the parasympathetic regulation of vascular function.

Inhibition of calcium current shows greater voltage-dependence with higher concentrations of G proteins (Lu et al. 2001). Therefore, it was hypothesized that there was a greater effective concentration of activated G protein subunits in whole-cell than in perforated patch recordings. To test this hypothesis, a somatostatin concentration – response curve was measured for both whole-cell and perforated patch recordings. Although there was no

significant difference in the percent inhibition at any single concentration tested, there was a slightly lower $IC_{50}$ and a trend towards greater percent inhibition at low somatostatin concentrations in whole-cell compared to perforated patch recordings. This difference is not unprecedented; there is an example of the dose – response relationship of calcium current inhibition differing between recording configurations, although in the opposite direction of what is reported here. Delmas et al. (1999) used both whole-cell and perforated patch to measure noradrenalin-mediated inhibition of N-type calcium current and found a significantly lower $IC_{50}$ in perforated patch recordings. Additionally, I measured the rate of reinhibition of calcium current following a maximal prepulse. This is hypothesized to occur due to rebinding of G protein subunits to the calcium channel and the rate of rebinding depends on the concentration of activated G proteins (Lopez and Brown 1991; Zamponi and Snutch 1998). The data show that the rate of re-inhibition is faster in whole-cell recordings, which is consistent with the hypothesis that there is a greater effectiveness of G proteins in whole-cell recordings. Because there is greater relief of inhibition during an action potential train in the whole-cell configuration, the greater voltage dependence of relief appears more important than the fast rebinding kinetics. Thus, the increased voltage dependence of inhibition relief dominates the rebinding and there is an increased net relief of inhibition during the AP train.

To further investigate the voltage dependence of somatostatin effect on calcium current, I studied calcium current modulation using a traditional 3-pulse-protocol, while varying the amplitude of the prepulse used to relieve inhibition. Based on the results using action potential trains to relieve inhibition, I hypothesized that inhibition would be more sensitive to relief at lower voltages in whole-cell recordings. As such, I expected that moderate depolarizations would relieve a greater percent of inhibition in whole-cell than in perforated patch recordings. In support of this hypothesis, prepulses to +25 mV caused significantly greater relief of inhibition in whole-cell than in perforated patch recordings. Furthermore, the voltage producing half-maximal relief was about 10 mV more hyperpolarized in whole-cell recordings. Interestingly, the

total amount of inhibition relieved with maximal prepulses was greater in perforated patch recordings. This suggests that a greater percentage of somatostatin-mediated inhibition is voltage-dependent when the cytoplasm is preserved. Thus, while relief of inhibition occurs at lower voltages in whole-cell recordings, there is more total voltage dependence in perforated patch recordings.

The effect of recording configuration also depends on the specific stimulus used to relieve inhibition and thus measure voltage dependence and the stimulus used to evoke calcium current after inhibition relief. While action potential trains are more effective at relieving inhibition in whole-cell recordings, strong square-shaped depolarizing prepulses are more effective at relieving inhibition in perforated patch recordings. This difference could be accounted for by the faster rate of G protein rebinding following a prepulse in whole-cell recordings. When measured using a traditional 3-pulse protocol, there is a 5-ms interval at -80 mV between the prepulse and the step depolarization that follows. Since rebinding of G protein to the calcium channel is favored at -80 mV (Elmslie & Jones, 1994), there could be appreciable recovery of inhibition during this interval. In contrast, the prepulse used at the end of the AP train, is followed by an interval to -60 mV, which may result in less G protein rebinding. Therefore, while the prepulse may relieve the same amount of inhibition in both recording configurations, the faster rebinding rate at -80 mV results in greater inhibition after the prepulse leading to an artificially low measurement of inhibition relief.

I considered alternative explanations for the observed difference in voltage-dependent relief of inhibition by AP trains when comparing modulated calcium currents recorded in whole-cell and perforated patch conditions. In comparing the two recording configurations, I first looked for differences in calcium current inactivation during a step depolarization and during an AP train. Although the method used to calculate relief of inhibition was designed to be independent of calcium current inactivation, differences in inactivation between whole-cell and perforated patch recordings could alter the appearance of calcium current and lead to incorrect

conclusions. For example, if whole-cell recordings of AP train-evoked calcium current inactivate less than perforated patch recordings, it may appear that the currents decrease less during the train, which could be interpreted as more relief of inhibition. However, no differences in inactivation between calcium currents recorded in whole-cell and perforated patch configurations were detected, whether measured using step depolarizations or trains of APs.

I also investigated charge screening effects that may occur due to the difference in internal chloride ion concentration between whole-cell and perforated patch solutions. This difference could result in charge screening that would alter the voltage seen by the cell. In turn, this could affect the voltage dependence of calcium channel gating and artificially create differences in the relief of inhibition seen between the two recording configurations. In fact there was a slight shift in the activation curve of currents recorded with low-chloride whole-cell solution, such that the voltage dependence of calcium current activation in these recordings was more similar to that of perforated patch recordings. Therefore the small difference in the voltage dependence of calcium current activation seen between whole-cell and perforated patch recordings could be explained by charge screening caused by internal chloride. Nonetheless, this does not account for the observed difference in AP train-induced relief of inhibition between whole-cell and perforated patch recordings since there was no difference in relief of inhibition produced by the AP train between regular whole-cell recordings and low-chloride whole-cell recordings.

Additionally, I tested the hypothesis that alterations in the microtubule structure affected the voltage-dependence of somatostatin-mediated inhibition. It is known that microtubules interact with both calcium channels and G protein βγ-subunits and that the magnitude of G protein-mediated inhibition can be effected by changes in microtubule structure (Gunderson and Cook, 1999; Unno et al. 1999). Therefore, if whole-cell dialysis results in altered microtubule structure, this in turn could affect the association between calcium channels and G protein βγ-subunits and explain the differences seen in inhibition relief between whole-cell and perforated

patch recordings. Disruption of microtubule structure by pretreatment with colchicine did not alter relief of inhibition produced by the choroid AP train or by a prepulse in perforated patch recordings. Thus, it seems unlikely that a change in microtubule structure underlies the increased voltage-dependence of somatostatin-mediated inhibition seen in whole-cell recordings. However, it is possible that the colchicine treatment was not sufficient to induce a measurable change in the association of calcium channels and G proteins. Alternatively, changes in other components of the cytoskeleton (such as microfilaments) could occur with whole-cell dialysis and may alter the association of calcium channels and G protein subunits.

An important consideration when comparing data obtained with whole-cell and perforated patch techniques is the difference in access resistance between the two recording configurations. In all experiments, access resistance was compensated for by at least 80%. However, the remaining access resistance could produce a voltage error of up to a few mV (Table 2). This could affect the voltage seen by the cell, especially during the fast changes in voltages produced during AP trains. To investigate the effect of access resistance on measurements of inhibition relief, I plotted relief of inhibition produced by a standard prepulse against access resistance. When the whole-cell data were plotted there was no significant relationship between inhibition relief and access resistance (Figure 15A). Data collected in perforated patch recordings also showed no correlation between inhibition relief and access resistance (Figure 15B). Similarly, data obtained using action potential trains to evoke calcium current were analyzed for access resistance. Figure 15C shows a plot of access resistance versus percent relief of inhibition by the AP train. As with the prepulse data, there was no significant relationship between access resistance and relief of inhibition. These comparisons argue that variations in relief of inhibition are not due to cell-to-cell differences in access resistance.

Functional alterations in the voltage sensitivity of G protein-mediated modulation may be due to changes in the hypothesized direct interaction between βγ-subunits of the activated G

**Table 2. Estimated voltage errors and time constants.**

| Parameter | Whole-cell | Perforated patch |
|---|---|---|
| Access Resistance (MΩ) | 7.18 | 14.05 |
| Uncompensated Access Resistance (MΩ) | 1.44 | 2.81 |
| Average Calcium Current Amplitude (pA) | 862 | 972 |
| Estimated voltage error (mV) | 1.24 | 2.73 |
| Capacitance (pF) | 20.6 | 20.3 |
| *tau* (μs) of membrance charging | 29.57 | 56.99 |

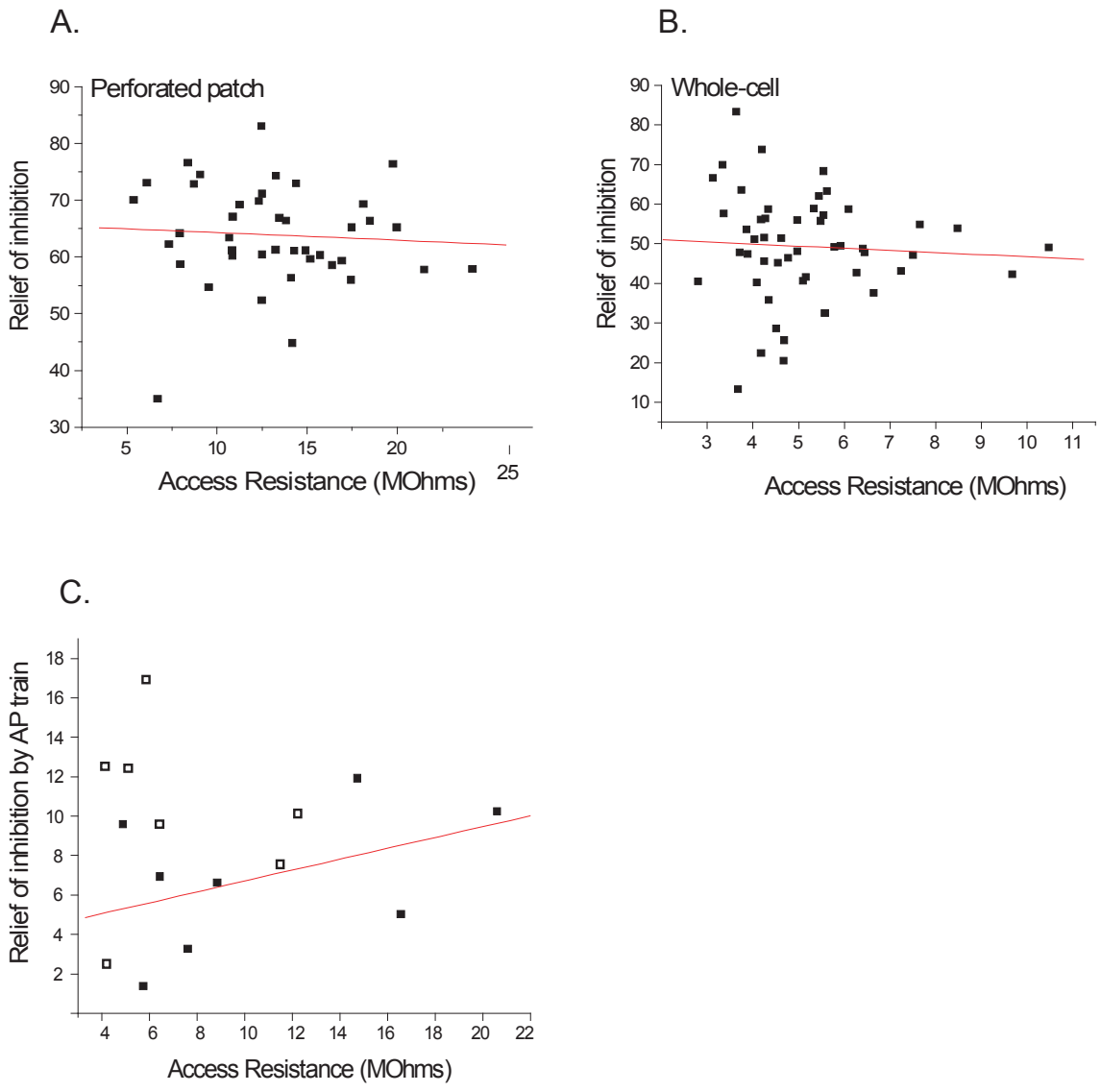


**Figure 15. Access resistance and prepulse relief of inhibition**. A. Plot of percent relief of somatostatin-mediated calcium current inhibition by a prepulse against access resistance for perforated patch recordings. B. Same plot as in A for whole-cell recordings. C. Plot of relief of somatostatin-mediated calcium current inhibition by a choroid action potential train against access resistance. This plot includes data from both whole-cell (open squares) and perforated patch (closed squares) recordings. Solid lines in all 3 plots are linear fits to the data. There were no significant correlations between relief of inhibition and access resistance in any of the data groups ($p > 0.05$).

protein and the N-type calcium channel. These could include: alterations in the voltage-dependent conformational changes in the calcium channel that are thought to lead to unbinding of the activated G protein subunit, altered affinity of the activated G protein subunit for the calcium channel binding site(s), or altered accessibility of the binding site for activated G protein. One or more of these conditions may alter the accessibility or affinity of activated G protein subunits for the calcium channel, and may lead to an accumulation of voltage-dependent relief during a train of APs. In general, the data presented here support a greater affinity of G protein subunits for the calcium channel in whole-cell recordings, although the mechanism underlying this affect has not yet been determined.

There are several potential mechanisms that could account for the difference in voltage-dependent inhibition reported here. The literature is replete with examples of manipulations that alter the voltage dependence of G protein-mediated calcium current inhibition. For example, as mentioned above, alterations in cytoskeletal components can affect calcium current modulation. When cytoskeleton integrity was compromised in embryonic stem cells, either by cytochalasin D or downregulation of β1 integrins, muscarinic inhibition of calcium current was disrupted due to displacement of $G_i$ (Bloch et al. 2001). Inhibition of microtubule polymerization also decreased muscarinic inhibition of barium current in muscle cells (Unno et al. 1999). Thus, if whole-cell dialysis can alter the structure and / or stability of the cytoskeleton, there may be increased accessibility of the calcium channel for G protein subunits and this could account for the increased voltage dependence of calcium current inhibition seen in whole-cell recordings.

Another mechanism affecting G protein modulation is variations in internal ion concentration. Specifically, an increase in intracellular sodium ions promotes dissociation of heterotrimeric G proteins (Rishal et al. 2003) and can inhibit N-type calcium channels in a G-βγ-dependent manner (Blumenstein et al. 2004). Both whole-cell and perforated patch recordings allow exchange of monovalent cations between the cell cytoplasm and internal pipette solution, although there is a more complete exchange in whole-cell recordings, which could lead to

differences in the concentration of internal ions. However, this is unlikely to be responsible for the differences in voltage dependence of inhibition seen in the present study as this mechanism requires very large changes in intracellular sodium concentration.

One potentially important difference between whole-cell and perforated patch recording conditions is the extent of calcium buffering. Whole-cell pipette solution is highly buffered (11 mM EGTA) while in perforated patch recordings endogenous calcium buffering is left intact. This difference in buffering would be expected to have a small effect on resting internal calcium concentration (~10 nM in whole-cell, 50-100 nM in perforated patch). Although this difference is small compared to local increases in calcium concentration that occur after activation of voltage-gated calcium channels, it may be enough to alter the cellular interactions involving phospholipids. $PIP_2$ has been shown to facilitate interactions between calcium channels and G protein βγ subunits and this effect is dependent on cytosolic calcium. Buffering the internal solution with BAPTA eliminates the ability of $PIP_2$ to enhance voltage dependent inhibition. Additionally, differences in buffering capacity between whole-cell and perforated patch recordings may alter the way in which the cell can handle large influxes of calcium such as those occurring with action potential stimulus or calcium tail currents. This difference may allow for differential activation of downstream calcium-dependent signaling pathways that may interact / alter direct G protein – calcium channel binding. One such pathway is the PI3 kinase pathway, which when activated results in phosphorylation of the calcium channel beta subunit (Viard et al. 1999). Therefore, calcium buffering conditions are a potentially important consideration when measuring voltage-dependent inhibition of calcium current. Alternatively, dialysis of the cytoplasm during whole-cell recordings may result in the loss of internal signaling molecules that may be active under normal circumstances to influence G protein inhibition of calcium current. One such pathway involves protein kinase C (PKC) which when activated antagonizes subsequent G protein-mediated inhibition of calcium current (Swartz, 1993; Lee et al. 2004; Doering et al. 2004). Thus, if whole-cell dialysis results in the loss of protein kinases, this

antagonism would be absent and G protein modulation would be more effective in these recordings. Another protein kinase pathway that may be involved is the cGMP-PKG pathway, which has been shown to increase kinetic slowing of somatostatin-modulated calcium currents in chick ciliary ganglion neurons (Meriney et al. 1994). Therefore, loss of this pathway during whole-cell dialysis may diminish the voltage-dependence of somatostatin-mediated calcium current inhibition.

Finally, alterations in various protein-protein interactions can influence calcium current modulation. One well-documented, although complex, example is the interaction of calcium channel beta subunits with G protein βγ-subunits. There is evidence that these two proteins compete for binding to the calcium channel alpha subunit and that downregulation of the calcium channel beta subunit increases calcium current sensitivity to G protein-mediated inhibition (Campbell et al. 1995; Bourinet et al. 1996). Syntaxin is another protein that is capable of affecting the calcium channel / G protein interaction. In chick sensory neurons expression of syntaxin 1A increases the sensitivity of N-type calcium channels to G-βγ modulation and shifts the inhibition towards greater voltage dependence (Lu et al. 2001). It has been shown that syntaxin 1A can interact simultaneously with the G-βγ subunit and the II-III linker of the calcium channel alpha subunit. Therefore, it is hypothesized that syntaxin 1A optimizes G protein modulation by co-localization of the G protein and calcium channel for more effective coupling (Jarvis et al. 2000). The specific proteins expressed in these studies may not necessarily be the proteins involved in the ciliary ganglion and the interactions described will depend on the specific proteins and subunits expressed in these cells. However, if whole-cell dialysis can alter the interaction or configuration of intracellular proteins, there may be an effect on the accessibility of calcium channels for G protein subunits. This may be a good candidate for changing the efficacy of G protein / calcium channel interactions in whole-cell recordings of calcium current.

The studies described above demonstrate that many cellular pathways and interactions can modulate the efficacy of G protein / calcium channel interactions. The ciliary ganglion preparation may provide a unique opportunity to investigate the effect of cytoplasmic components on voltage-dependent calcium current modulation. The experiments presented here have provided insight into the voltage dependence of somatostatin-mediated calcium current inhibition in chick ciliary ganglion neurons. When measured in whole-cell versus perforated patch recordings there is a difference in the voltage dependence of inhibition relief, the rate of reinhibition following a prepulse and the efficacy of activated G proteins. The differences in the voltage dependence of somatostatin-induced calcium current inhibition presented here vary depending on the protocol used to measure relief, but are consistent with the hypothesis that there is a greater affinity of calcium channels for G protein subunits in whole-cell recordings. Under conditions that favor relief of inhibition (G protein unbinding) such as during an action potential train, whole-cell recordings are more sensitive to relief of inhibition. However, under conditions that favor rebinding of G protein (repolarization to -80 mV), whole-cell recordings show enhanced reinhibition of calcium current during a step depolarization following a prepulse. Thus, different stimulus protocols allow different factors to dominate the calcium channel G protein interaction and result in different measurements of inhibition relief. In chick ciliary ganglion neurons, cytoplasmic components may alter the stability of the interaction of activated G protein subunits with the calcium channel and provide a mechanism of precise control over transmitter release during high frequency activity.

# III. CALCIUM CURRENT ACTIVATION: EFFECTS OF ACTION POTENTIAL SHAPE AND VARIATIONS IN ONSET OF ACTION POTENTIAL BROADENING

## A. SUMMARY

The voltage dependence and kinetic properties of stage 40 ciliary ganglion calcium currents were determined using 10 ms voltage steps. These properties aided the interpretation of the action potential-evoked calcium current described below and the comparison of the data with those observed in other preparations. Three different action potential waveforms were modeled by a series of ramps to generate voltage clamp commands. Calcium currents evoked by these model action potentials were compared before and after alterations in the repolarization phase of each action potential. Abrupt step repolarizations from various time points were used to estimate the time course of calcium current activation during each action potential. Calcium current evoked by fast action potentials (0.5 or 1.0 ms duration at half-amplitude) did not reach maximal activation until the action potential had repolarized by 40-50%. In contrast, calcium current evoked by a slow action potential (2.2 ms duration at half-amplitude) was maximally activated near the peak of the action potential. Slowing the rate of repolarization of the action potential (broadening) from different times was used to examine effect on peak and total calcium influx. With all three waveforms tested, broadening consistently increased total calcium influx. However, peak calcium current was either increased or decreased depending on the duration of the control action potential tested and the specific timing of the initiation of

broadening the repolarization phase. The opposite effects on peak calcium current observed with action potential broadening beginning at different time points in the repolarization may provide a mechanism for the variable effects of potassium channel blockers on transmitter release magnitude.

## B. INTRODUCTION

Action potential (AP) invasion of a nerve terminal is the natural stimulus that opens calcium channels critical for neurotransmitter release (Katz, 1969). The magnitude of transmitter released is tightly regulated by the magnitude of calcium entry into the nerve terminal at exocytotic release sites (Llinas et al. 1981; Augustine et al. 1991). The shape and time course of the AP that depolarizes nerve terminal membrane regulates the gating of calcium channels and the magnitude of the calcium flux. Under physiological conditions, modulation of AP shape and time course can fine tune the magnitude of transmitter released indirectly through changes in calcium influx characteristics (Klein & Kandel, 1980; Spencer et al. 1989).

In most preparations, calcium entry during an AP has been shown to begin just after the peak of the AP (Llinas et al. 1981; Toth & Miller, 1995; Sabatini & Regehr, 1996; Borst & Sakmann, 1998). The depolarizing phase of the AP initiates calcium channel opening, but the driving force for calcium entry through these open channels is relatively low at the peak of the AP (Llinas et al. 1981). Calcium influx increases during the repolarization of the AP because the open calcium channels pass more calcium ions as the driving force for calcium entry increases. As a result, the speed and magnitude of calcium entry during an AP is very sensitive to the time course of the repolarization phase of the AP.

Potassium channels are important because they shape the repolarization and after-hyperpolarization (AHP) phases of the AP. Blockade or inactivation of voltage-gated potassium channels has been shown to cause AP broadening that results in increased calcium influx and transmitter release (Augustine, 1990; Wheeler et al. 1996; Sabatini & Regehr, 1997). Calcium-activated potassium (BK) channels are of particular interest because they have been shown to be co-localized with calcium channels in the active zone (Yazejian et al. 1997). Blockade of BK channels has been shown to broaden somal APs at various times during AP repolarization and / or decrease the size of the AHP (Zhang & McBain, 1995; Davies et al. 1996).

At the synapse, BK channel blockade or *slowpoke* gene mutation has been shown to either increase (Robitaille & Charlton, 1992) or decrease (Warbington et al. 1996; Pattillo et al. 1997) transmitter release magnitude. A decrease in transmitter release magnitude resulting from a decrease in peak calcium influx has also been reported following AP broadening in the jellyfish, where these effects are attributed to A-type potassium current inactivation (Spencer et al. 1989). These opposite effects on transmitter release may result from subtle differences in the precise timing of effects on AP broadening and / or differences in BK and calcium channel gating.

This work was initiated to enhance understanding of the conditions under which the balance of influences that regulate calcium entry during APs is dominated by calcium channel activation or the driving force for calcium entry. The balance between these influences on peak and total calcium influx were examined using three different types of AP waveforms. The time course of calcium channel activation was estimated during each type of AP and the effects of broadening the repolarization phase from different points in the time course of the AP were measured. The time at which driving force becomes the dominant influence depends on the normal shape of the AP, because the time course of calcium channel activation is different with APs of differing shapes.

## C. METHODS

1. Cell culture

Ciliary ganglia were dissected from White Leghorn chicken embryos (quickly decapitated) at stage 40 (Hamburger and Hamilton, 1951) in Tyrode solution containing (in mM): 134 NaCl, 3 KCl, 3 $CaCl_2$, 1 $MgCl_2$, 12 glucose and 20 $NaH_2CO_3$, pH 7.3, adjusted with NaOH. Ganglia were incubated sequentially in collagenase type II (0.5 mg/mL) and trypsin (0.08%) in $Ca^{2+}$- and $Mg^{2+}$- free Tyrode solution at 37° C for 20 min. each. Trypsin was removed and inhibited by three washes in minimal essential medium (MEM) plus 10% heat-inactivated horse serum. Ganglia were dissociated mechanically by gentle trituration through a polished Pasteur pipette. The final suspension of cells was centrifuged at 100 *g* for 5 minutes and resuspended in MEM plus 10% chick embryo extract. Cells were plated onto poly-D-lysine-coated 35 mm plastic culture dishes, incubated at 37° C, and used for experiments after 1-6 hours of incubation.

2. Whole cell patch-clamp recordings

The traditional whole cell patch-clamp method was used for all experiments (Hamill et al. 1981). To isolate calcium currents, cells were bathed in an external saline of the following composition (in mM): 140 or 100 NaCl, 10 or 50 TEA-Cl, 10 HEPES, 5 glucose, 5 KCl, 5 $CaCl_2$, and 2 $MgCl_2$ plus 1 μM tetrodotoxin, pH = 7.3, adjusted by NaOH. Pipettes were pulled on a Flaming /Brown Micropipette Puller (Sutter Instruments Co.: Model P-97), coated with Sylgard (Dow Corning), and fire polished (electrode resistances ranged from 0.5 to 2 MΩ). Pipettes were filled with the following internal solution (in mM): 120 CsCl, 10 HEPES, 11 EGTA, 5 TEA-Cl, 1 $CaCl_2$ and 4 $MgCl_2$, with 4 Mg-ATP, 0.3 Na-GTP and 0.1 leupeptin added fresh daily. Access resistance was usually 3-4 MΩ. The -4.3 mV liquid junction potential was corrected for before all recordings

and series resistance was compensated by 80-90%. Currents were activated, acquired and leak-subtracted using a hyperpolarizing P/4 protocol by the pCLAMP software package (Axon Instruments) running on a Pentium processor-based microcomputer in concert with an Axopatch 200A patch-clamp amplifier (Axon Instruments). Voltage dependence and kinetics of ciliary ganglion calcium currents were determined using 10-ms voltage steps from -80 mV to potentials between -50 and +60 mV. After stepping back to -80 mV, the peak of the tail current was used as an estimate of the steady-state activation of calcium currents and these data were fitted with a Boltzmann function. To determine the time constant for activation of calcium currents, the calcium current was fitted to a single exponential beginning when inward current crossed baseline. To determine the time constant for deactivation, the decay of tail currents to baseline was fitted with a single exponential beginning 80 µsec after the peak of the tail current.

3. Action potential waveforms

The APs used in the construction of model AP waveforms were adapted from Borst et al. (1995) and Brody et al. (1997) for the calyx of Held AP, and from unpublished observations from this laboratory for the motoneuorn AP and the ciliary ganglion AP. The AHP was removed from the modeled AP waveforms since it did not affect significantly the peak calcium influx and only slightly affected the very late phases of total calcium influx. Further, removing the AHP simplified alterations to the repolarization phase. The descriptions of the time when alterations of the repolarization phase were begun are relative to the peak of the AP, defined as that time point at the end of the short plateau phase of the AP peak. Calcium currents were four-pole Bessel filtered at 5 kHz and digitized at 20 kHz. An average of four to six currents was used for all comparisons. Calcium currents activated by altered APs were compared with a control recording taken within 30 s of the experimental recording to control for the effects of calcium current run-down. Furthermore, the order in which the broadening protocols were used was

varied so that any potential error due to run-down would be minimized. The total calcium influx was measured as the integral of the calcium current bounded by the time point at which inward current initially deviated from baseline, and the time at which it returned to baseline. Nifedipine (RBI) was solubilized in DMSO at 1 mM and diluted into bath saline to 1 μM. All other reagents were obtained from Sigma and dissolved in saline. All values are expressed as means ± S.E.M. and all cells were studied at room temperature (22-23 °C).

## D. RESULTS

1. Calcium current in ciliary ganglion neurons

I have examined the time course of calcium channel activation during different action potential waveforms, and the effects of action potential broadening on calcium current. To aid in the interpretation of these data and to facilitate comparison with other studies, I have examined the voltage dependence of calcium current activation and the kinetics of calcium current activation and deactivation using traditional square voltage step commands (Figure 16). Stage 40 ciliary ganglion neuronal somata express predominantly N-type calcium channels (White et al. 1997). These currents began to activate at about –40 mV and maximal current flowed at about 0 mV (Figure 16 A-C). A boltzmann fit of the steady-state activation curve indicates half-maximal activation at -4 mV (Figure 16B). Activation and deactivation time constants are known to be voltage dependent (Hodgkin and Huxley, 1952). To estimate the speed of calcium current activation over the voltage range that is relevant to the action potential waveforms used in this study, we fitted (using a single exponential) the activation of calcium currents in response to voltage steps between -10 and +30 mV (Figure 16D). To estimate the speed of calcium current

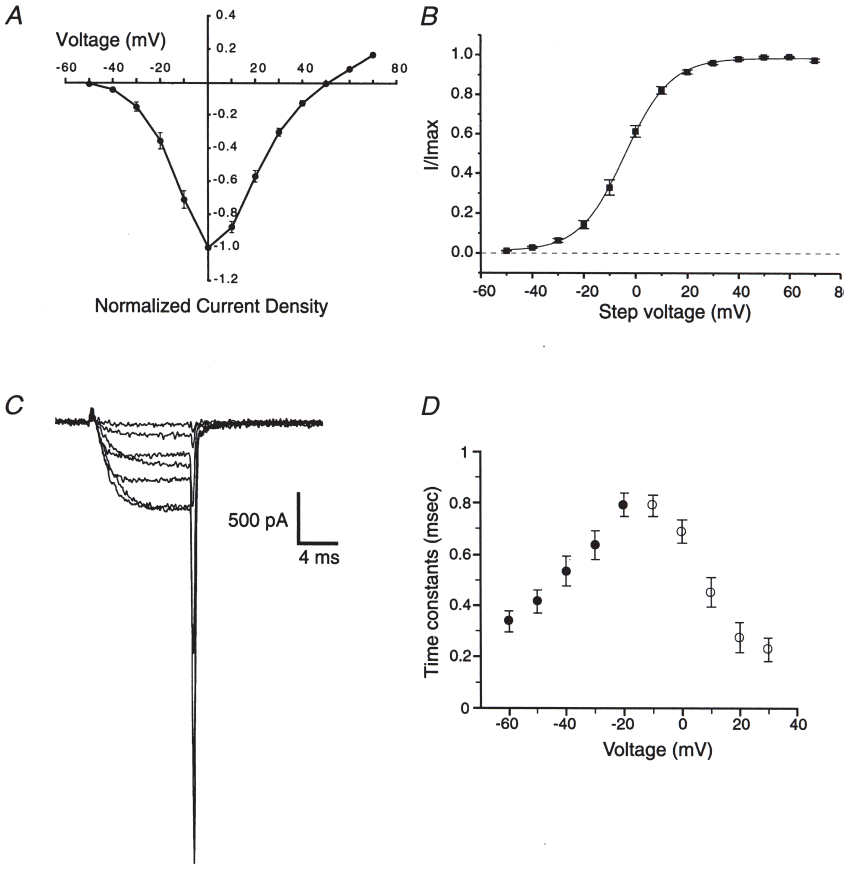


**Figure 16. Properties of calcium current in stage 40 ciliary ganglion neurons.** A. Current – voltage relationship obtained from six representative cells. To facilitate pooling of data from cells of different size, raw current amplitude values were divided by the capacitance recorded from each cell to obtain a current density (pA / pF) and these were normalized to the largest value from each cell. B. Voltage dependence of activation obtained by plotting normalized tail current amplitude ($I/ I_{max}$) against the step potential used to activate current (means ± S.E.M.; n = 6). The continuous line represents a Boltzmann fit to the data. C. Representative family of currents elicited by voltage steps between -30 and +30 mV in 10 mV increments ($V_{hold}$ = -80 mV). D. Time constants for calcium current activation (open circles) and deactivation (closed circles) at voltages to which calcium channels are exposed during AP waveforms (means ± S.E.M.; n = 6).

deactivation over the voltage range relevant to the action potential waveforms used in this study, we fitted (using a single exponential) the deactivation of tail currents following a repolarization step from +30 mV (peak of our action potential waveforms) to between -20 and -60 mV (Figure 16D).

2. Action potential waveforms

Three action potentials were selected to represent a wide range of action potential durations present in neurons. The fastest action potential examined ('fast') had a duration at half-amplitude of approximately 0.5 ms and was adapted from a study of the calyx of Held (Borst et al. 1995). The maximum slope of the rising phase of the fast action potential was 308 mV/ms. The second AP ('medium') was obtained from this lab's recordings of APs in the presynaptic varicosities of *Xenopus* motoneurons in culture (Pattillo and Meriney, unpublished observations). This AP had a duration at half-amplitude of 1 ms and a maximum rising phase slope of 94 mV/ms. The slowest AP examined ('slow'), with a duration at half-amplitude of 2.2 ms and a maximum rising phase slope of 83 mV/ms, was taken from our recordings of APs in cultured chick ciliary ganglion neurons (Artim and Meriney, 2000). These three AP waveforms were faithfully modeled with a series of voltage ramps (4-5) up to and including the small plateau at the peak (Figure 17). However, the repolarizing phase was simplified to a single ramp to allow for consistent modification. To isolate the effects of changing AP shape such that they included only changes in repolarization, all of the model APs were scaled in the voltage dimension so that they had a resting potential of -60 mV and peak amplitude of +30 mV. This is within 5 mV of the actual values for resting and peak potentials for the native APs on which the models were based, with the exception of the calyx of Held AP (natural resting potential = -80 mV; Borst et al. 1995). The three model AP waveforms derived from the calyx of Held, *Xenopus*

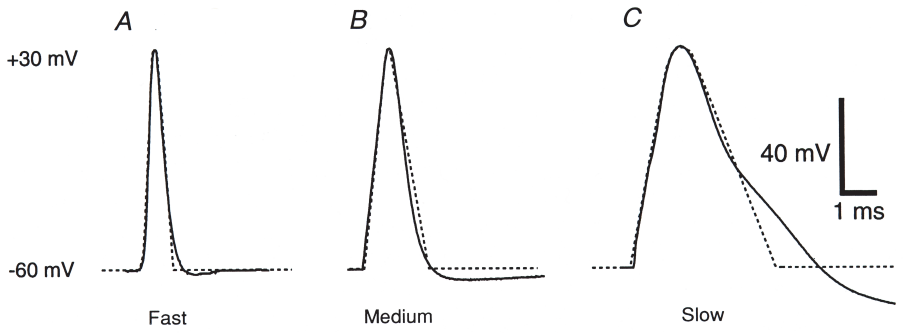


**Figure 17. Action potential waveforms.** To create the fast, medium, and slow model AP waveforms, three natural action potentials (continuous lines) of different durations were used. Action potentials were adapted from the presynaptic terminal of the calyx of Held (A), the presynaptic *Xenopus* motoneuron varicosity (B) and the soma of a chick ciliary ganglion neuron (C). To facilitate the use of these as voltage-clamp commands, the rising phase was modeled using a series of ramps (dotted lines), and the falling phase was simplified to a single ramp to allow consistent alterations in the repolarization phase. The action potentials were normalized to a resting potential of -60 mV and peak amplitude of +30 mV.

motoneuron varicosities and chick ciliary ganglion neurons will be referred to as the fast, medium and slow AP waveforms, respectively, from here forward.

3. Calcium current activation during an action potential.

To gain an understanding of the time course of calcium current activation during the repolarizing phase of different APs, we divided the repolarizing phase of into several time points at which the voltage command was stepped back to -80 mV (Figure 18). The peak of the resulting tail current was taken as a relative estimation of calcium channel activation at that point in the AP.

When the fast AP waveform was examined, the peak of calcium current activation occurred 230 μs after the peak of the AP, when the membrane potential had reached -15 mV, which corresponded to 50% of AP repolarization (Figure 18 C & D). The fast AP was so brief that even when the voltage returned to baseline (-60 mV), calcium current was still 75% of maximum activation. The peak calcium current activation during the medium AP occurred 400 μs after the peak of the AP when the membrane potential had reached -6 mV, corresponding to 40% of AP repolarization. The slower rate of repolarization of the medium AP allowed for significant deactivation of calcium current to occur as the membrane potential approached baseline. Thus, when repolarization was complete, calcium activation was only 38% of maximum. The slow AP waveform elicited calcium current that was near maximal at the peak of the AP. Calcium current activation peaked 400 μs after the peak of the AP, when the membrane potential had only repolarized to +21 mV (10% of AP repolarization). Calcium current activation during the slow AP appeared to plateau until the membrane potential dropped to below +5 mV, at which point deactivation began to reduce the number of active calcium channels. The slow repolarization rate of this AP allowed for calcium current to almost completely deactivate before the membrane potential reached baseline.

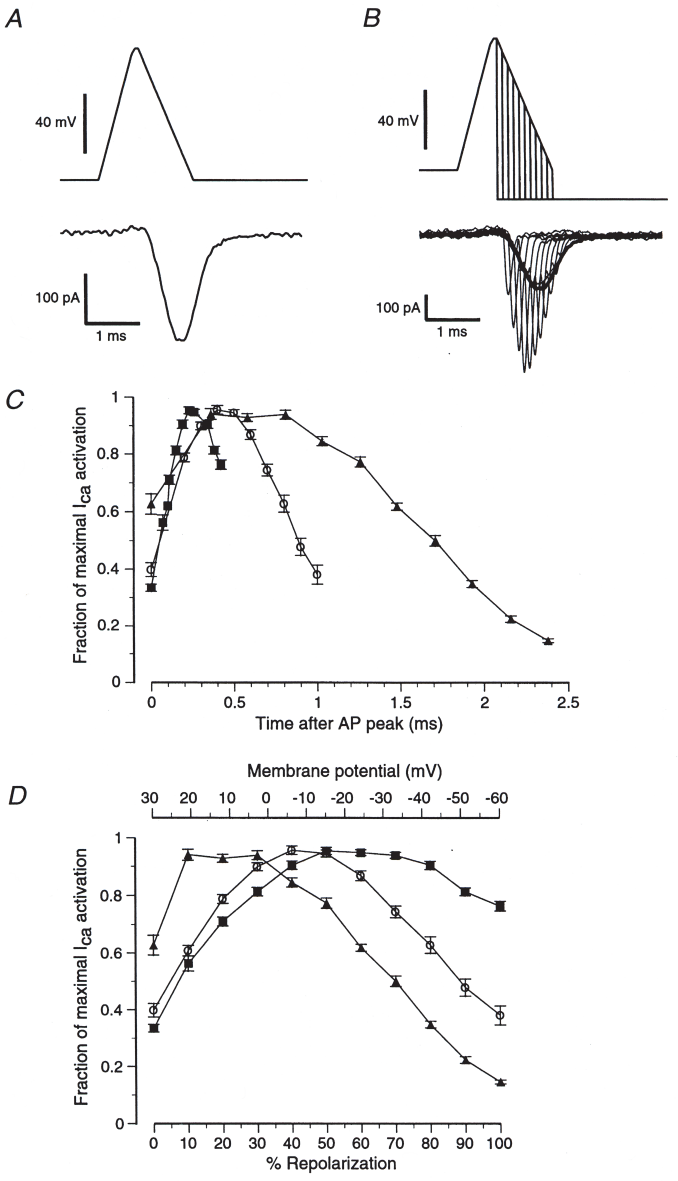


**Figure 18. Estimation of the timing of calcium current activation during an AP waveform.** A. Control calcium current evoked by the medium AP waveform. B. Family of tail currents (thin lines) evoked by instantaneous repolarizations of the medium AP waveform to -80 mV at various times during the AP repolarization. C and D. Summary of data obtained with the protocol outlined in B using the three different AP waveforms (fast AP – filled squares; medium AP – open circles; slow AP – filled triangles; means ± S.E.M; n > 16). C. Plot of maximal calcium current activation as a function of time following the peak of the AP waveform. D. Same data as in C, but plotted with membrane potential (top) or percentage AP repolarization (bottom) on the x-axis instead of time.

The ciliary ganglion neurons that were used for these experiments express both N- and L-type calcium channels, but N-type channels constitute about 75% of the total calcium current at this developmental stage (stage 40; White et al. 1997). For some experiments using the slow and medium AP waveforms, nifedipine (1μM) was added to the bath to block L-type calcium channels. In these experiments, there was no significant difference in the calcium current activation curves when compared with those collected in the absence of nifedipine (data not shown). Thus, although the data presented here reflect the effects of AP waveform alterations on both N- and L-type calcium channels, removal of the relatively minor L-type channel contribution did not significantly alter the data.

4. Timing of action potential broadening

To examine the effect of variations in the onset of AP broadening, the duration of each of the AP waveforms was increased by 1 ms, and the point in the repolarization at which the increase in duration began was varied (Figure 19). The peak and integral of the elicited calcium currents were then compared with those elicited by a control AP waveform.

The effect of AP broadening on the peak calcium current was dependent on the timing of the AP broadening. Broadening of the fast AP beginning at the peak of the AP caused the peak of the elicited calcium current to increase by 21 ± 2% relative to the control fast AP (Figure 19 Aa). However, when broadening was delayed to a point when repolarization was 20% complete, a small decrease in the peak of the calcium current was observed. Broadening from later time points further decreased the peak amplitude of the calcium current (Figure 20A). The greatest decrease in peak calcium current was 27 ± 1%, which occurred when broadening was delayed to a point when repolarization was 50% complete (Figures 19Ab and 20A). The effect of broadening on peak calcium current diminished as the time at which broadening began was delayed to 60% of repolarization or greater (Figure 20A). In contrast to the effects of

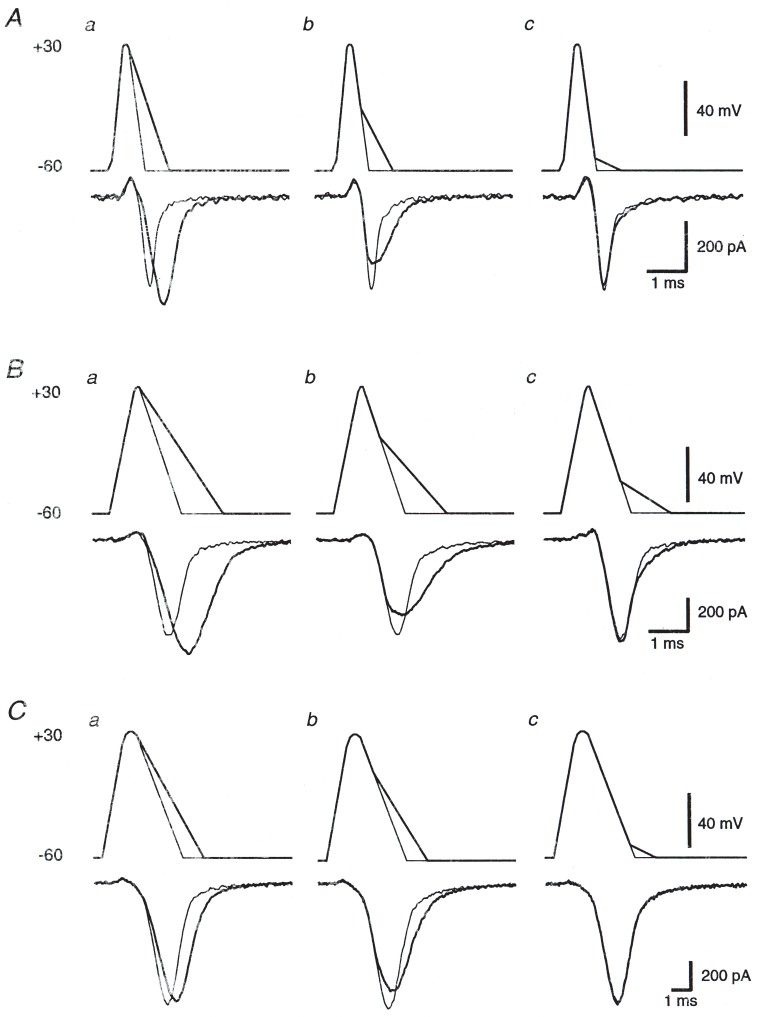


**Figure 19. Effects of action potential broadening on calcium current activation**. In all cases, the control AP and associated calcium current are shown using thin lines. Broadened action potentials and associated calcium currents are shown using thick lines. A. Fast action potential broadened from the peak (a), 50% of repolarization (b), and 90% of repolarization (c). B. Medium action potential broadened from the peak (a), 40% of repolarization (b), and 75% of repolarization (c). C. Slow action potential broadened from the peak (a), 30% of repolarization (b) and 90% of repolarization (c). Example action potential waveforms chosen for panel b in A-C were selected to represent maximal reductions in peak calcium current.

broadening on peak calcium current, broadening from any point led to increases in the total calcium influx (integral) relative to control (Figure 20B). This effect diminished steadily as broadening was delayed to later points in the repolarizing phase of the AP waveform. When the onset of broadening was delayed until 20% of repolarization or later, the increases in integral were largely confined to the latter half of the calcium influx (Figure 19Ab).

Broadening of the medium AP had a similar effect on peak calcium current (Figures 19B and 5). Broadening of the AP from the peak led to an 8 ± 4% increase in peak calcium current. The effect of broadening on peak calcium current reversed when broadening was delayed to a point when repolarization was 10% complete. The maximum decrease in peak calcium current of 19 ± 0.4% was observed when broadening was delayed until repolarization was 40% complete (Figures 19Bb and 20A). When broadening was delayed until later points in repolarization, the effect on peak calcium current diminished. As with the fast AP, broadening from any point in the repolarization phase of the AP caused an increase in the integral of calcium current (Figure 20B). The increase in total calcium influx (integral) was largely confined to the later phases of the AP-evoked calcium influx.

In contrast to the effects of broadening using the fast or medium APs, broadening of the slow AP from the peak resulted in a decrease in the peak of the evoked calcium current (6 ± 1%; Figures 19 Ca & 20A). This decrease in peak calcium current was most prominent (12 ± 1%) when broadening was delayed until repolarization was 30% complete. The effect of broadening on the peak of the AP-evoked current diminished when broadening was delayed to 40% of repolarization or greater. As with the fast and medium APs, broadening of the slow AP from any point in the repolarizing phase resulted in an increase in the integral of the calcium current (Figure 20B).

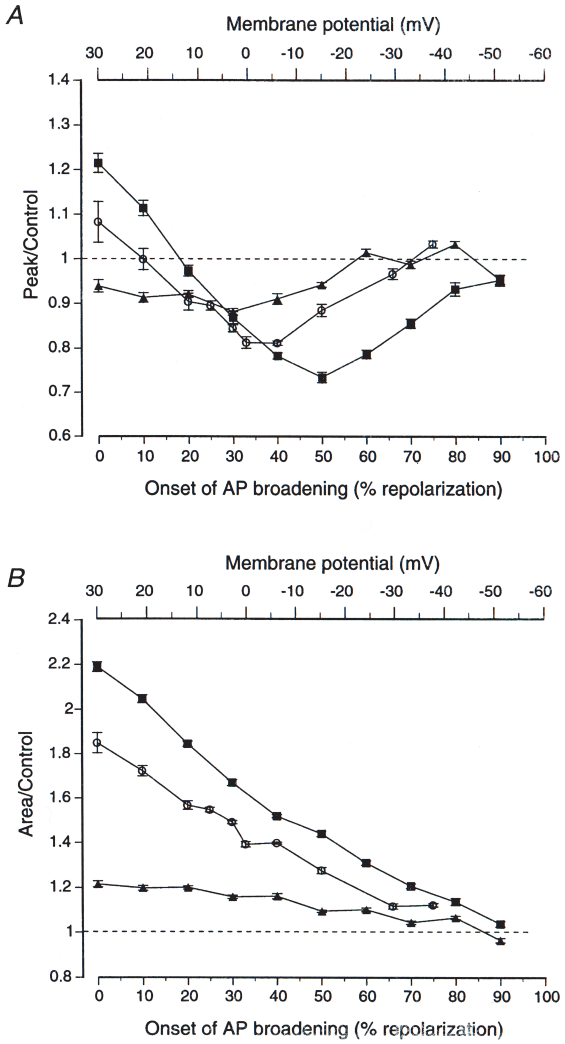


**Figure 20. Summary of action potential broadening effects.**
A. Effects of broadening the three action potential waveforms on peak calcium current. Broadening the slow AP resulted in decreases in peak calcium influx (triangles). Broadening the medium AP resulted in increases in peak calcium current when the broadening began before 10% of action potential repolarization (+21 mV), but in decreases in peak calcium current when it began later than 10% of repolarization (circles). Broadening the fast action potential resulted in increases in peak calcium current when the broadening began before 20% of action potential repolarization (+12 mV), but decreases in peak calcium current when it began later than 20% of repolarization (squares). B. Action potential broadening resulted in increased total calcium influx (measured as the integral of calcium current) for all three action potentials tested regardless of where in the repolarization phase broadening began. All values are the mean ± S.E.M. (n > 14).

## E. DISCUSSION

Calcium current during an AP will be determined by the balance between the number of open channels, the time the channels reside in the open state and the electrochemical driving force present when the channels are open. Any change in the shape of the AP will affect calcium current depending on the timing of that change and the specific balance of these three influences at that point in the AP. The data presented above have shown that the time course of activation of calcium current during an AP varies depending on the duration of the AP. In all three AP waveforms tested, the activation of calcium channels was not complete at the peak of the AP, but continued into repolarization. Depending on the AP waveform tested, calcium channel activation was maximal at between 10 and 50% of repolarization. Furthermore, the fast AP was so brief that, in the second half of repolarization, very little channel deactivation occurred. This may have been due to the unnatural abruptness of the repolarization in the simplified AP waveforms. Additionally, it is important to note that all of the experiments reported here were performed at room temperature. At physiological temperatures calcium channels would be expected to gate more rapidly and shift the time of maximal activation during AP repolarization to earlier in the AP waveform (Sabatini & Regehr, 1996). Additionally, the small charge screening effect of using 5 mM calcium, instead of the more physiological 2 mM, might also shift the time course of calcium channel gating slightly during an AP.

The differences in the time course of calcium current activation during the repolarizing phase of the three different APs used here are reflected in the effect on calcium current of variations in the onset of AP broadening. During the slow AP, calcium current activation was maximal soon after the peak of the AP. Thus, any AP broadening would not be likely to open more calcium channels, but would have its effects predominantly by decreasing the electrochemical driving force for calcium and delaying calcium channel deactivation. This is supported by the observation that broadening the slow AP beginning at the peak resulted in a

decrease in peak calcium current and an increase in the integral of calcium current. During faster APs, broadening from the peak might be expected to cause the opening of more calcium channels (Augustine, 1990). Since calcium current activation did not peak until the AP had repolarized by 40% for the medium AP and 50% for the fast AP, broadening these APs increased peak calcium current until the broadening began at 20% of repolarization or later. In fact, maximal decreases in peak calcium current occurred when broadening began near the time when calcium current activation had reached a maximum (40 and 50% of repolarization for medium and fast APs, respectively). Broadening-induced decreases in peak calcium current became less dramatic when broadening began very late in the AP repolarization, as driving force differences between the control AP and the broadened AP were diminished.

Regardless of the time of onset, AP broadening always increased the integral of calcium current in all three APs tested. When the two fastest APs were broadened beginning near the peak, the increase in the integral of calcium current observed can be explained as a combination of the effects of increased calcium channel activation and slowing of calcium channel deactivation. Broadening from later points where calcium current activation was maximal, or near maximal, led to an increase in the integral of calcium current primarily by slowing the deactivation of calcium channels. In the slow AP, where calcium current activation was maximal near the peak, increases in total calcium influx resulting from broadening AP repolarization were most likely to have been caused primarily by slowing of calcium channel deactivation.

Although the model APs allowed an examination of the effects of varying the onset of AP broadening during repolarization, they are not intended to reflect the precise time course of modifications seen in native neuron Action potentials. Selective BK channel blockade usually causes broadening that increases in magnitude as repolarization of the AP progresses and is not the linear function modeled here. This difference will certainly alter the specific timing of

when AP broadening causes an increase or decrease in peak calcium current, but not the general principle discussed here.

There are two possible mechanisms that could underlie an increase in peak calcium current following AP broadening. Single APs have been shown to effectively open the majority of available calcium channels (Borst & Sakmann, 1998), but the maximal activation of calcium channels during a brief AP does not occur until well after the peak of the AP. Therefore, by decreasing the rate of repolarization, AP broadening could slow calcium channel deactivation and cause maximal calcium channel activation to occur closer to the peak of the AP. This would result in a greater number of open channels contributing to the peak of the calcium current. Alternatively, if an AP does not activate all of the available calcium channels (Pumplin et al. 1981), AP broadening could open additional calcium channels, resulting in increased peak calcium current (Augustine, 1990).

Numerous studies have demonstrated that blockade of potassium channels leads to AP broadening. Application of potassium channel blockers that target voltage-gated potassium channels, or non-specific potassium channel blockers that target both voltage-gated and calcium activated potassium channels, typically results in broadening beginning at the peak of the AP (Augustine, 1990; Sabatini & Regehr, 1996; Wheeler et al. 1996). However, specific blockade of large-conductance calcium-activated potassium channels can result in AP broadening beginning at various times during repolarization (Solaro et al. 1995; Zhang & McBain, 1995; Davies et al. 1996). These effects are observed in many types of neurons with AP shapes that vary from fast time course APs in central interneurons (Zhang & McBain, 1995) to much slower APs in peripheral ganglion neurons and chromaffin cells (Solaro et al. 1995; Davies et al. 1996).

The effects of AP broadening have also been examined with reference to the magnitude of transmitter released and have been shown to be variable depending on the preparation examined. Blockade of voltage-gated potassium channels increases the magnitude of

transmitter release in many preparations (Llinas et al. 1981; Augustine, 1990; Wheeler et al. 1996; Sabatini & Regehr, 1996). Interestingly, inactivation of A-type potassium currents has been shown to decrease transmitter release in jellyfish neurons (Spencer et al. 1989). Selective BK channel blockade has been shown to increase transmitter release at adult neuromuscular synapses (Robitaille et al. 1993), but decrease transmitter release at cultured embryonic neuromuscular synapses (Pattillo et al. 1997). The mutation of the slowpoke gene in Drosophila, which codes for a BK channel, also decreases the magnitude of transmitter release (Warbington et al. 1996). These contrasting effects may result from variability in the timing of when these potassium channels contribute to the AP waveform and the shape of the natural waveform in the neurons studied. In neuronal somata, BK channels have been shown to be tightly coupled to specific types of calcium channels (Wisgirda & Dryer, 1994; Davies et al. 1996). Alterations in the precise coupling of BK channels to calcium channels, or the modulation of BK channel properties would be expected to alter the timing of their contribution to AP repolarization.

The increases or decreases in transmitter release that result from blockade, inactivation, or mutation of potassium channels, suggest that AP broadening at transmitter release sites can either increase or decrease the calcium influx that is relevant to the regulation of transmitter release magnitude. The data demonstrate that AP broadening invariably increases the total calcium influx (integral), but can have opposite effects on peak calcium current depending on the timing of broadening. The specific aspect of calcium influx (rate of influx or total calcium entry) that is most relevant to transmitter release magnitude may depend on the specific arrangement of calcium channels and docked vesicles at the synapse. Release of docked neurotransmitter vesicles is thought to be triggered by the intracellular microdomain of high calcium concentration near the mouth of active zone calcium channels (Naraghi & Neher, 1997). The largest and most transient changes in intracellular calcium concentration occur near the mouth of the channel. At sites further away from the mouth of the channel, calcium

concentration rises more slowly to an elevated level (Augustine et al. 1991). The large transient very close to the mouth of a calcium channel may be the most sensitive to small changes in the instantaneous driving force. Thus, release sites that are sensing calcium very near this large transient may be the most sensitive to the potentially opposite effects on the rate of calcium influx caused by AP broadening from different points in the repolarization.

The subtle control over the time course of AP repolarization is an important point of regulation over calcium influx and transmitter release. The influences that determine the consequences of alterations in potassium channel contribution to transmitter release regulation probably include the duration of the AP, the kinetics of calcium and potassium channel activation and deactivation, and the relationship between presynaptic calcium channels and the transmitter release apparatus.

# IV. G PROTEIN MODULATION OF ACTION POTENTIAL-EVOKED CALCIUM CURRENT

## A. SUMMARY

I have studied voltage-dependent inhibition of N-type calcium currents to investigate the effects of G protein modulation-induced alterations in channel gating on action potential-evoked calcium current. In isolated chick ciliary ganglion neurons, GTPγS produced voltage-dependent inhibition that exhibited slowed activation kinetics and was partially relieved by a conditioning prepulse. Using step depolarizations to evoke calcium current, I measured tail current amplitudes upon abrupt repolarization to estimate the time course of calcium channel activation from 1 to 30 ms. GTPγS prolonged significantly channel activation, consistent with the presence of kinetic slowing in the modulated whole-cell current evoked by 100 ms steps. Since kinetic slowing is caused by an altered voltage dependence of channel activation (such that channels require stronger or longer duration depolarization to open), I asked if GTPγS-induced modulation would alter the time course of calcium channel activation during an action potential. Using an action potential waveform as a voltage command to evoke calcium current, I abruptly repolarized to –80 mV at various time points during the repolarization phase of the action potential. The resulting tail current was used to estimate the relative number of calcium channels that were open. Using action potential waveforms of either 2.2 or 6 ms duration at half-amplitude, there were no differences in the time course of calcium channel activation, or in the percent activation at any time point tested during the repolarization, when control and

modulated currents were compared. It is also possible that modulated channels might open briefly, and that these reluctant openings would affect the time course of action potential-evoked calcium current. However, when control and modulated currents were scaled to the same peak amplitude and superimposed, there was no difference in the kinetics of the two currents. Thus, voltage-dependent inhibition did not alter the kinetics of action potential-evoked current. These results suggest that G protein modulated channels do not contribute significantly to calcium current evoked by a single action potential.

## B. INTRODUCTION

High voltage-activated, N-type calcium channels are involved in many cellular processes including neurotransmitter release. Inhibition of calcium current is a well-documented mechanism of presynaptic modulation of neurotransmitter release (Wu and Saggau 1997), and is induced by a variety of neurotransmitters and peptides that exert their effects via G protein coupled receptors (Hille 1994; Dolphin 1998). Many studies have investigated the mechanisms underlying G protein modulation of calcium channels and have revealed that a membrane-delimited pathway, likely involving the direct interaction of G protein $\beta\gamma$ subunits with the calcium channel, underlies many instances of calcium channel inhibition (Ikeda 1996; Herlitze et al. 1996).

An intriguing characteristic of this inhibition is that it is often voltage-dependent (reviewed by Jones and Elmslie 1997). This voltage dependence is typically demonstrated by the ability of a strong depolarizing step to relieve a large fraction of the inhibition (Elmslie et al. 1990). In whole-cell recordings of calcium current, voltage-dependent inhibition is characterized by a slowing of the activation kinetics, commonly referred to as kinetic slowing (Golard and

Siegelbaum 1993; Boland and Bean 1993; Elmslie and Jones 1994). It is thought that kinetic slowing is an example of relief of inhibition. The sustained stimulus provided by a long step depolarization moves modulated (reluctant) channels out of their reluctant mode into a willing mode from which they open. This kinetic change is caused by a positive shift in the voltage dependence of calcium channel activation such that modulated channels require a stronger or longer depolarization to move from a reluctant to willing state and then open (Bean 1989). So, while unmodulated channels activate normally, modulated channels will open more slowly, causing the appearance of slowed calcium current activation during prolonged depolarizations in whole-cell recordings. Consistent with this hypothesis, single-channel studies of calcium current inhibition have demonstrated that voltage-dependent inhibition results in an increase in the latency to first channel opening (Carabelli et al. 1996; Patil et al. 1996). This increase is correlated with the presence of kinetic slowing in ensemble currents and is relieved by a strong conditioning depolarization.

The fact that voltage-dependent inhibition can be relieved by strong depolarizations suggests that it may be sensitive to physiologically relevant voltage changes. In support of this idea, inhibition can be partially relieved by short trains of 1-2 ms step depolarizations designed to mimic AP stimulation (Womack and McCleskey 1995; Williams et al. 1997). Furthermore, it has been shown that trains of AP waveforms can relieve significantly inhibition of N-type channels in chick dorsal root ganglion (DRG) neurons (Park and Dunlap 1998), and of P/Q-type channels expressed in HEK 293 cells (Brody et al. 1997). Recently, it has been shown that relief of G protein-mediated inhibition of calcium current contributes to short-term synaptic facilitation in hippocampal autapses (Brody and Yue 2000). Thus, voltage dependent inhibition may represent an activity-dependent form of modulation that fine-tunes the degree of transmitter release based on neuronal activity.

It is also possible that calcium channels open directly from the modulated (reluctant) state. Recent studies have identified neurotransmitter-induced alterations in calcium channel

gating that provide evidence for calcium channel opening from the reluctant state (Lee and Elmslie 2000; Colecraft et al. 2000). These openings occurred without a delay in first latency, and with lower open probability and briefer open times than in the normal gating mode. These reluctant openings are induced by depolarizations within the range of membrane voltage typically reached during an AP (equivalent to 0 to 10 mV in physiological calcium concentration). Thus, modulation-induced changes in channel gating may alter the kinetics of calcium entry in two ways. The increase in first latency of channel opening (due to the slow transition between the reluctant and willing gating modes), and the presence of very brief, direct reluctant openings may both be important determinants for the time course of calcium entry.

The physiologic role of modulation-induced alterations in channel gating kinetics remains unclear. While there are studies, discussed above, that address this issue with respect to single channel events, there are no studies that focus on potential effects during a single AP. Modulated calcium channel gating could alter the timing of peak current, and thus the synaptic delay for transmitter release. Alternatively, if modulated channels gate too slowly or briefly, they may not contribute significantly to AP-evoked current. In this case, modulated channels would be effectively eliminated from contributing to transmitter release. To examine this issue, I have investigated the effects of voltage-dependent inhibition on calcium current evoked by single AP waveforms. Using GTPγS to induce G protein-mediated, voltage-dependent inhibition of calcium current, and AP waveforms as voltage commands, I have recorded N-type calcium current from the somata of chick ciliary ganglion neurons. Analysis of the tail currents resulting from abrupt repolarization at various times during the AP has enabled us to evaluate the time course of calcium channel activation during a single AP. The results demonstrate that the presence of voltage-dependent inhibition does not alter the time course of calcium channel activation during a single AP, suggesting that modulated channels do not contribute significantly to AP-evoked calcium current.

## C. METHODS

1. Cell culture

Ciliary ganglia from White Leghorn chicken embryos at stage 40 (Hamburger and Hamilton 1951) were dissected in oxygenated Tyrode containing (in mM): 134 NaCl, 3 KCl, 3 $CaCl_2$, 1 $MgCl_2$, 12 glucose and 20 $NaH_2CO_3$, pH 7.2. The ganglia were then incubated at 37°C in collagenase (0.5 mg/mL) followed by trypsin (0.08%) in $Ca^{2+}$- and $Mg^{2+}$- free Tyrode for 20 minutes each. The ganglia were mechanically dissociated by trituration through a fire polished Pasteur pipette in minimal essential media (MEM) with 10% heat-inactivated horse serum and the suspension of cells was centrifuged for 5 minutes at 500xg. The cell pellet was resuspended in MEM plus 10% chick embryo extract, plated onto poly-D-lysine coated 35-mm plastic culture dishes and incubated at 37 °C. Cells were used for experiments 1-8 hours after plating.

2. Electrophysiology

Calcium currents were recorded with an Axopatch 200B patch-clamp amplifier in the gigaohm-seal whole-cell configuration (Hamill et al. 1981). To isolate calcium currents the cells were bathed in a solution containing (in mM): 100 NaCl, 50 TEA-Cl, 10 HEPES, 5 Glucose, 5 $CaCl_2$, 2 $MgCl_2$, 5 KCl, 5 3,4-diaminopyridine and 500 nM TTX. Pipettes were pulled on a Flaming/Brown Micropipette Puller (Sutter Instruments Co.; Model P-97), coated with SYLGARD (Dow Corning, Midland, MI) and heat-polished. Electrode resistance ranged from 0.5 to 2 MΩ. Pipettes were filled with an internal solution containing (in mM): 120 CsCl, 10 HEPES, 11 EGTA, 5 TEA-Cl, 1 $CaCl_2$ and 4 $MgCl_2$, with 4 ATP-Mg, 0.3 GTP-Na and 0.1 leupeptin added fresh daily to retard run down of calcium currents. Series resistance was measured by changes in the area and

decay time constant of the capacitive transient associated with a 10 mV hyperpolarizing voltage step and was 3.9 ± 1.0 MΩ (mean ± SD; n = 129). Currents were activated, acquired and leak-subtracted using a standard P/4 protocol by the software package pClamp6 (Axon Instruments, Foster City, CA) running on a Pentium processor-based microcomputer. Currents were filtered using a four-pole Bessel filter at 5 kHz and digitized at 25 kHz. Series resistance was compensated by 80-90% and a 4.3-mV liquid junction potential was corrected before each recording. All cells were studied at room temperature (22-25°C) and, with the exception of the series resistance value reported in this section, all values are expressed as mean ± S.E.M.

3. Action potential waveforms

Two different APs were used to construct the model AP waveforms used here. The fast current-clamp mode of the Axopatch 200A (Magistretti et al. 1998), was used to evoke and record APs at room temperature from small neurons (cap. = 6-7 pF), presumed to be choroid neurons from the ciliary ganglion (see Smith and O'Dowd 1994). The perforated patch technique was used with $K_2SO_4$ and KCl replacing $Cs_2SO_4$ and CsCl in the internal pipette solution and Tyrode saline as the external solution. One of the choroid APs was used as a voltage command to evoke calcium current. The chick DRG neuron AP was adapted from Park and Dunlap (1998).

4. Pharmacological agents

Some experiments using GTPγS were performed in the presence of nifedipine (RBI; Natick, MA), which was solubilized in DMSO at 1 mM and diluted into bath saline at 1 μM (0.1% DMSO final dilution). This effectively isolates N-type calcium current in stage 40 ciliary ganglion neurons (White et al. 1997). There was no significant difference between data obtained in the

presence or absence of nifedipine; thus, the data were pooled. Somatostatin (SOM) and GTPγS were obtained from Sigma (St. Louis, MO). SOM was dissolved in deionized water to make a 10 μM stock solution, stored at -20°C, and diluted into bath saline to a final concentration of 100 nM. GTPγS was diluted into internal pipette saline to make 2 mM stock, stored at -80 °C, and used at a final concentration of 200 μM.

## D. RESULTS

1. GTPγS induced voltage-dependent inhibition of N-type calcium current.

Neurotransmitter-mediated modulation occurs via activation of G proteins following receptor-ligand binding. To activate G proteins directly, 200 μM GTPγS was included in the intracellular pipette solution. Upon obtaining the whole-cell configuration, GTPγS caused a rapid inhibition of calcium current that reached a steady state 5-10 minutes after obtaining whole-cell access. Current was evoked every 10 seconds by a 100 ms voltage step from –80 mV to +10 mV. GTPγS inhibited peak calcium current density by 54 ± 2.2% (Figure 22A) and the inhibition was partially voltage-dependent as measured by a double-pulse protocol (Figure 21A). During the first test pulse, the current exhibited slowed activation kinetics. A conditioning step given 10 ms before the second test pulse increased the current magnitude and restored normal activation kinetics. The effect of the conditioning step on relief of inhibition was expressed as the ratio of the magnitude of current evoked by a step depolarization 10 ms after the conditioning step, to the magnitude of current evoked without a preceding conditioning pulse. This ratio was calculated in all cells at the beginning and end of each experiment (Figure 21 B & C). The

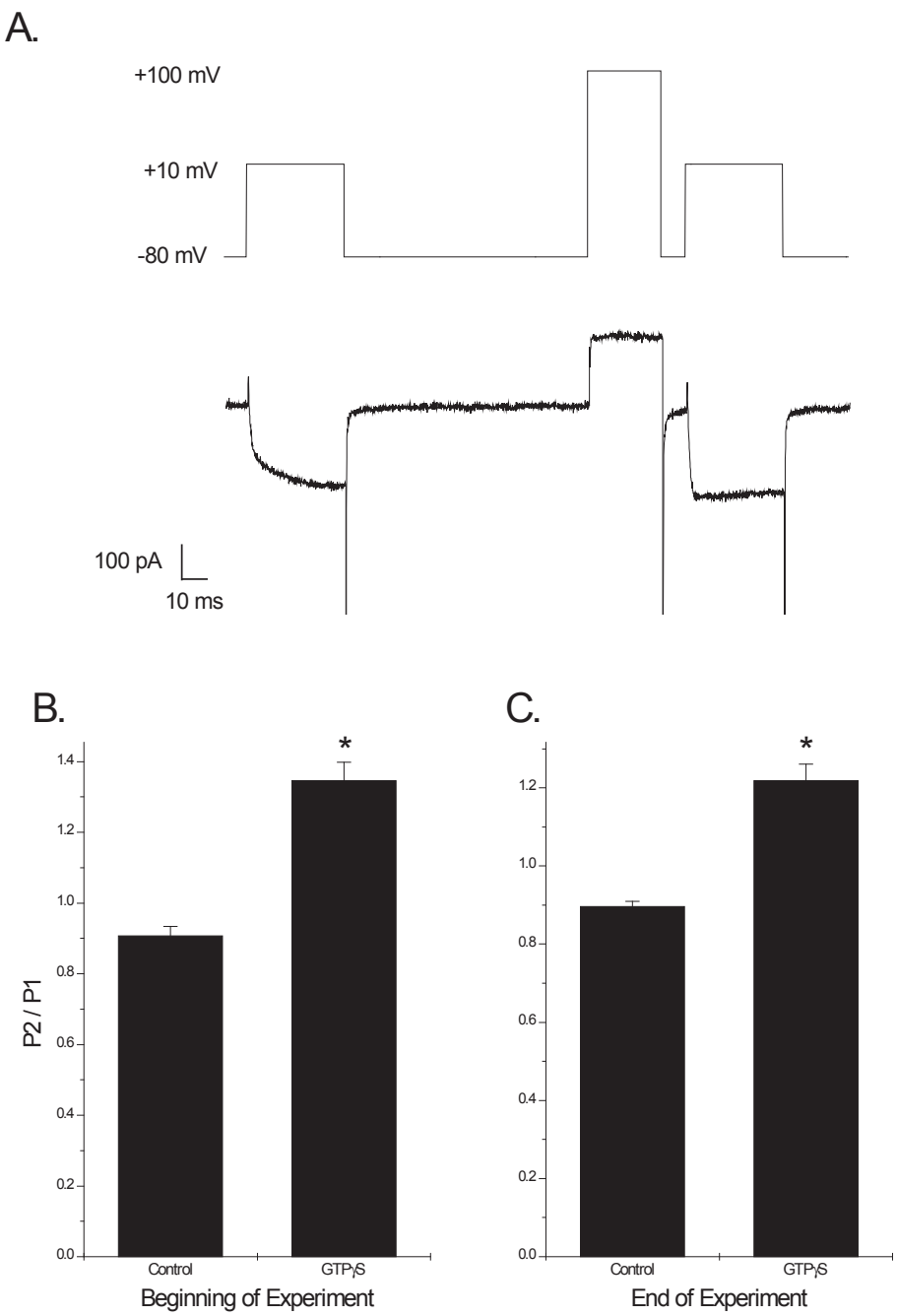


**Figure 21. Prepulse relief of GTPγS-induced calcium current inhibition**. A. The voltage-dependence of inhibition measured using a standard three-pulse protocol. A 30 ms conditioning pulse to +100 mV increased the current magnitude and reversed the slowed activation kinetics caused by GTPγS. B. and C. The degree of voltage-dependent inhibition was expressed as the ratio of current evoked by the second test pulse (after the conditioning pulse to +100 mV) to current evoked by the first test pulse. This was measured at the beginning of each experiment (after maximal GTPγS effect; B), and end of each experiment (after the completion of protocols shown in figures 22-26; C). The facilitation ratio (P2/P1) was significantly greater ($p < 0.001$) in the presence of GTPγS than in control experiments.

voltage-dependent inhibition induced by GTPγS is apparent by the significant increase in facilitation ratio (1.35 ± 0.05 for GTPγS vs. 0.90 ± 0.02 for control, $p < 0.001$). A significant increase in facilitation ratio persisted throughout the length of the recording, and was still present at the end of the experiment (1.22 ± 0.04 for GTPγS vs. 0.91 ± 0.03 for control, $p < 0.01$). Figure 22A shows the current-voltage relationship for calcium currents both in the control condition and with GTPγS included in the patch pipette. To facilitate comparisons among cells of varying size, current is expressed as current density (pA/pF). There was a robust inhibition of calcium current at potentials at or near the potential that evokes peak calcium current (0 mV), but little to no inhibition at more depolarized potentials (Figure 22B). GTPγS did not affect the potential that evoked peak calcium current or the measured calcium reversal potential. The voltage dependence of calcium channel activation was determined by stepping to various potentials for 5 ms. The tail currents that resulted from the repolarization to –80 mV were measured and normalized to the maximum tail current from each cell (Figure 22C). Each plot was fit by a Boltzmann function. As expected for voltage-dependent inhibition, GTPγS caused a shift in the voltage dependence of the channels to more depolarized potentials and a decrease in the steepness of the curve.

2. Time course of calcium channel activation during a step depolarization.

To investigate the effects of calcium channel inhibition on the time course of channel activation, I used square pulses to +30 mV of varying duration (0.5 - 30 ms) to evoke calcium current. Steps to + 30 mV were used here to facilitate comparison of these data with those obtained by AP waveforms that peaked at +30 mV. Tail currents resulting from the repolarization to –80 mV were measured and used as an estimate of the relative number of calcium channels open after that duration of depolarization. This protocol revealed a difference in calcium channel activation

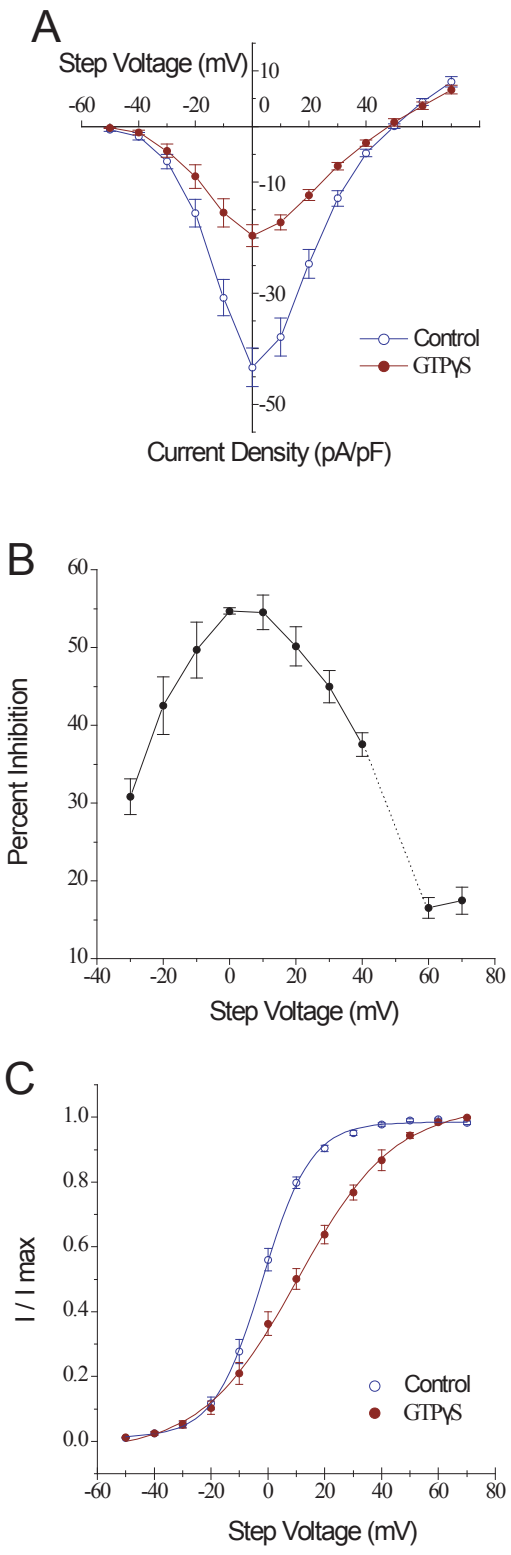


**Figure 22. Voltage-dependence of GTP-γ-S-induced calcium current inhibition.**

**Figure 22. Voltage-dependence of GTPγS-induced calcium current inhibition**. GTPγS (200 μM) was included in the patch pipette to induce voltage-dependent inhibition of calcium current. A. Current-voltage relationship from 26 representative cells. To facilitate pooling of data from cells of different sizes, raw current amplitude values were divided by the capacitance of each cell to obtain a current density (pA/pF). GTPγS produced a voltage-dependent inhibition of calcium current; inhibition was robust at moderate potentials but was minimal at more depolarized potentials. There was no effect on the voltage that evoked peak calcium current or on the apparent reversal potential. B. The voltage-dependence of inhibition is demonstrated by plotting mean percent inhibition of current density as a function of membrane voltage. C. The voltage-dependence of steady-state activation was obtained by plotting normalized tail current amplitude against the step potential used to activate current. Calcium current was evoked by 5 ms steps to –50 through +70 mV in 10 mV increments, and the tail current resulting from repolarization to –80 mV was measured and normalized to the maximum current evoked from that cell. Solid lines represent Boltzmann fits to the data. GTPγS shifted the activation curve to more depolarized potentials ($V_{½}$ = -2 mV for controls versus 11 mV for GTPγS) and decreased the steepness (dx = 8.6 for controls versus 17.1 for GTPγS) of the activation curve.

between control and GTPγS modulated conditions (Figure 23). Control calcium current activated rapidly with depolarization and reached peak activation after approximately 2-3 ms of depolarization. In contrast, modulated current activated more slowly, requiring at least 15 ms of depolarization to be activated maximally. The percent activation of calcium current differed between control and GTPγS conditions at each time point tested from 1 to 10 ms of depolarization (Figure 23). Thus, GTPγS slowed significantly the time course of calcium channel activation.

3. Time course of calcium channel activation during an action potential.

To investigate calcium channel activation during an AP, I used a series of modified AP waveforms as voltage commands to evoke calcium current (see Pattillo et al. 1999). The AP was recorded from a chick ciliary ganglion neuron (see methods), scaled to a peak of +30 mV and a resting potential of -60 mV, and was 2.2 ms in duration at ½ amplitude. The rising phase was modeled by a series of ramps that faithfully followed the actual AP waveform, and was followed by a brief plateau phase. The AP falling phase was simplified to a single ramp to allow for consistent alterations. The AP waveform was repolarized abruptly to –80 mV at various time points during the repolarizing phase (Figure 24A). I measured the tail current resulting from this step to –80 mV and used this measurement as an estimate of the relative number of calcium channels open at any given point of the AP (Figure 24). To correct for rundown during the course of the experiment, each tail current evoked by a modified AP was compared to a control recording taken within 30 seconds of the experimental recording. Additionally, the order in which the modified AP waveforms were applied was varied among experiments. These voltage commands were given either under normal whole-cell conditions, or with GTPγS included in the patch pipette to induce voltage-dependent inhibition of calcium current. Figure 25A shows the

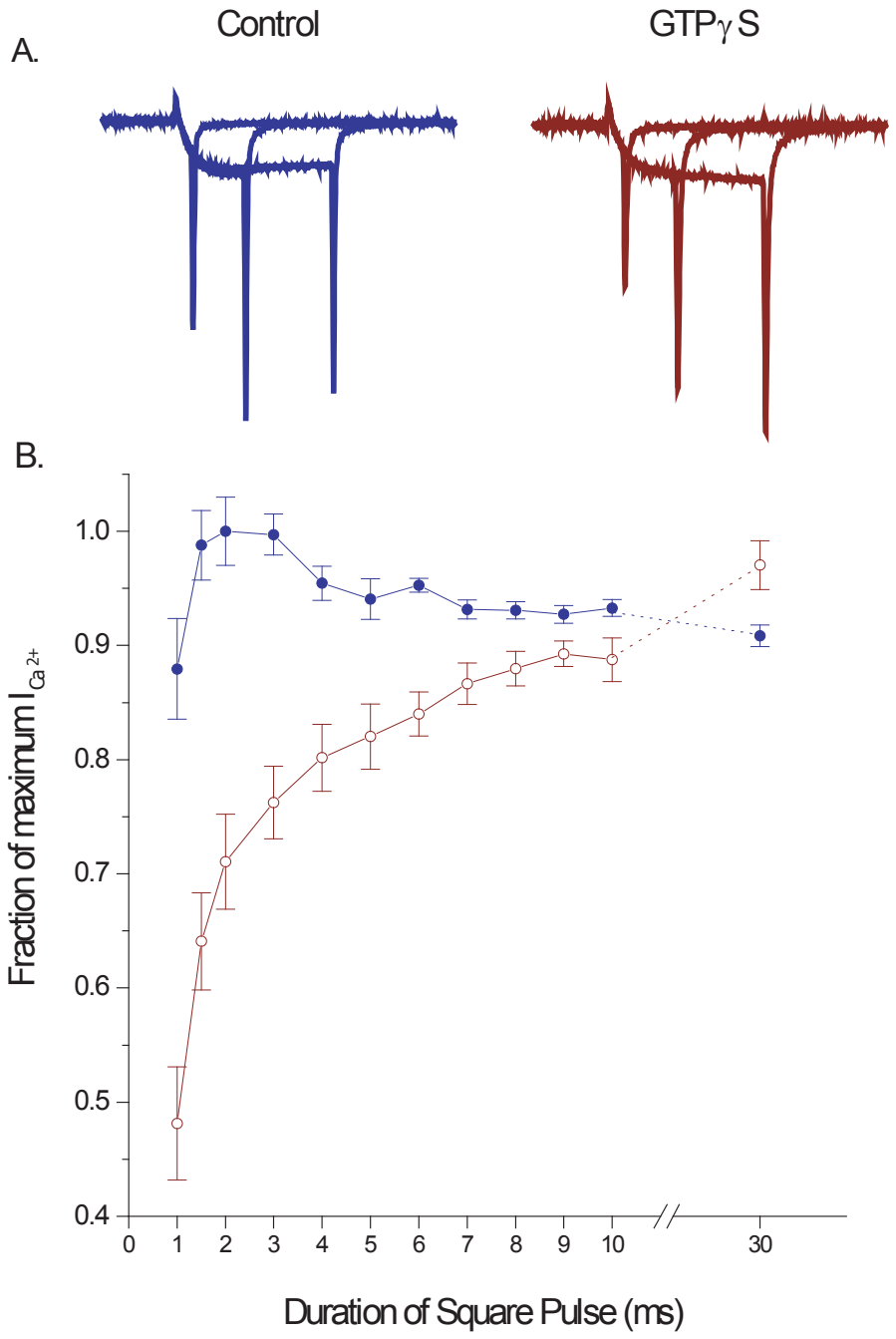


**Figure 23. GTPγS prolongs the time course of calcium channel activation**. To estimate the time course of calcium channel activation, calcium currents were evoked by step depolarizations to +30 mV. The tail current resulting from an abrupt repolarization to -80 mV was measured and normalized to the maximum tail current evoked in each cell. A. Representative traces from a control cell and a cell dialyzed with GTPγS, evoked by steps of 1, 4, and 9 ms duration. B. Plot of normalized tail current amplitude as a function of the duration of the step depolarization used to evoke current. Maximum channel activation occurred with ~2 ms of depolarization in control cells (filled circles) but required approximately 30 ms of depolarization in the presence of GTPγS (open circles).

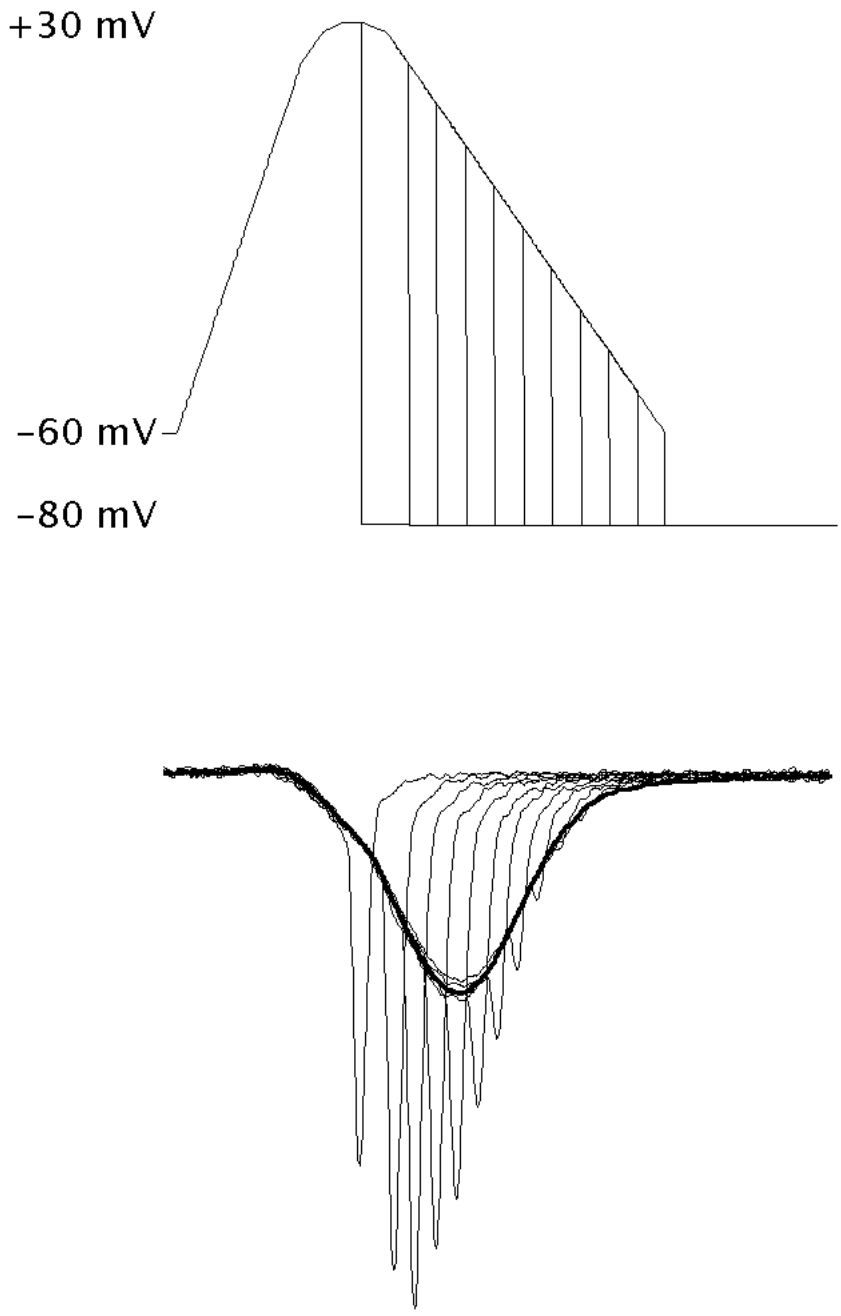


**Figure 24. Waveforms used to measure calcium channel activation during an action potential.** Modified AP waveforms (top) and a family of tail currents (bottom; thin lines) evoked by abrupt repolarization of the AP waveform at various times during the AP repolarization. The amplitude of the tail currents (normalized to the amplitude of an AP-evoked control calcium current; thick line) were used to estimate the relative number of calcium channels that were open at various points in the repolarization.

summary data obtained using this protocol. Each tail current ratio (experimental tail current / control current) was normalized to the maximum ratio recorded from each cell (defined as 100%). The ratios were then plotted as a function of percent AP repolarization. Plotting the data in this manner allows one to determine the time course of calcium current activation during the falling phase of an AP. GTPγS did not alter the time course of channel activation during the AP, as peak activation occurred at the same point in the AP in both control and inhibited conditions. Furthermore, there were no significant differences between control and GTPγS in the percent activation at any time during the repolarization. Since the proportion of current that displays kinetic slowing has been shown dependent on the amplitude of the test potential (Golard and Siegelbaum 1993), calcium current activation was also studied during AP waveforms that peaked at +10 mV. As with the AP waveforms to +30 mV, there were no significant differences in percent activation between control and GTPγS-modulated currents (Figure 25B).

4. Increasing action potential duration fails to reveal a change in activation time course.

To test the hypothesis that the choroid AP used above did not provide sufficient stimulus to convert reluctant channels to a willing gating mode, I tested the effects of an AP of longer duration. A chick DRG AP adapted from Park and Dunlap (1998; –80 mV resting potential, +24 mV peak amplitude and 6 ms duration at ½ amplitude) was used to create a series of modified AP waveforms as described above (Figure 26A). Again, I measured the tail currents that resulted from abrupt repolarization to –80 mV at various times during the repolarizing phase of the AP, and used this measure to estimate the proportion of calcium channels open at that time. I hypothesized that this AP waveform would provide sufficient depolarization to recruit kinetically slowed channels, and thus slow the time course of whole-cell current activation when channels

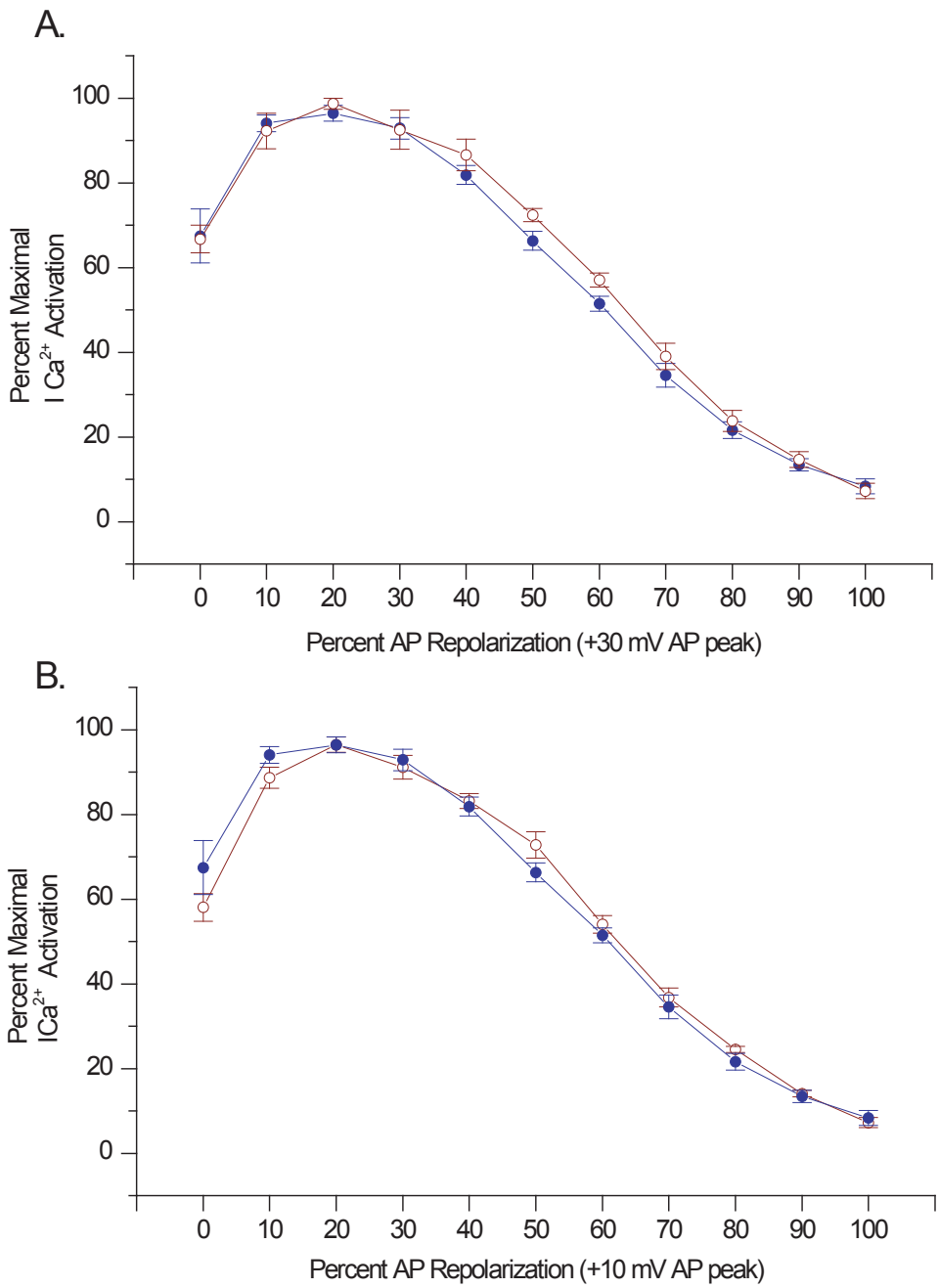


**Figure 25. Effect of GTPγS on calcium channel activation during a choroid action potential.** Summary of data obtained with the modified AP waveforms under control conditions and with GTPγS included in the patch pipette. Each tail current was expressed as a ratio to current evoked by the unmodified AP waveform and normalized to the maximum ratio recorded in each cell. A. Data obtained from the action potential waveforms that peak at +30 mV. B. Data obtained from AP waveforms scaled to peak at +10 mV. With both sets of waveforms, maximal calcium channel activation occurred at the same point of the AP repolarization in both control (filled circles) and GTPγS (open circles) conditions. Furthermore, there were no significant differences in the percent channel activation between control and GTPγS at any point during the repolarization phase.

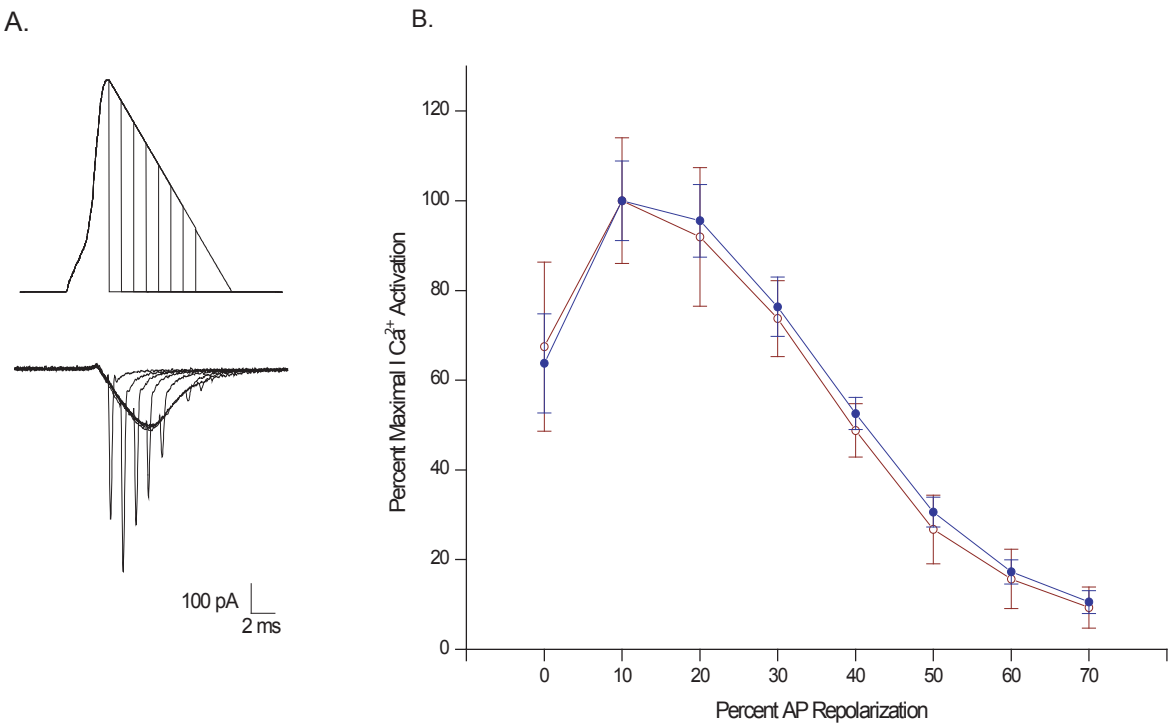


**Figure 26. Increasing action potential duration fails to reveal the recruitment of slowed channels.** To test the hypothesis that an action potential of longer duration might provide sufficient depolarization to recruit kinetically slowed calcium channels, a chick DRG action potential from Park and Dunlap (1998) adapted and used as a voltage-clamp command to evoke calcium current. This action potential was 6 ms in duration at half-amplitude with a –80 mV resting potential and +24 mV peak amplitude. A. The modified action potential waveforms, and a representative family of currents. The tail currents evoked by abrupt repolarization to –80 mV were measured, each current expressed as a ratio to current evoked by an unmodified AP waveform and normalized to the maximum ratio obtained from each cell. B. The graph plots the normalized ratios as a function of the percent of AP repolarization that had occurred before the step to –80 mV. In control (filled circles) and GTP$\gamma$S (open circles) recordings, maximum channel activation occurred at the same point in the repolarization phase in both recording conditions, and there were no significant differences between the percent channel activation at any point during the repolarization phase.

were modulated. However, as with the choroid AP waveforms, GTPγS did not change the time course of channel activation during this long AP (Figure 26B).

5. Relationship between kinetic slowing and voltage-dependence of inhibition.

Many groups have reported the presence of slowed activation kinetics together with voltage-dependent calcium channel inhibition (reviewed by Jones and Elmslie 1997). Although it is not necessarily apparent in all cases of voltage dependent inhibition, kinetic slowing is thought to be a marker of voltage dependent inhibition. Thus, I sought to look for a correlation between kinetic slowing and the degree of voltage dependent inhibition to determine the extent to which these two phenomena are coincident. Data for this experiment were obtained from 47 cells to which 100 nM somatostatin (SOM) was applied to produce voltage dependent inhibition. In these cells, SOM has been shown to inhibit preferentially N-type calcium channels in a voltage dependent manner, exhibiting kinetic slowing when measured using traditional whole-cell recording techniques (Meriney et al. 1994; White et al. 1997). Measures of kinetic slowing and of prepulse relief of inhibition were taken from each cell. Kinetic slowing was quantified as follows. Calcium current was evoked by a 100 ms step depolarization to +10 mV in the presence and absence of 100 nM somatostatin. The percent SOM-induced inhibition was measured at the time of peak control calcium current and after 95 ms of depolarization. Kinetic slowing was defined as the ratio of percent inhibition at the peak to the percent inhibition late in the depolarization (Figure 27A). The voltage dependence of inhibition was determined with a standard double-pulse protocol (see Figure 21A). A 30 ms depolarizing prepulse to +100 mV was applied 10 ms before a second test pulse. This prepulse relieved partially the SOM-induced inhibition, providing a robust measure of the voltage-dependence of inhibition. There was a significant correlation ($R = 0.51$, $p < 0.0003$) between kinetic slowing and the voltage dependence of inhibition (Figure 27C).

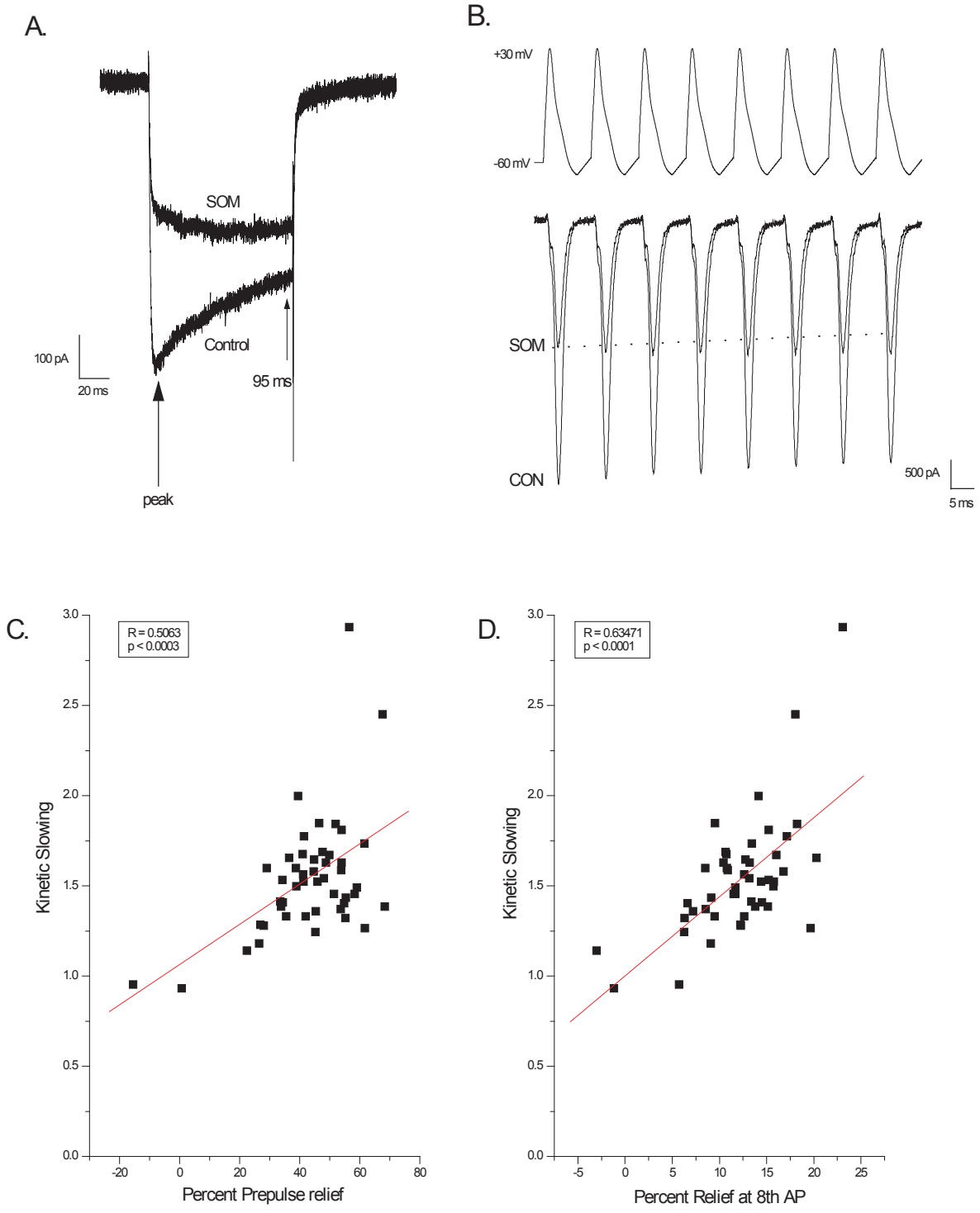


**Figure 27. Relationship between kinetic slowing and the votlage dependence of inhibition.**

**Figure 27. Relationship between kinetic slowing and the voltage dependence of inhibition.** 100 nM somatostatin was applied to produce voltage-dependent inhibition of calcium current. A. Kinetic slowing was defined as the ratio of inhibition of peak calcium current to percent inhibition of current after 95 ms of depolarization. In this example: peak inhibition = 53.9%; inhibition at 95 ms = 18.6%; kinetic slowing ratio = 2.8. Data from Figure 4A. B. To measure relief of inhibition by a burst of APs, calcium current was evoked by a train of 8 choroid AP waveforms delivered at 100 Hz. Application of 100 nM somatostatin inhibited calcium current and relief of inhibition was measured at the $8^{th}$ AP in the train. The dotted line represents the iso-inhibition level and is sloped to reflect the degree of inactivation recorded in the control currents. C. Correlation of kinetic slowing of the modulated current with the voltage-dependence of inhibition as measured by a standard conditioning pulse (30 ms to +100 mV). D. Kinetic slowing also correlated with the activity-dependent relief of inhibition produced by the choroid AP train.

I also investigated the relationship between kinetic slowing and relief of inhibition produced by an AP train. Kinetic slowing was quantified as described above, but now voltage dependence was defined as the inhibition relieved by a train of 8 choroid AP waveforms delivered at 100 Hz (Figure 27B). The percent inhibition of current evoked by the $8^{th}$ AP in the train was compared to the percent inhibition of current evoked by the $1^{st}$ AP in the train to produce a measure of inhibition relief, thus an indication of the voltage dependence of inhibition. Again, there was a significant correlation ($R = 0.63$, $p<0.0001$) between kinetic slowing and the degree of inhibition relief produced by the AP train (Figure 27D). Thus, kinetic slowing reliably predicted the extent of activity-dependent relief induced by this AP train.

6. Time course of action potential-evoked calcium current

To examine the effect of SOM-induced inhibition on the activation time course of AP-evoked currents, I compared control and SOM-modulated calcium currents evoked by the first and the eighth APs in a 100 Hz train. To facilitate a comparison of the activation of these currents, all traces were normalized to the maximum current evoked from each cell. Thus, control and modulated currents were scaled to be of identical amplitude. Data were pooled from 18 cells in which the kinetic slowing ratio was 1.53 or greater and expressed as the mean normalized current ± S.E.M. (Figure 28A). There was no difference in the time course of current activation between control and modulated currents. Examination of the calcium current evoked from the cell displaying the greatest degree of kinetic slowing (kinetic slowing ratio = 2.93; Figure 28B), also showed that the time course of current activation was identical for control and modulated currents when the currents were normalized to the same peak amplitude (Figure 28C).

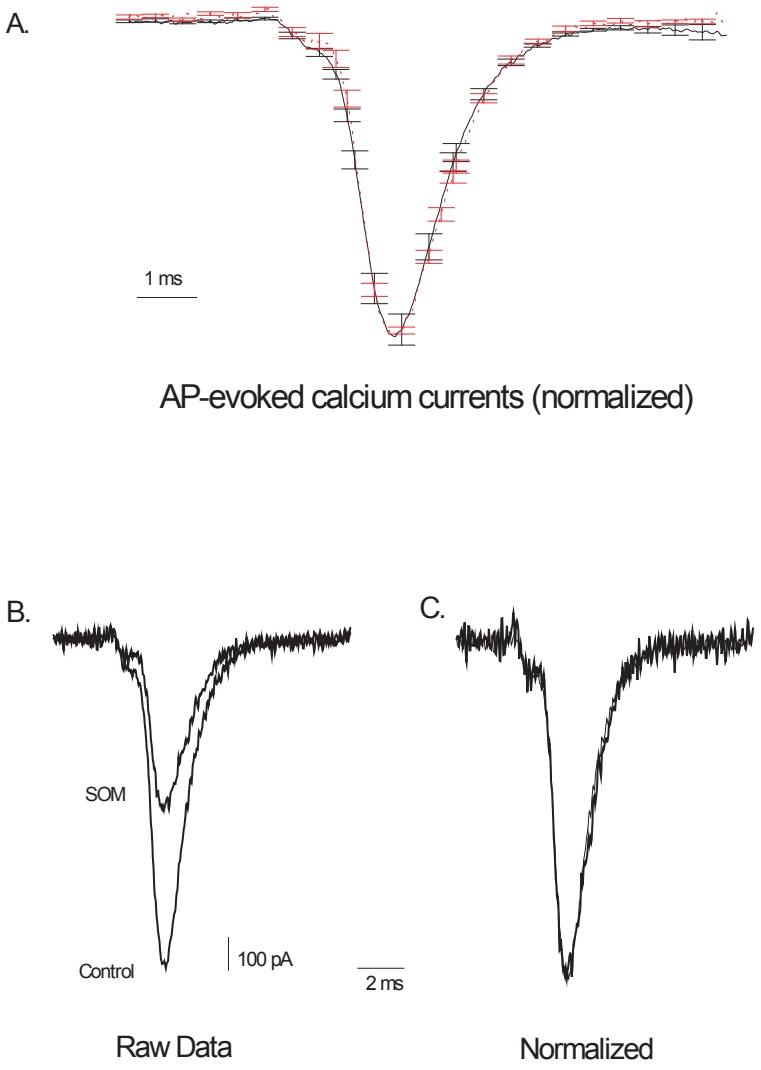


**Figure 28. Time course of action potential-evoked calcium currents.** Calcium current was evoked by choroid action potential waveforms in the absence and presence of somatostatin. To compare the time course of current activation, somatostatin-modulated current was scaled to the same amplitude as control current. A. Pooled data from 16 cells that exhibited significantly slowed activation kinetics in the presence of somatostatin (kinetic slowing ratio $\geq$ 1.53). Each current trace was normalized to the maximum current evoked from the cell. The normalized values were averaged and displayed (mean $\pm$ S.E.M.) for control (solid trace) and somatostatin modulated (dashed trace) currents. B. Raw current traces from the cell with the greatest degree of slowed activation kinetics (kinetic slowing ratio = 2.93). C. Data from B with the somatostatin-modulated current trace scaled to the same amplitude as the control current trace. There was no apparent difference in the time course of AP-evoked calcium current in the presence or absence of somatostatin.

## E. DISCUSSION

Calcium current inhibition is a well-documented mechanism underlying presynaptic inhibition (Wu and Saggau 1997). Thus, the voltage dependence of inhibition may provide a mechanism whereby transmitter release is modulated based on the level of neuronal activity. Our investigation has focused on the effects of G protein-mediated, voltage-dependent inhibition on AP-evoked calcium current. Using traditional whole-cell voltage clamp methods, I have used GTP$\gamma$S to induce voltage dependent inhibition of N-type calcium current in chick ciliary ganglion neurons. The inhibition displayed the characteristics common to voltage dependent inhibition: altered voltage dependence of steady-state activation, relief by a conditioning prepulse and kinetic slowing.

To address the physiologic relevance of calcium current inhibition, I have looked for functional consequences of G protein-mediated modulation that occur in a voltage-dependent manner in response to single AP stimulation. These are hypothesized to occur if significant numbers of modulated channels either open directly from the reluctant state (very brief opening), or convert to the willing gating mode (delayed opening) during a single AP depolarization. A significant current contribution from either of these types of openings would be expected to alter the time course of calcium entry during an AP and thus affect synaptic delay and/or the magnitude of transmitter released (Sabatini and Regehr 1996). The absence of a significant contribution argues that voltage-dependent modulation is relevant only during trains of APs, as has been suggested previously (Brody et al. 1997; Park and Dunlap 1998; Brody and Yue 2000).

Using APs of different duration and amplitudes, I did not find any evidence that voltage-dependent modulation altered the time course for calcium current entry during a single AP. Taken together, these results suggest that modulated N-type channels do not contribute significantly to calcium current evoked by a single AP in chick ciliary ganglion neurons. The

absence of measurable effects could be due to several issues. Studies that have focused on kinetic slowing, both at the whole-cell and single channel levels, have evoked calcium current using sustained step depolarizations (Bean 1989; Grassi and Lux 1989; Patil et al. 1996). This protocol reveals a robust change in activation kinetics associated with voltage-dependent inhibition of calcium current. However, comparably less is known about how channels behave when stimulated with APs. A ramp depolarization (as occurs during the AP rising phase) and a step depolarization may produce a different time course of channel activation. Additionally, an AP may not depolarize a cell to the same degree as a step depolarization of the same duration. In our recordings of modulated calcium current, there is evidence that modulated channels are partially recruited with a 2 or 6 ms step depolarization, but not with an AP of 2 or 6 ms duration at half-amplitude. However, the AP is not an equivalent stimulus, producing much less depolarization than a step pulse of similar duration. Given the dramatic increase in latency to first channel opening (10-20 fold) reported for modulated N-type calcium channels (Carabelli et al. 1996), modulated channels may not be recruited to convert to a willing gating mode by a single AP, even of relatively long duration.

Another issue to consider when interpreting these data is that if direct channel openings from the reluctant mode occur, they may in fact contribute a very small percentage of the total AP-evoked current. Single channel evidence of reluctant channel openings has shown them to be infrequent and to have a very brief mean open time (Colecraft et al. 2000). Thus, direct reluctant openings, if they occur, may contribute a very small amount of current that is not detectable in our whole-cell recordings.

Our results suggest that activated G proteins may result in the modulation of a proportion of the calcium channels such that during an AP, they are effectively closed and prevented from contributing to calcium influx. This could have both temporal and spatial consequences on transmitter release. Given the non-linear relationship between calcium influx and transmitter release (see Augustine and Charlton 1986), even small increases in calcium

influx caused by AP train-induced relief of inhibition could produce significant increases in the magnitude of transmitter released. In a temporal sense, relief of voltage-dependent inhibition has been shown to occur with a train or burst of APs (Brody et al. 1997; Park and Dunlap 1998), and this may contribute to short-term synaptic facilitation (Brody and Yue 2000). Activation of G proteins has also been shown to enhance paired-pulse synaptic facilitation (Dunwiddie and Hass 1985; Dittman and Regehr 1997). If modulated calcium channels are effectively not contributing to single AP-evoked release, but can be recruited with bursts of APs, this suggests that release sites positioned near modulated channels may become functional only near the end of a burst of APs. Thus, G protein modulation of calcium current could influence not only the degree of transmitter released with a single AP, but may also influence when transmitter release is facilitated during a burst of activity.

The spatial arrangement of modulated channels could also have profound effects on transmitter release. Models of calcium current inhibition and transmitter release have suggested that both the degree of inhibition and facilitation of release are greatly influenced by the location and distribution of modulated and unmodulated channels (Bertram and Behan 1999). For example, if modulated channels reside near release sites there would be increased facilitation of release during bursts of APs. This could be particularly relevant in nerve terminals if the calcium channels coupled to release sites (through interaction with syntaxin) are most susceptible to regulation by G proteins (see Stanley and Mirotznik 1997).

# V. SUMMARY AND GENERAL CONCLUSIONS

## A. CYTOPLASMIC DIALYSIS ALTERS THE VOLTAGE DEPENDENCE OF SOMATOSTATIN-MEDIATED CALCIUM CURRENT INHIBITION

### 1. Summary

Voltage-dependent inhibition of calcium current is an important mechanism underlying regulation of neuronal activity. In ciliary ganglion neurons, somatostatin causes G protein-mediated, voltage-dependent inhibition of N-type calcium current. The characteristics of inhibition are altered by cytoplasmic dialysis that occurs during whole-cell patch clamp recordings (Meriney et al. 1994), suggesting that the voltage dependence of somatostatin-mediated inhibition might be influenced by an intracellular component. I studied somatostatin-mediated inhibition using whole-cell and perforated patch recording techniques and found that the loss of cytoplasmic components during whole-cell recordings increased the sensitivity of calcium current inhibition to relief by action potential trains. Since the voltage dependence of calcium current inhibition increases with increasing concentration of activated G proteins, the increased action potential train-induced relief of inhibition seen in whole-cell recordings led me to hypothesize that there was a greater effective concentration of activated G proteins in whole-cell as compared to perforated patch recordings. The results of the experiments presented in Chapter 2 are consistent with this hypothesis. The concentration – response relationship for

somatostatin-mediated inhibition of calcium current showed a trend for lower $IC_{50}$ in whole-cell than in perforated patch recordings. The time course of reinhibition of calcium current following a prepulse (which is also concentration dependent) was faster in whole-cell than in perforated patch recordings. Additionally, whole-cell recordings had increased voltage-dependence of inhibition relief. Taken together, these results support the hypothesis that there is a greater effective concentration of G protein in whole-cell recordings of somatostatin-mediated calcium current inhibition.

The measurement of the voltage dependence of calcium current inhibition depended on the stimulus used to evoke calcium current and the stimulus used to produce inhibition relief. This is clearly illustrated in comparisons of the measurement of inhibition relief using the standard prepulse protocol versus the choroid action potential train protocol. Using the choroid action potential train as a voltage-clamp command, relief of inhibition was produced by an 8 action potential train followed by a prepulse to +70 mV, and percent relief of inhibition was measured during an action potential which followed the prepulse. Data collected with this protocol show that the total voltage dependence of somatostatin-mediated inhibition was similar for whole-cell and for perforated patch recordings. With the standard prepulse protocol, relief of inhibition was produced by a step to +100 mV and currents were evoked by step depolarization to +10 mV. Using this protocol, prepulse-induced relief of inhibition was significantly lower in whole-cell recordings than in perforated patch recordings, suggesting that the total voltage dependence of somatostatin-mediated inhibition was reduced by cytoplasmic dialysis. This difference may be explained by the time course of G-βγ rebinding following the prepulse. The prepulse is followed by a 5 ms interval at -80 mV, during which rebinding of G proteins would be favored. Since the time course of G protein rebinding following a prepulse is faster in whole-cell than in perforated patch recordings, there may be greater reinhibition during this 5 ms interval in whole-cell recordings, leading to an artificially low measurement of prepulse-induced relief of inhibition. In contrast, the prepulse following the choroid action potential train is followed by an

interval at -60 mV. This 20 mV difference in holding potential may alter appreciably the extent of G protein rebinding. If there is no difference in the rate of G protein rebinding at -60 mV between whole-cell and perforated patch recordings, this could explain why measures of total voltage dependence show no difference between whole-cell and perforated patch recordings using the choroid AP train, but greater voltage dependence in perforated patch than in whole-cell recordings using the standard prepulse protocol.

In considering relief of inhibition during the choroid action potential train, there are three parameters of G protein / calcium channel interactions relevant to relief of inhibition. First, the voltage dependence of inhibition relief is greater in whole-cell than in perforated patch recordings, favoring increased relief of inhibition during the train. The second parameter is the time course of G protein rebinding following relief of inhibition. Data from prepulse experiments in which the interval was varied show that the rate of reinhibition following a prepulse is faster in whole-cell than in perforated patch recordings, however, the holding potential was -80 mV in these experiments. As discussed above, a holding potential of -60 mV appears to reduce the difference in the time course of rebinding between whole-cell and perforated patch. This could be tested by altering the action potential train to have a holding potential of -80 mV and measuring relief of inhibition. In this case, the faster rebinding rate at -80 mV measured in whole-cell recordings may act to decrease the accumulation of inhibition relief during the train and minimize the difference in inhibition relief between whole-cell and perforated patch recordings. Nonetheless, regardless of the specific time constant governing G protein rebinding to the calcium channel, action potential train-induced relief of inhibition is greater in whole-cell recordings using the voltage protocol presented here. Thus, the voltage-dependence of inhibition relief must outweigh G protein rebinding during the train. There is a third factor to consider, the facilitation rate, which is the time course of G protein unbinding from the calcium channel during depolarization. This could be measured by using a 3-step prepulse protocol in which the duration of the prepulse was varied among experiments. Because of the increased

relief of inhibition produced by action potential trains in whole-cell recordings, and the data suggesting an increased effectiveness of G proteins in whole-cell recordings, one would expect the facilitation rate of G protein unbinding to be faster in whole-cell than in perforated patch recordings. Unfortunately, this experiment could not be preformed due to an unexpected complication in data collection (see Appendix 1). It should be a priority once this problem is overcome since it can aid significantly in the interpretation of the data.

There are a number of proteins and second messenger signaling cascades that can potentially modify the voltage dependence of G protein-mediated calcium current inhibition. It is not clear which mechanism(s) are responsible for the differences in the voltage dependence of calcium current inhibition between whole-cell and perforated patch recordings that are presented here. A particularly intriguing candidate is $PIP_{2,}$ which has been shown to stabilize interactions between the calcium channel and G-βγ. The molecular mechanism by which this occurs has not yet been determined, but it is hypothesized that $PIP_2$ stabilizes a binding site for G-βγ by direct binding to the C-terminus of the calcium channel $\alpha_1$ subunit (Rousset et al. 2004). Cytosolic calcium is an important co-factor in the actions of $PIP_2$ and the ability of $PIP_2$ to enhance voltage-dependent modulation is sensitive to calcium buffering. Because calcium buffering differs between whole-cell and perforated patch recording conditions, resting calcium concentration is expected to differ as well. Thus, $PIP_2$ is a viable candidate for influencing voltage-dependent inhibition in the ciliary ganglion preparation. The role of $PIP_2$ in voltage-dependent inhibition can be tested by injection of anti-$PIP_2$ antibodies into the neuron under study, or by treating the neurons with a phophoinositide-kinase inhibitor. It would be interesting to use these manipulations in ciliary ganglion neurons, and measure the ability of changes in calcium buffering or anti-$PIP_2$ treatments to inhibit voltage-dependent inhibition in whole-cell recordings of calcium current.

2. Implications of voltage dependent inhibition for ciliary ganglion function.

It has been shown previously that somatostatin inhibits release of ACh from choroid nerve terminals and from ciliary ganglion neuronal somata. Somatostatin is released preferentially during periods of high neuronal activity, and is hypothesized to provide negative feedback on subsequent ACh release (Gray et al. 1989, 1990). Choroid neurons fire action potentials of relatively long duration (2.2 ms at half-amplitude), thus they can relieve a significant portion of somatostatin-mediated calcium current inhibition. This may ensure reliable transmission of ciliary ganglion signaling during high frequency activity. Choroid neurons innervate smooth muscle and tend to fire action potentials in high frequency bursts to mediate contraction of the choroid tissue and the dilation of blood vessels (Fitzgerald et al., 1990). Thus, it is likely that somatostatin-mediated inhibition of ACh release is suppressed during action potential trains. Additionally, the degree of voltage dependence of somatostatin-mediated inhibition may be regulated. This may occur acutely, as seen with whole-cell recordings, by modulation of an intracellular signaling pathway. Alternatively, it may be regulated by differential expression of the modifying factor in various cellular compartments. For example, if $PIP_2$ interacts with calcium channels in ciliary ganglion neurons, it may promote voltage-dependent inhibition upon activation of G protein-coupled receptors. $PIP_2$ is enriched in specific membrane compartments (termed lipid rafts). Thus calcium channels that are colocalized with $PIP_2$ in these areas of the membrane would be more susceptible to G protein-mediated inhibition.

## B. THE SHAPE OF ACTION POTENTIAL WAVEFORMS AFFECT CALCIUM CURRENT

### 1. Action potential broadening

The magnitude and time course of calcium entry are critical determinants of transmitter release. As such, changes in action potential waveform shape will influence transmitter release indirectly through modulation of calcium influx. For example, at hippocampal mossy fiber boutons, a brief calcium transient triggers fast, synchronized glutamate release (Jonas et al. 1993). However, a longer duration calcium increase is necessary for peptide release (Williams & Johnston, 1996). The data presented in Chapter 3 demonstrate that the specific effects of action potential broadening on the magnitude of calcium influx depend both on the duration of the action potential and the time during action potential repolarization that broadening begins. Thus, the effect of inactivation or blockade of potassium channels may vary depending on the shape of the action potential for the cell under study, as well as the portion of the repolarization phase during which a modulated potassium channel contributes. Blockade of calcium-activated potassium channels, for example, may broaden action potentials late in the repolarization phase. This is likely to have a limited effect on calcium current amplitude relative to blockade of voltage-gated potassium channels, which contribute to action potential repolarization beginning at the peak of the action potential. Action potentials also undergo frequency-dependent broadening. When a neuron fires a train of action potentials, action potential waveforms late in the train tend to be of longer duration than those early in the train, largely due to inactivation of potassium channels. The extent of action potential broadening increases with increasing firing frequency (Park & Dunlap, 1998). Thus, the amplitude of calcium current influx may increase during an action potential train by two mechanisms. Specifically, a longer duration action potential may activate more calcium channels, leading to increased calcium current amplitude,

and will also be more effective at relieving G protein-mediated calcium current inhibition, also resulting in greater calcium influx.

2. G protein effects on action potential waveform.

The shape of action potential waveforms can also be altered by G protein-mediated pathways. The G protein-activated inwardly rectifying potassium channels (GIRKs) are a family of potassium channels activated by G proteins, which act to decrease the excitability of neuronal cells. Upon activation of G protein-coupled receptors, GIRK channels are activated by direct binding of G-βγ to the channel (Schreibmayer et al.1996). Thus, G-βγ can act through two mechanisms, inhibition of calcium current and activation of GIRK channels to inhibit neuronal activity. Other types of potassium channels can also be influenced by G protein activity. Somatostatin activates two types of potassium currents in hippocampal CA1 pyramidal cells by G protein-dependent mechanisms (Schweitzer et al. 1998). M current, a voltage-dependent, non-inactivating potassium current, is activated by somatostatin (Watson & Pittman, 1988) and develops upon neuronal depolarization (Schweitzer et al. 1988). Thus, it contributes to action potential repolarization, decreasing neuronal activity. Additionally, somatostatin activates a voltage-insensitive potassium leak current that is active at rest and results in hyperpolarization of the resting membrane potential, thus decreasing neuronal excitability.

Tetrodotoxin (TTX)-sensitive sodium channels are also targets of modulation by G protein-activated pathways. In rat dorsal root ganglion neurons, TTX-sensitive sodium channels are inhibited by ATP, which leads to a reduction in neuronal excitability (Choi et al. 2003). Also, the TTX-sensitive $Na_V1.7$ channels expressed in *Xenopus* oocytes are inhibited by two pathways, activation of protein kinase A and protein kinase C (Vijayaragavan, 2004). Both pathways converge on sodium channels to inhibit the amplitude of sodium influx, thereby reducing action potential amplitude.

Hence, the inhibitory actions of G proteins are not limited to effects on calcium channels. They may also act on sodium and potassium channels to inhibit transmitter release indirectly via changes in action potential waveform.

## C. G PROTEIN MODULATED CALCIUM CHANNELS DO NOT CONTRIBUTE TO ACTION POTENTIAL-EVOKED CALCIUM CURRENT

Based on the observation that G protein modulated calcium current has slowed activation kinetics, I hypothesized that the kinetics of action potential-evoked calcium current would be similarly altered by G protein activation. On the contrary, investigation of the time course of calcium current activation during an action potential showed no difference between control and G protein-modulated conditions. These rather non-intuitive results suggest that modulated calcium channels do not contribute to calcium current evoked by a single action potential. Thus, G protein-bound calcium channels would be effectively eliminated from contributing to action potential-evoked calcium current. This could have significant implications with regards to transmitter release regulation. The specific effects of G protein modulation on transmitter release will vary depending on the stoichiometry between open calcium channels and the sensor for transmitter release. If the active zone if flooded with calcium after each action potential stimulus and vesicle fusion is triggered by the combined action of calcium from multiple channel openings, G protein modulation would decrease the overall magnitude of the calcium influx. Under these circumstances G protein modulation would be expected to reduce transmitter release according to nonlinear relationship between calcium and transmitter release (Dodge & Rahamimoff, 1967). On the other hand, if transmitter release is triggered by calcium influx through a single calcium channel, G protein modulation may result in the elimination of

entire release sites and a linear reduction in transmitter release. This is potentially a powerful regulatory mechanism that could significantly vary the number of active transmitter release sites.

## D. STIMULUS EFFECTS ON CALCIUM CURRENT

The experiments investigating voltage-dependent calcium current inhibition demonstrate that the type of stimulus used to evoke calcium current can influence the data, and must be taken into consideration when interpreting the results of an experiment. For example, GTP-γ-S modulation of calcium current significantly slows the time course of current activation by a step depolarization to +10 mV, but not the time course of current activation by an action potential. Although these two results seem to be contradictory, they raise an interesting question of whether the rising phase of the action potential or the prolonged plateau of the step depolarization causes the difference in activation. One method to address this question is to use a protocol developed by Borst & Sackmann (1998) to investigate the proportion of calcium channels activated by an action potential. They used an action potential waveform with an extended plateau phase to evoked calcium current and compared this current to that evoked by a control action potential waveform. This allowed them to determine what proportion of channels that could be activated with a sustained stimulus was activated by the naturally occurring action potential. An adaptation of this protocol would be useful in determine the effect of G protein modulation on calcium current activation. This protocol would be applied to a choroid action potential waveform which would then be used to evoke calcium current in the presence and absence of GTP-γ-S to activate G proteins. If the time course of calcium current activation showed G protein-induced slowing, as with the step depolarization-evoked calcium

current, it would suggest that the plateau phase is critical in determining calcium channel activation. However, if the time course of calcium current activation showed no difference in the presence and absence of activated G proteins, it would suggest that the rising phase of the action potential is the critical determinant in calcium channel activation. The idea that the rising phase of an action potential waveform activates calcium channels differently than a step depolarization is suggested by data collected with the chick DRG action potential waveform. This action potential had 6 ms duration at half-amplitude, thus providing significant depolarization to the cell. Still, modulated calcium current evoked by this action potential waveform showed no difference in the time course of activation compared to control calcium current.

## E. COMPLEXITY OF CALCIUM CHANNEL REGULATION

The regulation of voltage-gated calcium channels is an amazingly complex process. The precise control of calcium influx into neurons is vital to proper functioning of the synapse and of the nervous system as a whole. Inhibition of calcium current by G proteins is an important mechanism by which calcium channels are modulated, and even this one pathway has tremendous complexity. Calcium current inhibition can be voltage-dependent, thus susceptible to relief by neuronal activity. The degree of inhibition relief depends on large number of protein / protein interactions and second messenger signaling cascades. Furthermore, the amount of inhibition that is relieved will depend on the duration of the action potential waveform stimulating the cell, and the frequency of action potential stimulation. The degree of voltage-dependent inhibition in a cell will also depend on which calcium channel subtypes are expressed, as various calcium channels are differentially susceptible to G protein-mediated modulation, and to

activity-dependent relief of inhibition. Also, the degree of kinetic slowing and prepulse-induced relief of inhibition depend on which specific G protein β and γ subunits are expressed. Additionally, the shape, duration, and repolarization of the action potential waveform that evokes calcium current in a cell will contribute to determining the time course and amplitude of calcium influx. The waveform in turn will be shaped by the presence and G protein modulation of a number of ion channels.

While we can continue to discover the general principles of calcium channel function and regulation, the precise control of calcium influx through any single channel will be tightly regulated by the immediate environment. Overall, calcium channels are regulated spatially by the specific proteins expressed and interactions among the channel and various proteins, and regulated temporally by the specific signaling pathways that are active at the time of channel activation.

# APPENDIX

## EFFECT OF PRESSURE ON CALCIUM CURRENTS RECORDED IN CHICK CILIARY GANGLION NEURONS

### A. INTRODUCTION

The purpose of this appendix is to report an unusual occurrence during data collection and the steps taken to prevent / solve this problem. Many experiments required extracellular application of drugs, which were applied locally to individual ciliary ganglion neurons in culture via glass pipette ~30-40 uM in diameter attached to a force transducer. Approximately 1 – 2 mmHg pressure was applied to the pipette to induce solution flow from the pipette into the bath solution. In some recordings, application of solution to the cell, even in the absence of a pharmacological agent (i.e. control calcium bath solution), caused a reduction in calcium current amplitude and altered the kinetics of the current such that it appeared to have enhanced inactivation during a voltage step to +10 mV (see Figure 29A). This effect was observed in both whole-cell and perforated patch recordings. There was significant cell-to-cell variability in the magnitude of the effect. In only about 10-15% of cells was the effect reversible upon removal of the drug pipette, and in these cells a second application of solution caused the same effect on

current amplitude and kinetics as the first application. In cells where a small effect was seen initially, increasing the applied pressure would result in a more robust effect, therefore it was impossible to separate the effect of pressure and of a pharmacological agent included in the drug pipette.

## B. CHARACTERISTICS

The effect of pressure on calcium current was clearly in contrast to the effects of somatostatin. In addition to the different effect on calcium current kinetics cause by pressure in comparison to somatostatin, the pressure effect had no discernable voltage dependence (Figure 29B). As opposed to the effects of somatostatin, a prepulse to +100 mV had no effect on the magnitude or kinetics of calcium current. To rule out the presence of a pressure-activated chloride conductance, the experiment was carried out with 1 mM SITS or 1 mM DIDS (chloride conductance blockers) in the bath solution. These blockers are effective against pressure-activated and calcium-dependent chloride currents. The inclusion of either SITS or DIDS did not prevent the pressure effect on calcium current. To determine if a secondary outward current was being activated, I included 200 μM cadmium in the bath solution to block calcium channels, repeated the voltage protocol used to evoke calcium current and applied bath solution to the cell via drug pipette. Application of bath solution did not induce any outward current, suggesting that the pressure effect was a direct effect on calcium current as opposed to activation of an additional conductance. While the pressure effect required current flow through calcium channels, it did not require specifically calcium ions, because substitution of 5mM barium for calcium in the bath solution did not prevent changes in current amplitude or kinetics upon drug pipette application of bath solution.

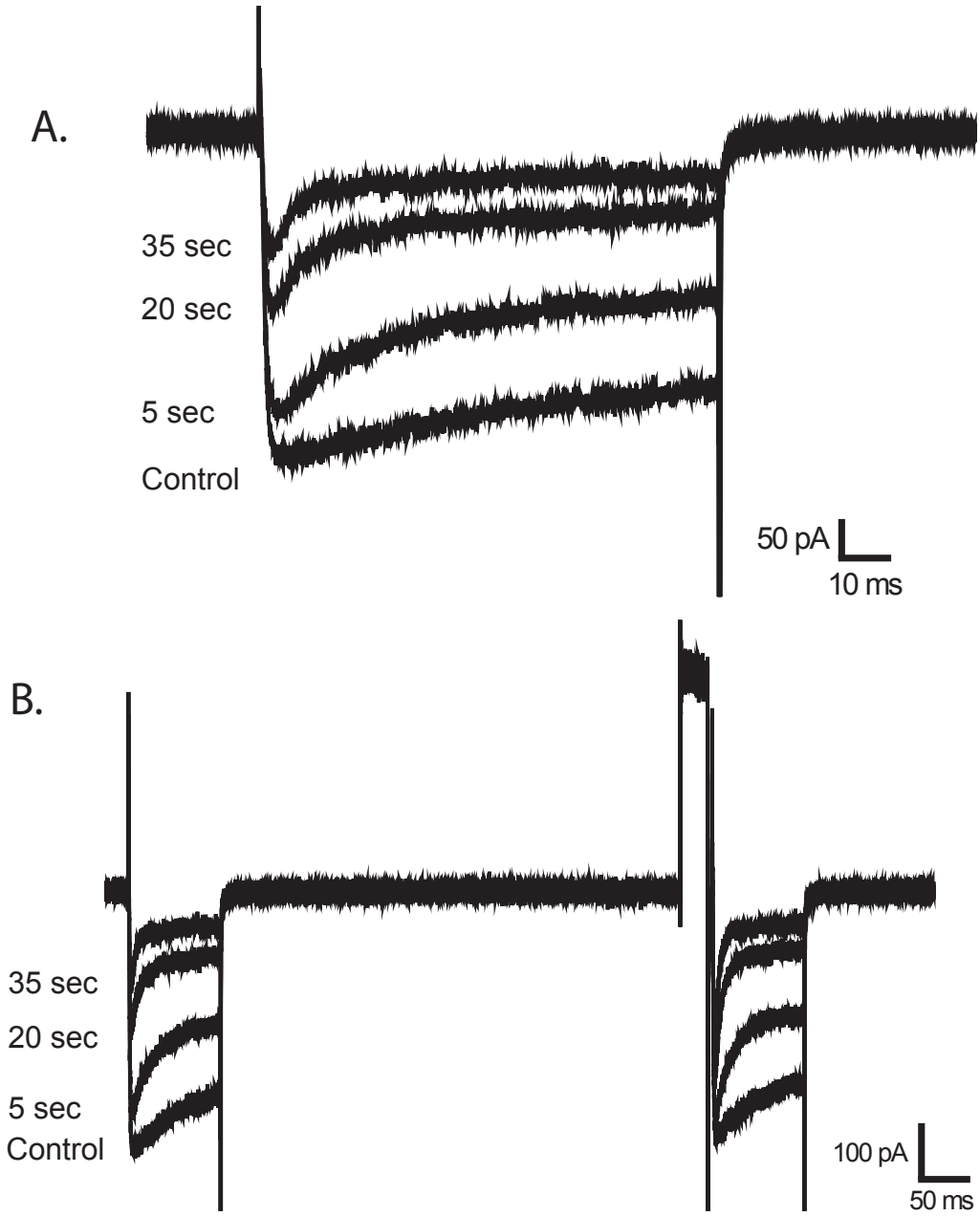


**Figure 29. Effect of pressure on calcium current.** Representative examples of pressure effect on calcium current magnitude and kinetics. Numbers to the left of the current traces indicate time after beginning of a continuous application of control bath solution to the cell under study, pressure applied via drug pipette. A. Calcium current was evoked by step depolarization from -80 mV to +10 mV. B. Calcium current evoked by standard prepulse protocol. The 30-ms step to +100 mV had no influence on the pressure effect on calcium current.

## C. TROUBLESHOOTING

Since I was unable to identify a specific current or mechanism underlying the apparent effect of pressure on calcium current, I attempted to eliminate the effect by altering various aspects of culture and experimental preparation. The following is a list of variables tested in an attempt to eliminate this anomalous effect.

- Enzymatic dissociation of ganglion sheath. I tried several enzyme protocols to treat ciliary ganglion prior to mechanical dissociation with two goals. On the one hand, I used a gentler enzyme treatment to prevent enzymatic damage to calcium channels. In the other direction, I used a stronger enzyme treatment to reduce the amount of mechanical dissociation needed to liberate neurons from the ganglion. The following is an example of enzyme protocols used (in addition to those presented in the preceded chapters)
    - Collagenase (type II) 15-30 minutes.
    - Trypsin (0.5 mg/mL)
    - Variations on the enzyme cocktail; different combinations of one to three of the four enzymes used.
- Culture dish substrates: I used various substrates on the 35 mM plastic culture dishes into which cells were plated.
    - Poly-D-lysine
    - Poly-L-lysine
    - Laminin
- I also plated cells onto glass coverslips with the various substrates.
- I used three sources of glass with which to make recording electrodes and drug pipettes. The type of glass and the company which supplied it was different. Also, I tried a different method of cleaning glass prior to making electrodes. Usually, the outside of the glass was

cleaned with 70% ethanol. In addition, I tried acid washing the glass and baking it overnight before making electrodes.

- I used a slow, gravity-fed perfusion system to bath apply drugs (or control bath solution) in an attempt to very gently expose the cell to the reagent. When the perfusion was turned on after beginning the recording, the pressure effect was still evident. If the perfusion was started prior to the beginning of the experiment, calcium current would be very small in amplitude and have kinetics similar to those observed after bath application, suggesting that the pressure effect occurred prior to the experiment.

- I used a variety of media supplements, or omitted usual supplements (horse serum and chick embryo extract) from the media.

- Bottled, filtered water from Sigma was used to make all necessary solutions for the dissection and for experiments (bath and pipette solution).

- Eggs were ordered from two different suppliers.

- I had typically used plastic containers for media and solutions each experimental day. I also tried using all autoclaved, glass containers to ensure there was no contaminant from the plastic.

Unfortunately, none of these precautions were sufficient to eliminate the effect of pressure on calcium currents. As such, this problem remains unsolved.

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