Ca²⁺- and Voltage-Dependent Inactivation of Ca²⁺ Channels in Nerve Terminals of the Neurohypophysis

Janet L. Branchaw, Matthew I. Banks, and Meyer B. Jackson

Department of Physiology, University of Wisconsin Medical School, Madison, Wisconsin 53706

Ca²⁺ channel inactivation was investigated in neurohypophyseal nerve terminals by using patch-clamp techniques. The contribution of intracellular Ca²⁺ to inactivation was evaluated by replacing Ca²⁺ with Ba²⁺ or by including BAPTA in the internal recording solution. Ca²⁺ channel inactivation during depolarizing pulses was primarily voltage-dependent. A contribution of intracellular Ca²⁺ was revealed by comparing steady-state inactivation of Ca²⁺ channels with Ca²⁺ current and with intracellular [Ca²⁺]. However, this contribution was small compared to that of voltage. In contrast to voltage-gated Ca²⁺ channels in other preparations, in the neurohypophysis Ba²⁺ substitution or intracellular BAPTA increased the speed of inactivation while reducing the steady-state level of inactivation. Ca²⁺ channel recovery from inactivation was studied by using a paired-pulse protocol. The rate of Ca²⁺ channel recovery from inactivation at negative potentials was increased dramatically by Ba²⁺ substitution or intracellular BAPTA, indicating

that intracellular Ca²⁺ inhibits recovery. Stimulation with trains of brief pulses designed to mimic physiological bursts of electrical activity showed that Ca²⁺ channel inactivation was much greater with 20 Hz trains than with 14 Hz trains. Inactivation induced by 20 Hz trains was reduced by intracellular BAPTA, suggesting an important role for Ca²⁺-dependent inactivation during physiologically relevant forms of electrical activity. Inhibitors of calmodulin and calcineurin had no effect on Ca²⁺ channel inactivation, arguing against a mechanism of inactivation involving these Ca²⁺-dependent proteins. The inactivation behavior described here, in which voltage effects on Ca²⁺ channel inactivation predominate at positive potentials and Ca²⁺ effects predominate at negative potentials, may be relevant to the regulation of neuropeptide release.

Key words: posterior pituitary; Ca²⁺ channels; neurosecretion; oxytocin; vasopressin; fura-2; frequency-dependent depression; synaptic plasticity

Inactivation of Ca²⁺ channels can limit the increases in intracellular Ca²⁺ concentration ([Ca²⁺]_i) produced by electrical stimulation, thereby limiting Ca²⁺-triggered processes such as exocytosis. A single action potential is rarely long enough to produce significant amounts of Ca²⁺ channel inactivation. Thus, Ca²⁺ channel inactivation is more likely to influence secretion from cells in which repetitive electrical activity plays an important role. Neurohypophyseal nerve terminals secrete oxytocin and vasopressin in response to bursts of action potentials (Dreifuss et al., 1971; Dutton and Dyball, 1979; Gainer et al., 1986) and show fatigue of secretion in response to sustained high-frequency stimulation (Bicknell et al., 1984; Gainer et al., 1986; Hobbach et al., 1988). Because these nerve terminals possess an inactivating Ca²⁺ current (Lemos and Nowicky, 1989; X. Wang et al., 1992), the neurohypophysis is an ideal system for investigating the role of Ca²⁺ channel inactivation in secretion.

Two general mechanisms of Ca²⁺ channel inactivation have been described, one depending on voltage and the other depending on Ca²⁺ (Chad and Eckert, 1984; Chad, 1989). Repetitive activity can engage either of these mechanisms: depolarization during bursts of action potentials can initiate voltage-dependent

inactivation, and Ca²⁺ entry can initiate Ca²⁺-dependent inactivation. The connection between these mechanisms and Ca²⁺ channel inactivation has yet to be established in a nerve terminal in which repetitive electrical activity triggers exocytosis. To address these issues, we have used voltage-clamp techniques and fluorometric [Ca²⁺]_i measurement to investigate Ca²⁺ channel inactivation in nerve terminals of the neurohypophysis. The results of these experiments suggest that Ca²⁺ channel inactivation occurs during physiological bursts of action potentials and that Ca²⁺ entry plays an important role by inhibiting the recovery of Ca²⁺ channels from inactivation. This feedback of intracellular Ca²⁺ on Ca²⁺ channel function may limit Ca²⁺ entry either to depress secretion or to protect nerve terminals from damage caused by excess intracellular Ca²⁺.

A preliminary account of this work has been presented in abstract form (Branchaw and Jackson, 1996).

MATERIALS AND METHODS

Slice preparation. Male rats weighing 240–300 gm were decapitated after CO₂-induced narcosis. The neurointermediate lobe of the pituitary was isolated and placed in ice-chilled 95% O₂/5% CO₂-saturated artificial cerebrospinal fluid (aCSF) containing (in mM): 125 NaCl, 4 KCl, 26 NaHCO₃, 1.25 NaH₂PO₄, 2 CaCl₂, 1 MgCl₂, and 10 glucose. Slices with a thickness of 70 μm were cut as described previously (Jackson et al., 1991; Jackson, 1993) in ice-cold aCSF with a vibratome. After cutting, slices either were stored in aCSF or were transferred to a recording chamber to be used immediately. Storage and recording were at room temperature (20–24°C). Slices remained viable for 2–3 hr.

Patch-clamp electrophysiology. Nerve terminals were identified with a Nomarski microscope on the slice surface as round structures of up to 15 μm in diameter (Jackson, 1993). Previous studies have demonstrated properties diagnostic of nerve terminals such as action potentials (Jackson et al., 1991), appended axons (Jackson, 1993), depolarization-

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Correspondence should be addressed to Dr. Meyer Jackson, Department of Physiology, SMI 129, University of Wisconsin Medical School, 1300 University Avenue, Madison WI 53706.

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induced increases in $[\text{Ca}^{2+}]$ (Jackson et al., 1991), and depolarization-induced increases in capacitance (Hsu and Jackson, 1996). These structures were voltage-clamped by the tight-seal patch-clamp technique (Hamill et al., 1981). Whole-terminal recordings were made with an Axopatch 200 patch-clamp amplifier (Axon Instruments, Foster City, CA). For recording Ca^{2+} current, the slices were superfused with a bathing solution consisting of (in mM): 121 NaCl, 4 CsCl, 20 TEA-Cl, 10 HEPES, 5 CaCl_2 , 1 MgCl_2 , 10 glucose, 2 4-aminopyridine, and 1 μM tetrodotoxin, titrated to pH 7.4 with NaOH. The patch pipette solution consisted of (in mM): 130 CsCl, 10 NaCl, 10 TEA-Cl, 10 HEPES, 4 MgATP, and 0.3 Na_2GTP , titrated to pH 7.2 with CsOH. No Ca^{2+} buffer other than 0.1 mM fura-2 was added to the pipette solution to minimize disturbance of Ca^{2+} -dependent processes. The decision to remove exogenous Ca^{2+} buffer from the control solution was made after a systematic study of $[\text{Ca}^{2+}]_i$ was conducted with fura-2 and different EGTA concentrations. This study showed that even 0.2 mM EGTA added to the patch pipette filling solution produced noticeable alterations in the time course of $[\text{Ca}^{2+}]_i$. To test the role of intracellular Ca^{2+} in Ca^{2+} channel inactivation, we either replaced CaCl_2 in the bathing solution with BaCl_2 to provide a permeant ion that substitutes poorly in many Ca^{2+} -dependent cellular processes, or included 10 mM BAPTA in the patch pipette solution to prevent rises in $[\text{Ca}^{2+}]_i$. The addition of BAPTA was compensated by reducing the CsCl concentration in the patch pipette solution to 120 mM.

Current signals were filtered at 5 kHz with a low-pass Bessel filter and read into a personal computer through an interface (Axon Instruments). Voltage stimulation and data acquisition were controlled by computer with the program pCLAMP v6.0 (Axon Instruments). Tight-seal whole-terminal recordings were made with SYLGARD-coated patch electrodes fabricated from thin-walled aluminosilicate glass (inner diameter, 1.15 mm; outer diameter, 1.5 mm). Electrode resistances ranged from 1.5 to 4 M Ω . The electrode capacitance artifact, whole-terminal capacitance transient, and series resistance were compensated with the patch-clamp amplifier circuitry. Whole-terminal capacitance ranged from 3 to 10 pF. Series resistance compensation ranged from 65 to 90%. Only recordings with uncompensated series resistances <15 M Ω were included in data analysis. Leak currents were subtracted by the P/4 procedure. Because these terminals often had long axonal processes attached to them, inadequate space clamp was sometimes a problem. Current recordings showing abrupt changes with delays of several milliseconds were interpreted as signs of inadequate space clamp and were not used for analysis. The cable properties of processes in this preparation are discussed in detail elsewhere (Jackson, 1993; Hsu and Jackson, 1996).

Calcium measurements. Intracellular Ca^{2+} concentration was measured in single nerve terminals with the fluorescent Ca^{2+} indicator fura-2 (Grynkiewicz et al., 1985). Membrane-impermeant fura-2 pentapotassium salt (100 μM) was added to the patch pipette filling solution from which it diffused into terminals during whole-terminal recordings (Jackson et al., 1991). A dual-wavelength excitation system (Photon Technology International, South Brunswick, NJ) was used to measure fura-2 fluorescence at the excitation wavelengths of 358 and 380 nm. The choice of wavelengths was based on an examination of excitation spectra obtained from single nerve terminals loaded with calibration solutions (see composition below). The fura-2 fluorescence at 358 nm changed very little with changes in $[\text{Ca}^{2+}]_i$, indicating that this wavelength is close to the isosbestic point of the dye within a nerve terminal. As a result, we could reduce the noise by using the average of the fluorescence signal recorded at 358 nm in the fluorescence ratio, $R = F_{380}/F_{358}$. Then $[\text{Ca}^{2+}]_i$ was calculated from the standard expression (Grynkiewicz et al., 1985).

$$[\text{Ca}^{2+}]_i = K_{\text{eff}} \cdot [(R - R_{\text{min}})/(R_{\text{max}} - R)]. \quad (1)$$

The calibration parameters were determined as $K_{\text{eff}} = 2.87 \mu\text{M}$, $R_{\text{min}} = 0.652$, and $R_{\text{max}} = 14.9$, using fluorescence ratios measured *in situ* with patch pipette calibration solutions (Neher, 1989). The compositions of these calibration solutions were (in mM): 120 CsCl, 10 NaCl, 10 TEA-Cl, 10 HEPES, 4 MgATP, and 0.3 Na_2GTP , titrated to pH 7.2 with CsOH. R_{min} was determined by using this solution with the addition of 10 mM EGTA and no CaCl_2 and with CsCl reduced to 110 mM. R_{max} was determined by using this solution without adding EGTA and with 1 mM CaCl_2 . A solution of intermediate free $[\text{Ca}^{2+}]$ was made with 10 mM EGTA and 7.84 mM CaCl_2 . The free $[\text{Ca}^{2+}]$ was calculated as 0.39 μM (Marks and Maxfield, 1991), and the R measurement with this solution enabled us to calculate K_{eff} .

Data analysis. Data were analyzed by the computer programs

pCLAMP and Origin (MicroCal, Northampton, MA). The time course of Ca^{2+} current inactivation, Ca^{2+} current recovery, and $[\text{Ca}^{2+}]_i$ were fit to a double exponential function of the form:

$$A_0 + A_{\text{fast}} \exp(-(t - t_0)/\tau_{\text{fast}}) + A_{\text{slow}} \exp(-(t - t_0)/\tau_{\text{slow}}). \quad (2)$$

The parameter t_0 was set as the time of peak current or peak $[\text{Ca}^{2+}]_i$. Steady-state inactivation curves were fit to a Boltzmann equation of the form:

$$I = I_2 + (I_1 - I_2)/(1 + \exp((V - V_{1/2})/k)). \quad (3)$$

Error bars in all figures represent SE. The Student's t test was used to determine the statistical significance of different means. Two-way ANOVA was performed by the computer program SigmaStat (Jandel, Corte Madera, CA).

RESULTS

Calcium current inactivation

Calcium currents were measured in response to single 500 msec pulses or to trains of 2 msec pulses. Currents elicited by a 500 msec pulse from -100 to 10 mV appeared to inactivate in two phases (Fig. 1A). Double exponential fits to the decay of these Ca^{2+} currents yielded the following parameters: $\tau_1 = 33.7 \pm 5.5$ msec, $\tau_2 = 365 \pm 12$ msec, and $I_{\text{ss}}/I_{\text{peak}} = 0.023 \pm 0.005$ ($n = 16$). Ca^{2+} currents also were elicited by physiological trains of 2 msec pulses from -100 to 50 mV designed to mimic bursts of action potentials (Jackson et al., 1991). Trains were applied at frequencies of 14 and 20 Hz, because these frequencies are relevant to neuropeptide secretion from the neurohypophysis (Poulain and Wakerley, 1982). As a 14 Hz train progressed, peak current activated by each 2 msec pulse was less than that activated by the preceding pulse. Plotting peak current versus time showed a monoexponential decay (Fig. 1B; $\tau_1 = 6.4 \pm 0.5$ sec; $n = 6$). After 15 sec the current was 15% below the current activated by the first pulse of the train. Ca^{2+} channel inactivation was stronger with 20 Hz trains, declining with a biexponential time course ($\tau_1 = 0.96 \pm 0.42$ sec and $\tau_2 = 5.8 \pm 1.3$ sec; $n = 11$) to 40% of the initial current after 15 sec. Thus, at the higher frequency a second, rapid component of inactivation became evident. The greater Ca^{2+} channel inactivation seen with 20 Hz trains may play a role in determining the optimal frequency for neuropeptide secretion. Further, the increase in Ca^{2+} channel inactivation with train frequency may be relevant to the fatigue of secretion, which is more pronounced at higher stimulation frequencies (Bicknell et al., 1984; Gainer et al., 1986; Hobbach et al., 1988).

Voltage- and Ca^{2+} -dependent inactivation

To evaluate the contribution made by Ca^{2+} to the inactivation of Ca^{2+} channels, we replaced Ca^{2+} in the bathing solution by Ba^{2+} , or we added 10 mM BAPTA to the patch pipette solution (see Materials and Methods). Normalized currents elicited by pulses from -100 to 10 mV for 500 msec are shown for each of these conditions in Figure 2A, with the Ca^{2+} current trace from Figure 1A included for comparison. Figure 2C shows representative normalized current-voltage (I - V) plots for control Ca^{2+} currents, Ba^{2+} currents, and Ca^{2+} currents with intracellular BAPTA. With Ba^{2+} as the charge carrier, the Ca^{2+} channel I - V relationship was shifted 20 to 30 mV to the left (more negative), as described previously in dissociated neurohypophysial nerve terminals (X. Wang et al., 1992).

The kinetics of inactivation of the Ca^{2+} currents shown in Figure 2A appear to be altered both by Ba^{2+} substitution and by intracellular BAPTA. The current decays were fit to a double exponential function (see Materials and Methods), and the re-

Figure 1. Calcium current inactivation. *A*, Calcium current recorded in response to a 500 msec pulse from -100 to 10 mV. The decay shown was fit with a biexponential function with the following parameters: $\tau_{\text{fast}} = 35$ msec, $\tau_{\text{slow}} = 126$ msec, $A_{\text{fast}} = -400$ pA, $A_{\text{slow}} = -336$ pA, and $A_0 = -32$ pA (see Materials and Methods, Eq. 2). *B*, Ca^{2+} currents were evoked by trains of 2 msec pulses from -100 to 50 mV. Peak currents measured during trains were normalized to the first current response, averaged, and plotted versus time. Error bars are plotted at 1 sec intervals to avoid obscuring the data. Inactivation was frequency-dependent and decayed monoexponentially at 14 Hz ($\tau_1 = 6.45$ sec; $n = 6$) and biexponentially at 20 Hz ($\tau_1 = 0.96$ and $\tau_2 = 5.79$ sec; $n = 11$). The fitted exponential functions were drawn in both *A* and *B*, but they are nearly concealed by the data.

sults of these fits showed that the change in inactivation kinetics results from a shortening of the time constants of both the fast and slow components of inactivation (Fig. 2*B*). The relative proportions of the two components of inactivation showed no significant differences among controls, Ba^{2+} substitution, and intracellular BAPTA (data not shown). Figure 2*B* shows the time constants for decay at 0 mV, a voltage at which Ca^{2+} current was near maximal. Inactivation kinetics also was analyzed over five voltage points in the range -20 to 20 mV, and a two-way ANOVA showed a statistically significant interaction between solution and voltage for τ_{slow} ($p = 0.002$) and for the ratio of final to peak current ($p = 0.024$; for control, $n = 16$; for Ba^{2+} , $n = 12$; for BAPTA, $n = 11$). More data will be presented below showing a decrease in steady-state inactivation of Ca^{2+} channels resulting from Ba^{2+} substitution and intracellular BAPTA.

It is interesting that the time constants for current inactivation were faster with Ba^{2+} substitution and intracellular BAPTA when compared with Ca^{2+} (Fig. 2*B*). Ca^{2+} channels in other

Figure 2. Current through Ca^{2+} channels with Ba^{2+} substitution and intracellular BAPTA. *A*, Extracellular Ca^{2+} was replaced by Ba^{2+} (left), or 10 mM BAPTA was included in the patch pipette filling solution (right). Current was activated by 500 msec pulses from -100 to 10 mV, as in Figure 1. Current traces were normalized to their peak values and displayed together with normalized control Ca^{2+} current from Figure 1. Inactivation was quantified by fitting the decay of the current to a sum of exponentials. This yielded the following parameters for the traces shown: $\tau_{\text{fast}} = 26$ msec, $\tau_{\text{slow}} = 144$ msec, $A_{\text{fast}} = -192$ pA, $A_{\text{slow}} = -247$ pA, and $A_0 = -91$ pA for Ba^{2+} substitution; $\tau_{\text{fast}} = 38$ msec, $\tau_{\text{slow}} = 152$ msec, $A_{\text{fast}} = -397$ pA, $A_{\text{slow}} = -171$ pA, and $A_0 = -29$ pA for intracellular BAPTA (see Materials and Methods, Eq. 2). *B*, Time constants for inactivation of Ca^{2+} channels are shown for pulses to 0 mV. The values for Ba^{2+} substitution and intracellular BAPTA differ significantly from controls ($p < 0.01$ for each pairwise comparison). For controls, $n = 16$; for Ba^{2+} , $n = 12$; for BAPTA, $n = 11$. *C*, Plots of peak current versus voltage for control Ca^{2+} current (■), Ba^{2+} current through Ca^{2+} channels (●), and Ca^{2+} current with intracellular BAPTA (▲).

preparations inactivate more slowly under these conditions (Chad and Eckert, 1984; Chad, 1989), and the increased speed of inactivation that we saw was the opposite of what one would expect for inactivation caused by intracellular Ca^{2+} . Because additional results are presented below that are consistent with Ca^{2+} -dependent inactivation, it is important to address this matter before proceeding. The situation can be clarified with the aid of a simple two-state model in which Ca^{2+} channels interconvert between an open (O) and inactivated (I) state with a forward rate constant, α , and a reverse rate constant, β .

$$\text{O} \xrightleftharpoons[\beta]{\alpha} \text{I} \quad (4)$$

For this model the time constant of inactivation and the steady-state fraction of inactivated channels are given by the expressions $1/(\alpha + \beta)$ and $\alpha/(\alpha + \beta)$, respectively. The more rapid time

constant and lower level of steady-state inactivation observed here can be explained by Ca^{2+} inhibition of both the forward and reverse rates, with a greater effect on the reverse rate. This interpretation is supported by the results presented below showing a strong inhibitory effect of Ca^{2+} on recovery from inactivation (β) at negative potentials (see Figs. 6, 7). Inhibition of recovery from inactivation by Ca^{2+} has been seen in a number of other preparations (Brehm et al., 1980; Yatani et al., 1983; Gutnick et al., 1989). More detailed kinetic studies of Ca^{2+} channel inactivation have been undertaken by others to explain a large body of data (Gutnick et al., 1989; Mazzanti et al., 1991; Fryer and Zucker, 1993; Imredy and Yue, 1994). The comparatively simple two-state analysis used here is presented solely to clarify two apparently conflicting results of increased rate of inactivation and reduced steady-state level of inactivation.

Steady-state inactivation

The contributions of voltage and Ca^{2+} to steady-state inactivation also were investigated by using a paired-pulse protocol to generate steady-state inactivation curves (Fig. 3). Long prepulses (500 msec) to different voltages were applied to allow inactivation to reach a steady state. Then the voltage was returned to a holding potential of -100 mV for 5 msec to close channels, after which a test pulse to 10 mV for 100 msec was applied to assess the state of inactivation of the channels. The peak current measured in response to this test pulse was normalized to the peak current of an identical control pulse given at least 1 min before the paired pulse protocol (Fig. 3A). Inactivation increased sharply as the prepulse potential increased from -60 to -20 mV, reaching a maximum of 93% at $+10$ mV (Fig. 3B). The data from -120 to 10 mV were fit to a Boltzmann function (see legend, Fig. 3B), and this curve was extended to 70 mV to emphasize the upward slope of the data at more positive voltages. For Ca^{2+} -dependent inactivation, an approximately U-shaped inactivation curve is expected, because as the prepulse voltage becomes more positive, Ca^{2+} influx first increases and then decreases (Fig. 2C). The data in Figure 3B indicate a relatively weak component of Ca^{2+} -dependent inactivation. Inactivation decreased from 93% at 10 mV to $\sim 65\%$ at 70 mV. The data above 10 mV were fit to a line with a slope of $0.0040 \pm 0.0003 \text{ mV}^{-1}$.

The possibility that positive prepulses may facilitate Ca^{2+} current (Hoshi et al., 1984) also was examined. When we used short prepulses of 25 msec to minimize inactivation, varied the prepulse potential from -40 to 100 mV, and varied the interpulse interval from 20 to 100 msec, we saw no evidence for facilitation of Ca^{2+} current in these nerve terminals (data not shown). Thus, the modest reduction in inactivation with increasing prepulse potential above 10 mV cannot be attributed to facilitation counteracting the effect of inactivation.

To determine whether the decline in steady-state inactivation at positive potentials in Figure 3B was accompanied by reduced Ca^{2+} entry, we made simultaneous measurements of Ca^{2+} current and $[\text{Ca}^{2+}]_i$, using the Ca^{2+} -sensitive fluorescent dye fura-2. Figure 4A shows representative changes in $[\text{Ca}^{2+}]_i$ evoked by 500 msec prepulses to -50 mV (a), 10 mV (b), and 50 mV (c), each of which was followed by a 100 msec test pulse to 10 mV to assess the state of the Ca^{2+} channels (as in Fig. 3). Note that in these experiments $[\text{Ca}^{2+}]_i$ rose as high as $1.5 \mu\text{M}$, which is considerably higher than that seen in previous studies (Stuenkel, 1990, 1994; Jackson et al., 1991). This difference reflects the lower amount of exogenous chelator used in the present experiments.

If intracellular Ca^{2+} contributes to Ca^{2+} channel inactivation,

Figure 3. Steady-state inactivation. *A*, The current traces shown were recorded by using the steady-state inactivation protocol indicated schematically by the inset. The prepulses were -50 mV (a), -10 mV (b), and 50 mV (c). Control pulses were similar each time and therefore are not labeled. Note the decrease in inactivation with a prepulse of 50 mV (c) when compared with a prepulse of -10 mV (b). *B*, Peak test pulse currents were normalized to peak control pulse currents and plotted against the prepulse voltage ($n = 21$). Points from -120 to 10 mV were fit to a Boltzmann equation ($I_1 = 0.98$, $I_2 = 0.07$, $V_{1/2} = -30.5$ mV, and $k = 12.1$ mV; see Materials and Methods, Eq. 3), and points from 10 to 70 mV were fit to a line (slope = $0.0040 \pm 0.0003 \text{ mV}^{-1}$). Inactivation decreased at prepulse potentials more positive than 10 mV.

an inverse relationship would be expected between $[\text{Ca}^{2+}]_i$ at the start of the test pulse and the noninactivated Ca^{2+} current evoked by the test pulse. Pooled data from seven nerve terminals show a correlation (Fig. 4B), but the decrease in $[\text{Ca}^{2+}]_i$ at voltages more positive than 10 mV is much greater than the decrease in inactivation. To explain this behavior entirely in terms of Ca^{2+} -dependent inactivation would require a very steep functional dependence. Further support for inactivation attributable primarily to voltage rather than to Ca^{2+} is provided by the result with a prepulse voltage of -30 mV, for which there is strong inactivation ($\sim 50\%$) with almost no increase in $[\text{Ca}^{2+}]_i$. Thus, Figure 4B suggests that positive voltages can inactivate Ca^{2+} channels without raising $[\text{Ca}^{2+}]_i$.

As an additional test of the Ca^{2+} dependence of steady-state inactivation of Ca^{2+} channels, the paired-pulse protocol was used with Ba^{2+} substitution and with intracellular BAPTA. The results are presented in Figure 5, with control data from Figure 3B

Figure 4. Steady-state inactivation is correlated with prepulse $[\text{Ca}^{2+}]_i$. *A*, $[\text{Ca}^{2+}]_i$ was measured during the steady-state inactivation protocol illustrated in Figure 3. Prepulse steps were given at $t = 0.5$ sec for 500 msec to the following voltages: -50 mV (*a*), 10 mV (*b*), and 50 mV (*c*). The arrow at the top indicates the $[\text{Ca}^{2+}]_i$ level at the end of the prepulse that is plotted in *B*. With no added Ca^{2+} buffer (other than 0.1 mM fura-2), resting $[\text{Ca}^{2+}]_i$ was 0.35 ± 0.04 μM ($n = 7$). *B*, Steady-state inactivation and $[\text{Ca}^{2+}]_i$ at the end of the prepulse (shown in *A*) are plotted as a function of prepulse voltage ($n = 7$). The fraction of noninactivated current and $[\text{Ca}^{2+}]_i$ is inversely related.

reproduced for comparison. These experiments showed that the Ca^{2+} -dependent component of steady-state inactivation, represented by the upward slope above 10 mV, was reduced but not eliminated by either Ba^{2+} substitution (Fig. 5*A*) or intracellular BAPTA (Fig. 5*B*). The slopes between 10 and 70 mV of 0.0030 ± 0.0011 mV^{-1} for Ba^{2+} and 0.0010 ± 0.0004 mV^{-1} for BAPTA were significantly different from zero. It is unclear whether Ba^{2+} influences this slope, but the slope in BAPTA is significantly less than the control value of 0.004 mV^{-1} . Although these results are consistent with our interpretation of the upward slope in steady-state inactivation in terms of Ca^{2+} (Fig. 3*B*), they suggest that neither Ba^{2+} nor BAPTA is completely effective in blocking this effect. Intracellular BAPTA had little effect on steady-state inactivation at prepulse potentials below 10 mV (Fig. 5*B*). However, substitution of Ca^{2+} by Ba^{2+} reduced the maximum level of inactivation at 10 mV from 93 to 81% and shifted $V_{1/2}$ by -11 mV (see legend for parameter values from Boltzmann fit, Fig. 5*A*). The decrease in maximum inactivation is consistent with a reduction in the Ca^{2+} -dependent component of inactivation. The leftward shift in $V_{1/2}$ may be related to the shift in the I - V relationship produced by Ba^{2+} (Fig. 2*C*).

Figure 5. Dependence of steady-state inactivation on $[\text{Ca}^{2+}]_i$. Steady-state inactivation of Ca^{2+} current was studied with the paired-pulse protocol shown in Figure 3, using Ba^{2+} substitution (*A*) and intracellular BAPTA (*B*). Points from -120 to -10 mV for Ba^{2+} and -110 to 10 mV for BAPTA were fit to a Boltzmann equation (see Materials and Methods, Eq. 3) to obtain the following parameters: for Ba^{2+} , $I_1 = 1.01$, $I_2 = 0.19$, $V_{1/2} = -41.9$ mV, and $k = 10.0$ mV; for BAPTA, $I_1 = 0.98$, $I_2 = 0.11$, $V_{1/2} = -27.4$ mV, and $k = 10.8$ mV (see Materials and Methods, Eq. 3). The curves based on these fits were extended to 70 mV to emphasize the upward slope at positive potentials. Points from 10 to 70 mV were fit to a line (see text for slopes). Control data from Figure 3*B* with 5 mM Ca^{2+} as the charge carrier are reproduced for comparison.

Ca^{2+} inhibition of recovery from inactivation

The results described above suggest relatively weak effects of intracellular Ca^{2+} on the rate and extent of inactivation at positive potentials. Recovery from inactivation is another process that can be influenced by Ca^{2+} (Brehm et al., 1980; Yatani et al., 1983; Gutnick et al., 1989). To study recovery, we inactivated most of the Ca^{2+} current with a 300 msec pulse to 10 mV, and after a variable recovery period at the holding potential of -100 mV, we applied a second 100 msec pulse to 10 mV to assess recovery. Results with control solutions, Ba^{2+} substitution, and

Figure 6. Recovery of Ca^{2+} current from inactivation. Ca^{2+} current was inactivated with 500 msec pulses from -100 to 10 mV. Then recovery was examined with 100 msec test pulses applied at various time intervals after the end of the inactivating pulse. *A*, Ba^{2+} currents are shown, and in these traces the noise appears greater than it actually is because the four traces selected do not superimpose perfectly. *B*, Normalized peak current from traces such as those in *A* was plotted versus the interpulse interval to show the time course of recovery. The current at the end of the first inactivating pulse was subtracted from the peak current, and this difference was plotted versus interpulse interval. The best-fitting double-exponential functions were drawn through the data (see text for parameter values and n values). In these fits we imposed the constraint $A_{\text{fast}} + A_{\text{slow}} = -A_0$ and set $t_0 = 0$ (see Materials and Methods, Eq. 2). *C*, Data in *B* are replotted with an expanded time scale to show the rapid component of recovery more clearly.

intracellular BAPTA show that recovery from inactivation is faster in the Ba^{2+} and BAPTA solutions (Fig. 6), implying that recovery is inhibited by intracellular Ca^{2+} . These experiments also resolved recovery into at least three distinct kinetic phases, the first two of which were analyzed by fitting to a double exponential function. The third component was too slow to quantify easily but appeared to require >10 sec.

The data in Figure 6C suggest that the rapid component of recovery is strongly inhibited by intracellular Ca^{2+} and that intracellular BAPTA is more effective at removing this inhibition than Ba^{2+} substitution. The time constant of the rapid component decreased from 0.17 ± 0.03 sec in Ca^{2+} ($n = 18$) to 0.10 ± 0.01 sec with Ba^{2+} substitution ($n = 12$) and 0.073 ± 0.018 sec with intracellular BAPTA ($n = 9$). The weight of the rapid

Figure 7. Inverse correlation between recovery and $[\text{Ca}^{2+}]_i$. The time course of recovery of Ca^{2+} current from inactivation ($\tau = 1.25$ sec) was similar to that for recovery of $[\text{Ca}^{2+}]_i$ ($\tau = 2.20$ sec). Data were pooled from five nerve terminals. *Inset*, $[\text{Ca}^{2+}]_i$ measured during a 300 msec pulse from -100 to 10 mV. Arrows indicate the times at which the test pulses were applied to produce points in the plot.

component increased from 0.42 ± 0.07 with Ca^{2+} to 0.67 ± 0.05 with Ba^{2+} substitution and 0.86 ± 0.06 with intracellular BAPTA. The intermediate time constant of recovery was less affected by Ca^{2+} removal: $\tau_{\text{intermediate}} = 1.58 \pm 0.36$ sec in Ca^{2+} and 1.82 ± 0.69 sec with Ba^{2+} substitution. With intracellular BAPTA the value seemed to be similar, but the weight of this component was too small to permit a quantitative determination of the time constant. In summary, Ba^{2+} substitution and intracellular BAPTA accelerated recovery by increasing both the speed and the weight of the fast component. Intracellular Ca^{2+} thus seems to inhibit the recovery of Ca^{2+} channels from inactivation at negative potentials. Recall that Ca^{2+} inhibition of the recovery rate, β , was invoked earlier in our analysis of inactivation at positive potentials in terms of a two-state model (Eq. 4). Thus, it seems that intracellular Ca^{2+} inhibits the reverse process at both positive and negative potentials.

Insight into the relationship between intracellular Ca^{2+} and the recovery of Ca^{2+} current from inactivation can be gained by inspection of our fluorometric $[\text{Ca}^{2+}]_i$ measurements (Fig. 4). The return of $[\text{Ca}^{2+}]_i$ to baseline requires seconds and is clearly too slow to account for the fastest component of recovery of Ca^{2+} current. However, the intermediate component of recovery from inactivation appeared to have a similar rate. To compare these two processes further, we made simultaneous measurements of $[\text{Ca}^{2+}]_i$ and Ca^{2+} current, using pairs of pulses as in Figure 6. In this experiment interpulse intervals of 1–10 sec were used, so the rapid component of Ca^{2+} current recovery was complete at the start of the test pulse. Current recovery approximately mirrored the return of $[\text{Ca}^{2+}]_i$ to baseline (Fig. 7). The recovery of current and $[\text{Ca}^{2+}]_i$ each was fit to single exponentials, yielding comparable time constants of $\tau = 1.25 \pm 0.77$ and $\tau = 2.20 \pm 0.31$ sec, respectively ($p = 0.14$). (Note that the time constants here are similar to the intermediate time constant of recovery of Ca^{2+} current of 1.58 sec from Fig. 6.) Thus, the intermediate component of recovery of Ca^{2+} current from inactivation could reflect Ca^{2+} removal from the nerve terminal. This is consistent with the finding that, when Ca^{2+} rises were prevented by intracellular

Figure 8. Ca^{2+} dependence of inactivation during trains. *A*, Ca^{2+} current was evoked by a train of 2 msec pulses, as in Figure 1*B*, but with BAPTA included in the patch pipette solution. BAPTA reduced inactivation during a 20 Hz train to that seen with a 14 Hz train. Normalized peak Ca^{2+} currents were averaged and plotted versus time with error bars at 1 sec intervals. The time constants from fits of double exponential functions (see Materials and Methods, Eq. 2) were $\tau_1 = 0.69$ and $\tau_2 = 26.3$ sec at 14 Hz ($n = 4$), and $\tau_1 = 0.40$ and $\tau_2 = 24.7$ sec at 20 Hz ($n = 5$). *B*, $[\text{Ca}^{2+}]_i$ rises to a plateau during 14 and 20 Hz trains (with no intracellular BAPTA). The increases and decreases were both faster at 20 Hz. Baseline $[\text{Ca}^{2+}]_i$ was 300 nM in this terminal, and the plateau was 0.7 μM for both frequencies (see text for averages). The inset shows two selected $[\text{Ca}^{2+}]_i$ traces from different terminals on an expanded time scale to illustrate the different rates of rise for 14 and 20 Hz trains. The time constants in these two traces were 3.2 and 0.5 sec, respectively (see text for average time constants for both rises and falls in $[\text{Ca}^{2+}]_i$).

BAPTA (Fig. 6), the weight of the intermediate component was reduced from 0.58 to only 0.14.

Frequency dependence of Ca^{2+} -dependent inactivation

The data in Figure 1*B* showed that Ca^{2+} current inactivation during trains of brief pulses increased when the train frequency was increased from 14 to 20 Hz. To determine whether Ca^{2+} influences inactivation during trains, we performed experiments like those in Figure 1 by using intracellular BAPTA. Intracellular BAPTA rather than Ba^{2+} substitution was chosen because of its greater efficacy in accelerating recovery from inactivation (Fig. 6) and in removing the upward slope in steady-state inactivation above 10 mV (Fig. 5). These experiments showed that intracellular BAPTA reduced inactivation during 20 Hz trains (Fig. 8*A*).

In contrast to the control data in Figure 1, the time constants of inactivation with BAPTA in response to trains of stimulation were the same for 14 and 20 Hz. This suggests that the frequency-dependent component of inactivation is mediated by Ca^{2+} entry. The results above obtained with long-duration pulses indicated that at positive potentials intracellular BAPTA accelerated both inactivation and recovery, and at negative potentials intracellular BAPTA accelerated recovery. The reduction of Ca^{2+} channel inactivation by intracellular BAPTA during high-frequency trains, therefore, would appear primarily to be attributable to an acceleration of recovery of Ca^{2+} current from inactivation during the intervals between pulses.

If, as suggested by the data in Figure 8*A*, the greater inactivation of Ca^{2+} current with 20 Hz trains in Figure 1*B* is attributable to a greater rise in $[\text{Ca}^{2+}]_i$, we would expect that the $[\text{Ca}^{2+}]_i$ level reached during a 20 Hz train would be significantly higher than that reached during a 14 Hz train. However, we found no significant difference in the level of $[\text{Ca}^{2+}]_i$ reached during trains at these two frequencies (Fig. 8*B*). The increase in $[\text{Ca}^{2+}]_i$ was $0.46 \pm 0.06 \mu\text{M}$ at 14 Hz ($n = 5$) and $0.55 \pm 0.13 \mu\text{M}$ at 20 Hz ($n = 5$). Although the plateau level of $[\text{Ca}^{2+}]_i$ was not significantly different, the rate of rise and the rate of decay of $[\text{Ca}^{2+}]_i$ were faster during 20 Hz bursts. The comparison of the rates of rise can be seen in the inset of Figure 8*B*. At 20 Hz we found $\tau_{\text{rise}} = 0.62 \pm 0.08$ sec and $\tau_{\text{decay}} = 1.82 \pm 0.17$ sec ($n = 5$). At 14 Hz we found $\tau_{\text{rise}} = 1.92 \pm 0.41$ sec and $\tau_{\text{decay}} = 3.18 \pm 0.61$ sec ($n = 5$). The threefold increase in rate of rise in $[\text{Ca}^{2+}]_i$ during 20 Hz trains suggests that recovery from inactivation between pulses will be inhibited much earlier than during a 14 Hz train, and this is consistent with the faster time course of Ca^{2+} current inactivation seen during a 20 Hz train (Fig. 1*B*). It is still difficult to reconcile such a large change in Ca^{2+} -dependent Ca^{2+} current inactivation with such small differences in $[\text{Ca}^{2+}]_i$, and possible explanations are considered in Discussion.

Ca^{2+} -dependent enzymes

The Ca^{2+} -dependent protein phosphatase calcineurin (protein phosphatase 2B) has been proposed to mediate Ca^{2+} -dependent inactivation of Ca^{2+} channels by dephosphorylation of the channel or of a closely associated protein (Chad and Eckert, 1986; Armstrong, 1989). To evaluate the role of calcineurin in the inactivation of neurohypophysial Ca^{2+} channels, we examined Ca^{2+} current during 20 Hz trains in the presence of two enzyme inhibitors. The choice of 20 Hz trains was based on the fact that this stimulus protocol revealed Ca^{2+} -dependent Ca^{2+} channel inactivation especially clearly (Fig. 1*B* vs 8*A*). After 15 sec, peak Ca^{2+} current was inactivated by $40 \pm 4\%$ ($n = 11$) in control recordings (Fig. 1*B*), and intracellular BAPTA reduced this inactivation to $20.0 \pm 1.5\%$ ($n = 5$) (Fig. 8*A*). We first tested RS-20, a 20-amino-acid peptide that binds calmodulin with nanomolar affinity and blocks most of the known Ca^{2+} -calmodulin-dependent enzymes (Lukas et al., 1986), including calcium/calmodulin-dependent protein kinase II and calcineurin. Because the concentration of calmodulin in cells can be quite high, we added 50 μM RS-20 to the patch pipette filling solution and waited 5 min to allow this compound to diffuse into the cell. Ca^{2+} channels were seen to inactivate by $35 \pm 6\%$ ($n = 6$), which was indistinguishable from controls ($p = 0.24$), and different from that seen with intracellular BAPTA ($p = 0.027$).

Calcineurin can be activated weakly by direct binding of Ca^{2+} to its regulatory domain (Stemmer and Klee, 1994; Perrino et al., 1995). Therefore, cyclosporin A, a direct inhibitor of calcineurin

(Liu, 1993), also was tested. With 50–100 nM cyclosporin A in the patch pipette, Ca^{2+} current still inactivated $35\% \pm 9\%$ after 15 sec ($n = 5$; $p = 0.28$ vs control and $p = 0.07$ vs intracellular BAPTA). Experiments with cyclosporin A must be interpreted in light of the fact that the receptor for this agent is a small soluble protein (Liu, 1993), which could wash out in whole-terminal patch recordings such as these. However this drug was effective in experiments of similar design in hippocampal neurons (Tong et al., 1995), suggesting that the negative result here is meaningful. These results therefore suggest a mechanism of Ca^{2+} -dependent inactivation of Ca^{2+} current that is independent of calmodulin and calcineurin.

DISCUSSION

Calcium- and voltage-dependent inactivation

The experiments presented here show that Ca^{2+} channel inactivation in neurohypophyseal nerve terminals is sensitive to both voltage and intracellular Ca^{2+} . The effect of intracellular Ca^{2+} was very prominent at negative potentials at which an inhibition of the recovery of Ca^{2+} channels was seen (Figs. 6, 7). Inhibition of recovery from inactivation by Ca^{2+} has been reported for other Ca^{2+} channels as well (Brehm et al., 1980; Yatani et al., 1983; Gutnick et al., 1989). Intracellular Ca^{2+} also influenced Ca^{2+} channel inactivation at positive potentials, but the effect at positive potentials was overshadowed in large part by voltage-dependent inactivation (Fig. 4). At positive potentials we were surprised to see that removing Ca^{2+} made inactivation faster. In many other preparations Ba^{2+} substitution and intracellular chelators reduced the rate of current decay during sustained depolarization (Chad and Eckert, 1984; Chad, 1989). Therefore, the increase in the rate of Ca^{2+} channel inactivation shown here for both Ba^{2+} substitution and intracellular BAPTA (Figs. 2A,B) represents a property unique to the Ca^{2+} channels of the neurohypophysis. Other unique properties of neurohypophyseal N-type Ca^{2+} channels have been described, including lower single channel conductance and more rapid inactivation, as compared with N-type channels in cell bodies (Lemos and Nowycky, 1989; Wang et al., 1993).

The decrease in steady-state inactivation with prepulse potentials above 10 mV (Fig. 3) was correlated with the decrease in Ca^{2+} current (Figs. 2, 4). A U-shaped steady-state inactivation curve is a hallmark of Ca^{2+} -dependent inactivation (Chad and Eckert, 1984; Imredy and Yue, 1994), but in other preparations this shape has been attributed to voltage-dependent facilitation (Slesinger and Lansman, 1991). Using voltage pulses too brief to inactivate channels, we saw no evidence for facilitation. Thus, the fact that intracellular BAPTA reduced the upward slope in the steady-state inactivation plot (Fig. 5B) strengthens the case for a Ca^{2+} -dependent component of inactivation. Neither Ba^{2+} substitution nor intracellular BAPTA removed Ca^{2+} -dependent inactivation completely, and this is consistent with studies in other preparations showing that Ba^{2+} could substitute weakly for Ca^{2+} in Ca^{2+} -dependent inactivation (Brehm and Eckert, 1978; Tillotson, 1979; Gutnick et al., 1989; Mazzanti et al., 1991; Imredy and Yue, 1994) and that intracellular EGTA reduced but did not abolish the effect (Brehm and Eckert, 1978; Bechem and Pott, 1985).

Mechanism of Ca^{2+} -dependent inactivation

Early work in *Helix* neurons suggested dephosphorylation of Ca^{2+} channels by calcineurin as the mechanism of Ca^{2+} -dependent Ca^{2+} channel inactivation (Chad and Eckert, 1986;

Armstrong, 1989). However, more recent studies favor a mechanism involving a direct action of Ca^{2+} ions. Studies with enzyme inhibitors failed to reveal a role for protein phosphatases in Ca^{2+} channel inactivation (Fryer and Zucker, 1993; Imredy and Yue, 1994). Further, Ca^{2+} channels reconstituted into artificial bilayers could be cycled through Ca^{2+} -dependent inactivation and reactivation without ATP (Haack and Rosenberg, 1994). Finally, deletion of a segment of the Ca^{2+} channel that contains a putative Ca^{2+} binding site removes Ca^{2+} -dependent inactivation (de Leon et al., 1995). Our finding that RS-20 and cyclosporin A had no influence on Ca^{2+} channel inactivation in neurohypophyseal nerve terminals during trains of pulses is consistent with these diverse results on other Ca^{2+} channels and argues against an enzymatic mechanism for inactivation.

An enzymatic mechanism seems unlikely, but a simple binding process seems to be at odds with our finding that 14 and 20 Hz trains raised $[\text{Ca}^{2+}]_i$ to similar levels (Fig. 8B), because these two train frequencies produced very different amounts of Ca^{2+} channel inactivation (Fig. 1B). Stuenkel (1994) saw increases in $[\text{Ca}^{2+}]_i$ with train frequency, but his plots showed relatively small changes in the 14–20 Hz range as well. Although the $[\text{Ca}^{2+}]_i$ levels were similar for these two frequencies, the dynamics of the rises and falls in $[\text{Ca}^{2+}]_i$ were quite different. The more rapid rise in $[\text{Ca}^{2+}]_i$ may be responsible for the rapid component of Ca^{2+} channel inactivation seen with 20 Hz trains. Furthermore, these dynamic aspects lead one to consider the possibility that $[\text{Ca}^{2+}]_i$ around channels rises and falls very rapidly during a train and that the time course of these changes varies with frequency. Such changes would not be evident in our measurements of the spatial average of $[\text{Ca}^{2+}]_i$. It is well known that $[\text{Ca}^{2+}]_i$ is very different within domains around single channels and clusters of channels (Chad and Eckert, 1984; Augustine and Neher, 1992; Llinás et al., 1995; Tucker and Fettiplace, 1995). The domains around individual Ca^{2+} channels are not likely to be relevant in the present study because they collapse within <1 msec after channel closure and therefore would disappear at negative potentials at which a large effect of Ca^{2+} on recovery from inactivation is seen. However, domains around clusters of Ca^{2+} channels are thought to be relevant to Ca^{2+} -dependent Ca^{2+} channel inactivation (Imredy and Yue, 1992), and these larger domains should decay more slowly. Fixed Ca^{2+} buffers (Zhou and Neher, 1993) and Ca^{2+} -dependent cytoskeletal interactions (Johnson and Byerly, 1993) also could help Ca^{2+} domains to persist after channel closure. Estimates of $[\text{Ca}^{2+}]_i$ levels that inactivate Ca^{2+} channels vary widely among preparations and are often highly model-dependent (Gutnick et al., 1989; Romanin et al., 1992; Fryer and Zucker, 1993; Hirano and Hiroaki, 1994), but many of these studies suggest that $[\text{Ca}^{2+}]_i$ is $>1 \mu\text{M}$ and well above the spatial averages we observed during trains ($\sim 0.5 \mu\text{M}$). If the effect of Ca^{2+} on Ca^{2+} channels has a kinetic delay and if the time course of $[\text{Ca}^{2+}]_i$ within domains depends on train frequency, then this could help to explain why 20 Hz trains produce greater Ca^{2+} channel inactivation than 14 Hz trains while raising average $[\text{Ca}^{2+}]_i$ to similar levels.

Calcium currents in nerve terminals and the relevance to secretion

The Ca^{2+} currents described in the present study in slices of the neurohypophysis were similar in many respects to those described in dissociated neurohypophyseal nerve terminals (Lemos and Nowycky, 1989; X. Wang et al., 1992, 1993). In this preparation the slow time constant of inactivation ranged from 0.07–1.25 sec

and the $V_{1/2}$ of steady-state inactivation ranged from -75 to -60 mV. Our slow time constant of inactivation fell in the range of values from neurosecretosomes, but our $V_{1/2}$ value of -30 mV was significantly different. This difference may be attributable in part to experimental conditions. In neurosecretosomes longer inactivating pulses were used, and exogenous Ca^{2+} buffer was added to the patch pipette solutions. The Ca^{2+} dependence of Ca^{2+} channel inactivation has yet to be investigated in neurosecretosomes, but Ca^{2+} channels in two other nerve terminal preparations, retinal bipolar nerve terminals (von Gersdorff and Matthews, 1996) and rat brain synaptosomes (Tareilus et al., 1994), have been shown to inactivate by a Ca^{2+} -dependent mechanism.

Neurosecretosomes contain channels closely related to N-type and L-type Ca^{2+} channels (Lemos and Nowycky, 1989; Wang et al., 1993). Evidence also has been presented for P-type Ca^{2+} channels in the neurohypophysis (Salzberg et al., 1990; Wang and Lemos, 1994). On the basis of these other studies we can assign the rapidly inactivating component of Ca^{2+} current to N-type Ca^{2+} channels and the slowly inactivating component to L-type and P-type Ca^{2+} channels. Most studies showing Ca^{2+} -dependent inactivation were conducted on L-type channels (Chad, 1989), but Ca^{2+} -dependent inactivation has been reported in N-type (Cox and Dunlap, 1995) and P-type (Tareilus et al., 1994) Ca^{2+} channels as well. N-type Ca^{2+} channels are thought to play the greater role in secretion from the neurohypophysis (Dayanithi et al., 1988; Obaid et al., 1989; von Spreckelsen et al., 1990), as well as from most other nerve terminals (Dunlap et al., 1995). However, L-type Ca^{2+} channels also contribute to secretion, not only in the neurohypophysis (Nowycky, 1991; Bicknell et al., 1993; Stuenkel and Nordmann, 1993; Raji and Nordmann, 1994) but in a number of other preparations (Perney et al., 1986; Artalejo et al., 1994; Loechner et al., 1996). L-type Ca^{2+} channels will be especially important during trains because of the rapid inactivation of N-type Ca^{2+} channels. The variable contributions made by different Ca^{2+} channel types to secretion partly may reflect dependence on stimulation protocol. Ca^{2+} channel inactivation behavior such as that described here may account for some of this.

Ca^{2+} channel inactivation was evident during physiological trains of action potential-like pulses. A higher train frequency produced more inactivation (Fig. 1B), and intracellular BAPTA abolished this frequency dependence (Fig. 8A). Vasopressin and oxytocin have optimal frequencies for stimulation of secretion (Dutton and Dyball, 1979; Poulain and Wakerley, 1982; Gainer et al., 1986) and exhibit use-dependent depression of secretion as the frequency is increased (Gainer et al., 1986; Hobbach et al., 1988). The results presented here suggest that Ca^{2+} current inactivation would vary significantly, depending on stimulation frequency and duration, and these variations in Ca^{2+} channel activity could influence neuropeptide secretion. Other membrane processes also have been proposed to contribute to the fatigue of secretion, including the activation of a Ca^{2+} -dependent K^+ channel (Bielefeldt et al., 1992; G. Wang et al., 1992; Bielefeldt and Jackson, 1993), the activation of a slowly activated K^+ channel termed the D channel (Bielefeldt et al., 1992), and accumulation of extracellular K^+ (Leng and Shibuki, 1987). It is likely that a variety of mechanisms comes into play, acting together to determine how neurosecretion will vary with different forms of electrical activity. Diverse mechanisms such as these can serve to integrate many features of a complex electrical stimulus, including burst frequency, duration, and interburst interval, to determine the amount of hormone released.

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