Effects of Spike Parameters and Neuromodulators on Action Potential Waveform-Induced Calcium Entry Into Pyramidal Neurons

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Stewart, Ansalan E. and Robert C. Foehring. Effects of spike parameters and neuromodulators on action potential waveform-induced calcium entry into pyramidal neurons. J Neurophysiol 85: 1412–1423, 2001. Neocortical pyramidal neurons express several different calcium channel types. Previous studies with square voltage steps have found modest biophysical differences between these calcium channel types as well as differences in their modulation by transmitters. We used acutely dissociated neocortical pyramidal neurons to test whether this diversity extends to different activation by physiological stimuli. We conclude that 1) peak amplitude, latency to peak, and the total charge entry for the Ca2+ channel current is dependent on the shape of the mock action potential waveforms (APWs). 2) The percent contribution of the five high-voltage-activated currents to the whole cell current was not altered by using an APW as opposed to a voltage step to elicit the current. 3) The identity of the charge carrier affects the amplitude and decay of the whole cell current. With Ca2+, there was a greater contribution of T-type current to the whole cell current. 4) Total Ba2+ charge entry is linearly dependent on the number of spikes in the stimulating waveform and relatively insensitive to spike frequency. 5) Current decay was greatest with Ca2+ as the charge carrier and with minimal internal chelation. 6) Voltage-dependent neurotransmitter-mediated modulations can be reversed by multiple spikes. The extent of the reversal is dependent on the number of spikes in the stimulating waveform. Thus the neuronal activity pattern can determine the effectiveness of voltage-dependent and -independent modulatory pathways in neocortical pyramidal neurons.

INTRODUCTION

Electrophysiological studies of calcium channel currents using prolonged square voltage steps to evoke the current have revealed that the soma/proximal dendrites of neocortical pyramidal neurons express robust currents of at least six types. These include T-type (Sayer et al. 1990; Tarasenko et al. 1998), and five high-voltage-activated (HVA) currents: L, N, P, Q, and R type (Brown et al. 1994; Foehring et al. 2000; Lorenzon and Foehring 1995a; Mermelstein et al. 1999; Sayer et al. 1990; Ye and Akaike 1993).

Ca2+ influx through voltage-gated Ca2+ channels serves an important role in neuronal integration by regulating Ca2+-dependent second messengers, gene expression, neurotransmitter release, and repetitive firing behavior (Bertolino and Llinas 1992; Bito et al. 1997; Kasai and Peterson 1994; Mermelstein et al. 2000; Pineda et al. 1998). For example, Ca2+-dependent transcription factors are preferentially supported by L-type currents in hippocampal pyramidal neurons (Bito et al. 1997; Mermelstein et al. 2000). Neocortical pyramidal cell Ca2+ currents are differentially involved in repetitive firing, activation of Ca2+-dependent K+ currents underlying afterhyperpolarizations (AHPs), and spike frequency adaptation (Pineda et al. 1998). N-, P-, and Q-type channels activate AHPs as well as provide inward current, but L-type currents act only as an inward charge carrier (Pineda et al. 1998). HVA currents in neocortical pyramidal neurons are also differentially modulated by transmitters (Choi and Lovinger 1996; Foehring 1996; Sayer 1998; Sayer et al. 1992; Stewart et al. 1999). It is unknown, however, whether Ca2+ channel types are activated differently in response to physiological stimuli.

Under physiological conditions, voltage-gated Ca2+ channels are activated by excitatory postsynaptic potentials and action potentials (APs). Since AP parameters show variation under physiological conditions in pyramidal neurons (Connors et al. 1982; Lorenzon and Foehring 1993; McCormick and Prince 1987; Stafstrom et al. 1984; Wheeler et al. 1996), we used AP waveforms (APWs) as the voltage stimuli to elicit Ca2+ channel currents in acutely dissociated neocortical pyramidal neurons (cf. Brody et al. 1997; Jackson et al. 1991; Llinas et al. 1982; McCobb and Beam 1991; Park and Dunlap 1998; Patil et al. 1998; Pennington et al. 1992; Schiller et al. 1995; Scroggs and Fox 1992; Toth and Miller 1995; Williams et al. 1997). We tested hypotheses concerning the effects of pyramidal cell AP parameters on Ca2+ entry, the role of Ca2+ current inactivation and facilitation in regulating Ca2+ entry during trains of spikes, and the influence of APs on modulation by transmitters.

METHODS

Acute isolation of pyramidal cells

Two- to 6-week-old Sprague-Dawley rats were anesthetized with methoxyflurane. Under anesthesia, the rats were decapitated and the brains extracted. The brains were then sectioned into 400-μM slices using a vibrating tissue slicer (Cambden Instruments) in an oxygenated high-sucrose solution that contained (in mM) 250 sucrose, 2.5 KCl, 1 NaH2PO4, 11 glucose, 4 MgSO4, 0.1 CaCl2, and 15 HEPES (pH = 7.3 adjusted with 1 N NaOH; 300 mOsml). The slices were held for a minimum of 1 h in a carboxygen (95% O2-5% CO2) bubbled artificial spinal fluid (ACSF). The sensorimotor cortex (com-
bined primary motor and primary somatosensory cortices) was placed in ice-cold ACSF and dissected from the slices with the aid of a stereomicroscope. The ACSF contained (in mM) 125 NaCl, 3 KCl, 2 CaCl₂, 2 MgCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, 20 glucose, 1 kynurenic acid, 1 pyruvic acid, 0.1 nitro-arginine, and 0.05 glutathione (pH = 7.4 adjusted with 1 N NaOH; 310 mOsml/l). The dissected cortex was then incubated at 32°C for 20–30 min in an oxygenated ACSF containing Pronase E (Sigma protease type XIV, 1.0 mg/ml) (modified from Lorenzon and Foehring 1995a). Following the incubation period, the tissue was first rinsed in a sodium isethionate solution that contained (in mM) 140 Na isethionate, 2 KCl, 1 MgCl₂, 23 glucose, 15 HEPES, 1 kynurenic acid, 1 pyruvic acid, 0.1 nitro-arginine, and 0.05 glutathione (pH = 7.3, adjusted with 1 N NaOH; 310 mOsml/l), then triturated in the same solution using fire-polished Pasteur pipettes. The supernatant was collected and poured into a 310 mOsml/l), then triturated in the same solution using fire-polished Pasteur pipettes. The supernatant was collected and poured into a plastic petri dish (Lux) positioned on the stage of an inverted microscope (Nikon Diaphot 300). The cells were allowed several minutes to adhere to the petri dish, and then the background flow of HEPES-buffered saline solution (HBSS) was initiated (~1 ml/min). HBSS contained (in mM) 10 HEPES, 138 NaCl, 3 KCl, 1 MgCl₂, and 2 CaCl₂ (pH = 7.3, adjusted with 1 N NaOH, 300 mOsml/l).

Recording solutions and pharmacological agents

The external recording solution used to isolate the Ca²⁺ channel currents (TEA-free solution) consisted of (in mM) 125 NaCl, 20 CsCl, 1 MgCl₂, 10 HEPES, 5 BaCl₂, 0.001 TTX, and 10 glucose (pH 7.2, adjusted with 0.1 N H₂SO₄; 265–275 mOsm/l). Ten to 20 mM CaCl₂, 2 MgCl₂, 1.25 NaH₂PO₄, 26 NaHCO₃, 20 glucose, 1

Voltage steps (~30 ms) elicited robust Ba²⁺ currents through Ca²⁺ channels in neocortical pyramidal cells (Lorenzon and Foehring 1995a,b; Sayer et al. 1990), and the current evoked with 5 mM BaCl₂ has similar I-V relationships to currents using the more physiological 2 mM CaCl₂ (Lorenzon and Foehring 1995a). In this study, we examined Ca²⁺ and Ba²⁺ currents in response to action potential waveforms.

Whole cell recordings were acquired at room temperature using a DAGON 8900 or an Axopatch 200A electrometer. The recordings were monitored and controlled by pCLAMP6 (Axon Instruments) installed on a 486 computer. The electrodes were pulled from 7052 glass (Garner) and fire polished. Leak currents and capacitative artifacts were subtracted on-line with a p/4 procedure. In addition, currents obtained in the presence of 400 µM Cd²⁺ were subtracted from the data. Series resistance compensations of 70–80% were employed. The average whole cell capacitance for recorded cells was 14.6 ± 0.7 (SE) pF (median = 13.6 pF, n = 64). Uncompensated series resistance averaged 1.7 ± 0.1 MΩ. For a typical peak current of 2–6 pA, this would lead to an estimated series resistance error of 3–10 mV (calculated using Ohms Law, V = IR; uncompensated series resistance multiplied by peak current). Uncompensated series resistance was estimated from dial readings for series resistance and percent compensation on the Axopatch 200A. Voltage control was also assessed by observing tail currents after brief voltage steps (Lorenzon and Foehring 1995a). Cells with broad or variable tail current decay were discarded. A gravity-fed parallel array of glass tubes was used to apply the drugs. Voltage data were uncorrected for liquid junctional potential (8 mV).

SYSTAT (SYSTAT, Evanston, IL) software was used to carry out all statistical calculations. Unless otherwise stated, the sample data are represented as median and mean ± SD. Data are also presented graphically as scatter plots or box plots. In the box plots, the internal line represents the median while the outer edges of the box represent the inner quartiles of the data set. The bars extending from the box depict the two outer quartiles. Data points more than two times the difference between the box edges were considered outliers and are indicated by a small asterisk in the plots (Tukey 1977). Statistical differences were determined with the Mann-Whitney U test (α = 0.05) unless otherwise stated.

RESULTS

In response to either digitized or mock APs, the Ba²⁺ current was first evident during the rising phase of the AP or APW, peaked during the repolarization phase, and decayed rapidly during the AHP phase (Fig. 1, n = 91). These results are in agreement with similar studies in various other neuron types (e.g., Linhas et al. 1982; McCobb and Beam 1991; Park and Dunlap 1998; Scroggs and Fox 1992; Spencer et al. 1989; Wheeler et al. 1996).

**Effect of APW parameters on Ba²⁺ entry**

The shape of the AP is altered under various physiological conditions (i.e., ontogeny, repetitive spiking, modulation, cell injury). Therefore, to test how changing the shape of the AP affects the amplitude and timing of Ba²⁺ entry, we systematically varied the parameters (e.g., holding potentials, widths, and depolarization/repolarization rates) of the mock AP. Experimental estimates of Ca²⁺ channel availability under a...
range of physiological conditions are necessary for modeling postsynaptic integration by pyramidal cells.

**HOLDING POTENTIAL.** We first compared the current evoked from a holding potential (HP) of −70 mV to that from an HP of −50 mV. These potentials were chosen to reflect the “up” and “down” states observed in in vivo cortical recordings from anesthetized (Cowan and Wilson 1994; Stern et al. 1997) or sleeping (Steriade et al. 1993) rats. The cells were held for >1 min at a given holding potential before testing for currents. Voltage protocols are shown in Fig. 2. Peak currents were smaller (significant: \( P < 0.04 \)) in all cells tested when elicited from a HP of −50 versus −70 mV (Fig. 2, A and B; \( n = 5 \); median peak at −70 mV: 1.6 nA; median peak at −50 mV: 1.5 nA). Total charge entry (measured as the time integral of the median peak at −70 mV) was also reduced at a HP of −50 versus −70 mV (median area at −70 mV: 2.5 pC; median area at −50 mV: 2.3 pC).

**AP SHAPE.** We next examined the effects of spike width and rates of change in voltage (depolarization and repolarization). The rates were chosen to reflect an approximate physiological range of values (adjusted for temperature). Over the range examined, as the rate-of-rise was increased (repolarization rate kept the same: Fig. 3A1), there was a linear decrease in the peak current (Fig. 3B1) and total charge entry decreased exponentially (Fig. 3B2; \( n = 16 \)). (In Fig. 3, “normalized” = all data points divided by the largest value.) Accelerating the repolarization rate (rise rate kept the same) led to a linear increase in the peak amplitude (Fig. 3B1) and an exponential decrease in the total charge entry for the range of values examined (Fig. 3B2; \( n = 19 \)). The faster the repolarization rate, the more rapid the change in driving force, thus the greater the peak amplitude of the current. The largest currents were obtained with an instantaneous jump from peak depolarization to the final voltage (cf., tail currents after voltage steps). The peak also came earlier in time with increasing repolarization rate (Fig. 3A2). Total charge entry decreased as the repolarization rate was increased because the spikes became narrower.

Peak current was sensitive to the rate of change of AP polarization and repolarization (Fig. 3C1). In contrast, total charge entry was primarily a function of spike width; the linear relationship between normalized charge entry and AP width over this physiological range was similar whether width was varied by changing polarization or repolarization (Fig. 3C2).

The early peak (shoulder) on some of the current traces (Figs. 2–5; see \( \rightarrow \) in Fig. 2A; also Fig. 5A) represents HVA current influx during the rising phase of the APW. This shoulder was only seen with slower rise rates \( \geq 55 \text{ mV/ms} \). We hypothesize that the current activates during the rising phase but is small due to the low driving force. The large increase in current does not occur until the AP falls and driving force increases. Consistent with this hypothesis, if following the upstroke of the APW the voltage is kept at +30 mV for 1.8 to 3 ms before repolarizing to −90 mV, the current remains steady (and small) in size until repolarization, then increases in synchrony with repolarization (\( n = 2 \), data not shown).

The shoulder was not due to T-type current because it was not significantly reduced by holding the cell at −50 mV for several minutes (\( n = 7 \)). Furthermore this early peak was partially blocked by all of the organic blockers of HVA chan-

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**FIG. 1.** 
Ba\(^{2+}\) current evoked by action potential (AP) waveforms. A: current in response to an AP digitized from a recording of a layer II/III pyramidal cell in the slice preparation (room temperature). Top: the AP used as a voltage protocol. This AP had a half-width of 1.8 ms and base width of −3.5 ms. The maximum \( dV/dt \) for spike polarization was 277 mV/ms (average = 60 mV/ms) and for the repolarization, peak \( dV/dt \) was 60 mV/ms (average = 28 mV/ms). Bottom: the resulting Ba\(^{2+}\) current. B: current in response to mock AP waveform (APW). Top: the voltage protocol composed of 3 voltage ramps [depolarization −70 mV to +30; repolarization: +30 mV to −90 mV; after-hyperpolarization (AHP): −90 mV to −70 mV]. The half-width was 1.8 ms and base width was 3.6 ms (average \( dV/dt = 55.6 \text{ mV/ms} \) polarization and 66.7 mV/ms repolarization). Bottom: the Ba\(^{2+}\) current in response to the APW. C: current in response to a different mock AP waveform (APW, no AHP) in a different cell. Top: the voltage protocol composed of 2 voltage ramps (depolarization −70 mV to +30; repolarization: +30 mV to −70 mV). The half-width was 1.2 ms and base width was 2.4 ms (average \( dV/dt = 166.7 \text{ mV/ms} \) polarization and 55.6 mV/ms repolarization). Bottom: the Ba\(^{2+}\) current in response to the APW. D: partial block by 50 nM Cd\(^{2+}\) led to little change in the time course of the current, suggesting adequate voltage control. ---, peak current in the absence of Cd\(^{2+}\) (Glu). Similar data were obtained from 5 cells tested. Voltage protocol as in B.

**FIG. 2.** Effects of holding potential. A: representative traces demonstrating the effect of changing the holding potential on APW-induced Ba\(^{2+}\) current. →, the early influx of current (shoulder; see text). Top: voltage protocol (half-width was 1.8 ms and base width was 3.6 ms). Bottom: the current trace for holding potential (HP) = −70 mV is the black line. The current elicited from −50 mV is indicated by the gray line. B: scatter plot of the peak current for 5 cells held at a holding potentials of −70 or −50 mV. Peak currents for a given cell at the 2 HPs are connected by —.
The amplitude of the peak current depends on the rates of both rise and fall. Depolarization or repolarization rate, the greater the charge entry (mean $\pm$ SE). The slower the rise rate, the larger the peak amplitude. The slower the fall rate, the smaller the peak current. Data were normalized by dividing the peak current evoked by each APW by the peak current evoked by the 1st APW. B: plot of average normalized charge entry vs. rate-of-rise ($\circ$) or repolarization (■) showing that the slower the depolarization or repolarization rate, the greater the charge entry (mean $\pm$ SE). C1: the amplitude of the peak current depends on the rates of both rise and fall. Symbols and error bars as in B. C2: charge entry depends on the width of the APW. As the duration of the APW is increased, total charge entry is increased. Symbols and error bars as in B.

**Ca$^{2+}$ current types**

We have previously used voltage steps to study the kinetics and voltage dependence of activation and inactivation for specific Ca$^{2+}$ current types (Lorenzon and Foehring 1995a,b; Mermelstein et al. 1999). In neocortical pyramidal neurons, we found only modest differences among N-, P-, and L-type Ba$^{2+}$ currents in terms of current time-to-peak, voltage dependence of activation, inactivation kinetics, percent inactivation, and deactivation kinetics (Foehring et al. 2000; Lorenzon and Foehring 1995a; Mermelstein et al. 1999). The largest differences in neocortical pyramidal cells were that Q-type current inactivated faster than N-, P-, and L-type (Mermelstein et al. 1999) and R-type current activated at more negative potentials and inactivated faster and more completely than L-, P-, N-, and Q-type Ca$^{2+}$ currents (Foehring et al. 2000).

Since the biophysical differences are small and primarily related to inactivation kinetics (which are slow compared with an APW), we hypothesized that the percentage that each current type contributes to the peak whole cell current would not differ between currents elicited by APWs or 30-ms steps. We used selective pharmacological antagonists to determine the percent that each current type contributed to the whole cell current evoked by either an APW or a voltage step (Fig. 4). The Ca$^{2+}$ channel blockers were added sequentially in the order: 5 µM nifedipine, 25 nM AgTX, 1 µM CgTx GVIA, 1 µM CgTx MVIIC, and Cd$^{2+}$ to block L-type (nifedipine sensitive), P-type (AgTX sensitive), N-type (CgTx GVIA sensitive), Q-type (CgTx MVIIC sensitive, after prior block of N and P type), and R-type (resistant to organic blockers, Cd$^{2+}$ sensitive) HVA currents, respectively. A 30-ms voltage step from $-90$ to $-10$ mV was used to evoke the current during the application of each toxin. Peak currents were measured and plotted versus time. Once the block by the antagonist reached steady state (- - - in Fig. 4B), the stimulating waveform was switched to an APW (from $-70$ to $+30$ mV, 3.6-ms base width). This procedure allowed for the comparison of current types in the same cell evoked by both steps and APWs. We found that although individual cells showed considerable variability in the percentage of each current subtype, on average a similar percentage of each Ba$^{2+}$ current type constituted the whole cell current when either stimuli was used (Fig. 4; $n = 7$).

The currents carried by each of the specific channel types were obtained by subtracting the antagonist-sensitive current from the whole cell current. For the APW, the five HVA currents were then compared with respect to their base width, half-width, latency-to-peak, half decay time, and amplitude at 10 and 90% of rise. For the step-induced current, we measured the activation time constant and the decay time constant. On average, we found no significant differences between calcium channel types for these measures (Kruskal-Wallis test; data not shown). The biophysical differences between the current types are apparently too small to alter the percent contribution to the peak current or affect the contribution to different temporal portions of a brief stimulus such as an AP.

**Ba$^{2+}$ versus Ca$^{2+}$**

Ba$^{2+}$ (5 mM) was used as the charge carrier in the previous experiments to avoid complications of Ca$^{2+}$-dependent inactivation or activation of Ca$^{2+}$-dependent currents. After substituting 2 mM Ca$^{2+}$ for 5 mM Ba$^{2+}$, the peak current evoked by an APW was reduced, and a second slowly decaying component was evident in the current decay (21% of total decay; Fig. 5A; $n = 7$). We compared 2 mM Ca$^{2+}$ to 5 mM Ba$^{2+}$ because these concentrations display a similar voltage dependence (Lorenzon and Foehring 1995a) and because physiological [Ca$^{2+}$], is thought to be 1–2 mM. The peak amplitude of the current was larger when Ba$^{2+}$ was used as the charge carrier because Ba$^{2+}$ is more permeant than Ca$^{2+}$ through HVA Ca$^{2+}$ channels (Tsien et al. 1988) and a smaller concentration of Ca$^{2+}$ than Ba$^{2+}$ was used.

Since T-type Ca$^{2+}$ currents have slower deactivation kinetics than HVA currents (Matteson and Armstrong 1986) and T-type currents are more prominent in Ca$^{2+}$ than Ba$^{2+}$ in pyramidal cells (Lorenzon and Foehring 1995a,b; Sayer et al. 1990), we hypothesized that the slow component to the Ca$^{2+}$ current decay represented T-type current. If this hypothesis is correct, holding the cell at $-50$ mV should eliminate this component since T-type channels in pyramidal cells are inac-
tivated at this voltage (Huguenard 1996; Sayer et al. 1990; Tarasenko et al. 1998; Ye and Akaike 1993). We tested five cells that displayed both fast and slow components of the Ca$^{2+}$ current decay following an APW from a holding potential of −70 mV. When the holding potential was maintained at −50 mV for >1 min, the slow component was eliminated in three of five cells and reduced (53%, 86%) in the remaining cells (Fig. 5B). Nickel ions (Ni$^{2+}$: 50 μM), a relatively specific antagonist for T-type currents of the a1H type (Lee et al. 1999; Sayer et al. 1990; Tsien et al. 1988), blocked the slow component in four of five cells tested, yet only caused a minor change in the peak current (Fig. 5C). These results are consistent with the slow tail current being due to T-type channels.

Facilitation and inactivation

The preceding results suggest that single APWs are too brief for differences in biophysical properties of different types of HVA currents to be evident in our cells. We next examined whether multiple spikes lead to either facilitation or inactivation of Ca$^{2+}$ currents. These processes could potentially alter the additive effects of multiple spikes on Ca$^{2+}$ entry during prolonged firing at physiological rates.

**FACILITATION.** Facilitation refers to an increase in the current recorded during a test pulse that was preceded by a depolarizing prepulse (Dolphin 1996; Elmslie and Jones 1994; Ikeda 1991; Kasai 1992). We tested whether multiple APWs in sequence would lead to facilitation, defined here as the percent increase in the peak amplitude of the current evoked by the final spike over that evoked by the first spike in the train. We observed no increase in peak Ba$^{2+}$ or Ca$^{2+}$ current (no facilitation) during trains of 2, 4, 8, or 16 spikes at 50, 100, or 200 Hz (n = 12, Fig. 6). This result is consistent with data obtained with voltage steps where in the absence of a G-protein-mediated modulation, Ba$^{2+}$ current can be facilitated by only ~12% in pyramidal cells of the sensorimotor cortex (Foehring 1996; Stewart et al. 1999).

**INACTIVATION.** Percent current decay was determined by comparing the peak amplitude of the current entering in response to the first spike to that entering with the final spike in trains of APWs at various frequencies. Both voltage-dependent and Ca$^{2+}$-dependent inactivation of calcium channel currents occur in various types of neurons (e.g., Forsythe et al. 1998; Gutnick et al. 1989; Jones and Marks 1989; Nagerl and Mody 1998). We tested five cells with internal EGTA (10 mM) to emphasize voltage-dependent inactivation. We compared these data to that from cells where Ca$^{2+}$ was the charge carrier and minimal chelation (0.1 mM BAPTA) was employed to allow both Ca$^{2+}$- and voltage-dependent processes.

In response to 16 spikes at 100 Hz, there was minimal current decay with Ba$^{2+}$ as the charge carrier (Fig. 7). On average, the current decayed by only 9 ± 2% (median = 6.5%; n = 24). Furthermore 20/24 cells tested displayed <10% current decay (median = 5%). Four of 24 cells had currents which decayed by >10% (Fig. 7C, median = 30%; • in Fig. 7A), suggesting that a subset of pyramidal cells may show stronger inactivation. Percent inactivation was similar when a frequency of 200 Hz was used (Fig. 8A; n = 11; median decay = 8%).

The observation that little voltage-dependent inactivation (or facilitation) occurs in response to repetitive APWs (≤16 spikes at 200 Hz) in these cells suggests that each spike contributes the same amount of charge entry, i.e., Ba$^{2+}$ entry would depend on the number of spikes but would be independent of spike frequency. We tested this hypothesis by comparing the total charge entry when 2, 4, 8, or 16 spikes (50 and 100 Hz) were used as stimuli. Total charge entry increased linearly with increasing number of spikes (n = 12; Fig. 6A). When the same number of spikes was used, charge entry was the same regardless of the frequency (50 Hz or 100 Hz). Neither increasing the number of spikes nor changing their frequency affected the peak amplitude of the current (there was no inactivation or facilitation; data not shown; n = 12).

Ca$^{2+}$-dependent inactivation depends on both the amount of Ca$^{2+}$ entry and Ca$^{2+}$ buffering (Eckert and Chad 1984); it should be greatest when Ca$^{2+}$ entry is high and Ca$^{2+}$ chelation is low. Under conditions where Ca$^{2+}$-dependent inactivation...
was possible (0.1 mM BAPTA and 2 mM Ca\(^{2+}\)), percent decay during a train of 16 spikes at 200 Hz was more than doubled (20 \(\pm\) 2%; median = 20%; \(n = 6\); Fig. 8C; significantly different at \(P < 0.05\)) compared with 10 mM EGTA and Ba\(^{2+}\) (Fig. 8, A and D). Using a train of 16 spikes at 100 Hz yielded similar results in two cells (20 and 26% decay). An intermediate amount of current decay was seen with Ca\(^{2+}\) as charge carrier and 10 mM internal EGTA (Fig. 8, B and D), suggesting incomplete Ca\(^{2+}\) chelation. These findings suggest that Ca\(^{2+}\)-

depended inactivation may be important for regulating Ca\(^{2+}\) entry during spike trains in pyramidal neurons. However, at this time we cannot rule out other Ca\(^{2+}\)-dependent processes, such as unblocked outward currents or accumulation of intracellular Ca\(^{2+}\).

**Modulation**

N- and P-type Ca\(^{2+}\) currents in various cell types are modulated via voltage-dependent, membrane-delimited pathways (Hille 1992; Jones and Elmslie 1997). In neocortical pyramidal cells, N- and P-type Ca\(^{2+}\) channels are modulated by both serotonin (5HT\(_{1A}\)) and muscarinic (M\(_2\)) receptor activation via membrane-delimited pathways (Foehring 1996; Stewart et al. 1999). Such modulations have been proposed to play important roles in regulating neurotransmitter release at synaptic terminals (Wu and Saggau 1997).

In many voltage-dependent, membrane-delimited modulations, activation kinetics are slowed. When this slowing is present and a voltage step is used to evoke the current, the modulation is greatest early in the trace and reduced later in the step (Bean 1989). Thus when a brief stimulus like an APW is used, the modulation should be larger compared with that determined at the end of a long voltage step.

We previously found that activation kinetics were not slowed in the presence of muscarine (\(\leq 100 \mu M\)) (Stewart et al. 1999), and slowing was only noted at high concentrations of the 5HT\(_{1A}\) agonist 8-OH-DPAT (e.g., 100 \(\mu M\)) (Foehring 1996). Percent modulation was determined by calculating the absolute amplitude of current blocked by agonists, then divid-
ing by the amplitude of the control current. Our criteria for kinetic slowing were the presence of two τs to current activation or a slower single τ (Foehring 1996; Stewart et al. 1999).

We first compared the magnitude of the modulation by 2 μM muscarine (n = 6) or 2 μM 8-OH-DPAT (n = 8) when current was evoked by an APW or by a voltage step (measured at peak current). At this dose, slowing of activation kinetics was not evident (Fig. 9, A and C), and the Ca2+ channel current elicited by an APW was modulated to a similar extent as the current evoked by a voltage step (Fig. 9, B and D; no significant difference). Interestingly, one cell exhibited slowed activation kinetics in 2 μM 8-OH-DPAT caused slowing of activation kinetics, and APW-induced currents were modulated to a greater extent than peak currents elicited by a 30-ms square wave (n = 6; Fig. 9, E and F; P < 0.05). The differences in percent modulation were lost if step-induced currents were compared at 1.8 ms into the step (corresponding to peak current in response to an APW; data not shown).

Another characteristic of voltage-dependent, membrane-delimited modulations is that they can be reversed by long (e.g., 30 ms) depolarizing prepulses (e.g., +100 mV) (Bean 1989; Elrlich and Elmslie 1995; Kasai 1992). In a previous study in cortex, the 5HT1A-mediated modulation was reversed by such prepulses by ~50% (Foehring 1996) and the M2-like modulation was 100% voltage dependent (Stewart et al. 1999).

AP-like steps were reported to reverse G-protein-mediated modulations in basal forebrain neurons (Williams et al. 1997) and chick DRG cells (Park and Dunlap 1998) but not dorsal raphe neurons (Pennington et al. 1991). We asked whether a train of APs at physiological firing rates can effectively reverse membrane-delimited, voltage-dependent modulations in neocortical pyramidal neurons (Figs. 10 and 11).

We tested the voltage-dependent 5HT1A and M2-receptor-mediated modulations (target N- and P-type currents) and the voltage-independent M1 pathway (targets L-type current) (Stewart et al. 1999). The 5HT1A effect was elicited with the specific agonist 8-OH-DPAT. The rapid M2-mediated modulation could be isolated using 10 mM EGTA in the internal recording solution to block the M1 pathway (the modulation is Ca2+ dependent) (Stewart et al. 1999). The M1 modulation was examined by using minimal chelation (0.1 BAPTA) and 50 μM N-ethylmaleimide (blocks the M2 pathway) (Stewart et al. 1999).

We used two different protocols. In the first protocol, we used a 15 ms test step to ~30 mV (HP = −90 mV). Multiple brief voltage steps with parameters similar to an AP (holding potential of ~70 mV, peak of +30 mV, 2-ms duration) were employed as the prepulse. We measured the peak current response to the test pulse following 0, 10, 20, or 50 AP-like steps (at 100 Hz) and found that the 5HT1A and M2 modulations were reduced by the AP-like steps (Fig. 10B) and that the
amplitude of the modulation depended on the number of steps used in the prepulse. Following a 50-step prepulse, the 5HT1A- (Fig. 10B; n = 5) and M2-mediated modulations (n = 4; data not shown) were almost completely reversed [for both transmitters, median reversal = 100% (e.g., median modulation after steps = 0%)] (see also Williams et al. 1997). In contrast, the isolated M1 pathway was not affected by a prepulse of multiple AP-like steps (n = 4, data not shown).

Our second protocol elicited current with 16 APWs at 200 Hz. The amplitude of current entering in response to the first spike in control solution was compared with the amplitude of the current entering with the first and the 16th spike in the presence of the modulator. The modulation was considered completely reversed if the amplitude of the current entering in response to spike 16 in the presence of transmitter was equal in amplitude to the current entering with spike 1 under control conditions. We found the 5HT1A-mediated modulation to be completely reversed by this protocol in five of six cells tested and partially reversed in the sixth cell (by 44%; Fig. 10A). The fast (M2) muscarinic modulation was likewise completely reversed in four of five cells and partially reversed in the fifth (by 30%; Fig. 11, A and B). The slow muscarinic modulation (M1) was not reversed in any of the four cells tested (Fig. 11, C and D).

**DISCUSSION**

Calcium-dependent mechanisms influence the integrative behavior (e.g., repetitive firing, gene regulation) of neocortical pyramidal neurons (Bito et al. 1997; Connors et al. 1982; Lorenzon and Foehring 1993; Pineda et al. 1998; Schwindt et al. 1988). Understanding these mechanisms requires knowledge of Ca2+ entry in response to physiological stimuli. Accordingly, we varied the parameters of mock APWs in the...
physiological range to examine how variations in holding potential, and action potential shape, numbers, and frequency influence Ca\(^{2+}\) entry into acutely dissociated neocortical pyramidal neurons. In addition, we examined currents during spike trains and the influence of spike number and frequency on the modulation of Ca\(^{2+}\) currents by transmitters.

**HOLDING POTENTIAL.** In vivo, neocortical pyramidal cells shift between two preferred membrane potential ranges, −45 to −55 mV ("up state") and −60 to −90 mV ("down state") (Cowen and Wilson 1994; Steriade et al. 1993; Stern et al. 1997). In slices, resting potentials are typically about −65 to −80 mV (Lorenzon and Foehring 1993; McCormick et al. 1985). We found that AP-induced Ca\(^{2+}\) channel currents were somewhat sensitive to holding potential in these potential ranges: current amplitude and charge entry were greater from negative potentials, indicating that more Ca\(^{2+}\) channels are available for activation when the neuron resides in the down state versus the up state.

**SPIKE PARAMETERS.** During prolonged repetitive firing in neocortical pyramidal cells, action potentials become smaller and broader (rise rate decreases) (Connors et al. 1982; Stafstrom et al. 1984). During postnatal development, pyramidal cell action potentials become narrower and increase in amplitude and in rates of rise and repolarization with age (Lorenzon and Foehring 1984). During postnatal development, pyramidal cell action potentials become smaller and (Al. 1984). During postnatal development, pyramidal cell action potentials become smaller and in amplitude additionally depended on rates of spike rise and repolarization (Fig. 3C1). The faster the repolarization, the larger the peak current because driving force is rapidly increased while conductance (determined by the level of depolarization) is high. Faster rise rates result in smaller peak current because there is less time to activate current.

When action potentials repolarize quickly, the large, rapid Ca\(^{2+}\) transient may trigger Ca\(^{2+}\)-dependent processes before intrinsic Ca\(^{2+}\) buffers lower internal [Ca\(^{2+}\)]. With slow repolarization, Ca\(^{2+}\) entry occurs over a longer time period, resulting in a decreased peak but increased charge entry (Park and Dunlap 1998; Toth and Miller 1995; Wheeler et al. 1996). A slow rate of polarization also promotes larger charge entry by allowing more time for channel activation. The total Ca\(^{2+}\) entry may be most closely related to influence on gene regulation (Bito et al. 1997; Mermelstein et al. 2000) or activation of Ca\(^{2+}\)-dependent K\(^{+}\) currents (Pineda et al. 1998) in pyramidal cells. Increased Ca\(^{2+}\) entry through broad, slowly repolarizing APs may also partially compensate for low density of Ca\(^{2+}\) channels early in development (Lorenzon and Foehring 1995b) or inactivation of Ca\(^{2+}\) channels during prolonged spiking.

When spike rate of rise was slow, we observed an early current shoulder (Figs. 2 and 3). In cultured *Xenopus* nerve-muscle synapses, Ca\(^{2+}\) entering during a similar early peak activated Ca\(^{2+}\)-dependent K\(^{+}\) currents (BK-type channels) (Yazejian et al. 1997), which repolarized the membrane potential and regulated spike width. In pyramidal cells, Ca\(^{2+}\)-dependent K\(^{+}\) currents do not contribute to the repolarization of an AP (Kang et al. 2000; Pineda et al. 1998; Schwindt et al. 1988).

**PHARMACOLOGY.** We found that HVA Ca\(^{2+}\) current types contribute a similar percentage to the whole cell current when evoked by either a voltage step or an APW. Furthermore we found no significant differences on average between the properties of APW-evoked currents for the five HVA currents, suggesting that the biophysical differences found with step protocols are too slight to be detected with short stimuli. In contrast, in cultured hippocampal pyramidal cells, L-type current comprises a much higher proportion of currents in response to voltage steps or EPSP-like waveforms versus APWs (underlying greater activation of CREB phosphorylation by EPSPs vs. APs) (Mermelstein et al. 2000). In those cells, (unlike neocortical pyramidal neurons) (Lorenzon and Foehring 1995a), L-type currents activate more slowly and at more negative potentials than other HVA current types (Mermelstein et al. 2000).

**CHARGE CARRIER.** Previous studies using voltage steps and Ba\(^{2+}\) found little T-type current in acutely dissociated neocortical pyramidal cells (Lorenzon and Foehring 1995a,b; Ye and Akaikhe 1993; but see Sayer et al. 1990). With Ca\(^{2+}\) as the charge carrier, we found that T-type current contributed to a prolonged tail current after APWs. This finding is consistent.
with data from dorsal root ganglion (DRG) neurons (McCobb and Beam 1991; Scroggs and Fox 1992), where T-type current contributed more than expected to the whole cell current elicited by an APW and caused prolonged decay of the current. In neocortical pyramidal cells, T-type current activation is first detected at about −65 mV (in the subthreshold range for Na⁺ spikes). T-type currents can be completely inactivated by holding the cell at potentials depolarized to about −70 mV (Sayer et al. 1990). Therefore T-type current would contribute to subthreshold inward current when action potentials depolarize the membrane from the down state, but not from the up state.

SPike Trains. Pyramidal cells in awake animals in vivo exhibit highly irregular low firing rates (<10 Hz) which are increased to 30–50 Hz with sensory stimulation (Mountcastle et al. 1969; Simons et al. 1992). In response to sensory input, they often fire at rates of >100 Hz for prolonged periods (>1 s) in awake behaving rats (Burne et al. 1984) and can sustain firing rates of 300 Hz in monkeys (Cheney and Fetz 1980; Knierim and Van Essen 1992; Newsome et al. 1989). We examined the effects of spike trains at 50–200 Hz.

Unlike chick DRG cells (Park and Dunlap 1998), striatal medium spiny cells (Song and Surmeier 1996), pituitary terminals (Jackson et al. 1991), or α1A and α1B calcium channel subunits in expression systems (Brody et al. 1999; Patil et al. 1999), we did not observe facilitation of Ba²⁺ or Ca²⁺ current by multiple spikes at physiological rates (±200 Hz). These data are consistent with the low level of facilitation seen with voltage steps in these cells (Foehring 1996; Stewart et al. 1999) and may reflect a low level of tonic G-protein inhibition in these cells (Bean 1989; Ikeda 1991).

We also found little evidence for voltage-dependent inactivation in most cells, even in response to sustained high-frequency stimuli (16 spikes at 200 Hz), although a few cells do show greater current decay (Fig. 6). This differs from other cell types, which show pronounced Ba²⁺ current decay during spike trains (e.g., Brody et al. 1997; Park and Dunlap 1998), and is consistent with the modest inactivation seen with long current steps in neocortex (Foehring et al. 2000; Lorenzon and Foehring 1999a). As a result of minimal facilitation and inactivation, charge entry increased linearly with the number of spikes in pyramidal cells, and this relationship was not altered by the frequency of the stimulus. Interestingly, in vitro studies of neocortical pyramidal neurons indicate that proximal dendritic [Ca²⁺]i (steady-state) is linearly related to firing frequency (up to ~30 Hz) (Helmchen et al. 1996). This has been proposed to be an indicator of firing rate for the cell even during interspike intervals (Helmchen et al. 1996; Wang 1996). Real spike trains in vivo may show changes in spike width and rates of polarization and repolarization during the train. The linear relationship between spike number and total charge entry would not hold under such conditions.

Furthermore, we found that significant Ca²⁺-dependent decay of the current accrued in response to multiple spikes. Ca²⁺-dependent inactivation may therefore shape Ca²⁺-mediated events in response to repeated activity. It is not known which Ca²⁺ current types display Ca²⁺-dependent inactivation in neocortical pyramidal cells. L- and P-type Ca²⁺ currents are reported to show Ca²⁺-dependent inactivation in various cell types (de Leon et al. 1995; Forsythe et al. 1998; Imredy and Yue 1994). Alternatively, other Ca²⁺-dependent processes (e.g., intracellular Ca²⁺ accumulation or activation of Ca²⁺-dependent outward current) could contribute to the increased current decay in Ca²⁺.

Modulation. Voltage-dependent and -independent modulation of Ca²⁺ currents has been observed in many cell types (Jones and Elmslie 1997). Activation of 5HT1A and M3 receptors leads to reduction in N- and P-type currents in neocortical pyramidal cells (Foehring 1996; Stewart et al. 1999). We found that peak modulation was greater for APW- versus step-evoked currents when kinetic slowing was evident (high doses of 8-OH-DPAT). If slowing did not occur, peak modulation was similar for each protocol. These data are similar to findings in dorsal raphe and sympathetic neurons (Pennington et al. 1992; Toth and Miller 1995). A recent study on chick ciliary ganglion (Artim and Meriney 2000) reported no change in the kinetics of APW-induced Ca²⁺ currents loaded with GTPγS, suggesting that modulated channels are not activated during single APWs.

In neocortical pyramidal cells, the K⁺ current responsible for the sAHP is activated by Ca²⁺ entering through N-type and P-type channels (Pineda et al. 1998), but activation of 5HT1A or M3 receptors does not reduce the sAHP (Araneda and Andrade 1991; Dutar and Nicoll 1988). This paradox could be explained by our finding that a high-frequency train of APs, such as that necessary to generate the sAHP, would reverse voltage-dependent modulations occurring via these receptors. The voltage-independent block of L-type currents (which do not couple to the AHPs) (Pineda et al. 1998) would remain under these conditions, leading to a decrease in firing (Pineda et al. 1998) (and possibly increased CREB phosphorylation) (Merz and Kandel 2000). The voltage-dependent modulations would only be effective at lower rates of firing.

Conclusion. We found that several parameters of APWs influence Ca²⁺ entry in neocortical pyramidal cells. With Ba²⁺ as the charge carrier, there was no evidence for differential involvement of Ca²⁺ channel subtypes in the AP or for current facilitation or inactivation. With Ca²⁺ as charge carrier, T-type current was unmasked and current decay was increased, suggesting a possible role for Ca²⁺-dependent inactivation. The magnitude of membrane-delimited modulations mediated by 5HT1A or M3 receptors were greater for AP-induced currents (vs. peak currents in steps) if activation slowing was evident. APWs were efficient in reversing these voltage-dependent pathways (see also Brody et al. 1997; Park and Dunlap 1998; Williams et al. 1997) but not the voltage-independent M1 pathway, suggesting that neuronal activity can selectively alter the effectiveness of different modulatory pathways.

In synaptic terminals, G-protein modulation of Ca²⁺ channels is thought to be a major mechanism underlying presynaptic inhibition (Wu and Saggau 1997). Even small changes in spike parameters (e.g., width, amplitude) would cause large changes in neurotransmitter release (Sabatini and Regehr 1997) because of the power relationship between Ca²⁺ entry and release (Dodge and Rahamimoff 1967). Variable relief of this modulation by spike trains may underlie some forms of synaptic facilitation (Brody and Yue 2000). If similar Ca²⁺ channels are expressed in synaptic terminals of pyramidal neurons (Choi and Lovoeger 1996; Wheeler et al. 1996) as we have observed in soma/proximal dendrites, our findings would also
have implications for transmitter release. Calcium channel inactivation would seem to play little role for single APs and a limited role during repetitive activity. Likewise, facilitation appears unimportant unless transmitters activate G-protein 

modulation of Ca2+ channels. Finally, reduction of transmitter release by voltage-dependent modulatory pathways would only be effective at low rates of activity.

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REFERENCES


