G-Protein-Modulated Ca\textsuperscript{2+} Current With Slowed Activation Does Not Alter the Kinetics of Action Potential-Evoked Ca\textsuperscript{2+} Current

DEBRA E. ARTIM AND STEPHEN D. MERINEY  
Department of Neuroscience, University of Pittsburgh, Pittsburgh, Pennsylvania 15260

Received 13 March 2000; accepted in final form 31 July 2000

Artim, Debra E. and Stephen D. Meriney. G-protein-modulated Ca\textsuperscript{2+} current with slowed activation does not alter the kinetics of action potential-evoked Ca\textsuperscript{2+} current. J Neurophysiol 84: 2417–2425, 2000. We 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, GTP\textgamma S produced voltage-dependent inhibition that exhibited slowed activation kinetics and was partially relieved by a conditioning prepulse. Using step depolarizations to evoke calcium current, we measured tail current amplitudes on abrupt repolarization to estimate the time course of calcium channel activation from 1 to 30 ms. GTP\textgamma S 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), we asked if GTP\textgamma S-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, we 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 effect 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.

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 (Dolphin 1998; Hille 1994). 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 \textbeta \gamma subunits with the calcium channel, underlies many instances of calcium channel inhibition (Herritz et al. 1996; Ikeda 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 (Boland and Bean 1993; Elmslie and Jones 1994; Golard and Siegelbaum 1993). 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- to 2-ms step depolarizations designed to mimic action potential (AP) stimulation (Williams et al. 1997; Womack and McCleskey 1995). 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. 1998).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Address for reprint requests: S. D. Meriney, Dept. of Neuroscience, University of Pittsburgh, 446 Crawford Hall, Pittsburgh, PA 15260 (E-mail: Meriney@imap.pitt.edu).
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 (Colecraft et al. 2000; Lee and Elmslie 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 0–10 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 in the preceding text, 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, we 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, we 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.

METHODS

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 CaCl2, 1 MgCl2, 12 glucose, and 20 NaH2CO3, pH 7.2. The ganglia were then incubated at 37°C in collagenase (0.5 mg/ml) followed by trypsin (0.08%) in deionized water to make a 100 nM stock solution, stored at –80°C, and diluted into bath saline at a final concentration of 100 nM. GTPγS was obtained from Sigma (St. Louis, MO). SOM was dissolved in deionized water to make 10 μM stock solution, stored at –20°C, and diluted into bath saline to a final concentration of 10 μM. GTPγS was diluted into internal pipette solution to make 2 mM stock, stored at –20°C, and used at a final concentration of 200 μM.

RESULTS

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 intra-
cellular pipette solution. On obtaining the whole cell configuration, GTPγS caused a rapid inhibition of calcium current that reached a steady state 5–10 min after obtaining whole cell access. Current was evoked every 10 s by a 100-ms voltage step from −80 to +10 mV. GTPγS inhibited peak calcium current density by 54 ± 2.2% (Fig. 2A), and the inhibition was partially voltage-dependent as measured by a double-pulse protocol (Fig. 1A). 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 (Fig. 1, B and C). The 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 < 0.001). Figure 2A 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 (Fig. 2B). GTPγS did not effect 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 (Fig. 2C). 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.

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, we 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 between control and GTPγS-modulated conditions (Fig. 3, A–C). 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 (Fig. 3C). Thus GTPγS slowed significantly the time course of calcium channel activation.

Time course of calcium channel activation during an AP

To investigate calcium channel activation during an AP, we 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 half-amplitude (Fig. 4A). 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. We 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 (Fig. 4B). To correct for rundown during the course of the experiment, each tail current evoked by a modified AP was compared with a control recording taken within 30 s 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 5A shows the 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 (Fig. 5B).

Increasing AP duration fails to reveal a change in activation time course

To test the hypothesis that the choroid AP used in the preceding text did not provide sufficient stimulus to convert reluctant channels to a willing gating mode, we 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 half-amplitude) was used to create a series of modified AP waveforms as described in the preceding text (see Fig. 6A). Again, we 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

---

**FIG. 2.** 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. —, Boltzmann fits to the data. GTPγS shifted the activation curve to more depolarized potentials (V_{1/2} = −2 mV for controls vs. 11 mV for GTPγS) and decreased the steepness (dz = 8.6 for controls vs. 17.1 for GTPγS) of the activation curve.
calcium channels open at that time. We 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 were modulated. However, as with the choroid AP waveforms, GTP\(_{\gamma}S\) did not change the time course of channel activation during this long AP (Fig. 6B).

**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 we 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 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 SOM. The percent SOM-induced inhibition was measured at the time of peak
Kinetic slowing was defined as the ratio of percent inhibition at the peak to the percent inhibition late in the depolarization (Fig. 7A). The voltage dependence of inhibition was determined with a standard double-pulse protocol (see Fig. 1A). 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 (Fig. 7C).

We also investigated the relationship between kinetic slowing and relief of inhibition produced by an AP train. Kinetic slowing was quantified as described in the preceding text, but now voltage dependence was defined as the inhibition relieved by a train of eight choroid AP waveforms delivered at 100 Hz (Fig. 7B). The percent inhibition of current evoked by the eighth AP in the train was compared with the percent inhibition of current evoked by the first 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 (Fig. 7D).

**FIG. 5.** GTPγS does not alter time course of 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 waveforms (as shown in Fig. 4B), which 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 and GTPγS conditions. Furthermore there were no significant differences in the percent channel activation between control and GTPγS at any point during the repolarization phase.
Thus kinetic slowing reliably predicted the extent of activity-dependent relief induced by this AP train.

**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, we have used GTP\_yS 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, we 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).

Using APs of different duration and amplitudes, we 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- to 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 nonlinear 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 (Dittman and Regehr 1997; Dunwiddie and Hass 1985). 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). We thank J. Simples, R. Poage, J. Pattillo, D. King, and J. Dilmore for many helpful discussions and critical evaluation of the manuscript. This work was supported by National Institutes of Health Grants NS-32345 (S. D. Meriney) and MH-18273 (D. E. Artim) and by a Grant-in-Aid from the American Heart Association (S. D. Meriney).

REFERENCES


We thank J. Simples, R. Poage, J. Pattillo, D. King, and J. Dilmore for many helpful discussions and critical evaluation of the manuscript. This work was supported by National Institutes of Health Grants NS-32345 (S. D. Meriney) and MH-18273 (D. E. Artim) and by a Grant-in-Aid from the American Heart Association (S. D. Meriney).


