Ca^{2+}/Calmodulin-Dependent Facilitation and Inactivation of P/Q-Type Ca^{2+} Channels

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Trains of action potentials cause Ca^{2+}-dependent facilitation and inactivation of presynaptic P/Q-type Ca^{2+} channels that can alter synaptic efficacy. A potential mechanism for these effects involves calmodulin, which associates in a Ca^{2+}-dependent manner with the pore-forming α_{1A} subunit. Here, we report that Ca^{2+} and calmodulin dramatically enhance inactivation and facilitation of P/Q-type Ca^{2+} channels containing the auxiliary β_{2a} subunit compared with their relatively small effects on channels with β_{1b}. Tetanic stimulation causes an initial enhancement followed by a gradual decline in P/Q-type Ca^{2+} currents over time. Recovery of Ca^{2+} currents from facilitation and inactivation is relatively slow (30 sec to 1 min). These effects are strongly inhibited by high intracellular BAPTA, replacement of extracellular Ca^{2+} with Ba^{2+}, and a calmodulin inhibitor peptide. The Ca^{2+}/calmodulin-dependent facilitation and inactivation of P/Q-type Ca^{2+} channels observed here are consistent with the behavior of presynaptic Ca^{2+} channels in neurons, revealing how dual feedback regulation of P/Q-type channels by Ca^{2+} and calmodulin could contribute to activity-dependent synaptic plasticity.

Key words: calcium channel; calmodulin; synaptic plasticity; inactivation; facilitation; action potential

Ca^{2+} entry through presynaptic voltage-gated Ca^{2+} channels links membrane depolarization and exocytosis of synaptic vesicles in the nerve terminal. The amount of neurotransmitter released is steeply dependent on presynaptic Ca^{2+} concentrations (Dodge and Rahamimoff, 1967; Mintz et al., 1995) such that increases or decreases in Ca^{2+} influx can powerfully alter neurotransmission. At many central and peripheral synapses, transmission is mediated by N- and P/Q-type Ca^{2+} channels (Dunlap et al., 1995). These channels are inhibited by G-protein $\beta\gamma$ subunits (Herlitze et al., 1996; Ikeda, 1996), and relief of this inhibition can produce short-term synaptic facilitation (Brody and Yue, 2000). In addition, P/Q-type channels are subject to feedback regulation by Ca^{2+}. We have shown that Ca^{2+} influx through P/Q-type channels enhances inactivation, increases recovery from inactivation, and causes a long-lasting facilitation of the Ca^{2+} current, effects that require direct association of Ca^{2+}/calmodulin with the pore-forming α_{1A} subunit (Lee et al., 1999).

High-frequency activation of presynaptic axons at a brainstem auditory synapse accelerates inactivation of presynaptic P/Q-type Ca^{2+} channels leading to post-tetanic depression of EPSPs, an effect that is enhanced by extracellular and intracellular Ca^{2+} (Forsythe et al., 1998). Both tetanic and paired-pulse stimulation also cause a transient facilitation of P/Q-type Ca^{2+} currents that depends on incoming Ca^{2+} and is reduced by intracellular dialysis with BAPTA (Borst and Sakmann, 1998; Cuttle et al., 1998). The modulation of recombinant P/Q-type channels by Ca^{2+}/calmodulin (Lee et al., 1999) is consistent with, but smaller than, these effects of Ca^{2+} on inactivation and facilitation of native presynaptic Ca^{2+} channels.

Brain presynaptic Ca^{2+} channels formed from α_{1A} subunits are often characterized by little voltage-dependent inactivation (Mintz et al., 1992; Usowicz et al., 1992). Slowly inactivating P/Q-type channels can be reproduced in heterologous systems by expression of a recently recognized splice variant of α_{1A}, $\alpha_{1A\text{-}b}$ (Bourinet et al., 1999), or by coexpression of α_{1A} with the auxiliary Ca^{2+} channel β subunit β_{2a} (Stea et al., 1994; De Waard and Campbell, 1995). Channels comprised of α_{1A}, β_{1b}, and $\alpha_2\delta$ subunits exhibit strong voltage-dependent inactivation that might occlude Ca^{2+}-dependent modulation (Lee et al., 1999). Therefore, Ca^{2+}-dependent inactivation and facilitation may be more prominent in P/Q-type Ca^{2+} channels containing β_{2a} subunits, which are likely physiological partners of α_{1A} in many regions of the brain (Stea et al., 1994; Tanaka et al., 1995). Here we demonstrate that substitution of β_{1b} with β_{2a} unmasks a surprisingly large Ca^{2+}-dependent facilitation and inactivation of P/Q-type Ca^{2+} channels in response to step depolarizations and high-frequency activation with characteristics similar to those observed for presynaptic Ca^{2+} channels in the brain. Both effects are mediated in part by Ca^{2+} and calmodulin but differ in their kinetics and sensitivity to Ca^{2+}. These results reveal a complex feedback regulation of P/Q-type channels by Ca^{2+} that may contribute to both the enhancement and depression of synaptic transmission.

MATERIALS AND METHODS

cDNA expression constructs. Mammalian expression constructs of rat Ca^{2+} channel subunits were α_{1A} (rbA), β_{1b}, and β_{2a} that were subcloned in pMT2XS and $\alpha_2\delta$ that was subcloned in pZEM228 (Stea et al., 1994). Deletion of the α_{1A} calmodulin-binding domain (CBD; amino acids 1960–2000) was accomplished by amplifying by PCR an *Eco*RV/*Pml*I fragment that incorporated the deletion and subcloning into the corresponding sites of rbA in a pBluescript SK^+ shuttle vector. From this construct, a *Sgr*aI/*Mlu*I fragment containing the deletion was subcloned into rba/pMT2XS. The adenylate cyclase I (ACI) expression construct was generated by amplifying amino acids 481–575 of adenylyl cyclase type I by PCR and subcloning into pCEP4 (Invitrogen, San Diego, CA). The same strategy was used for the ACI(F-R) construct used in control experiments except that the PCR template was adenylyl cyclase type I containing a single phenylalanine to arginine mutation that disrupts calmodulin binding (Wu et al., 1993). Both mutant and wild-type ACI cDNAs were provided by Dr. Daniel Storm (University of Washington, Seattle, WA).

Cell culture and transfection. tsA-201 cells were maintained in DMEM/Ham's F12 (1:1) supplemented with 10% fetal bovine serum (Life Technologies, Rockville, MD) at 37°C under 10% CO_2. Cells plated in 35 mm tissue culture dishes were grown to ~70% confluency and transfected by the calcium phosphate method with a total of 5 μg of DNA including a 1:1 molar ratio of Ca^{2+} channel subunits and 0.3 μg of a CD8 expression plasmid for identification of transfected cells. ACI peptide constructs were expressed at a 10:1 molar ratio with Ca^{2+} channel subunits.

Electrophysiological recordings. At least 48 hr after transfection, tsA-201 cells were incubated with CD8 antibody-coated microspheres (Dynal, Oslo, Norway) to allow visual identification of transfected cells. Ca^{2+} currents were recorded in the whole-cell configuration of the patch-clamp technique using a List EPC-7 patch-clamp amplifier and were filtered at 5 kHz. Voltage protocols were applied, and data were acquired using Fastlab software (Indec Systems). Leak and capacitive transients were subtracted

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using a P/-4 protocol. Extracellular recording solutions contained 150 mM Tris, 1 mM $MgCl_2$, and 10 mM $CaCl_2$ or $BaCl_2$. Intracellular solutions consisted of 120 mM *N*-methyl-D-glucamine, 60 mM HEPES, 1 mM $MgCl_2$, 2 mM Mg-ATP, and 0.5 or 10 mM EGTA. GDPβS (1 mM) and GTPγS (0.5 mM) (Sigma, St. Louis, MO) were included in some intracellular solutions. The pH of intracellular and extracellular recording solutions was adjusted to 7.3 with methanesulfonic acid. Because extracellular Ba^{2+} and intracellular BAPTA caused shifts in the voltage dependence of activation of 10 mV negative and positive, respectively, voltage protocols were adjusted to compensate for this difference as noted. All averaged data represent the mean ± SEM.

RESULTS

Ca^{2+}-dependent inactivation of P/Q-type Ca^{2+} channels during step depolarizations

P/Q-type Ca^{2+} channels (α_{1A}, β_{1b}, and $\alpha_2\delta$) transfected into tsA-201 cells inactivate faster and more completely when Ca^{2+} rather than Ba^{2+} is the permeant ion and when Ca^{2+} accumulates intracellularly with reduced concentrations of Ca^{2+} chelators in intracellular recording solutions (Lee et al., 1999). However, inactivation is not caused solely by incoming Ca^{2+} ions because P/Q-type Ca^{2+} currents inactivate rapidly at positive voltages even in the presence of high intracellular EGTA (Fig. 1*A*). Because Ca^{2+}-dependent inactivation of P/Q-type Ca^{2+} channels may be occluded by a voltage-dependent mechanism of inactivation, we tested whether Ca^{2+}-dependent inactivation was more significant in P/Q-type channels containing the β_{2a} subunit, which exhibit relatively little voltage-dependent inactivation (Stea et al., 1994). Unlike channels containing β_{1b} subunits, Ca^{2+} currents (I_{Ca}) through P/Q-type channels containing β_{2a} subunits inactivate slowly with high intracellular EGTA or BAPTA and when extracellular Ca^{2+} is replaced by Ba^{2+} (Fig. 1*A*). However, when intracellular EGTA is reduced to 0.5 mM, I_{Ca} inactivates almost completely during a 1 sec step depolarization (Fig. 1*A*). To estimate the magnitude of Ca^{2+}-dependent inactivation, residual current at the end of the test pulse (I_{res}) was compared with the peak current (I_{pk}) (Fig. 1*B*). With 0.5 mM intracellular EGTA, inactivation of I_{Ca} was approximately three times more complete ($I_{res}/I_{pk} = 0.24 \pm 0.02$; $n = 12$) than that with 10 mM EGTA ($I_{res}/I_{pk} = 0.66 \pm 0.08$; $n = 9$) or 10 mM BAPTA ($I_{res}/I_{pk} = 0.64 \pm 0.02$; $n = 10$) or when Ba^{2+} was used as the permeant ion ($I_{res}/I_{pk} = 0.66 \pm 0.04$; $n = 10$). As shown for P/Q-type channels containing β_{1b} (Lee et al., 1999), calmodulin is important for Ca^{2+}-dependent inactivation, because it is greatly diminished by deletion of the α_{1A} CBD (ΔCBD; $I_{res}/I_{pk} = 0.45 \pm 0.03$; $n = 8$) and by overexpression of a peptide from type I adenylyl cyclase that competes with Ca^{2+} channels for binding to calmodulin (Wu et al., 1993) ($I_{res}/I_{pk} = 0.40 \pm 0.04$ [ACI; $n = 12$] vs 0.16 ± 0.04 [ACI(F-R), control peptide; $n = 9$]) (Fig. 1). The prominent Ca^{2+}- and calmodulin-dependent inactivation revealed by the β_{2a} subunit suggests that, under physiological conditions, the extent to which P/Q-type Ca^{2+} channels are regulated by Ca^{2+} depends critically on subunit composition. The robust effects of Ca^{2+} and calmodulin, combined with the limited voltage-dependent inactivation conferred by β_{2a}, facilitate detailed analysis of Ca^{2+}-dependent modulation of P/Q-type Ca^{2+} channels. Therefore, further studies were restricted to channels containing the β_{2a} subunit.

To confirm further the Ca^{2+} dependence of P/Q-type channel inactivation, we tested the effects of a conditioning prepulse (1 sec) to various voltages on Ca^{2+} currents elicited by a test pulse to +20 mV. If P/Q-type channel inactivation depends on previous Ca^{2+} entry, then inactivation of the test pulse current should be greatest at the prepulse voltage eliciting the peak inward Ca^{2+} current. As shown in Figure 2, *A* and *B*, I_{Ca} inactivation increased concomitantly with the amplitude of the prepulse-induced current and declined as the prepulse voltage approached the reversal potential for Ca^{2+}. Unlike the proposed mechanism of preferential closed-state inactivation (Patil et al., 1998), inactivation measured here depended on Ca^{2+} entry and intracellular accumulation during the prepulse because no inactivation of the test current was observed when BAPTA was included in the recording pipette (Fig. 2*B*).

Similar to presynaptic Ca^{2+} channels in rat brainstem (Forsythe et al., 1998), Ca^{2+} currents through P/Q-type Ca^{2+} channels expressed in tsA-201 cells recover slowly from inactivation. The time course of recovery from inactivation induced by a 2 sec prepulse to +10 mV was described by a single exponential ($\tau = 22.9 \pm 3.5$ sec; $n = 6$), and full recovery took >1.5 min (Fig. 2*C*,*D*). The time constant reflects primarily recovery from Ca^{2+}-dependent inactivation because the 2 sec prepulse caused relatively little inactivation in cells recorded with intracellular BAPTA. The

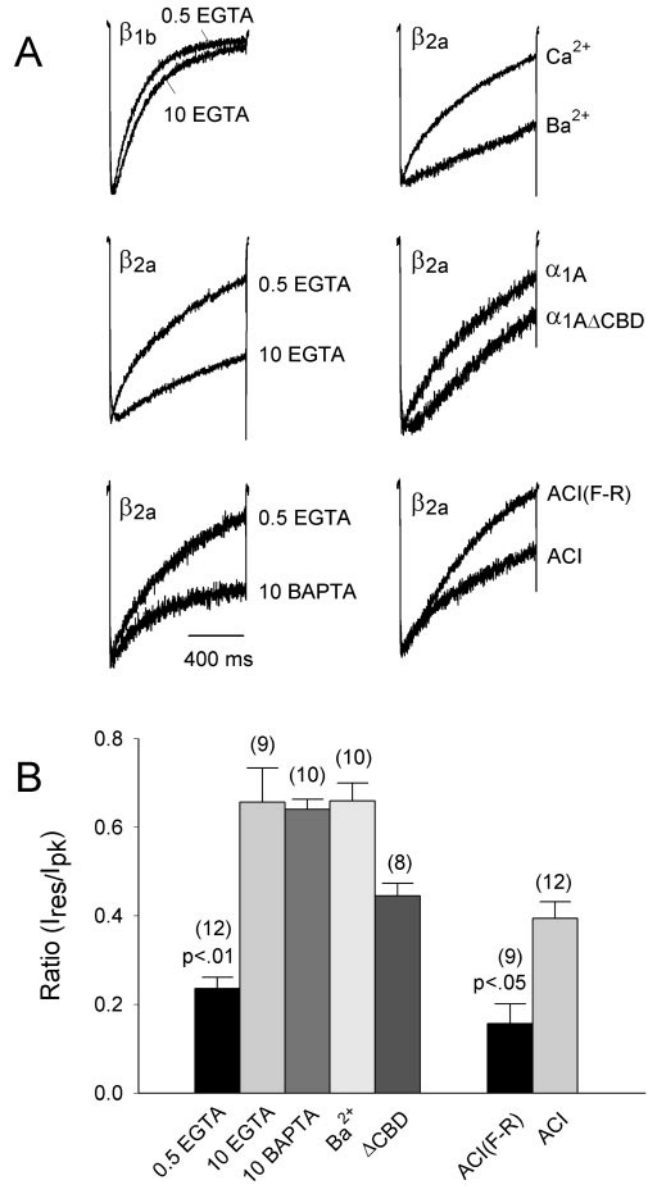

Figure 1. Ca^{2+}- and calmodulin-dependent inactivation of P/Q-type Ca^{2+} channels in tsA-201 cells. *A*, Normalized Ca^{2+} currents recorded from cells cotransfected with α_{1A}, β_{1b} or β_{2a} (as indicated), and $\alpha_2\delta$ subunits are shown. *Left*, The extracellular solution contained 10 mM Ca^{2+}, and the intracellular solution contained EGTA (0.5 or 10 mM) or BAPTA (10 mM) as indicated. *Right*, The intracellular solution contained 0.5 mM EGTA. Current *traces* are shown from channels containing α_{1A} subunits recorded with 10 mM extracellular Ca^{2+} or Ba^{2+} (*top*), from channels containing α_{1A} or the α_{1A} ΔCBD deletion mutant (*middle*), or from channels containing α_{1A} with overexpression of either a calmodulin-binding inhibitor peptide (ACI) or an inactive mutant form of the peptide [ACI(F-R)] (*bottom*). Currents were elicited by a 1 sec step depolarization to +10 mV from a holding potential of −80 mV, except when BAPTA or Ba^{2+} was used and depolarization was stepped to +20 and 0 mV, respectively, to compensate for shifts in the voltage dependence of activation. *B*, Inactivation expressed as the ratio of residual current at the end of the 1 sec depolarization (I_{res}) to the peak current (I_{pk}) is shown. *Numbers* in *parentheses* indicate the number of cells recorded in each condition.

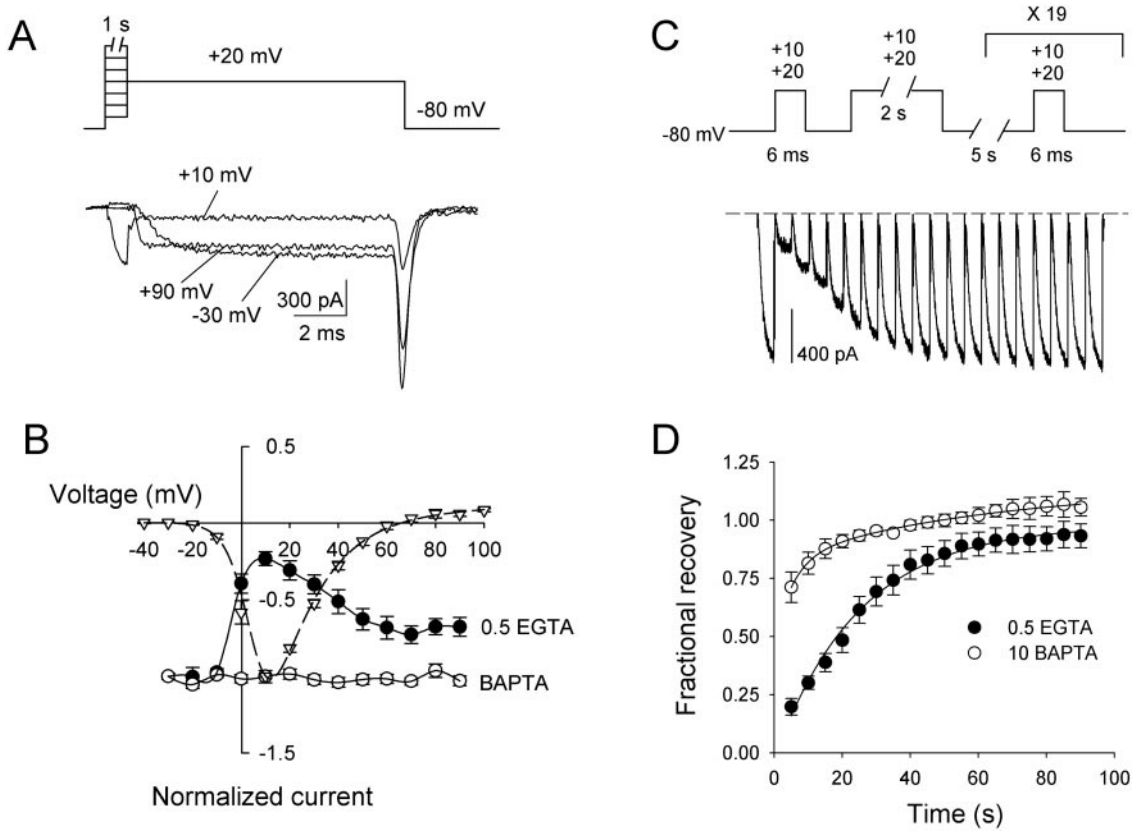

Figure 2. Voltage dependence of inactivation and recovery from inactivation of P/Q-type Ca^{2+} currents. *A*, The voltage protocol for measuring inactivation caused by a 1 sec conditioning prepulse is shown above representative Ca^{2+} currents evoked by a 10 msec test pulse to +20 mV after prepulses to the indicated voltages. *B*, The relationship between the inactivating prepulse voltage and peak Ca^{2+} currents is shown. Peak Ca^{2+} currents elicited by the +20 mV test pulse after the conditioning prepulse were recorded with intracellular solutions containing 0.5 mM EGTA (*closed circles*; $n = 6$) or 10 mM BAPTA (*open circles*; $n = 9$) and were normalized to Ca^{2+} currents evoked after a −30 mV prepulse. *Open inverted triangles* represent the current–voltage relationship for Ca^{2+} currents (0.5 mM intracellular EGTA) elicited by test pulses to the indicated voltages without a prepulse and normalized to the current amplitude of the +10 mV test pulse ($n = 8$). *C*, Recovery from inactivation induced by a 2 sec conditioning pulse to +10 mV (+20 mV for BAPTA) was monitored using +10 mV test pulses (+20 mV for BAPTA) (6 msec) at 0.2 Hz. Test currents were normalized to the noninactivated Ca^{2+} current evoked before the inactivating prepulse. The voltage protocol is shown *above* the sample current records. The current during the 5 sec interval between test pulses was not recorded. *D*, Fractional recovery from inactivation obtained by plotting Ca^{2+} current amplitudes normalized to the noninactivated test current against time after the inactivating prepulse is shown. Intracellular recording solutions contained either 0.5 mM EGTA ($n = 6$) or 10 mM BAPTA ($n = 5$).

slow recovery of I_{Ca} inactivation would lead to cumulative inactivation of P/Q-type Ca^{2+} channels during repetitive stimulation, a proposed mechanism for post-tetanic depression at some synapses (Branchaw et al., 1997; Forsythe et al., 1998).

Ca^{2+}-dependent inactivation and facilitation during repetitive stimulation

To determine the significance of Ca^{2+}-dependent modulation of P/Q-type channels during physiological stimuli, Ca^{2+} currents were elicited by 100 Hz trains of 5 msec test pulses. With 0.5 mM intracellular EGTA, the amplitude of I_{Ca} increased ~30% (facilitation) during the first five depolarizations and then inactivated below the initial current level over the next 800 msec (Fig. 3*A*). Both the facilitation and inactivation of I_{Ca} required Ca^{2+} and calmodulin because neither effect was observed for Ba^{2+} currents or Ca^{2+} currents with intracellular BAPTA, and both were inhibited by overexpression of the calmodulin inhibitor peptide (Fig. 3). Surprisingly, raising intracellular EGTA to 10 mM blocked inactivation but not facilitation of I_{Ca} during repetitive stimulation, suggesting a difference in the Ca^{2+} sensitivity of the two processes (Fig. 3*A*). Because facilitation was blocked by 10 mM BAPTA, but not by EGTA that is a slower Ca^{2+} buffer, facilitation may result from rapid local increases in Ca^{2+} that are ineffectively buffered by 10 mM EGTA. By contrast, inactivation may require longer-lasting and/or more global increases in Ca^{2+} that are readily prevented by both EGTA and BAPTA.

This explanation is strengthened by the strong facilitation of I_{Ca} without inactivation during a train of 2 msec depolarizations, in which the cumulative Ca^{2+} influx would be considerably less than that during repetitive 5 msec test pulses (Fig. 3*C*). Facilitation of I_{Ca} accumulated and was maintained during the train with 0.5 mM intracellular EGTA but not BAPTA. Because action potentials typically do not exceed 2 msec in duration, facilitation of I_{Ca} through P/Q-type Ca^{2+} channels would predominate during short, high-frequency bursts of action potentials, whereas inactivation of I_{Ca} would develop during prolonged trains causing a progressive accumulation of intracellular Ca^{2+}. This could explain why tetanic stimulation causes an initial facilitation followed by inactivation of presynaptic P/Q-type channels in neurons (Forsythe et al., 1998), whereas short paired pulses cause only facilitation of I_{Ca} (Borst and Sakmann, 1998; Cuttle et al., 1998).

Ca^{2+}-dependent facilitation in double-pulse protocols

The potentially large impact of P/Q-type Ca^{2+} channel facilitation on synaptic function motivated further analysis of this process using double-pulse protocols. Facilitation induced by a 50 msec prepulse to a variable voltage was measured by comparing I_{Ca} elicited by a test pulse before and after the conditioning prepulse (Fig. 4*A*). If facilitation depended on Ca^{2+} influx during the prepulse, then facilitation should be greatest at prepulse voltages eliciting the largest inward Ca^{2+} current. With 0.5 mM intracellular EGTA, facilitation of I_{Ca} increased with prepulse voltages positive to −20 mV, reached a maximum of more than twofold near +20 mV, and declined at more positive prepulse voltages (Fig. 4*B*). The

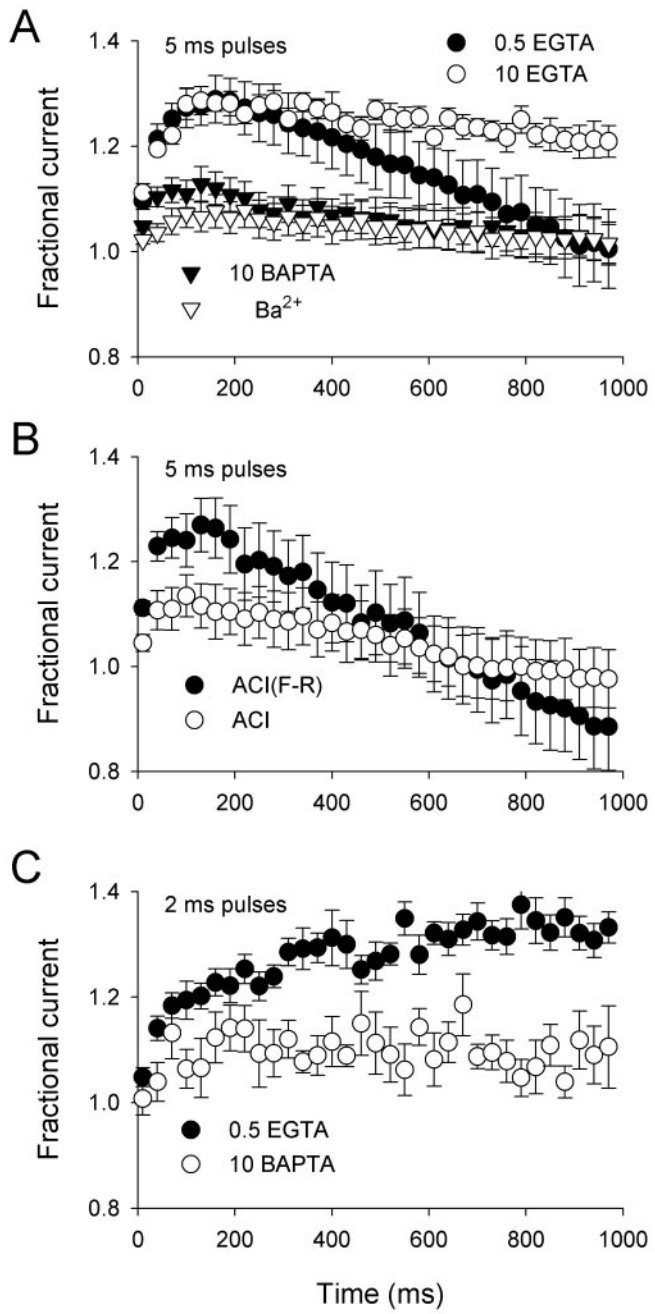

Figure 3. Ca^{2+}- and calmodulin-dependent facilitation and inactivation of P/Q-type Ca^{2+} channels in response to repetitive depolarizations. *A*, Effects of intracellular Ca^{2+} chelators (0.5 mM EGTA; $n = 7$; 10 mM EGTA; $n = 6$; 10 mM BAPTA; $n = 11$) and extracellular Ba^{2+} ($n = 8$) on currents elicited by 100 Hz trains of 5 msec pulses to +10 mV (+20 mV for BAPTA; 0 mV for Ba^{2+}). *Points* represent averaged peak currents normalized to the peak current elicited by the first pulse of the train. Every third point of the train is plotted. *B*, Effect of a calmodulin inhibitor (ACI; $n = 8$) and control peptide [ACI(F-R); $n = 7$] on fractional current measured as described in *A* with 0.5 mM intracellular EGTA and 10 mM extracellular Ca^{2+}. *C*, The same voltage protocol described in *A* except that the duration of test pulses during the train was reduced to 2 msec. Recordings were with 0.5 mM intracellular EGTA ($n = 9$) or 10 mM BAPTA ($n = 8$).

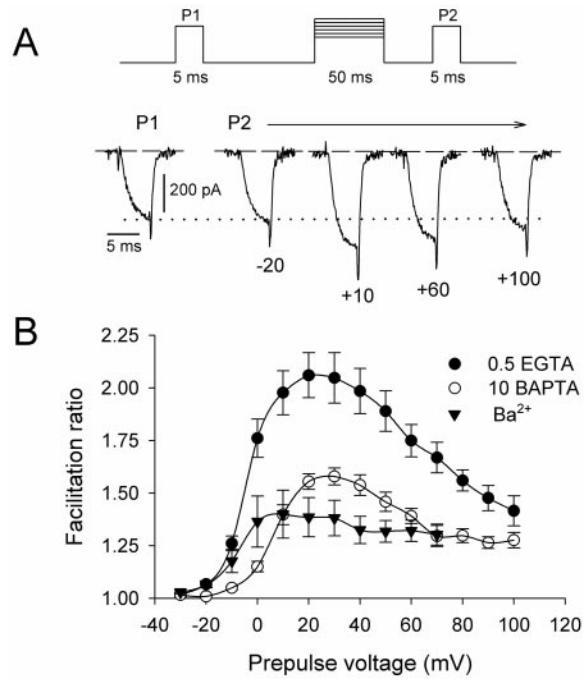

Figure 4. Prepulse voltage dependence of P/Q-type Ca^{2+} channel facilitation. *A*, The voltage protocol shown above representative Ca^{2+} currents recorded with 0.5 mM intracellular EGTA. Shown are currents elicited by a test pulse to 0 mV before (*P1*) and 5 msec after (*P2*) a 50 msec conditioning prepulse to the voltages (in millivolts) indicated *below* each current *trace*. *B*, Effects of intracellular Ca^{2+} chelators (0.5 mM EGTA; $n = 8$; 10 mM BAPTA; $n = 6$) and extracellular Ba^{2+} ($n = 6$) on facilitation as a function of prepulse voltage. The facilitation ratio was obtained by normalizing the peak current from *P2* to that from *P1*.

peak of this biphasic voltage dependence of facilitation correlated with the peak of the *I–V* relationship for I_{Ca} during the prepulse (see Fig. 2*B*), underscoring the importance of prepulse-induced Ca^{2+} influx for the enhancement of the test current. Furthermore, I_{Ca} facilitation during the test pulse was significantly but incompletely reduced when extracellular Ba^{2+} was substituted for Ca^{2+} and when 10 mM BAPTA was included in the intracellular solution. A proportion of the residual facilitation seen with intracellular BAPTA may result from insufficient Ca^{2+} buffering because its voltage dependence corresponded to current activation during the prepulse (Fig. 4*B*). However, the small voltage-dependent facilitation of Ba^{2+} currents (Fig. 4*B*) suggests that facilitation of P/Q-type Ca^{2+} channels may be initiated by a Ca^{2+}-independent mechanism that is greatly enhanced by Ca^{2+} influx and calmodulin binding to the channel. This Ca^{2+}-independent facilitation, revealed during double-pulse protocols using long (50 msec) prepulses, may be relatively insignificant under physiological conditions, in which Ca^{2+} is the charge carrier and action potentials typically do not surpass 2 msec in duration (see Fig. 3).

Presynaptic calcium currents are reduced by activation of G-proteins, and G-protein-modulated currents can be facilitated by double-pulse protocols (Dolphin, 1996). Such facilitation superficially resembles the Ca^{2+}-dependent facilitation observed here. However, several lines of evidence argue against the involvement of G-proteins in the facilitation of I_{Ca} in our experiments. First, activation of G-proteins with intracellular GTPγS is necessary for significant G-protein-dependent facilitation of P/Q-type Ca^{2+} channels (Herlitze et al., 1996). Prepulses to +10 mV produce near maximal Ca^{2+}-dependent facilitation of I_{Ca} (Fig. 4*B*), but GTPγS is not required for, and does not enhance, facilitation by this prepulse voltage (Fig. 5*A*, *top, middle*). Second, inhibiting basal G-protein activity by intracellular dialysis with GDPβS does not reduce facilitation of I_{Ca} (Fig. 5*A*, *bottom*). Third, a strong depolarizing prepulse to +100 mV causes robust G-protein-dependent facilitation when intracellular GTPγS is included in the pipette (Fig. 5*B*, *middle*) but little facilitation of I_{Ca} when GTPγS is absent (Fig. 5*B*, *top*) or when G-proteins are inhibited by GDPβS (Fig. 5*B*, *bottom*). Taken together, these results are inconsistent with an essential role for G-proteins in Ca^{2+}-dependent facilitation of P/Q-type channels. Although the G-protein- and Ca^{2+}-dependent forms of facilitation are clearly separated by prepulses to +100 and +10 mV, respectively, both would contribute to facilitation at the

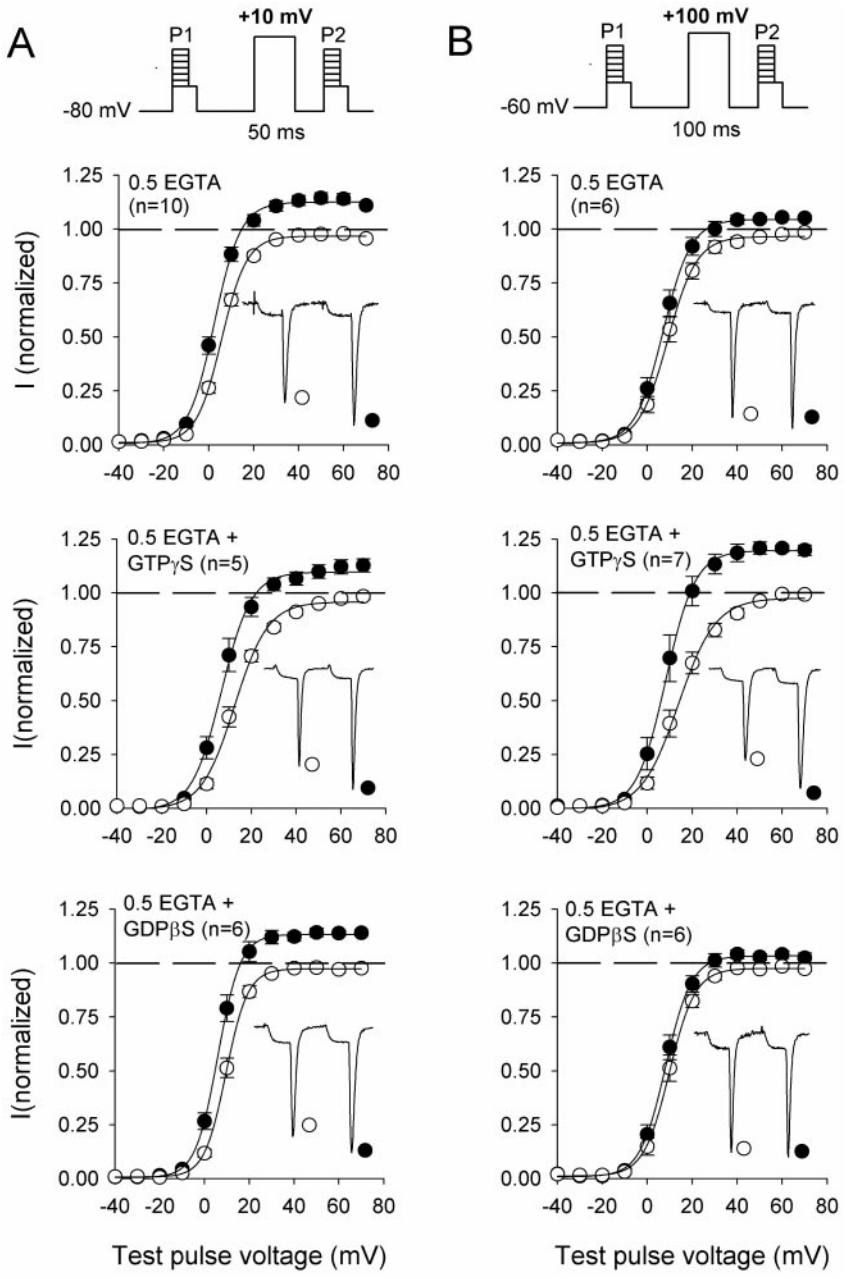

Figure 5. Effect of guanine nucleotides on prepulse facilitation of P/Q-type Ca^{2+} channels. *A*, Voltage dependence of activation of Ca^{2+} currents before (*open circles*; *P1*) and after (*closed circles*; *P2*) a depolarizing prepulse from −80 to +10 mV with intracellular solutions containing 0.5 mM EGTA (*top*), 0.5 mM EGTA and 0.5 mM GTPγS (*middle*), or 0.5 mM EGTA and 1 mM GDPβS (*bottom*). Tail currents were measured by holding at −40 mV for 5 msec after test pulses (*P1*, *P2*) to variable voltages. Peak tail currents were normalized to the largest tail current measured during the nonfacilitated prepulses (*P1*) and plotted against the test pulse voltage. *Insets*, Representative currents elicited by test pulses to +40 mV before and after the prepulse. *B*, Voltage dependence of activation measured as described in *A* except that *P1*, a +100 mV conditioning pulse, and *P2* were stepped from a holding potential of −60 mV. Intracellular solutions contained 0.5 mM EGTA (*top*), 0.5 mM EGTA and 0.5 mM GTPγS (*middle*), or 0.5 mM EGTA and 1 mM GDPβS (*bottom*). *Symbols* and *insets* are as described in *A*.

peak of the neuronal action potential (approximately +40 mV) under physiological conditions.

Onset and decay of Ca^2-dependent facilitation

The effect of Ca^{2+} on enhancing I_{Ca} facilitation (Fig. 4*B*) may be caused by a Ca^{2+}-dependent acceleration of the onset of facilitation and/or a slowing of its decay. To characterize the mechanism by which Ca^{2+} entry and intracellular accumulation promote facilitation of P/Q-type Ca^{2+} channels, the rates of onset and decay of I_{Ca} facilitation were determined with various degrees of intracellular Ca^{2+} buffering with or without the calmodulin inhibitor peptide.

In a double-pulse protocol, the onset of facilitation was determined by plotting facilitation of I_{Ca} as a function of prepulse duration (Fig. 6*A*). With 0.5 mM intracellular EGTA, facilitation increased with prepulse duration according to a single-exponential time course (τ = 13.3 ± 1.3 msec; n = 8), with I_{Ca} inactivation becoming evident with prepulses longer than 60 msec (Fig. 6*B*). With 10 mM intracellular BAPTA, facilitation of I_{Ca} was reduced, but its time course was similar to that in 0.5 mM EGTA (τ = 16 ± 1.3 msec; n = 7). At various prepulse voltages, the time constants for the onset of I_{Ca} facilitation were not significantly different ($p > 0.05$) with either Ca^{2+} buffer (Fig. 6*C*). These results are consistent with a role for Ca^{2+} in controlling the amount but not the rate of onset of I_{Ca} facilitation.

Although Ca^{2+} entry during the prepulse did not affect the rate of onset of I_{Ca} facilitation, it did significantly slow its decay. As the interval between the 50 msec conditioning prepulse and the second test pulse was increased, facilitation of I_{Ca} decreased with a single-exponential time course (τ = 112.7 ± 17 msec; n = 9) with 0.5 mM intracellular EGTA. The time course was sensitive to Ca^{2+} influx during the prepulse because the decay of facilitation was almost twice as slow (τ = 221.5 ± 48.4 msec; n = 6) with a longer 200 msec prepulse and twice as fast (τ = 43.5 ± 5.6 msec; n = 6) with a 5 msec prepulse (Fig. 7*A,B*). These data suggest that the persistence of facilitation is caused by the intracellular accumulation of Ca^{2+} ions that had entered during the prepulse.

Consistent with this idea, the decay of facilitation depended on intracellular Ca^{2+} buffering. After a 50 msec prepulse, the decay of

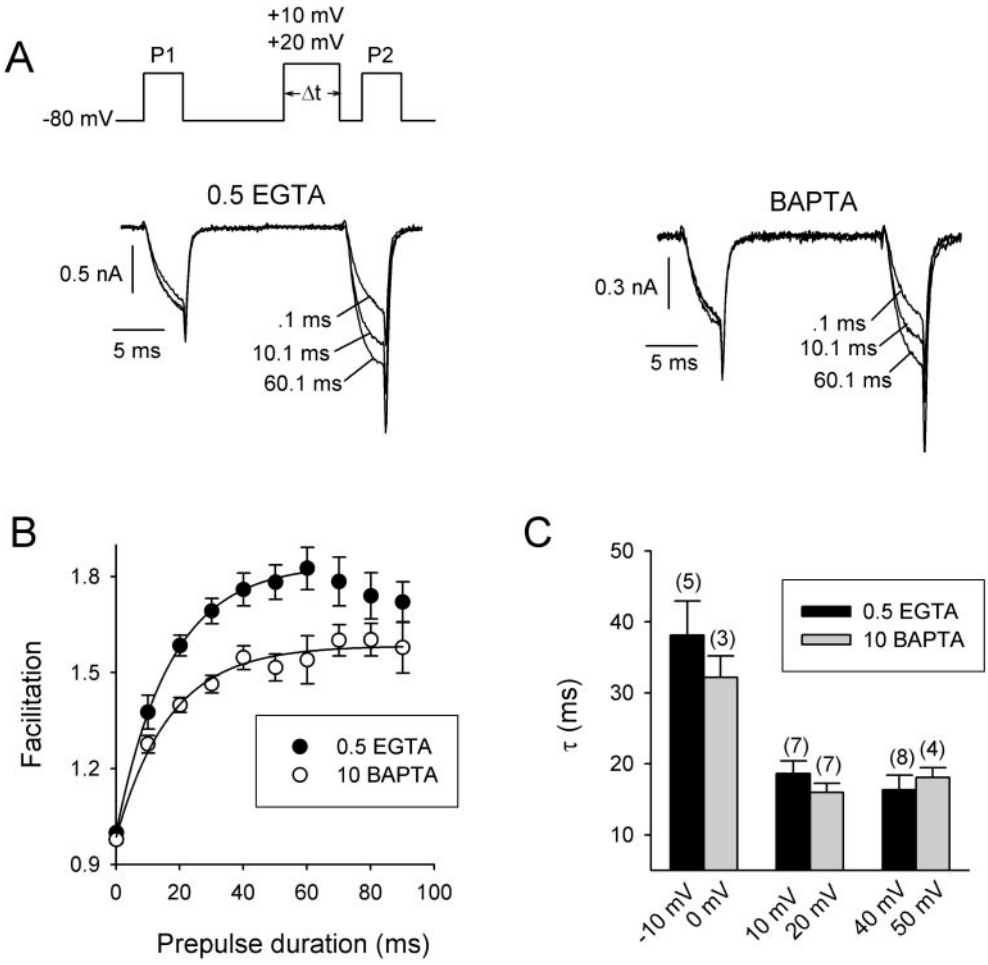

Figure 6. Onset of facilitation of P/Q-type Ca^{2+} channels. *A*, The voltage protocol (*top*) and representative current *traces* (*bottom*) recorded with 10 mM extracellular Ca^{2+} and 0.5 mM EGTA or 10 mM BAPTA in the intracellular recording solution. Currents were elicited by test pulses to 0 mV (+10 mV for BAPTA) before (*P1*) and 5 msec after (*P2*) conditioning prepulses (+10 mV for 0.5 mM EGTA; +20 mV for 10 mM BAPTA) of the indicated durations. *B*, Effect of intracellular Ca^{2+} chelators (0.5 mM EGTA; $n = 8$; 10 mM BAPTA; $n = 7$) on facilitation as a function of prepulse duration. Facilitation was obtained by normalizing the peak current from *P2* to that from *P1*. Single-exponential fits of the data are shown; only the first seven points of the data obtained with 0.5 mM EGTA were included in the fit because of the onset of inactivation after longer prepulses. *C*, Effect of prepulse voltage on time constants for the onset of facilitation. Mean time constants were obtained from single-exponential fits to the data in *B*. Shown are results obtained with intracellular solutions containing 0.5 mM EGTA or 10 mM BAPTA at the indicated prepulse voltages. The *number* of cells recorded for each condition is indicated in *parentheses*.

I_{Ca} facilitation with 10 mM intracellular BAPTA was significantly faster ($\tau = 47.6 \pm 3.6$ msec; $n = 10$) than that with 0.5 mM EGTA, and the time course changed little with prepulse duration ($\tau_{200msec} = 43.4 \pm 3.9$ msec; $n = 7$; $\tau_{5msec} = 46.7 \pm 7.4$ msec; $n = 5$) (Fig. 7*A,B*). The Ca^{2+}-dependent slowing of the decay of facilitation was also diminished by overexpression of the calmodulin inhibitor peptide ($\tau = 67.7 \pm 9.2$ msec; $n = 6$) (Fig. 7*C*) and by substitution of extracellular Ca^{2+} with Ba^{2+} (data not shown). Interestingly, 10 mM intracellular EGTA, which supported facilitation equal to that in 0.5 mM EGTA (see Fig. 7*C*, $\tau = 0$ intercept), also greatly accelerated its decay ($\tau = 55.3 \pm 2.7$ msec; $n = 7$).

In addition to slowing the decay of I_{Ca} facilitation, Ca^{2+} influx and intracellular accumulation prevented complete recovery of I_{Ca} to initial levels. With 0.5 mM EGTA, I_{Ca} was still potentiated by $26.1 \pm 8.3\%$ ($n = 9$) 400 msec after a 50 msec conditioning prepulse. In contrast, little facilitation of I_{Ca} remained at this time point with intracellular BAPTA ($4.6 \pm 1.6\%$; $n = 9$) or 10 mM EGTA ($0.5 \pm 1.2\%$; $n = 7$) or with overexpression of the calmodulin inhibitor peptide ($5.5 \pm 2.2\%$ msec; $n = 6$) (Fig. 7*C,D*). Complete decay of I_{Ca} to initial levels was also observed when the prepulse was limited to 5 msec (Fig. 7*B*). Thus, Ca^{2+} entry during a conditioning prepulse both enhances and prolongs facilitation of I_{Ca} for >0.5 sec after a depolarizing stimulus. I_{Ca} remained facilitated for at least 10 sec but had decayed completely by 30 sec, the interval between trials (data not shown). This prolonged facilitation would greatly enhance Ca^{2+} influx in presynaptic nerve terminals over time and may have important implications for relatively long-lasting changes in synaptic strength.

DISCUSSION

We have shown that incoming Ca^{2+} triggers both a negative and positive feedback regulation of subsequent Ca^{2+} entry through P/Q-type Ca^{2+} channels. Repetitive stimuli and step depolarizations enhance facilitation and accelerate inactivation of P/Q-type Ca^{2+} currents. Both effects are suppressed by intracellular dialysis with BAPTA, extracellular Ba^{2+} in place of Ca^{2+}, and overexpression of a calmodulin-binding inhibitor peptide. These modulatory effects of Ca^{2+} and calmodulin are distinct from previously described mechanisms of facilitation and inactivation of P/Q-type Ca^{2+} channels (Brody et al., 1997; Patil et al., 1998) and are consistent with the behavior of presynaptic P/Q-type Ca^{2+} channels in nerve terminals (Borst and Sakmann, 1998; Cuttle et al., 1998; Forsythe et al., 1998).

Auxiliary β subunits and Ca^{2+}-dependent modulation of P/Q-type Ca^{2+} channels

Differences in the properties of P/Q-type Ca^{2+} channels in the nervous system arise in part from the association of α_{1A} with distinct β subunits. Unlike β_1, β_3, and β_4 subunits, which accelerate inactivation of P/Q-type Ca^{2+} channels, β_{2a} significantly slows inactivation kinetics and causes a large depolarizing shift in the voltage dependence of steady-state inactivation (Stea et al., 1994; De Waard and Campbell, 1995). However, in P/Q-type Ca^{2+} channels containing β_{2a}, the relatively limited voltage-dependent inactivation unmasks powerful negative and positive feedback regulation by Ca^{2+}. Ca^{2+}-dependent modulation of strongly inactivating P/Q-type Ca^{2+} channels, such as those containing β_{1b} (Lee et al., 1999), is modest compared with that of Ca^{2+} channels with β_{2a}. Because α_{1A} is widely distributed throughout the nervous system (Stea et al., 1994; Westenbroek et al., 1995), the significance of Ca^{2+}-dependent inactivation and facilitation of P/Q-type Ca^{2+} channels in neurons would depend critically on coexpression with a specific β subunit. The prominence of Ca^{2+}-dependent regulation of P/Q-type channels may also depend on the splice variant of α_{1A}

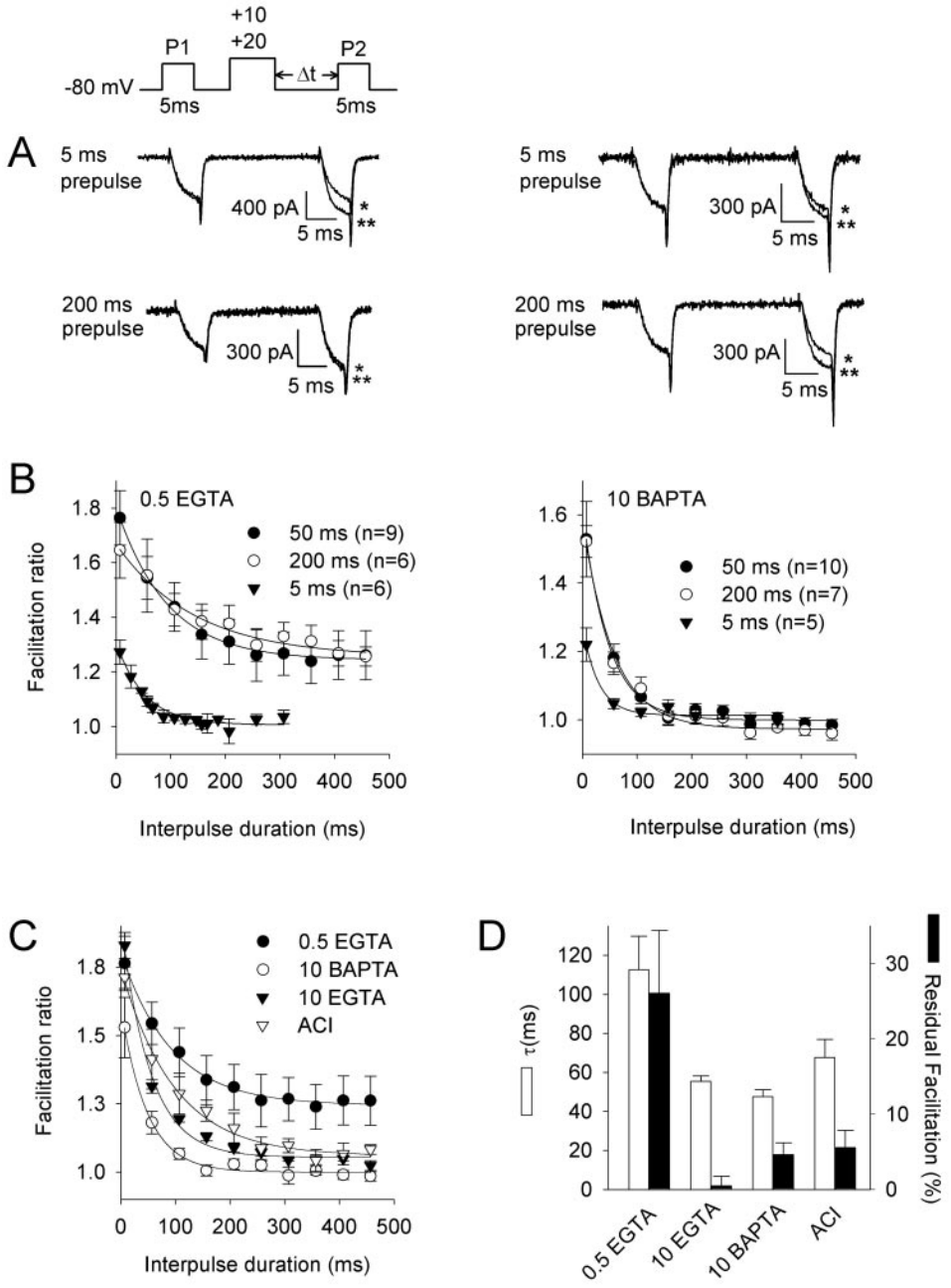

Figure 7. Effects of Ca^{2+} and calmodulin on decay of facilitation of P/Q-type Ca^{2+} channels. *A*, The voltage protocol (*top*) for measuring decay of facilitation and representative Ca^{2+} currents (*bottom*) elicited by test pulses to 0 mV (+10 mV for 10 mM BAPTA) before (*P1*) and after (*P2*) a conditioning prepulse to +10 mV (+20 mV for BAPTA) for 5 or 200 msec. Superimposed are currents in which *P2* was given 0.1 msec (*double asterisks*) or 300 msec (*single asterisk*) after the conditioning prepulse. Intracellular recording solutions contained either 0.5 mM EGTA (*left traces*) or 10 mM BAPTA (*right traces*). *B*, Effect of prepulse duration and Ca^{2+} buffering on decay of facilitation. The facilitation ratio was obtained by normalizing the peak current from *P2* to that from *P1* and was plotted against the interval between the conditioning prepulse and *P2*. Shown are results obtained with 5, 50, and 200 msec conditioning prepulses with 0.5 mM EGTA (*left*) or 10 mM BAPTA (*right*) in the recording pipette. *C*, Data obtained as described in *B* with a 50 msec conditioning prepulse and intracellular solutions containing 0.5 mM EGTA ($n = 9$), 10 mM BAPTA ($n = 10$), or 10 mM EGTA ($n = 7$) and with overexpression of the calmodulin inhibitor peptide (ACI; $n = 5$) with a 0.5 mM EGTA intracellular solution. *D*, Time constants (τ;msec) for the decay of facilitation obtained from single-exponential fits of the data in *C*. Residual facilitation is the percentage of the maximum facilitated current remaining 400 msec after the conditioning prepulse.

expressed. Voltage-dependent inactivation of the $\alpha_{1A\text{-}b}$ variant coexpressed with either β_{1b} or β_{2a} is minimal (Bourinet et al., 1999). Based on our findings, this should reveal a marked Ca^{2+}-dependent modulation of $\alpha_{1A\text{-}b}$ channels, regardless of the β subunit expressed. Thus, the functional diversity of P/Q-type Ca^{2+} channels is potentially quite complex, and various combinations of α_{1A} and β subunits will yield channels distinguished by their biophysical properties as well as their regulation by Ca^{2+}.

Mechanism of Ca^{2+}-dependent inactivation and facilitation of P/Q-type Ca^{2+} channels

Ca^{2+} entry through P/Q-type Ca^{2+} channels promotes the binding of calmodulin to an atypical α_{1A} CBD in the cytoplasmic C-terminal domain of the α_{1A} subunit (Lee et al., 1999). This interaction underlies Ca^{2+}-dependent inactivation and facilitation of P/Q-type channels with β_{1b}, because these effects are reversed by deletion of the α_{1A} CBD. Our studies of P/Q-type channels with β_{2a} reveal important new insights into the role of Ca^{2+} and calmodulin in the feedback regulation by Ca^{2+}. First, deletion of the α_{1A} CBD strongly reduces but does not eliminate Ca^{2+}-dependent inactivation and facilitation (Fig. 1; data not shown). Also, the ACI peptide, which should eliminate the influence of Ca^{2+}/calmodulin, does not completely abolish the effects of Ca^{2+} on inactivation or facilitation (Figs. 1, 3). The incomplete blockade of Ca^{2+}-dependent inactivation and facilitation by these two manipulations suggests additional modulation by Ca^{2+}, independent of calmodulin. Such calmodulin-independent modulation might be caused by Ca^{2+} ions binding to an EF-hand motif in the C-terminal domain of α_{1A} (De Leon et al., 1995) or to other, as yet unrecognized, Ca^{2+}-binding sites. In addition, Ca^{2+} entry through P/Q-type channels could activate second messenger-regulated kinases, such as protein kinase C, that potentiate I_{Ca} through P/Q-type channels (Stea et al., 1995; Bourinet et al., 1999) and could produce effects additive to those of calmodulin on facilitation.

A second surprising feature of P/Q-type Ca^{2+} channel modulation by Ca^{2+} was revealed in the block of inactivation but not facilitation by 10 mM intracellular EGTA (Figs. 1, 3), which suggests a difference in the Ca^{2+} dependence of the two processes. An intriguing explanation for the distinct effects of Ca^{2+} on facilita-

tion and inactivation is provided by differences in Ca^{2+} binding to the N- and C-terminal lobes of calmodulin (James et al., 1995). Ca^{2+} binding to calmodulin is highly cooperative with Ca^{2+} binding first to the C-terminal EF-hands, which have the highest intrinsic affinity for Ca^{2+}, followed by Ca^{2+} binding to lower affinity sites in the N-terminal lobe (Wang, 1985). Thus, I_{Ca} facilitation and inactivation could result from conformational changes in calmodulin after Ca^{2+} binding to the C- and N-terminal lobes, respectively. That the two lobes of calmodulin can differentially regulate ion channel function is evident from analyses of calmodulin mutants in *Paramecium* (Kink et al., 1990), Ca^{2+} activation of K^+ channels (Keen et al., 1999), and Ca^{2+}-dependent inactivation of L-type Ca^{2+} channels (Peterson et al., 1999).

Finally, paired-pulse protocols reveal some prepulse-induced facilitation, even when intracellular Ca^{2+} is strongly buffered with BAPTA (Figs. 4, 6, 7) and when extracellular Ca^{2+} is replaced by Ba^{2+} (Fig. 4). This residual, Ca^{2+}-independent facilitation may result from a voltage-dependent enhancement of channel activation. Membrane depolarization might cause the initial facilitation of the current because of activation of one or more voltage sensors. The association of Ca^{2+} and calmodulin with α_{1A} could promote and stabilize the activated conformation of the sensor or sensors such that these partially activated channels would activate more rapidly and at lower voltages, causing facilitation in response to subsequent depolarizations. This mechanism would explain the longer lifetime of the facilitated state of the channel in the presence of Ca^{2+} and calmodulin (Fig. 7) and the accumulation and maintenance of Ca^{2+}- and calmodulin-dependent facilitation of P/Q-type channels during trains of pulses (Fig. 3*C*).

Ca^{2+}-dependent modulation of P/Q-type Ca^{2+} channels and synaptic plasticity

Inactivation and facilitation of Ca^{2+} currents through P/Q-type channels expressed in tsA-201 closely resemble the behavior of presynaptic P/Q-type channels such as those recorded at the calyx of Held synapse in the rat brainstem (Borst and Sakmann, 1998; Cuttle et al., 1998; Forsythe et al., 1998). In both cases, trains of depolarizations cause an initial facilitation followed by inactivation of the Ca^{2+} current over time, recovery from inactivation is relatively slow, and facilitation is strongly dependent on incoming Ca^{2+} ions. At the calyx of Held, inactivation of P/Q-type channels, along with other presynaptic mechanisms (Wang and Kaczmarek, 1998; Borst and Sakmann, 1999; Wu and Borst, 1999), contributes to post-tetanic depression of synaptic transmission (Forsythe et al., 1998). However, the effects of extracellular Ba^{2+} and intracellular BAPTA in slowing inactivation are significantly weaker for native than for recombinant P/Q-type channels. This could be explained by the existence of multiple β subunits in rat auditory neurons that would produce Ca^{2+} channels that are modulated by both Ca^{2+}-dependent and -independent mechanisms. In addition, a Ca^{2+}-independent regulation of native Ca^{2+} channels could be mediated by signaling molecules not present in the tsA-201 cells recorded in our study. Nevertheless, inactivation of P/Q-type channels at the calyx of Held is strongest for Ca^{2+} currents in the absence of strong intracellular Ca^{2+} buffers (Forsythe et al., 1998), suggesting a role for Ca^{2+} and calmodulin in the negative feedback of P/Q-type channels at this and other synapses.

Considering its rapid onset (Figs. 3, 6), Ca^{2+}-dependent facilitation of P/Q-type channels may contribute to the short-term enhancement of synaptic transmission that depends on elevated intracellular Ca^{2+} during the second of two depolarizing pulses (Zucker, 1999). Although synaptic enhancement occurs in the absence of measurable increases in presynaptic Ca^{2+} influx (Tank et al., 1995; Zucker, 1999), fluorometric techniques typically used for measuring intracellular Ca^{2+} signals are relatively insensitive and may not detect very high but local changes in Ca^{2+} caused by facilitation of Ca^{2+} channel opening. At the calyx of Held synapse, where the contribution of presynaptic Ca^{2+} currents to neurotransmitter release can be assessed by simultaneous presynaptic and postsynaptic recording, paired-pulse stimulation causes facilitation of presynaptic P/Q-type channels and, occasionally, the enhancement of the postsynaptic response when the effects of synaptic depression are minimized (Borst and Sakmann, 1998). Facilitation of presynaptic Ca^{2+} channels at this synapse is attenuated when extracellular Ca^{2+} is replaced by Ba^{2+} and by presynaptic dialysis with BAPTA, but not by GTPγS, GDPβS, or pharmacological blockade of Ca^{2+}-dependent kinases and phosphatases (Cuttle et al., 1998). These results are consistent with the facilitation of P/Q-type channels caused by the Ca^{2+}-dependent association of calmodulin with the α_{1A} subunit. Because P/Q-type channels are important in the regulation of neurotransmitter release at numerous synapses, Ca^{2+}-dependent facilitation of these channels may fundamentally contribute to the enhancement of synaptic function in the nervous system.

Ca^{2+} and calmodulin have long been implicated in mechanisms of synaptic plasticity. That P/Q-type Ca^{2+} channels are significantly regulated by these molecules is unexpected, in part because current knowledge is primarily limited to the behavior of Ba^{2+} currents through these channels. However, the importance of P/Q-type channel modulation by Ca^{2+} and calmodulin is underscored by the physiological consequences of Ca^{2+}-dependent inactivation and facilitation of presynaptic P/Q-type channels in neurons. Elucidating how Ca^{2+}/calmodulin, G-proteins, and other regulatory influences coordinately control Ca^{2+} influx through P/Q-type channels promises further insight into mechanisms leading to altered synaptic function.

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