β -Adrenergic Stimulation Selectively Inhibits Long-Lasting L-Type Calcium Channel Facilitation in Hippocampal Pyramidal Neurons

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L-type calcium channels are abundant in hippocampal pyramidal neurons and are highly clustered at the base of the major dendrites. However, little is known of their function in these neurons. Single-channel recording using a low concentration of permeant ion reveals a long-lasting facilitation of L-type channel activity that is induced by a depolarizing prepulse or a train of action potential waveforms. This facilitation exhibits a slow rise, peaking 0.5–1 sec after the train and decaying over several seconds. We have termed this behavior “delayed facilitation,” because of the slow onset. Delayed facilitation results from an increase in opening frequency and the recruitment of longer duration openings. This behavior is observed at all membrane

potentials between -20 and -60 mV, with the induction and magnitude of facilitation being insensitive to voltage. β -Adrenergic receptor activation blocks induction of delayed facilitation but does not significantly affect normal L-type channel activity. Delayed facilitation of L-type calcium channels provides a prolonged source of calcium entry at negative membrane potentials. This behavior may underlie calcium-dependent events that are inhibited by β -adrenergic receptor activation, such as the slow afterhyperpolarization in hippocampal neurons.

Key words: calcium channel; L-type; modulation; facilitation; hippocampus; single channel

A number of high-voltage-activated Ca channel subtypes are found in CNS neurons, including the dihydropyridine-sensitive L-type Ca channel, which was first described in ventricular heart muscle (for review, see Tsien et al., 1988; McCleskey, 1994). Recent work has identified possible roles for some Ca channel subtypes in neurons (Takahashi and Momiyama, 1993; Wheeler et al., 1994), but little is known of the function of L-type Ca channels in the CNS or their modulation by endogenous neurotransmitters.

L-type Ca channels are prevalent in hippocampal pyramidal neurons, contributing ~ 30 – 50% of total calcium current (Mintz et al., 1992; Swartz and Bean, 1992; McDonough et al., 1996). The majority of L-type channels are distributed on the surface of both the cell body and the proximal dendrites (Westenbroek et al., 1990). The somatic distribution of these channels is highly uneven, with the majority being localized to the base and proximal portions of both the apical and basal dendrites (Ahlijanian et al., 1990; Westenbroek et al., 1990). Their abundance and location suggest that L-type calcium channels likely play a crucial role in control of dendritic excitability and in addition provide calcium for intracellular effectors. For example, activation of L-type calcium channels provides the intracellular calcium increase for generation of the slow afterhyperpolarization (AHP) in these neurons (Rascol et al., 1991; Moyer et al., 1992). Therefore, modulation of L-type Ca channel activity may have dramatic effects on pyramidal cell excitability.

Cardiac L-type Ca channels exhibit distinct gating modes,

characterized either by short openings and low open probabilities [$P(o)$] or by long openings and high $P(o)$ (Hess et al., 1984). High $P(o)$ activity is promoted by the dihydropyridine (DHP) calcium channel agonist BAY K 8644, and low $P(o)$ behavior is favored by DHP antagonists (Hess et al., 1984). In addition, strong prepulse depolarizations can drive the L-type channel from its normal rapid gating pattern to one characterized by long openings and high $P(o)$ (Pietrobon and Hess, 1990). Such behavior, termed prepulse facilitation, has also been observed for L-type channels in CNS neurons. For example, cerebellar granule cells possess an L-type calcium channel that exhibits reopening behavior after membrane depolarization (Slesinger and Lansman, 1991, 1996; Forti and Pietrobon, 1993). Prepulse facilitation of L-type calcium channels has also been observed in hippocampal pyramidal neurons (Fisher et al., 1990; Thibault et al., 1993; Kavalali and Plummer, 1994, 1996).

Prepulse facilitation of L-type Ca channels in cerebellar and hippocampal neurons is complete within 100–200 msec after the prepulse. This relatively slow process can be modestly prolonged by lowering the permeant ion concentration from 110 to 20 mM (Thibault et al., 1993). In this study we report a longer-lasting form of facilitated L-type calcium channel activity that is induced by either a depolarizing prepulse or a train of action potential waveforms. Unlike previously described examples, the facilitation is slow to rise and is extremely long-lasting, decaying over several seconds. We have termed this phenomenon “delayed facilitation.” In addition, we show that induction of delayed facilitation is blocked by activation of β -adrenergic receptors. Delayed facilitation provides a long-lasting source of calcium entry at negative membrane potentials. This behavior may underlie prolonged calcium-dependent events that are inhibited by β -adrenergic stimulation, such as the slow AHP.

MATERIALS AND METHODS

Acutely dissociated hippocampal CA1 neurons were obtained as described previously (McDonough et al., 1996). Briefly, Sprague Dawley

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rats (9–14 d old) were anesthetized with halothane and decapitated. Hippocampi were rapidly dissected and cut into 300–400- μ m-thick slices. Slices were incubated at 37°C in saline containing (in mM): Na_2SO_4 , 82; K_2SO_4 , 30; HEPES, 10; and MgCl_2 , 5, pH 7.4, with added protease type XXIII (3 mg/ml) for nine min, bubbled with O_2 . Tissue slices were then transferred to solution containing trypsin inhibitor (1 mg/ml) and bovine serum albumin (1 mg/ml) for 1 min and finally rinsed in saline solution containing no enzyme. The CA1 region was microdissected and triturated into Falcon Primaria dishes as needed.

Experiments using the membrane-permeant cAMP analog 8-(4-chlorophenylthio)-adenosine 3':5'-cyclic monophosphate (8-CPT) cAMP used cultured hippocampal neurons. These experiments were completed before acutely dissociated neurons were used routinely. Delayed facilitation was observed with these neurons (Craig and Marrion, 1995) and did not differ from that reported in this study. Hippocampal cells were dissociated from neonatal (1–3 d) Sprague Dawley rats and maintained in culture as described previously (Legendre and Westbrook, 1990).

Before recording, cells were incubated for 20–30 min in a modified Krebs' solution containing (in mM): NaCl , 150; KCl , 5; CaCl_2 , 1; MgCl_2 , 1; HEPES, 10; and glucose, 5, pH 7.4, supplemented with ω -conotoxins MVIIC (5 μ M) and GVIA (1 μ M) to block P-, Q-, and N-type Ca channels (McCleskey et al., 1987; McDonough et al., 1996). During recording, cells were superfused (15 ml/min) with an external solution containing (in mM): potassium aspartate, 125; KCl , 35; MgCl_2 , 5; HEPES(Na), 10; EGTA, 10; and CaCl_2 , 5.64 (to give an estimated free concentration of 60 nM) (Fabiato and Fabiato, 1979), pH 7.4, with CsOH. Cells in this solution had $\sim$ 0 mV membrane potential. All potentials are expressed as the negative of the potential imposed on the pipette. Cell-attached patch recordings (Hamill et al., 1981) were made using thick-walled (1.5 mm outer diameter, 0.5 mm inner diameter) quartz electrodes (7–10 M Ω) containing (in mM): BaCl_2 , 10; tetraethylammonium chloride, 135; and HEPES, 10, pH 7.4. Single-channel currents were recorded with an Axopatch 200A amplifier (Axon Instruments) and digitized onto video (94 kHz sampling frequency, 37 kHz bandwidth; VR10B, Instrutech Corp.) for later analysis. Single-channel records were filtered at 1–2 kHz with an eight pole Bessel filter (Frequency Devices) and acquired at 100 μ sec intervals for analysis using Pulse (Heka, distributed by Instrutech Corp.). Single channels were analyzed using MacTAC (Skalar Instruments, distributed by Instrutech Corp.). The "50% threshold" technique was used to estimate event amplitudes and durations, with each transition inspected visually before being accepted. Open duration histograms, constructed from openings to level -1 , were binned logarithmically (20 bins/decade) plotted against the square root transformation of the ordinate (number of events/bin), and the distribution was fitted by a sum of exponential probability density functions using the maximum likelihood method. With this type of representation, peaks in the histogram correspond to the time constant of the exponential (Sigworth and Sine, 1987). Events <166 μ sec were missed, because after filtering they did not reach the 50% threshold. These missed events were not corrected for.

Channel $P(o)$ was first estimated as $NP(o)$, the product of the open probability $\times$ the number of channels. $NP(o)$ was calculated as the sum of (dwell time $\times$ level number) divided by the total time. N was estimated as the number of simultaneously open channels after induction of delayed facilitation, and finally $P(o)$ was obtained by dividing $NP(o)$ by N . $P(o)$ was calculated within 200 msec time segments using ReadEvents 1.36 (Dr. Scott Eliasof, Vollum Institute). However, it is apparent from data such as those illustrated in Figure 2 that an estimate of N is difficult. We have observed patches that did not display superimposed openings on induction of delayed facilitation or during standard activation (by a depolarizing voltage pulse from -60 mV to 0 mV) but exhibited multiple openings after addition of BAY K 8644. However, because we have found that BAY K 8644 could not be completely washed from the experimental rig, its use was limited to a period of experiments demonstrating that the channel class underlying delayed facilitation is L-type. Thus, the value of N is probably an underestimate, thereby giving an overestimate of patch $P(o)$ but with accurate determinations of the reported changes in $P(o)$. This may be reflected in the range of $P(o)$ values observed under control conditions in Figures 1, 3, and 6. However, L-type channels are known to exhibit activity at negative membrane potentials (Marchetti et al., 1995; Magee et al., 1996; Rubart et al., 1996). For example, whole-cell Ca current and intracellular Ca measurements in hippocampal CA1 pyramidal neurons have demonstrated a DHP antagonist-sensitive Ca current active at membrane potentials negative to -60 mV (Magee et al., 1996). The DHP-sensitive Ca channels were found to be located most

densely on the soma and proximal apical dendrite of these neurons (Magee et al., 1996), the subcellular location of L-type Ca channels (Ahljian et al., 1990; Westenbroek et al., 1990). These data suggest that L-type channels exhibit a low level of activity at very negative membrane potentials.

Delayed facilitation was evoked by three different voltage protocols: a 200 msec prepulse to $+40$ mV, a 100 Hz train of 10 rectangular voltage pulses to $+40$ mV (5 msec duration, separated by 5 msec intervals), and a 50 Hz train of 10 action potential waveforms. Each action potential waveform consisted of a 200 μ sec ramp from -60 mV to a peak of $+50$ mV, followed by a 400 μ sec ramp to $+20$ mV, a 400 μ sec ramp to -10 mV, and ending with a 2 msec ramp to -70 mV. The potential was returned to -60 mV by an additional 17 msec ramp (to mimic an afterhyperpolarization) before the next waveform was initiated. The half-maximal duration of each waveform was 1 msec (see Fig. 2, inset).

Single channel conductance was estimated as the slope of the current-voltage relationship for L-type Ca channels recorded with 10 mM Ba^{2+} as the charge carrier. Current-voltage curves were generated using the current amplitude determined from gaussian fits to amplitude histograms from individual patches (see above). Open times of L-type channels in the absence of a DHP agonist were extremely short (see Figs. 6 and 8), and only events that exceeded a certain duration were used to provide an estimate of single-channel amplitude. For example, when data were Bessel-filtered at 1 kHz, the rise time of the eight pole Bessel filter was ~ 400 μ sec. Under these conditions, only events that exceeded a 1 msec duration were included, thereby excluding those events in which the full amplitude was not resolved. All statistical tests were unpaired, two-tailed Student's t test or ANOVA (Origin, Microcal Software Inc.). Values are given as mean $\pm$ SEM.

All reagents were obtained from Sigma, except ω -conotoxin MVIIC (Bachem), ω -conotoxin GVIA (Peninsula Laboratories), nimodipine and isoproterenol (Research Biochemicals), CaCl_2 (Fluka), BAY K 8644 and HEPES (Calbiochem), and CsOH (Aldrich, WI).

RESULTS

Delayed facilitation of L-type channel activity

Delayed facilitation of L-type calcium channels was identified using cell-attached patch recordings from acutely dissociated CA1 hippocampal pyramidal neurons with a low concentration of Ba^{2+} or Ca^{2+} as the permeant ion (5–10 mM). Prepulse depolarization ($+40$ mV) dramatically enhanced L-type channel activity at negative membrane potentials (27 of 55 patches) (Fig. 1). In the absence of a prepulse (control), channels exhibited a very low $P(o)$. After a 200 msec depolarization to $+40$ mV, the peak $P(o)$ was increased 14.2 ± 3.6 -fold ($n = 17$). When measured over a 6 sec sweep, prepulses increased total patch $P(o)$ by 7.4 ± 1.5 ($n = 22$). It is not known whether channel activity was required during the prepulse for delayed facilitation to be observed, because openings could not be resolved at $+40$ mV (during the prepulse).

Delayed facilitation required a low concentration of permeant ion (5–10 mM) and was never seen with 110 mM Ba^{2+} in the recording solution (also see Thibault et al., 1993). Whole-cell current activation curves were used to measure the effect of charge screening of Ba^{2+} on L-type channel voltage dependence. An increase of Ba^{2+} concentration from 15 to 110 mM produced a depolarizing shift of 14.5 ± 2.7 mV ($n = 5$) in the position of the activation curves. Correction of this voltage shift did not reveal "hidden" delayed facilitation of L-type channels ($n = 26$). Therefore, delayed facilitation was only observed when low, more physiological concentrations of permeant ion were used.

Delayed facilitation was often preceded by a more rapidly decaying form of facilitated L-type channel activity (26 of 55 patches). This fast facilitation consisted of longer duration openings than observed in the absence of a prepulse and decayed within 100–200 msec (not shown). Fast facilitation appeared to be kinetically distinct from delayed facilitation and decayed before the peak of delayed facilitation was observed. In addition, they

Figure 1. Delayed facilitation of L-type calcium channel activity by a depolarizing prepulse. *A*, Stability plot of L-type channel activity in a cell-attached patch, showing $P(o)$ for each 6 sec sweep. The patch appeared to contain a single channel. The patch potential was stepped to -30 mV for 6 sec from a holding potential of -60 mV, either directly (*Control*, thin bar) or immediately after a prepulse to $+40$ mV (200 msec duration) (*Prepulse*, thick bar). Channel activity was low throughout the control sweeps and was dramatically augmented after the depolarizing prepulse. Sweeps were considered facilitated if the patch $P(o)$ measured over the entire 6 sec sweep exceeded two times the mean $P(o)$ of control sweeps (dashed line). *B*, Selected sweeps as indicated in *A*. Little channel activity was observed on changing the patch potential from -60 to -30 mV (control, left traces). After the prepulse to $+40$ mV, there was a large increase in L-type activity. Short duration events were observed throughout the 6 sec sweep.

frequently occurred in separate patches, with 13 patches exhibiting only delayed facilitation, whereas 14 patches displayed both forms. Of those patches that did not exhibit delayed facilitation, $\sim 45\%$ displayed fast facilitation (12 of 28 patches). Fast facilitation possesses a time course similar to that described previously for L-type channels (Kavalali and Plummer, 1994, 1996) and was not examined in detail in this study. All subsequent data are presented with analysis started 250 msec after the prepulse or termination of the burst of action potential waveforms, thereby excluding this form of facilitation from analysis (see Fig. 6 legend).

Delayed facilitation of L-type calcium channels could be evoked either by a long rectangular voltage step (200 msec) (Fig. 1) or by a train of short (5 msec) duration voltage pulses (see Fig. 5). Both protocols featured a voltage excursion to $+40$ mV, which is close to the peak of the action potential measured in hippocampal neurons (Lancaster and Adams, 1986). Delayed facilitation was also induced by a 50 Hz train of action potential waveforms (Fig. 2) (9 of 15). This result is of major physiological significance.

It indicates that a train of only 10 action potential waveforms, at a lower frequency than the likely maximum firing frequency (100 Hz; Lancaster and Adams, 1986), evokes robust delayed facilitation at -20 mV. In three of three patches that did not exhibit delayed facilitation with a 200 msec rectangular prepulse, we were able to produce it when the protocol was switched to a train of action potential waveforms. Figure 3 shows an example of such a patch in which repeated depolarizing prepulses ($+40$ mV, 200 msec duration) failed to evoke delayed facilitation. However, after the protocol was changed to the train of action potential waveforms (see Fig. 2, *inset*) delayed facilitation was observed at -50 mV in $\sim 30\%$ of sweeps (Fig. 3). Induction of delayed facilitation at -60 mV by action potential waveforms was also observed using 5 mM Ca^{2+} as the charge carrier (two of three patches) (data not shown). Delayed facilitation with Ca^{2+} was indistinguishable from that recorded using Ba^{2+} ; i.e., peak $P(o)$ occurred with a delay and decayed over the 6 sec voltage excursion. There was also an increase in the relative contribution of longer duration openings in facilitated sweeps, as observed with

Figure 2. A train of action potential waveforms induce delayed facilitation of L-type channels. Selected traces of 5 sec sweeps of channel activity for control (*left*) and after a train of action potential waveforms (*right*) at -20 mV. The patch appeared to contain two channels. Low $P(o)$ activity was seen in control sweeps. Delayed facilitation was observed after the 50 Hz. train of an action potential waveform (*inset*). Each action potential waveform consisted of a ramp to a peak of $+50$ mV, followed by successive ramps to $+20$ and -10 mV. The potential was returned to -70 mV by an additional ramp (to mimic an afterhyperpolarization). The membrane potential was slowly ramped back to -60 mV before the next waveform was initiated (see Materials and Methods for details). Delayed facilitation was evoked by 10 action potential waveforms in 200 msec, giving a frequency of 50 Hz. Openings with a curtailed amplitude reflect short duration openings with apparent amplitude that is clipped at the bandwidth used (2 kHz). Expanded traces for behavior observed in control jumps and after the train of action potential waveforms are shown below. The expanded traces were taken from the sweeps marked with a bar and *. As is seen in open duration histograms (see Fig. 6), delayed facilitation is caused by an increase in opening frequency and an increase of longer duration openings.

Ba^{2+} (see below). These data indicate that the physiological charge carrier through L-type Ca channels can sustain delayed facilitation, and that changes in membrane potential that occur during a burst of action potentials may be more suited to promoting L-type channel facilitation than standard rectangular voltage pulses.

N-, P-, and Q-type channel currents were blocked by preincubating cells with ω -conotoxins GVIA and MVIIC (McCleskey et al., 1987; McDonough et al., 1996). Under these conditions the channel activity observed during delayed facilitation was blocked by the DHP antagonist nimodipine ($1 \mu\text{M}$; $n = 4$) (results not shown). In addition, the channel activity observed after preincubation with ω -conotoxins GVIA and MVIIC was sensitive to the DHP agonist BAY K 8644 ($5 \mu\text{M}$; $n = 7$) (Fig. 4). Before addition of BAY K 8644, channel openings were observed during depolarizing voltage pulses from -60 to 0 mV (Fig. 4*Ai*) and after a

burst of action potential waveforms (Fig. 4*Cii*). Short duration channel activity was elicited by a 200 msec voltage pulse to 0 mV (holding potential, -60 mV) and was observed throughout the pulse. Generation of an ensemble current gave a waveform that showed some decay during the voltage pulse (Fig. 4*Aiii*). Measurement of channel openings >1 msec in duration (see Materials and Methods) over a voltage range of -20 to 10 mV gave a slope conductance of 10.5 pS (Fig. 4*B*). Delayed facilitation was evoked by a burst of action potential waveforms, revealing that this patch contained at least two channels (Fig. 4*Ci,ii*) of similar amplitude to events evoked by a voltage pulse (Fig. 4*B*). After addition of BAY K 8644 ($5 \mu\text{M}$), voltage pulses to 0 mV (holding potential, -60 mV) produced superimposed channel openings of long duration (Fig. 4*Aii*). Generation of an ensemble current showed that BAY K 8644 had greatly augmented the channel activity, producing a large current that showed little decay during the voltage

Figure 3. Trains of action potential waveforms are more suited to promoting delayed facilitation than a depolarizing prepulse. *Top*, Time plot of patch $P(o)$ for each 6 sec postpulse potential of -50 mV. Holding potential was -60 mV. The patch appeared to contain two channels. Delayed facilitation was not observed with a 200 msec prepulse to $+40$ mV but was observed with a 50 Hz train of an action potential waveform (see Fig. 2, *inset*, for waveform schematic and Materials and Methods for description). *Bottom*, Selected traces of the 6 sec time segment used to calculate patch $P(o)$. Low $P(o)$ activity was seen for both control and after a prepulse. Delayed facilitation was observed after the 50 Hz train of an action potential waveform.

pulse (Fig. 4*Aiv*). Long duration openings were also observed during 5 sec pulses either with or without a preceding burst of action potential waveforms (Fig. 4*Ciii,iv*). Estimation of channel slope conductance in the presence of BAY K 8644 was 15.5 pS (Fig. 4*B*) (mean conductance, 14.3 ± 0.9 pS; $n = 4$). The increase in single-channel conductance observed in the presence of BAY K 8644 has been reported previously (Mantegazza et al., 1995). The conversion of short duration channel activity to long duration openings characteristic of DHP agonist-modified channels indicates that the channel underlying delayed facilitation is the L-type channel. However, because DHP agonists affect the gating of this channel (Fig. 4), all remaining data were obtained in the absence of BAY K 8644.

The slope conductance of L-type channels exhibiting delayed facilitation and recorded in the absence of DHP agonist was 10.7 ± 1.9 pS ($n = 3$) (see Materials and Methods), close to that observed for L-type calcium channels in ventricular heart muscle (Hess et al., 1986). No significant difference in conductance was observed between L-type channels in patches that exhibited delayed facilitation and those that did not ($p > 0.05$). In addition, channel activity observed in control sweeps (i.e., without the prepulse) and those activated by a voltage step from -60 mV (200 msec duration) had conductances identical to the channels that

displayed delayed facilitation. Delayed facilitation was observed only in patches that possessed L-type channels activated by depolarization from -60 mV to 0 mV, which suggests that delayed facilitation results from an increase in activity of the standard L-type channel, rather than recruitment of a novel channel type (see Discussion).

Time-dependent and voltage-independent properties of delayed facilitation

The time course of delayed facilitation can be best described by a generated ensemble current. Figure 5 illustrates an ensemble of L-type channel activity, induced by a train of short-duration rectangular voltage pulses. The onset of facilitated channel activity was slow to rise, peaking ~ 600 msec after termination of the train (Fig. 5). The behavior subsequently decayed with an exponential time course ($\tau \sim 1.6$ sec). Although the time course of delayed facilitation varied between patches, it consistently peaked > 500 msec after the prepulse and decayed by the end of the 6 sec test potential.

Delayed facilitation was attributable to a time-dependent increase in $P(o)$ (Fig. 6) and was observed at membrane potentials between -20 and -60 mV. Under control conditions, at both -20 (Fig. 6*A*) and -50 mV (Fig. 6*B*), the patch $P(o)$ was low

Figure 4. Channels underlying delayed facilitation are sensitive to the DHP agonist BAY K 8644. *A*, Inward channel openings evoked by repeated depolarizing voltage pulses from -60 mV to 0 mV recorded from a cell-attached patch with 10 mM Ba^{2+} as the charge carrier. Channel openings are downward. This patch appeared to contain two channels. *i*, In the absence of a DHP agonist, single-level channel openings were brief and were observed throughout the 200 msec depolarization. Generation of an ensemble current (average of 23 sweeps) gave a waveform that showed little decay during the depolarization (*iii*). *ii*, In the presence of the DHP agonist BAY K 8644 (5 μM), channel openings were of long duration and were observed to the second level. Generation of an ensemble current (average of 27 sweeps) showed that BAY K 8644 had greatly augmented channel activity (*iv*). *B*, Current-voltage relationship of channel amplitude observed in the absence and presence of BAY K 8644. Channel amplitude was obtained by gaussian fits to amplitude histograms obtained by visual inspection of each opening (see Materials and Methods) evoked by a family of depolarizing voltage pulses to -40 to 10 mV (holding potential, -60 mV). Continuous lines represent the least squares fit to the data. In the absence of BAY K 8644 ($\bullet$), channel slope conductance was 10.5 pS. In the presence of BAY K 8644 ($\blacklozenge$), the channel slope conductance increased to 15.5 pS (see Results). Channel amplitude observed in the absence of BAY K 8644 during 5 sec pulses to -20 (see below), with ($\blacktriangle$) or without ($\blacktriangledown$) a train of action potential waveforms superimposed on the control *I/V*. In the presence of BAY K 8644, the increase in channel amplitude was also observed for openings evoked during 5 sec pulses to -20 (see below), with ($\triangle$) or without ($\triangledown$) a train of action potential waveforms. *C*, *i*, *iii*, Records evoked by a 5 sec voltage pulse from -60 mV to -20 mV (see Fig. 2 for protocol) in the absence (*i*) and presence (*iii*) of BAY K 8644 (5 μM). *ii*, *iv*, Records evoked by a 5 sec voltage pulse to -20 mV (holding potential, -60 mV) preceded by the train of action potential waveforms (see Fig. 2 for protocol) in the absence (*ii*) and presence (*iv*) of BAY K 8644 (5 μM). *i*, *ii*, Delayed facilitation was evoked by a train of action potential waveforms and was observed at -20 mV, with few openings seen in the absence of the action potential waveform train. Induction of delayed facilitation demonstrated that the patch contained two channels. *iii*, *iv*, In the presence of BAY K 8644, long duration openings were observed during 5 sec pulses to -20 (see below), with or without a train of action potential waveforms. Note the addition of BAY K 8644 caused the short duration openings to be replaced by openings characteristic of DHP-agonist modified channels. In addition, note that normal channel gating was observed between bursts of DHP-modified behavior (*iv*, upper trace).

Figure 5. Ensemble current of delayed facilitation induced by a train of voltage pulses. Ensemble current generated by a 100 Hz train of rectangular voltage pulses to $+40$ mV of 5 msec duration, separated by 5 msec intervals. Inset, Voltage protocol in more detail. Holding potential was -60 mV, and post-train voltage was -30 mV. Ensemble is an average of 27 sweeps. A slow rise of inward current was observed, peaking ~ 600 msec after the termination of the train. The rising phase of the waveform was interpolated with a continuous line, and the decay was fit with an exponential time course (the arrow marks the start point of the exponential fit; $\tau \sim 1.6$ sec). The dashed line represents zero current.

throughout a 5 sec sweep. After a prepulse to $+40$ mV (200 msec duration), patch $P(o)$ slowly increased to a plateau of ~ 0.1 – 0.15 and decayed back toward control levels with an exponential time course ($\tau \sim 1.5$ sec) (Fig. 6). This waveform was observed at each voltage tested, with no obvious effect of membrane potential on the time to peak or the decay rate of delayed facilitation.

The time-dependent increase in patch $P(o)$ resulted both from an increased channel opening frequency (evident in Figs. 1–4) and an increase in the proportion of long duration openings. Under control conditions, patches exhibited short duration channel open times that were best described by a single exponential ($\tau \sim 0.4$ msec) or by two exponentials with a small percentage of longer duration openings ($\tau \sim 3$ msec) (Fig. 6*A,B*). On facilitation by a voltage prepulse ($+40$ mV prepulse, 200 msec duration), an increase in the contribution of longer duration openings was observed. Open time histograms of all events in facilitated sweeps were best described by the sum of two exponentials ($\tau \sim 0.4$ and 2 – 5 msec), with a significant increase in openings (ANOVA, $p < 0.01$) described by the longer time constant (Fig. 6*A,B*). This observation was the same at each membrane potential tested (-20 to -50 mV) and was unaffected by the membrane potential (see below).

The induction of delayed facilitation was not affected by the postprepulse potential (Fig. 6). In addition, the membrane potential at which delayed facilitation was observed did not have a marked effect on the contribution of longer duration openings. Figure 7*A* shows the contribution of the longer time constant to the open duration histogram of facilitated openings. At each potential, either a 200 msec voltage prepulse to $+40$ mV or a train of action potential waveforms evoked a significant increase over control activity in the proportion of longer duration openings (ANOVA, $p < 0.01$). In contrast, there was no significant differ-

Figure 6. Time-dependent increase in $P(o)$ results partly from an increase in long duration openings. *A, Top*, Open probability in 200 msec time segments averaged over a 5 sec voltage pulse to -20 mV. Under control conditions (mean of 10 sweeps) the $P(o)$ was low throughout the voltage pulse (*left*). A 200 msec prepulse to $+40$ mV (*right*) caused a dramatic increase in $P(o)$ with a rise time of ~ 600 msec and a subsequent decay (mean of nine facilitated sweeps). The rising phase of the waveform was interpolated with a continuous line, and the decay was fit with an exponential function ($\tau \sim 1.5$ sec). *Bottom*, Open duration histograms show that openings under control conditions were of very short duration ($\tau \sim 0.4$ msec) (0% of long openings), and that the prepulse recruited longer duration openings (13% of events), best fit by an exponential of $\tau \sim 4$ msec. *B, Top*, $P(o)$ plots of control (mean of 14 sweeps) and after facilitation (mean of 10 sweeps) measured at -50 mV. As seen at -20 mV, a prepulse induced a time-dependent increase in $P(o)$. The rising phase of the waveform was interpolated with a continuous line, and the decay of facilitated $P(o)$ increase was fit with an exponential function ($\tau \sim 1.3$ sec). *Bottom*, Open duration histograms show that the prepulse-induced increase in $P(o)$ was accompanied by an increase in longer duration openings (long duration openings contributed 6% of events in control sweeps, which increased to 11% in facilitated sweeps). Holding potential was -60 mV for both patches. $P(o)$ measurement began 250 msec after the prepulse to exclude the fast decaying form of prepulse facilitation (see text).

ence in the contribution of longer duration openings at each membrane potential (ANOVA, $p > 0.1$) (Fig. 7A).

The effect of the postpulse membrane voltage on induction of delayed facilitation was assessed by quantitation of the peak $P(o)$ increase. This was measured as the ratio of the facilitated peak $P(o)$ and the mean $P(o)$ observed in control sweeps. Figure 7B shows that there was no significant difference in the magnitude of the peak $P(o)$ change at each potential (ANOVA, $p > 0.1$). In addition, the number of sweeps within an experiment that dis-

played delayed facilitation was unaffected by the postpulse membrane potential (ANOVA, $p > 0.1$) (Fig. 7C).

L-type calcium channels exhibited a biexponential open time distribution, with prepulse depolarization promoting more long open time events. Mean open time constants were voltage-insensitive in both control and facilitated sweeps (Fig. 7D). The open time kinetics of control and facilitated behavior were compared with those of L-type channels activated by a standard activation protocol (150 msec depolarizing voltage steps from -60 mV). Open duration histograms of L-type channel activity evoked by both protocols showed two exponential components, with comparable time constants. As was observed for control and prepulse-induced activity, time constants obtained from standard activation steps were also voltage-insensitive (Fig. 7D; see Fig. 9 for schematic representation of protocols). This indicates that delayed facilitation does not affect L-type channel open times.

In summary, the data presented in Figures 6 and 7 show that the induction of delayed facilitation, the magnitude and time course of the change in $P(o)$, and the observed increase in long duration openings are insensitive to the postpulse voltage.

Modulation of delayed facilitation

The hippocampus receives projections of noradrenaline-containing fibers from the locus coeruleus. The diffuse nature of these projections implicates noradrenaline as a neuromodulator. Activation of β -adrenergic receptors in dentate gyrus granule cells augments the whole-cell calcium current, an effect mimicked by membrane-permeant cAMP analogs (Gray and Johnston, 1987). In contrast, application of the β -adrenergic receptor agonist isoproterenol ($1 \mu\text{M}$) to cell-attached patches in this study inhibited delayed facilitation of L-type channel activity (five of six patches) (Fig. 8). Before application of isoproterenol (control), a 200 msec prepulse to $+40$ mV induced delayed facilitation of L-channel activity. Analysis of channel open times after the prepulse showed that the distribution was best fitted by the sum of two exponentials (Fig. 8; see Fig. 6 for comparison). Under control conditions, channel open time was best described by a single exponential ($\tau \sim 0.25$ msec) (not shown). Approximately 1 min after the application of isoproterenol, prepulse-induced facilitation was inhibited (Fig. 8). Channel open time was now best described by a single exponential with a short open time constant, similar to control sweeps without the prepulse. A reversal of the effect of isoproterenol was observed after addition of the β -adrenergic receptor antagonist propranolol ($10 \mu\text{M}$) in the continued presence of isoproterenol (two of three patches). Recovery was apparent by the return of high $P(o)$ behavior after the prepulse and a return of the longer time constant in the open duration histogram (Fig. 8). Open state kinetics in the presence of isoproterenol were the same as those without prepulse, suggesting that application of isoproterenol prevented the induction of delayed facilitation by a depolarizing prepulse.

Isoproterenol selectively inhibited delayed facilitation of L-type channel activity. Figure 9 shows mean results of the effect of isoproterenol and the membrane-permeant analog of cAMP (8-CPT cAMP; 1 mM) on the $P(o)$ of control and delayed facilitated channels and the effect of isoproterenol on channel activity evoked by a standard activation protocol. In paired experiments, a 200 msec prepulse to $+40$ mV evoked either a 5.0 ± 1.3 -fold (isoproterenol experiments, $n = 5$) or a 7.2 ± 0.83 -fold (8-CPT cAMP experiments, $n = 4$) $P(o)$ increase during 6 sec sweeps to -40 or -50 mV. Both isoproterenol ($1 \mu\text{M}$) and 8-CPT cAMP (1 mM) completely abolished induction of delayed facilitation, leav-

Figure 7. Delayed facilitation of L-type channel activity is not markedly voltage-dependent. *A*, The contribution of the exponent with the longer time constant is plotted for control and facilitated sweeps. Longer duration openings contributed $\sim 1\%$ of events in control sweeps. After facilitation by a 200 msec prepulse to $+40$ mV, longer openings constituted $\sim 5\%$ of all events. The increase in the number of longer duration openings was significant at each voltage (one-way ANOVA, $p < 0.01$). This increase in longer duration openings was the same at each membrane voltage ($n = 3$ –5 for each voltage). *B*, The magnitude of the increase in peak $P(o)$, induced by a prepulse to $+40$ mV (200 msec duration), or a train of action potential waveforms was not obviously voltage-sensitive. The ratio of the peak $P(o)$ observed in 200 msec time segments to the mean $P(o)$ observed during the 6 sec control sweep is plotted as mean $\pm$ SEM. The effect of postprepulse displayed no obvious voltage dependence (one-way ANOVA, $p > 0.1$). *C*, The induction of delayed facilitation was not dependent on the postprepulse potential. The ratio of facilitated sweeps relative to the total number of sweeps is plotted. There was no significant difference in the number of facilitated sweeps observed at each potential (one-way ANOVA, $p > 0.1$). *D*, Open-state kinetics of L-type channels are not voltage-dependent. Plotted are time constants (short and long) obtained from the fitting of open duration histograms (for example, see Fig. 6). Time constants were obtained from control sweeps (open triangles, open circles superimposed with closed circles), facilitated sweeps (closed triangles, closed circles), and standard activation voltage pulses from a holding potential of -60 mV (closed and open diamonds). Channel open times obtained under all conditions were voltage-insensitive ($n = 3$ –6 for each voltage).

ing a $P(o)$ that was not significantly different from control sweeps ($p > 0.05$, paired Student's t test) (Fig. 9). In contrast, both isoproterenol and 8-CPT cAMP had no effect on control L-type channel activity observed in the six second sweeps. Finally, isoproterenol ($1 \mu\text{M}$) produced a small, but not significant, enhancement of activity evoked by a 200 msec voltage pulse from -60 to 0 mV (Fig. 9). These data demonstrate that stimulation of β -adrenergic receptors inhibits delayed facilitation of L-type calcium channels, while having little effect on standard L-type channel activity.

DISCUSSION

Delayed facilitation of L-type channel activity possesses some unusual properties. In addition to being observed at very negative membrane potentials, close to the resting membrane potential of the cells, delayed facilitation exhibited a very slow time course, not reaching a peak until 0.5–1 sec after termination of the

prepulse (Fig. 5). It was evoked by a single voltage prepulse, close to the peak of the action potential, and may be preferentially induced by a short train of action potentials (Figs. 2, 3). The time-dependent change in $P(o)$ occurred at all membrane potentials and was caused by an increase in channel opening frequency and the recruitment of longer duration events (Figs. 6, 7). Finally, the induction of delayed facilitation was blocked by activation of β -adrenergic receptors (Figs. 8, 9).

The slow rise and longevity of delayed facilitation makes this behavior distinct from previously described examples of prepulse facilitation of L-type channels in hippocampal neurons (Fisher et al., 1990; Thibault et al., 1993; Kavalali and Plummer, 1994, 1996). Two mechanisms have been proposed for the more rapid decaying form of prepulse facilitation: either an activation of a distinct DHP-sensitive channel (Kavalali and Plummer, 1994, 1996) or a channel-related phenomenon such as relief of inactivation or loss

Figure 8. Inhibition of delayed facilitation by β -adrenergic receptor stimulation. Cell-attached patch recording of delayed facilitation of L-type channel activity. The patch appeared to contain two channels. *A, Top*, Under control conditions, a 200 msec prepulse to +40 mV induced facilitation of L-type channel openings at -40 mV. *Bottom*, Open duration histogram of all events after the prepulse. The distribution was best fit by the sum of two exponentials with time constants 0.3 and 2.5 msec. *B, Top*, In the presence of the β -adrenergic agonist isoproterenol ($1 \mu\text{M}$), the prepulse failed to evoke high $P(o)$ L-type channel activity. *Bottom*, Open state analysis of events after the prepulse were best fit by a single exponential distribution ($\tau \sim 0.3$ msec). *C, Top*, The β -adrenergic receptor antagonist propranolol ($10 \mu\text{M}$) was applied in the continued presence of isoproterenol. Approximately 5 min after the antagonist was added, delayed facilitation was observed after the prepulse. *Bottom*, Open duration histogram of events after the prepulse, in the presence of isoproterenol and propranolol. Recovery from the effect of isoproterenol was apparent with the return of the longer time constant ($\tau \sim 3.1$ msec).

of a blocking ion (Thibault et al., 1993). In this study, the conductance of channels underlying delayed facilitation was not significantly different from the conductance of channels evoked by short depolarizing voltage pulses (Fig. 4). Delayed facilitation was observed in four patches that showed openings to a single level. This data would suggest that delayed facilitation represents an increase in activity of the standard L-type channel, rather than the recruitment of a novel DHP-sensitive channel. However, this suggestion must be considered with caution, because it is extremely difficult to determine the number of channels present in a patch (see Materials and Methods). The marked delay and slow rise time of delayed facilitation suggest that second messenger-mediated modulation of the channel may be responsible. This hypothesis is supported by the block of delayed facilitation by β -adrenergic receptor activation. This inhibition was mimicked by a membrane-permeant cAMP analog, suggesting that inhibition is mediated by a phosphorylation event. Therefore, delayed facilitation may be promoted by dephosphorylation. It is proposed that the presence of a phosphate group prevents induction of delayed facilitation, and that the channel is tonically phosphorylated at a site inaccessible to a phosphatase. A train of action potentials or a voltage prepulse would induce a conformational

change, allowing access to the phosphatase. The decay of delayed facilitation may reflect the action of an associated kinase. However, it is also possible that delayed facilitation does not require either kinase or phosphatase activity, and that β -adrenergic receptor activation simply prevents induction of delayed facilitation.

Inhibition of delayed facilitation by β -adrenergic stimulation is *opposite* to what is expected, because both the L-type calcium current recorded in ventricular myocytes (Bean et al., 1986) and high-voltage-activated (HVA) calcium current in dentate granule cells (Gray and Johnston, 1987) were found to be enhanced by β -adrenergic agonists. These effects were mimicked by membrane-permeant analogs of cAMP and were assumed to result from activation of PKA. In addition, a voltage-dependent facilitation of L-type calcium currents in skeletal muscle required activation of PKA and was blocked by its specific inhibitor PKI (Sculptoreanu et al., 1993). However, delayed facilitation exhibits a slow rising phase and is much longer-lasting than facilitation in skeletal muscle, suggesting that the two systems are not comparable. The reported effect of β -adrenergic stimulation on HVA calcium current in dentate granule cells (Gray and Johnston, 1987) has not been repeated. In addition, attempts to repeat this effect using hippocampal neurons maintained in culture have not been successful

Figure 9. Selective inhibition of delayed facilitation by β -adrenergic receptor activation or addition of a cAMP analog. *A*, The $P(o)$ measured in 6 sec sweeps at -40 or -50 mV after a prepulse to $+40$ mV is normalized to control (without the prepulse). In the absence of isoproterenol, the prepulse induced a 5.0 ± 1.3 -fold increase in $P(o)$ during facilitated sweeps ($n = 5$). In different paired experiments (before addition of 8-CPT cAMP), the prepulse induced a 7.2 ± 0.83 -fold increase in $P(o)$ during facilitated sweeps ($n = 4$). The increase in patch $P(o)$ was inhibited by the addition of the β -adrenergic receptor agonist isoproterenol ($1 \mu\text{M}$) or the membrane-permeant analog of cAMP 8-CPT cAMP (1 mM). Results are from paired data, with the effect of either isoproterenol or 8-CPT cAMP being compared with delayed facilitation evoked before their application. The two control bars, in isoproterenol and in 8-CPT cAMP, have been normalized to the first control (in the absence of treatment), showing that neither isoproterenol nor 8-CPT cAMP had an effect on control behavior. *B*, The $P(o)$ observed during a 200 msec voltage pulse from -60 mV to 0 mV in the presence of isoproterenol was normalized to the $P(o)$ observed in the absence of agonist. Isoproterenol had no significant effect on the channel activity observed during this standard activation protocol ($n = 4$).

(R. A. Craig and N. V. Marrion, unpublished observation). The reason for this discrepancy is unclear. However, two separate gene products [C (Snutch et al., 1991) and D class (Williams et al., 1992)] encode subtypes of neuronal L-type channels, and both of these classes of channels are present in hippocampal neurons (Hell et al., 1993a). Furthermore, two isoforms of the $\alpha 1$ subunit of the C class channel co-exist in rat brain, and only one is a substrate for PKA (Hell et al., 1993b). It is possible that this variability underlies the observed differences in modulation. However, it should be noted that a small, but not statistically significant, enhancement of activity evoked by a standard activation protocol was observed (Fig. 9). It is possible that this observation is related to the effect reported by Gray and Johnston (1987).

The time course of delayed facilitation is remarkably close to that of the slow AHP in hippocampal pyramidal neurons. L-type channels are activated by somatically generated action potentials (Magee and Johnston, 1995), whereas the slow AHP is observed after a train of these action potentials. Under voltage clamp, both are activated by the same voltage protocol and display a slow rising phase and decay at similar rates (Lancaster and Adams, 1986; Sah and Issacson, 1995). Previous studies have suggested that calcium-activated small conductance potassium channels (SK channels) underlying the slow AHP are intimately linked to L-type Ca channels. The slow AHP is blocked by DHP antagonists (Rascol et al., 1991; Moyer et al., 1992), indicating that L-type Ca channels provide the calcium for activation of the slow AHP. The number of L-type channels (Thibault and Landfield, 1996) and the amplitude of the slow AHP (Moyer et al., 1992) both increase with age. In addition, learning-induced reductions in the slow AHP are re-

ported (de Jonge et al., 1990), and block of L-type channels with nimodipine enhances associative learning (Deyo et al., 1989).

The present finding that delayed facilitation is blocked by β -adrenergic receptor activation may shed new light on the modulation of the slow AHP in hippocampal neurons. The time course of the slow AHP is suggested to be controlled by diffusion of intracellular calcium accumulated during the preceding burst of action potentials (Lancaster and Adams, 1986; Lancaster and Nicoll, 1987). β -Adrenergic modulation of the slow AHP, which is mediated by activation of PKA (Pedarzani and Storm, 1993), has been assumed to occur at a step subsequent to the entry of Ca (Nicoll, 1988; Knöpfel et al., 1990). This assumption results from the observations that both calcium action potentials and the resulting intracellular calcium transients are unaffected by agonists that suppress the slow AHP (Madison and Nicoll, 1982; Knöpfel et al., 1990). However, the intracellular calcium transients are only 10–50 nM in amplitude (Knöpfel et al., 1990). The three classes of SK channels cloned, which include SK1, the mRNA for which is present in hippocampal pyramidal neurons, are half-maximally activated by ~ 700 nM Ca (Köhler et al., 1996). This value is similar to the Ca sensitivity of the apamin-sensitive SK channel found in rat adrenal chromaffin cells ($K_{0.5}$, ~ 800 nM) (Park, 1994). Therefore, the measured intracellular calcium transients are probably too small to account for activation of SK channels that underlie generation of the slow AHP. It seems likely that local intracellular calcium increases are responsible for activation of SK channels in hippocampal neurons, with the measured intracellular calcium transients representing residual calcium not removed by cellular sequestration and pump processes. Therefore, it seems likely that if these calcium transients principally arise from calcium entry during the depolarization used to evoke the slow AHP (with only a minor component arising from delayed facilitation), they would not be apparently affected by the receptor activation that suppresses the slow AHP. However, activation of potassium channels by the intracellular photolytic release of Ca from DM-nitrophen is apparently sensitive to β -adrenergic receptor stimulation (Lancaster and Zucker, 1994). It is not known whether the potassium channels activated by this method were SK channels. In addition, because repeated photolysis causes depletion of Ca-loaded DM-nitrophen, and calcium release was not monitored (Lancaster and Zucker, 1994), the lack of a reversible effect of the applied agonist makes it possible that the apparent response could result from this depletion. The results presented here raise the possibility that the time course of the slow AHP is determined by delayed facilitation. The selective modulation of delayed facilitation by β -adrenergic receptor activation, with little or no effect on the channel activity evoked during membrane depolarization (Fig. 9), would mean that an effect on calcium spikes and residual intracellular calcium transients would not be expected. Therefore, modulation of delayed facilitation of L-type calcium channels may underlie the suppression of the slow AHP by β -adrenergic receptor activation.

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