

# Voltage-dependent facilitation of cardiac L-type Ca channels expressed in HEK-293 cells requires $\beta$ -subunit

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**Kamp, Timothy J., Hai Hu, and Eduardo Marban.** Voltage-dependent facilitation of cardiac L-type Ca channels expressed in HEK-293 cells requires  $\beta$ -subunit. *Am. J. Physiol. Heart Circ. Physiol.* 278: H126–H136, 2000.—The activity of native L-type Ca channels can be facilitated by strong depolarizations. The cardiac Ca channel  $\alpha_{1C}$ -subunit was transiently expressed in human embryonic kidney (HEK-293) cells, but these channels did not exhibit voltage-dependent facilitation. Coexpression of the Ca channel  $\beta_{1a}$ - or  $\beta_{2a}$ -subunit with the  $\alpha_{1C}$ -subunit enabled voltage-dependent facilitation in 40% of cells tested. The onset of facilitation in  $\alpha_{1C}$  +  $\beta_{1a}$ -expressing HEK-293 cells was rapid after a depolarization to +100 mV ( $\tau = 7.0$  ms). The kinetic features of the facilitated currents were comparable to those observed for voltage-dependent relief of G protein inhibition demonstrated for many neuronal Ca channels; however, intracellular dialysis with guanosine 5'-O-(2-thiodiphosphate) and guanosine 5'-O-(3-thiotriphosphate) in the patch pipette had no effect on facilitation. Stimulation of G protein-coupled receptors, either endogenous (somatostatin receptors) or coexpressed (adenosine A<sub>1</sub> receptors), did not affect voltage-dependent facilitation. These results indicate that the cardiac Ca channel  $\alpha_{1C}$ -subunit can exhibit voltage-dependent facilitation in HEK-293 cells only when coexpressed with an auxiliary  $\beta$ -subunit and that this facilitation is independent of G protein pathways.

patch clamp; electrophysiology; G protein; somatostatin receptor; adenosine receptor

L-TYPE CALCIUM CHANNELS are present in a wide variety of cell types and are essential for various cellular processes, including excitation-contraction coupling in muscle and excitation-secretion coupling in neurons and endocrine cells. These channels are multimeric protein complexes that include a central pore-forming  $\alpha_1$ -subunit in combination with auxiliary subunits:  $\beta$ ,  $\alpha_2\delta$ , and sometimes  $\gamma$  (28, 45). L-type Ca channels can be encoded by three different  $\alpha_1$ -subunit genes:  $\alpha_{1C}$ ,  $\alpha_{1D}$ , and  $\alpha_{1S}$  (28, 45). In addition, splice variants of these  $\alpha_1$ -subunit genes have been identified, which in the case of  $\alpha_{1C}$ -isoforms, exhibit differential expression in cardiac muscle, smooth muscle, and brain. Four distinct genes encode Ca channel  $\beta$ -subunits, each having

multiple splice variants (7). Three  $\alpha_2\delta$  genes have been identified (20, 39). The cell- and tissue-specific combination of Ca channel subunits leads to distinct functional properties of the channels.

The opening and closing of L-type Ca channels is dependent on membrane potential similar to other voltage-dependent channels, including many K and Na channels. In addition, L-type Ca channels have been demonstrated to have an additional level of voltage- or use-dependent regulation of channel activity called facilitation, which can be broadly defined as an increase in Ca channel activity resulting from single or multiple preceding depolarizations (17). It is a form of positive feedback and represents an apparently unique property of Ca channels. The proposed physiological roles of facilitation vary greatly among different tissues (17). In the case of cardiac muscle, facilitation has been suggested to play an important role in the normal increase in contractile force that accompanies physiological increases in heart rate, referred to as the positive force-frequency relationship (41, 49, 72). In addition, facilitation of L-type Ca channels may be important in the genesis of certain cardiac arrhythmias (66). In skeletal muscle, facilitation may be of critical importance in tetanic stimulation, when frequent depolarizations result in a greatly increased force of contraction (62). In neuronal preparations, facilitation of L-type Ca channels has been suggested to contribute to the control of neuronal excitability and to be involved in processes such as long-term potentiation (31, 34, 37, 51). In adrenal chromaffin cells, voltage-dependent facilitation of L-type Ca channels has been proposed to contribute to stress-induced catecholamine release (3, 29).

Multiple molecular mechanisms have been suggested to underlie facilitation of L-type Ca channels. In cardiac myocytes, a Ca-dependent facilitation and a voltage-dependent (Ca-independent) facilitation of L-type Ca channel activity have been described (21, 41, 42, 55). Previous investigations using cardiac and smooth muscle myocytes have suggested that Ca-dependent facilitation is due to the activation of Ca-calmodulin protein kinase II, which phosphorylates the Ca channel or a closely associated protein, leading to an increase in Ca current ( $I_{Ca}$ ) (2, 44, 67, 68). Alternatively, voltage-dependent (Ca-independent) facilitation of L-type Ca channels in cardiac myocytes and neurons has been suggested to be due to cAMP-dependent protein

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kinase phosphorylation of the channel complex and/or a voltage-dependent conformational switch of the channel protein, leading to altered gating (mode switching) (55, 62).

Studies using heterologous expression systems have provided additional mechanistic information on voltage-dependent facilitation. Expression of the neuronal splice variant of  $\alpha_{1C-C}$  with various  $\beta$ -subunits in *Xenopus* oocytes results in L-type Ca channels capable of demonstrating long-lasting facilitation that requires protein kinase A (PKA) phosphorylation (9). Further studies demonstrated that the rat  $\beta_{2a}$ -subunit does not support this facilitation (11) because of a unique palmitoylation at its amino terminus that prohibits facilitation (12, 57). Other studies using different splice variants of  $\alpha_{1C}$  or different expression systems have produced conflicting results on the necessity of PKA phosphorylation as well as the need for  $\beta$ -subunit coexpression to observe facilitation (13, 19, 38, 62). These results suggest that voltage-dependent facilitation may result from multiple underlying mechanisms dependent on the exact subunits and splice variants as well as the expression system used.

Many neuronal non-L-type Ca channels exhibit apparent voltage-dependent facilitation as a result of relief of G protein-mediated inhibition of the channels (18, 32). Receptor-stimulated G protein activation leads to the direct binding of G protein  $\beta\gamma$ -subunits to the Ca channel to produce the inhibition that can be relieved transiently by strong depolarization (16, 26, 30). G protein-mediated inhibition of Ca channels has been extensively studied for several neuronal Ca channel isoforms, but it has not been demonstrated for L-type Ca channels, perhaps because of the lack of the putative G protein  $\beta\gamma$ -subunit binding sites in the linker between domains I and II (QXXER consensus) or the carboxy terminus (27, 50, 58, 70).

Our studies expressing the rabbit cardiac  $\alpha_{1C}$ -subunit in HEK-293 cells demonstrate a rapid-onset voltage-dependent facilitation distinct from the more slowly developing and persistent facilitation observed in previous oocyte studies (9). In addition, the facilitation in this system is independent of PKA, inasmuch as this regulatory pathway does not modulate  $\alpha_{1C}$  channels in the absence of other regulatory proteins (23, 71). This mammalian expression system using a cardiac  $\alpha_{1C}$  splice variant may recapitulate the facilitation observed in native ventricular myocytes (55). The purpose of the present work was 1) to determine the subunit dependence of facilitation in HEK-293 cells and 2) to determine whether G protein modulation is required for this facilitation.

## MATERIALS AND METHODS

**HEK-293 cell transfection.** HEK-293 cells (a human embryonic kidney cell line) were cultured using standard techniques in DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, and 2 mM L-glutamine. The HEK-293 cells were transiently transfected using the calcium phosphate method (kit from GIBCO-BRL, Gaithersburg, MD), as previously described (53). Channel subunits to be studied were subcloned into pGW1H, an expression vector

using a cytomegalovirus promoter (British Biotechnology, Oxford, UK). The expressed  $\alpha_{1C}$ -subunit was identical to the originally cloned full-length rabbit cardiac  $\alpha_{1C}$ -subunit (47), except for alternative splicing in domain IV S3 (64). The  $\alpha_{1C}$ -subunit was expressed alone or in a 1:1 molar combination with rabbit  $\beta_{1a}$ - (56) or rat  $\beta_{2a}$ -subunit (54). In a subset of control experiments the rabbit brain  $\alpha_{1B}$ -subunit (22) was expressed instead of the  $\alpha_{1C}$ -subunit (a kind gift of Dr. Y. Mori, National Institute for Physiological Sciences, Okazaki, Japan). The channel subunits were cotransfected with pSV40Tag to increase expression levels. In addition, to allow detection of transfected cells, the S65T bright green fluorescent protein (GFP) mutant (25) was coexpressed in the vector GFPpRK5 (a kind gift of Dr. Jeremy Nathans, Johns Hopkins University). In a subset of experiments, the human  $A_1$  adenosine receptor ( $A_1$ AR) was coexpressed with the Ca channel by use of p $A_1$ AR (59) (a kind gift from Dr. Gary Stiles, Duke University) in a 1:1 molar ratio with Ca channel subunits. Cells were then studied on the day after transfection.

**Epifluorescence detection of transfected cells.** Cells were transfected on 15-mm glass coverslips (no. 1, Carolina Biological Supplies, Burlington, NC), which were then used to form the bottom of a perfusion chamber (model RC22, Warner Instrument, Hamden, CT). Epifluorescence microscopy was performed using a 100-W mercury lamp and a standard FITC filter set (Nikon, Tokyo, Japan). Cells expressing S65T GFP were easily visualized by their prominent green fluorescence. Single isolated green fluorescent cells were then studied as described below.

**Whole cell electrophysiology.** The whole cell configuration of the patch-clamp technique was employed as previously described (36, 53) with the addition of GFP to detect transfected cells (43). Over 95% of the green fluorescent cells exhibited L-type Ca channel currents. The composition of the pipette solution was (in mM) 114 CsCl, 10 EGTA, 10 HEPES, and 5 MgATP (pH adjusted to 7.20 with CsOH). In a subset of experiments, 0.3 mM lithium guanosine 5'-O-(3-thiotriphosphate) (GTP $\gamma$ S; Sigma Chemical, St. Louis, MO) or 3.0 mM guanosine 5'-O-(2-thiodiphosphate) (GDP $\beta$ S; Boehringer Mannheim, Mannheim, Germany) was included in the pipette solution where indicated. The cells were initially bathed in a solution composed of (in mM) 1.8 Ca, 142 NaCl, 5.4 KCl, 1.0 MgCl<sub>2</sub>, 0.33 Na<sub>2</sub>HPO<sub>4</sub>, 5 HEPES, and 5 glucose (pH 7.40) to allow formation of a gigaseal. The bath solution was connected to ground via a 3 M KCl-agar bridge and an Ag-AgCl electrode. A liquid junction potential of  $-5.3 \pm 0.1$  mV was measured between the pipette solution and the bath solution, and the data are not corrected for this offset. All experiments were carried out at room temperature (20–22°C). After the whole cell configuration was obtained, the cells were superfused with buffers containing (in mM) 2 BaCl<sub>2</sub>, 147 CsCl, and 10 HEPES (pH adjusted to 7.40 with CsOH) or 10 BaCl<sub>2</sub>, 125 CsCl, and 10 HEPES (pH adjusted to 7.40 with CsOH). In some experiments, cells were perfused with the above buffers including 300 nM somatostatin (SST; Calbiochem-Novabiochem, San Diego, CA) or 10  $\mu$ M 2-chloro-N<sup>6</sup>-cyclopentyladenosine (CCPA; Research Biochemical International, Natick, MA). Whole cell currents were recorded using an Axopatch 200B amplifier sampled every 40 ms and filtered at 5 kHz, with pCLAMP 6.0 (Axon Instruments, Foster City, CA) to drive data acquisition. The holding potential was  $-90$  mV for most experiments. The capacity transients were analog compensated, and series resistance was compensated by 50–80%. Data were leak subtracted using a P/4 protocol. Voltage protocols are described with the particular data set. Cells were allowed  $\geq 5$  min to equilibrate after whole cell access was obtained. The subset of experi-

ments examining the tail currents on repolarization to  $-50$  mV required rapid voltage control for accurate measurement of the peak tail currents. Therefore, the following objective criteria were used to exclude data from analysis: 1) a time constant  $>100$   $\mu$ s determined from the uncompensated capacity transient resulting from a 10-mV hyperpolarization and 2) an estimated compensated series resistance error  $>5$  mV.

**Data analysis and statistics.** Data were analyzed using PClamp 6.0 software and plotted using Origin 4.1 software (Microcal, Northampton, MA). A standard three-pulse voltage protocol was used to measure voltage-dependent facilitation (Fig. 1). The percent facilitation was calculated by measuring the percent increase in the test current relative to the control current at the end of the pulses. To determine the amount of facilitation for a given cell, facilitation was calculated from the average of 10–20 records repeated at 10-s intervals. These averaged data were collected 5–20 min after whole cell access was obtained, which represents a time period during which average facilitation was stable for a given cell.

The measured peak tail currents on repolarization from a family of voltage steps were fit to the sum of two Boltzmann distributions according to the following equation

$$I = I_{\max A} / [1 + e^{-(V_{1/2A} - V)/k_A}] + I_{\max B} / [1 + e^{-(V_{1/2B} - V)/k_B}]$$

where  $I_{\max A}$  and  $I_{\max B}$  are the maximal components of current activating in the A and B fractions, respectively,  $V_{1/2A}$  and  $V_{1/2B}$  are the voltage midpoints for the distributions, and  $k_A$  and  $k_B$  are the slope factors.

For experiments examining the kinetics of current activation or the onset of facilitation, the data were fit to a single- or double-exponential association model. The data were fit to the following equation

$$y = y_0 + A_1(1 - e^{-t/\tau_f}) + A_2(1 - e^{-t/\tau_s})$$

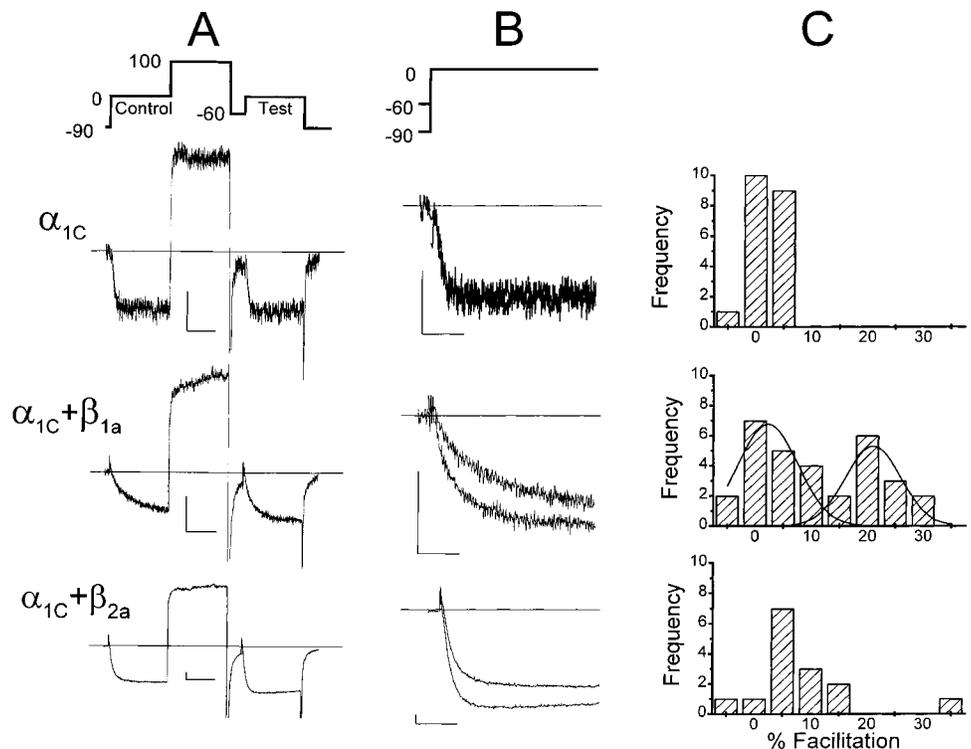
where  $y$  is the measured current magnitude or calculated percent facilitation,  $y_0$  is the amount of current or percent facilitation at time 0,  $A_1$  and  $A_2$  are the fast and slow

components of current activation or facilitation, and  $\tau_f$  and  $\tau_s$  are the fast and slow time constants. The data were fit to these equations by use of nonlinear least-squares regression analysis with the Levenberg-Marquardt or Simplex methods available with Origin 4.1. Pooled data are expressed as means  $\pm$  SD. Statistical comparisons were performed using unpaired two-tailed Student's  $t$ -test, and  $P < 0.05$  was considered statistically significant.

## RESULTS

**Voltage-dependent facilitation requires  $\beta$ -subunit co-expression.** HEK-293 cells transfected with the  $\alpha_{1C}$ -subunit of the Ca channel with or without an auxiliary  $\beta$ -subunit were tested for their ability to demonstrate voltage-dependent facilitation. The voltage protocol is shown in Fig. 1A. A control 20-ms pulse to 0 mV was followed by a strong conditioning depolarization to  $+100$  mV for 20 ms, a 5-ms repolarization to  $-60$  mV, and then the test depolarization to 0 mV for 20 ms. The percent facilitation was determined as the percent increase in peak Ba current ( $I_{Ba}$ ), comparing the test pulse with the control pulse. For cells expressing the  $\alpha_{1C}$ -subunit alone, the control and test pulses were typically superimposable, as shown in Fig. 1B. A histogram representing the responses for all the cells examining the  $\alpha_{1C}$ -subunit expressed alone is shown in Fig. 1C, and these cells did not demonstrate voltage-dependent facilitation, with average facilitation for 20 cells of  $0.6 \pm 2.8\%$ . Next, the effect of coexpression of the  $\beta$ -subunit with the  $\alpha_{1C}$ -subunit on voltage-dependent facilitation was examined. Coexpression of the  $\beta_{1a}$ -subunit with the  $\alpha_{1C}$ -subunit resulted in  $I_{Ba}$  that activates more slowly, as previously demonstrated (6, 53). In the cell expressing  $\alpha_{1C} + \beta_{1a}$  shown in Fig. 1, the test pulse  $I_{Ba}$  was 26% larger than the control  $I_{Ba}$ . The

Fig. 1. Subunit dependence of voltage-dependent facilitation. **A:** representative current records in response to indicated voltage protocol for human embryonic kidney (HEK-293) cells expressing  $\alpha_{1C}$ -subunit alone and  $\alpha_{1C} + \beta_{1a}$  and  $\alpha_{1C} + \beta_{2a}$  Ca channel subunits. Percent increase in amplitude of test current that follows conditioning depolarization to  $+100$  mV relative to control current is a measure of voltage-dependent facilitation. **B:** overlay of test and control current records for data in A. **C:** results for all cells tested with different subunit combinations. For 20 cells expressing  $\alpha_{1C}$ -subunit alone, average facilitation was  $0.6 \pm 2.8\%$ . For  $\alpha_{1C} + \beta_{1a}$ , 31 cells were studied, and a Gaussian fit of results to 2 peaks centered at 2.3 and 21.3% facilitation, encompassing 67.8 and 42.2% of data, respectively. For 15 cells expressing  $\alpha_{1C} + \beta_{2a}$ , facilitation could also be observed, but again this result was variable, with average facilitation of  $8.6 \pm 9.4\%$ . In experiments with  $\alpha_{1C}$ -subunit, 10 mM Ba was used; in experiments with  $\alpha_{1C} + \beta_{1a}$  and  $\alpha_{1C} + \beta_{2a}$ , 2 mM Ba was used. Vertical scale bars, 200 pA; horizontal bars, 10 ms (A) and 5 ms (B).



activation kinetics during the test pulse were also accelerated relative to the control pulse. The average calculated percent facilitation for the 31 cells expressing  $\alpha_{1C} + \beta_{1a}$  was  $10.7 \pm 10.8\%$ , which was significantly different from the null hypothesis by use of a single-population two-tailed  $t$ -test ( $P < 0.00001$ ). The facilitation observed in cells expressing the  $\alpha_{1C}$ -subunit compared with that in cells expressing  $\alpha_{1C} + \beta_{1a}$  was also statistically different ( $P < 0.005$ ). However, the response to this voltage protocol in  $\alpha_{1C} + \beta_{1a}$ -expressing cells was variable, as shown in the histogram of all experiments done vs. observed percent facilitation shown in Fig. 1C. Gaussian fits of the histogram to two peaks reveal that the cells apparently segregated into two response types: 1) approximately three-fifths of cells demonstrated little or no facilitation (centered at 2.3% facilitation) and 2) the remaining two-fifths of cells exhibited voltage-dependent facilitation (centered at 21.3% facilitation). To confirm that this behavior was not simply due to slowed activation kinetics in the presence of the  $\beta_{1a}$ -subunit or unique to that  $\beta$ -subunit, cells were transfected with  $\alpha_{1C} + \beta_{2a}$ . The cells expressing  $\alpha_{1C} + \beta_{2a}$  showed more rapid activation kinetics than the  $\alpha_{1C} + \beta_{1a}$ -expressing cells: time to 70% peak current for depolarization to 0 mV was  $2.6 \pm 0.5$  ( $n = 15$ ) vs.  $5.3 \pm 1.8$  ( $n = 31$ ) ms ( $P < 0.0001$ ). Some of the  $\alpha_{1C} + \beta_{2a}$ -expressing cells exhibited a clear pattern of voltage-dependent facilitation, as shown in a representative  $\alpha_{1C} + \beta_{2a}$ -expressing cell in Fig. 1 by the 35% increase in peak  $I_{Ba}$  in a comparison of control and test pulses. However, this response was also variable, and the histogram in Fig. 1C shows the range of results for 15 cells.

In the experiments studying expression of the  $\alpha_{1C}$ -subunit alone, 10 mM Ba was used as the charge carrier to have a favorable signal-to-noise ratio; in experiments studying  $\alpha_{1C} + \beta_{1a}$  or  $\alpha_{1C} + \beta_{2a}$  expression, 2 mM Ba was used because of the substantially higher density of channel expression (14, 36). Therefore, to confirm that the observed voltage-dependent facilitation was not simply due to changes in ionic conditions, using 10 mM Ba, we tested six  $\alpha_{1C} + \beta_{1a}$ -expressing cells, and the average facilitation was  $21.7 \pm 13.6\%$ . Overall, cells expressing the  $\alpha_{1C}$ -subunit alone were never observed to demonstrate voltage-dependent facilitation, which was seen in approximately two-fifths of cells expressing  $\alpha_{1C} + \beta_{1a}$  or  $\alpha_{1C} + \beta_{2a}$ .

*Density of  $I_{Ba}$  does not correlate with facilitation.* The level of expression of  $I_{Ba}$  is variable from cell to cell by use of transient transfection techniques in HEK-293 cells. Certain properties of expressed Ca channels, such as activation rate and sensitivity to inhibition by G proteins, have been found to correlate with channel density (1, 46). A comparison of  $I_{Ba}$  density, reflecting Ca channel density, and the observed voltage-dependent facilitation for the 31 cells expressing  $\alpha_{1C} + \beta_{1a}$  showed no correlation between current density and observed percent facilitation, as shown in the scatterplot in Fig. 2. Coexpression of the  $\beta_{1a}$ -subunit was evident in all cells by the current magnitude (significantly larger than in cells expressing the  $\alpha_{1C}$ -subunit

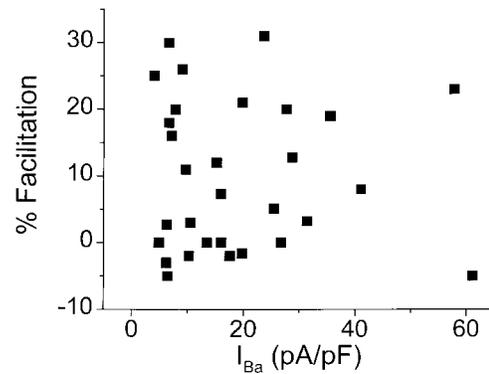


Fig. 2. Ba current ( $I_{Ba}$ ) density vs. percent facilitation. Peak  $I_{Ba}$  density after a depolarization from  $-90$  mV to test potential of 0 mV is plotted vs. percent facilitation calculated for each cell, as described in Fig. 1 for 31 cells expressing  $\alpha_{1C} + \beta_{1a}$ . There is no correlation between  $I_{Ba}$  density measured as peak current at 0 mV and facilitation.

alone) and the characteristic slow activation kinetics for all cells.

*Voltage-dependent activation of  $I_{Ba}$ .* Voltage-dependent facilitation was observed only in  $\beta$ -subunit-coexpressing cells, which suggests that voltage-dependent activation of  $I_{Ba}$  may be substantially different between cells expressing the  $\alpha_{1C}$ -subunit alone and those expressing  $\alpha_{1C} + \beta$ . Instantaneous current-voltage ( $I$ - $V$ ) relationships were examined in the transfected cells by depolarizing the cells for 25 ms to a family of potentials from  $-50$  to  $+120$  mV in 10-mV steps. The cells were then repolarized to  $-50$  mV, evoking an inward tail current through the open Ca channels. Representative current records are shown in Fig. 3A for a cell expressing the  $\alpha_{1C}$ -subunit alone and in Fig. 3B for a cell expressing  $\alpha_{1C} + \beta_{1a}$  for depolarizations to 0, 60, and 120 mV. As quantitated in our previous study, peak current density of  $I_{Ba}$  is larger in cells expressing  $\alpha_{1C} + \beta_{1a}$ , and despite the use in the present study of a fivefold lower permeant Ba concentration, cells expressing  $\alpha_{1C} + \beta_{1a}$  typically had larger currents than those expressing the  $\alpha_{1C}$ -subunit alone (36, 53). The peak inward tail currents evoked on repolarization to  $-50$  mV after the test depolarizations were measured and normalized to the tail current resulting after the test pulse to  $+120$  mV. Figure 3C shows the average normalized instantaneous  $I$ - $V$  values for five cells expressing the  $\alpha_{1C}$ -subunit alone and for eight cells expressing  $\alpha_{1C} + \beta_{1a}$ . These data demonstrate that activation of  $I_{Ba}$  cannot be adequately described by a single Boltzmann distribution, but rather a sum of two Boltzmann distributions is required to fit the data for expression of the  $\alpha_{1C}$ -subunit alone and  $\alpha_{1C} + \beta_{1a}$ . This was true not only for the pooled data, but data from individual cells also required the sum of two Boltzmann distributions to fit. A previous study examining activation of  $I_{Ca}$  in rat ventricular myocytes similarly found that the tail current  $I$ - $V$  curves were best described by the sum of two Boltzmann distributions (5). Direct comparison of the voltage dependence of activation for the cells expressing the  $\alpha_{1C}$ -subunit and  $\alpha_{1C} + \beta_{1a}$  is difficult because of the use of different

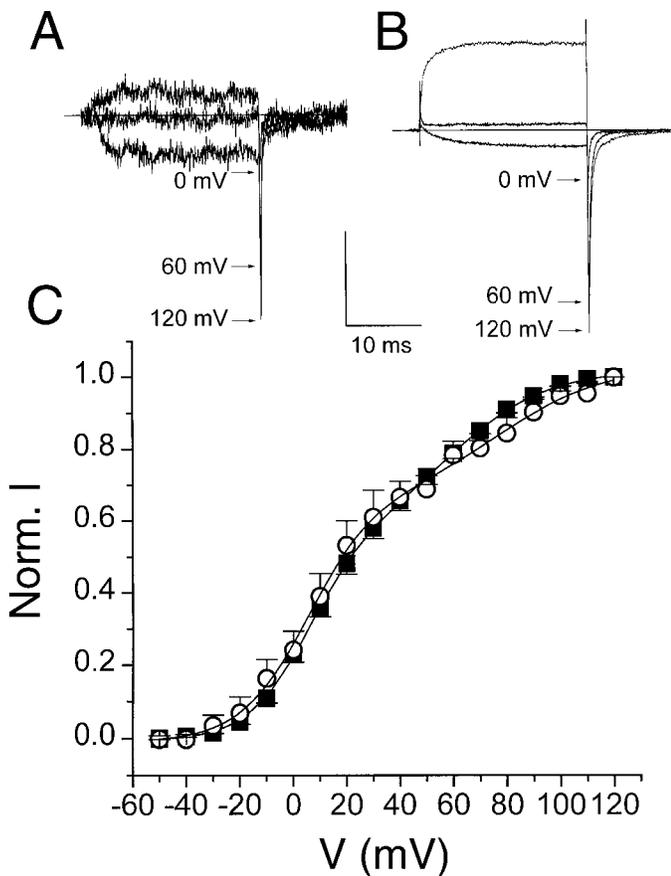


Fig. 3. Instantaneous  $I_{Ba}$ -voltage ( $V$ ) relationships. *A* and *B*: current traces of  $I_{Ba}$  evoked from a holding potential of  $-90$  mV to test potentials of 0, 60, and 120 mV in cells expressing  $\alpha_{1C}$ -subunit alone and  $\alpha_{1C} + \beta_{1a}$ , respectively. For cells expressing  $\alpha_{1C}$ -subunit alone, bath contained 10 mM Ba; for cells expressing  $\alpha_{1C} + \beta_{1a}$ , bath contained 2 mM Ba. Vertical scale bar, 200 pA (*A*) and 2,500 pA (*B*). *C*: average normalized (Norm)  $I$ - $V$  relationships for 5 cells expressing  $\alpha_{1C}$ -subunit ( $\circ$ ) and 8 cells expressing  $\alpha_{1C} + \beta_{1a}$  ( $\blacksquare$ ). Upward error bars, SE of  $\alpha_{1C}$ -subunit data; downward error bars, SE of  $\alpha_{1C} + \beta_{1a}$  data. Data were fit to sum of 2 Boltzmann distributions:  $I_{maxA} = 0.67$ ,  $I_{maxB} = 0.37$ ,  $V_{1/2A} = 5.4$  mV,  $V_{1/2B} = 77$  mV,  $k_A = 12.5$  mV, and  $k_B = 20.1$  mV ( $\alpha_{1C}$ ) and  $I_{maxA} = 0.55$ ,  $I_{maxB} = 0.47$ ,  $V_{1/2A} = 5.2$  mV,  $V_{1/2B} = 58.3$  mV,  $k_A = 10.7$  mV, and  $k_B = 18.3$  mV ( $\alpha_{1C} + \beta_{1a}$ ), where  $I_{maxA}$  and  $I_{maxB}$  are maximal components of current activating in A and B fractions,  $V_{1/2A}$  and  $V_{1/2B}$  are voltage midpoints for distributions, and  $k_A$  and  $k_B$  are slope factors.

Ba concentrations, which affects membrane surface charge and possibly even channel gating. Comparing  $\alpha_{1C} + \beta_{1a}$  cells, which did and did not exhibit facilitation, revealed no clear difference in tail current  $I$ - $V$  curves (data not shown).

Inasmuch as voltage-dependent facilitation may be related to changes in macroscopic channel deactivation, we examined tail current kinetics during repolarization. Tail currents were measured at  $-50$  mV after a range of depolarizations, as shown in Fig. 3, and the tails were well described by a double-exponential decay process throughout this voltage range. However, the relatively small amplitude of tail currents of cells expressing the  $\alpha_{1C}$ -subunit made uniquely fitting these data difficult. Therefore, we systematically compared the tail current kinetics after a strong depolarization to

$+100$  mV in  $\alpha_{1C} + \beta_{1a}$ -expressing cells that did ( $>10\%$ ) and did not ( $<10\%$ ) exhibit facilitation. For 10  $\alpha_{1C} + \beta_{1a}$ -expressing cells that exhibited facilitation, tail currents were described by two time constants ( $\tau_f = 0.40 \pm 0.06$  ms and  $\tau_s = 4.7 \pm 0.9$  ms), with  $90.4 \pm 2.7\%$  of the tail current decay occurring with the fast time constant. For six  $\alpha_{1C} + \beta_{1a}$ -expressing cells that did not exhibit facilitation, we obtained comparable results:  $\tau_f = 0.41 \pm 0.08$  ms and  $\tau_s = 4.98 \pm 1.84$  ms with  $91.1 \pm 3.3\%$  of the current described by the fast component. These results were not significantly different when cells that did and did not exhibit facilitation were compared.

**Onset of facilitation.** To characterize the voltage-dependent facilitation of  $I_{Ba}$  of the  $\alpha_{1C} + \beta_{1a}$  channels, the kinetics of the onset of facilitation were studied. The standard pulse protocol previously described was used, but the duration of the conditioning depolarization to  $+100$  mV was varied from 0 to 20 ms. Figure 4, *A* and *B*, shows current records from a representative cell studied using this protocol. The amplitude of the inward tail current on repolarization to  $-60$  mV after the conditioning depolarization to  $+100$  mV increases significantly with increasing duration of the conditioning pulse, as plotted in Fig. 4*C* for the average data from four  $\alpha_{1C} + \beta_{1a}$ -expressing cells. The activation of this additional  $I_{Ba}$  by the conditioning depolarization can be described by a double-exponential process with  $\tau_f = 0.34$  and  $\tau_s = 5.0$  ms. In distinction, the onset of facilitation measured as the percent facilitation comparing the test pulse with the control pulse is described by a single-exponential process with  $\tau = 7.0$  ms, similar to the slow component of current activation. Increasing the duration of the conditioning pulse from 20 to 200 ms failed to elicit additional facilitation (data not shown). These results demonstrate that the onset of facilitation does not simply reflect voltage-dependent activation of  $I_{Ba}$  given the distinct kinetics. However, the slower component of tail current activation and voltage-dependent facilitation may reflect a common gating process.

**Decay of facilitation.** To determine the time course of the decay of the facilitation of  $I_{Ba}$ , the repolarizing step to  $-60$  mV that follows the conditioning depolarization was varied in duration from 6 to 71 ms, as shown in Fig. 5. Figure 5*A* shows representative current records from a cell studied with this protocol. In Fig. 5*B*, the average percent facilitation from four  $\alpha_{1C} + \beta_{1a}$ -expressing cells is plotted as a function of the repolarization interval at  $-60$  mV. The average data were fit by a single-exponential decay curve ( $\tau_f = 34.6$  ms). The decay of facilitation is significantly slower than the rate of channel deactivation, as shown by the relatively fast tail currents. This suggests that voltage-dependent facilitation represents a distinct gating process separable from traditional channel deactivation.

**Lack of role of G proteins in L-type Ca channel facilitation.** Several classes of Ca channels found primarily in neurons can be directly inhibited by G proteins in a voltage-dependent fashion (18, 32). This inhibition is evident with small depolarizations, but

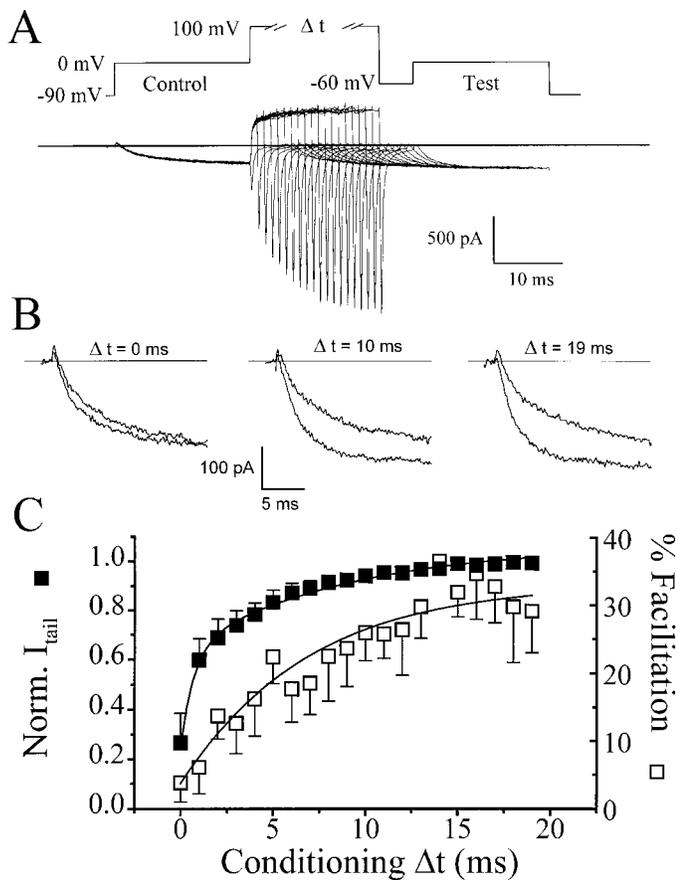


Fig. 4. Onset of voltage-dependent facilitation. *A*: voltage protocol, which varies duration of conditioning depolarization ( $\Delta t$ ) from 0 to 19 ms, and resulting current records from a representative cell. *B*: data in *A* for  $\Delta t = 0, 10,$  and  $19$  ms overlaps control (smaller) and test currents to illustrate facilitation. Straight line, 0 current. *C*: average peak amplitude of normalized tail currents after repolarization to  $-60$  mV (■) and calculated average percent facilitation from control and test pulses to  $0$  mV (□) for 4 cells expressing  $\alpha_{1C} + \beta_{1a}$ . Error bars, SE. Peak tail current data were fit to a double-exponential growth function:  $y_0 = 0.26, A_1 = 0.26, \tau_f = 0.34$  ms,  $A_2 = 0.48,$  and  $\tau_s = 4.97$  ms; percent facilitation data were fit to a single-exponential growth function:  $y_0 = 3.7\%, A_1 = 29.7\%,$  and  $\tau = 7.0$  ms, where  $y_0$  is amount of current or percent facilitation at *time 0*,  $A_1$  and  $A_2$  are fast and slow components of current activation or facilitation, and  $\tau_f$  and  $\tau_s$  are fast and slow time constants.

strong depolarizing pulses can remove it. A basal level of tonic G protein-mediated inhibition has been described when recombinant  $\alpha_{1A}$ - or  $\alpha_{1B}$ -type Ca channels are expressed in *Xenopus* oocytes (60). Therefore, it is possible that the voltage-dependent facilitation that has been observed in this study is due to relief of tonic G protein-mediated inhibition of the expressed  $\alpha_{1C}$ -type channels. Furthermore, the kinetics of the facilitation observed in this study are similar to those described for G protein interactions with non-L-type Ca channels (8, 24, 32, 46, 61). To test for this possibility, the nonhydrolyzable guanine nucleotides GDP $\beta$ S and GTP $\gamma$ S were included in the pipette solution. Cells expressing  $\alpha_{1C} + \beta_{1a}$  were compared with cells expressing  $\alpha_{1B} + \beta_{1a}$ , inasmuch as  $\alpha_{1B}$ - or N-type channels have been demonstrated to exhibit voltage-dependent G protein inhibition (30, 46, 52, 65, 70). The voltage protocol described

in Fig. 1 was used to measure voltage-dependent facilitation, and the pooled results are shown in Fig. 6. The results of these experiments are presented in a box plot to display the full range of the data, inasmuch as the response to this voltage protocol was variable among cells (Fig. 1). Inclusion of GDP $\beta$ S would be predicted to blunt or block the activation of endogenous G proteins and, therefore, to minimize any tonic G protein-mediated inhibition of the expressed  $\alpha_{1C}$ -subunit and, hence, minimize measured voltage-dependent facilitation. The data presented in Fig. 6 show that GDP $\beta$ S had no clear effect on the facilitation observed in  $\alpha_{1C} + \beta_{1a}$ -expressing cells relative to control pipette solution. In contrast,  $\alpha_{1B} + \beta_{1a}$ -transfected cells showed no facilitation (voltage-dependent relief of G protein-mediated inhibition) when GDP $\beta$ S was in the pipette. Inclusion of GTP $\gamma$ S in the pipette would be predicted to activate endogenous G proteins and produce maximal G protein-mediated inhibition of expressed Ca channels and, thus, maximal voltage-dependent relief of inhibition. As shown in Fig. 6, this intervention likewise did not alter the observed voltage-dependent facilitation relative to control for  $\alpha_{1C} + \beta_{1a}$ -expressing cells. All seven  $\alpha_{1B} + \beta_{1a}$ -expressing cells demonstrated clear facilitation when GTP $\gamma$ S was included in the pipette. These results demonstrate that

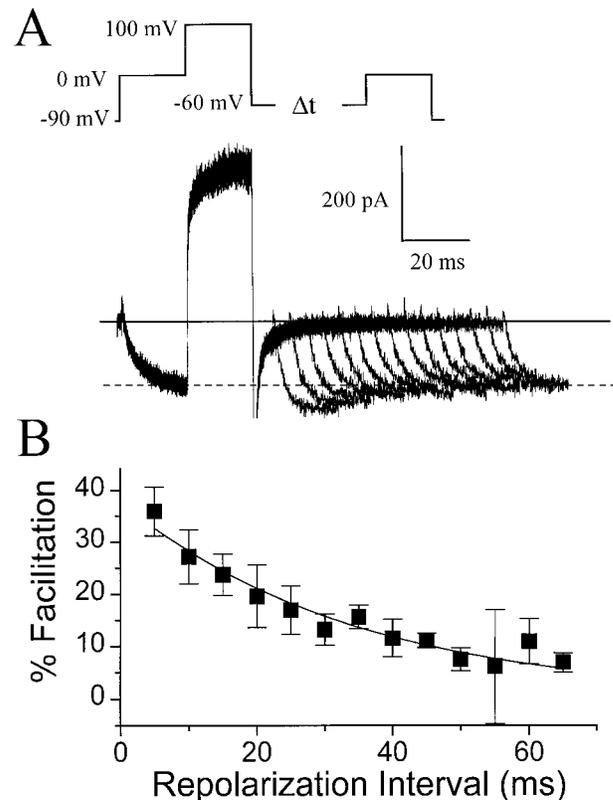


Fig. 5. Decay of voltage-dependent facilitation. Voltage protocol shown in *A* varies duration at  $-60$  mV between conditioning depolarization and test pulse. *A*: current records from a representative cell. Solid line, 0 current. *B*: average percent facilitation calculated by comparing test and control pulses as a function of repolarization interval for 4 cells expressing  $\alpha_{1C} + \beta_{1a}$ . Data are fit with a single-exponential decay ( $\tau = 34.6$  ms).

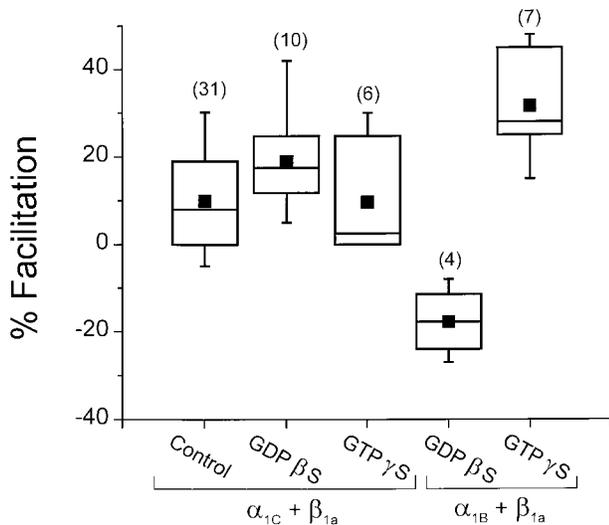


Fig. 6. Effect of nonhydrolyzable guanine nucleotides on facilitation. Experiments including nonhydrolyzable GTP analogs guanosine 5'-O-(2-thiodiphosphate) (GDP $\beta_S$ , 0.3–2.0 mM) and guanosine 5'-O-(3-thiotriphosphate) (GTP $\gamma_S$ , 0.3 mM) in pipette solution are summarized for cells expressing  $\alpha_{1C} + \beta_{1a}$  and  $\alpha_{1B} + \beta_{1a}$ . Percent facilitation was determined using pulse protocol described in Fig. 1. Data are presented as box plots: bottom vertical line, 5th percentile; bottom of box, 25th percentile; median line, 50th percentile; top of box, 75th percentile; top vertical line, 95th percentile; ■, mean. Numbers in parentheses indicate number of cells tested.

endogenous G proteins in HEK-293 cells can be modulated by nonhydrolyzable guanine nucleotides given the clear effects on  $\alpha_{1B}$ -type channels, but these G proteins do not similarly affect  $\alpha_{1C}$ -type channels.

Inasmuch as close interactions of G protein-coupled receptors and G proteins with Ca channel may be necessary for maximal facilitation, we tested the effect of activation of the endogenous SST receptor SSTR2 present on HEK-293 cells (40) and, second, activation of the heterologously expressed adenosine receptor ( $A_1AR$ ) (59). Activation of the endogenous SSTR2 receptor, which is coupled to  $G_{\alpha_{i3}}$  (40), has been shown to result in G protein-mediated inhibition of  $\alpha_{1A}$ - and  $\alpha_{1B}$ -type Ca channels expressed in these cells by others (65, 69). When 300 nM SST was applied to an HEK-293 cell expressing  $\alpha_{1C} + \beta_{1a}$ , there was a 30% reduction in the peak inward current (Fig. 7). However, this reduction was voltage independent, in that the percent facilitation did not change when the standard three-pulse protocol was applied (Fig. 7C). For five  $\alpha_{1C} + \beta_{1a}$ -expressing cells exposed to 300 nM SST, there was an average  $25 \pm 5.9\%$  decrease in peak  $I_{Ba}$  at 0 mV without any change in the observed facilitation: average facilitation was  $4 \pm 5\%$  before SST and  $3 \pm 6\%$  after SST. This suggests that the heterologously expressed  $\alpha_{1C}$ -type Ca channels are not directly inhibited by G proteins in a voltage-dependent fashion. Rather, these channels are sensitive to modulation by endogenous second messenger systems in the HEK-293 cells that are regulated by SSTR2.

Inasmuch as the SSTR2 is expressed in low density in HEK-293 cells and is prone to rapid desensitization, we sought to confirm these results using heterologously

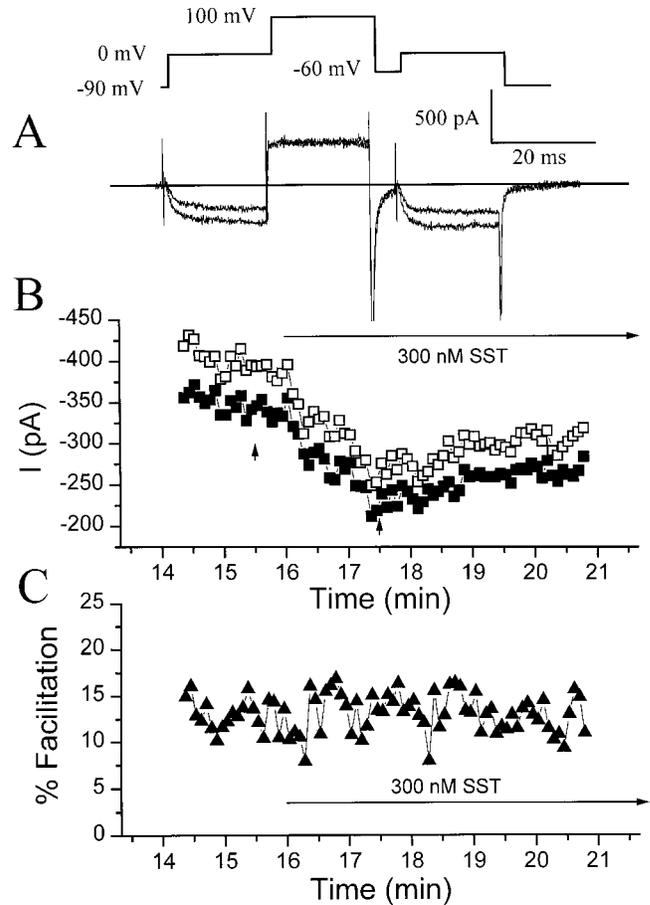


Fig. 7. Somatostatin inhibits  $I_{Ba}$  without affecting facilitation. *A*: voltage protocol repeated every 5 s during experiment and representative current records before (larger currents) and during application of 300 nM somatostatin (SST) at time points indicated by arrows in *B*. *B*: peak amplitude of control current (■) and test current (□); SST was added to bath where indicated. At *time 0*, access is achieved for cell. *C*: calculated percent facilitation for same data.

expressed adenosine receptors ( $A_1AR$ ) (59). A cell expressing  $\alpha_{1C} + \beta_{1a}$  and  $A_1AR$  was treated with the specific  $A_1AR$  agonist CCPA at 10  $\mu M$  (Fig. 8). There was no significant change in current density, and no facilitation was observed in this cell before or during exposure to 10  $\mu M$  CCPA. In four cells expressing  $\alpha_{1C} + \beta_{1a}$  and  $A_1AR$ , no change was observed in facilitation after treatment with 10  $\mu M$  CCPA:  $-5.6 \pm 2.3$  and  $-7.2 \pm 2.5\%$  before and after CCPA, respectively.

## DISCUSSION

Voltage-dependent facilitation of L-type Ca channel activity has been demonstrated in a number of different preparations, and the present study is one of the first to demonstrate facilitation with recombinant L-type Ca channels expressed in HEK-293 cells. Two previous studies using HEK-293 cells and a related rabbit  $\alpha_{1C}$ -isoform coexpressed with  $\beta_{2a}$  or  $\beta_3$ , respectively, failed to detect voltage-dependent facilitation (46, 71). Perhaps subtle differences in  $\alpha_{1C}$ -isoforms studied may underlie the contrasting results, because the present study used a rabbit cardiac  $\alpha_{1C}$ -isoform that has al-

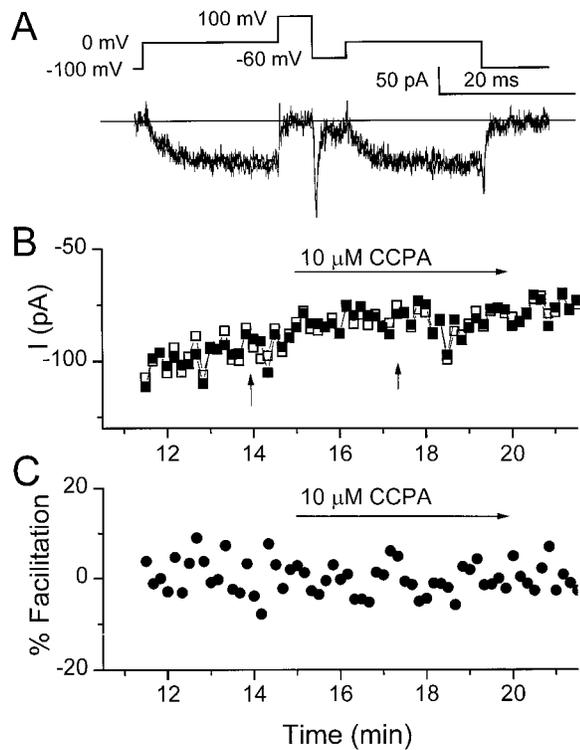


Fig. 8. Stimulation of heterologously expressed adenosine  $A_1$  receptors has no effect on facilitation. In *A*, voltage protocol was applied every 6 s. Current records before and during application of  $10 \mu\text{M}$  2-chloro- $N^6$ -cyclopentyladenosine (CCPA) are superimposed in *A*, as indicated in *B* by arrows. *B*: time sequence of peak amplitude of control current (■) and test current (□). CCPA was applied where indicated. At *time 0*, electrical access was obtained for cell. *C*: calculated percent facilitation for same data.

ternative splicing in domain IV S3 compared with the rabbit cardiac  $\alpha_{1C}$ -isoform used in previous studies (46, 71). Alternatively, differences in experimental protocols or conditions can mask facilitation. Likewise, voltage-dependent facilitation has not been uniformly observed when the *Xenopus* oocytes are used to express the  $\alpha_{1C}$ -isoform (9–11, 57). These contrasting results suggest that voltage-dependent facilitation is a complex behavior of the channel that may be modulated by multiple factors. In fact, even in a given expression system, HEK-293 cells, we observed variable results, with facilitation being observed in some cells but not in others. This variability occurred between cells and not within cells, inasmuch as facilitation was stable over time in a given cell. Therefore, even in a given expression system, differences in channel regulation may dramatically alter the ability of the expressed channels to exhibit voltage-dependent facilitation.

*Activation of PKA is not required for voltage-dependent facilitation.* Conflicting results exist regarding the necessity of PKA-dependent phosphorylation for voltage-dependent facilitation (9, 19, 38, 62). One advantage of using the HEK-293 cells for the present studies is that previous investigations have demonstrated that PKA does not regulate L-type Ca channels expressed in these cells unless additional regulatory proteins are present, such as AKAP79 (23, 71). This

was also confirmed by our initial experiments. Therefore, the present study examined voltage-dependent facilitation under conditions where voltage-dependent phosphorylation by PKA should not occur. In agreement with that prediction is the finding that the onset of facilitation is more rapid ( $\tau = 7.5$  ms) than would be predicted for a phosphorylation event. This faster onset contrasts with the one to two order of magnitude slower onset of facilitation observed in systems where PKA has been implicated (9, 62). Another contrast with results in systems that demonstrated a role for PKA is the magnitude of the effect of facilitation, which was  $\sim 20\%$  in our study compared with 250–350% in studies demonstrating an effect of PKA (9, 62). Therefore, we hypothesize that there exist at least two distinct forms of voltage-dependent facilitation of L-type Ca channels, one that requires PKA and another that is independent of PKA.

*$\beta$ -Subunit is required for voltage-dependent facilitation.* Expression of the  $\alpha_{1C}$ -subunit of the L-type Ca channel alone in various heterologous systems results in functional L-type Ca channels, as demonstrated in previous studies (36, 48, 53, 63). However, coexpression of the auxiliary  $\beta$ -subunit with the  $\alpha_{1C}$ -subunit significantly modifies many properties of the expressed channels (14, 33, 36, 48, 53, 63). In the present study, it was only when we coexpressed the auxiliary  $\beta_{1a}$ - or  $\beta_{2a}$ -subunit with the  $\alpha_{1C}$ -subunit that we observed voltage-dependent facilitation. Although the presence of facilitation was variable in  $\beta$ -subunit-coexpressing cells, it was never observed in cells expressing the  $\alpha_{1C}$ -subunit alone. The finding that the  $\alpha_{1C}$ -subunit alone cannot support facilitation contradicts the results obtained by others using Chinese hamster ovary cells to express the  $\alpha_{1C}$ -subunit (38, 62). The conflict with Kleppisch et al. (38) is most surprising, inasmuch as their voltage-dependent facilitation was PKA independent, in agreement with the present data. That study used a different isoform of the  $\alpha_{1C}$ -subunit derived from smooth muscle, which may contribute to the differences. Conversely, studies using *Xenopus laevis* oocytes to study facilitation of expressed neuronal  $\alpha_{1C}$ -subunit splice variant Ca channels have demonstrated a necessity for  $\beta$ -subunit coexpression (9). Interestingly, the rat  $\beta_{2a}$ -subunit does not support facilitation in the oocyte expression system (11), and this has been recently linked to an amino-terminal palmitoylation site unique to that  $\beta$ -subunit isoform (12, 57). The fact that our results demonstrate facilitation with the rat  $\beta_{2a}$ -subunit (Fig. 1) again argues that we are studying a distinct PKA-independent form of voltage-dependent facilitation compared with oocyte expression studies. Additionally, consideration of isoform variations, endogenous subunits, and regulatory state of the recombinant systems (e.g., phosphorylation state) may be important to reconcile the different results obtained in different systems.

*Activation of G proteins is not required for facilitation of  $\alpha_{1C}$ -type channels.* The biophysical characteristics of voltage-dependent facilitation described here are comparable to the behavior of neuronal, non-L-type Ca channels directly inhibited by activated G protein

$\beta\gamma$ -subunits. Such G protein-mediated inhibition can display a prominent voltage-dependent component, in that block can be relieved by strong depolarizations, resulting in apparent voltage-dependent facilitation (17, 18, 32). The acceleration of activation kinetics by a strong depolarizing prepulse is observed in this study and in studies of G protein-mediated inhibition of non-L-type Ca channels (17, 32). The rapid kinetics of the onset of facilitation in this study and those examining this effect in non-L-type Ca channels inhibited by G proteins are described by  $\tau = 5\text{--}10$  ms (8, 24, 32, 46, 61). The extent of enhancement by the conditioning pulse is also comparable between our studies and many others examining voltage-dependent G protein inhibition (17). Additionally, the biphasic nature of the voltage-dependent activation of the channels demonstrated by the instantaneous  $I\text{-}V$  relationships is comparable to that observed in studies of G protein-inhibited N- and P/Q-type channels (17, 18, 32). Finally, a study of  $\alpha_{1A}$ - or  $\alpha_{1B}$ -type channels expressed in *Xenopus* oocytes demonstrated a tonic G protein  $\beta\gamma$ -subunit inhibition of the channels that suggested possible tonic inhibition of  $\alpha_{1C}$ -type channels in the present study (60). Nevertheless, using high concentrations in the patch pipette of an activator (GTP $\gamma$ S) or an inhibitor (GDP $\beta$ S) of endogenous heterotrimeric G proteins, we found no evidence for a role of G proteins in facilitation of  $\alpha_{1C}$ -type channels. To further support this conclusion, we tested activation of heptahelical G protein-coupled receptors by stimulating the endogenous SST receptors or a heterologously expressed  $A_1$ AR. We observed only a voltage-independent inhibition of the expressed  $\alpha_{1C} + \beta_{1a}$  channels by SST and no voltage-dependent inhibition in either case. This lack of a role for G proteins in facilitation of  $\alpha_{1C}$ -encoded Ca channels confirms previous studies (27, 46, 57, 70). The lack of G protein effects on  $\alpha_{1C}$ -type channels has been attributed to the lack of a consensus QXXER G protein  $\beta\gamma$ -binding site in the I-II linker region, but the QXXER site alone is not sufficient for G protein modulation of  $\alpha_{1C}$ -type channels (27, 46, 58, 70). These findings suggest that  $\alpha_{1C} + \beta$ -expressing channels in HEK-293 cells, independently of G proteins, exhibit a voltage-dependent gating behavior similar to  $\alpha_{1A}$ - or  $\alpha_{1B}$ -type channels inhibited by G protein  $\beta\gamma$ -subunits.

**Mechanistic implications for voltage-dependent facilitation.** Voltage-dependent facilitation of L-type Ca channels expressed in HEK-293 cells can occur independently of voltage-dependent PKA phosphorylation of the channels and independently of G protein binding. We propose that the observed gating behavior is intrinsic to the Ca channel complex itself. The voltage-dependent activation of channels expressing the  $\alpha_{1C}$ -subunit alone and those expressing  $\alpha_{1C} + \beta_{1a}$  can be described by the sum of two Boltzmann distributions, with complete activation of the channels requiring strongly positive voltages. However, with use of a three-pulse protocol as shown in Fig. 1, only channels expressing  $\alpha_{1C} + \beta$  and not those expressing the  $\alpha_{1C}$ -subunit alone exhibited voltage-dependent facilitation. This suggests that the  $\beta$ -subunit slows the transitions among closed states during deactivation of the

channels after strong positive conditioning depolarizations. However, this effect must involve deep closed states, inasmuch as there is no observable change in the initial open-to-closed transition reflected by macroscopic current deactivation. Understanding the gating responsible for this facilitation in more detail will require future single-channel studies and gating current studies. Does this voltage-dependent facilitation result in a marked increase in mode 2 gating behavior with its long openings and high open probability, as shown originally in native cardiac cells (55)? Alternatively, this behavior could also potentially result from prolonged first latency of the channels, which is reduced by a strong depolarization, as described for  $\alpha_{1B}$ -type channels expressed in HEK-293 cells (52). Does greater charge movement occur after a strong conditioning depolarization? These important questions have yet to be explored.

**Physiological significance.** Given the critical role of L-type Ca channels in multiple cellular processes, any modulation of this current can have important functional importance. For example, in neuronal cells a rapid train of action potentials could result in increasing  $I_{Ca}$  through this channel by the process of voltage-dependent facilitation described here. In the case of cardiac muscle, where the  $\alpha_{1C}$ -isoform is predominantly expressed, repetitive action potentials can result in a positive force-frequency relationship, which may in part be due to voltage-dependent facilitation of L-type Ca channels. Given the kinetics of facilitation observed in the present study, it is difficult to extrapolate these results to significant effects on L-type Ca channels at physiological heart rates. However, differences in myocardial cells and HEK-293 cells may obviously alter the kinetics of this process. It is possible that prolonged action potentials can lead to facilitation of L-type Ca channels, which may promote afterdepolarizations and associated arrhythmias (4, 66). Additionally, changes in the activation kinetics for cardiac Ca channels can have significant effects on the kinetics and magnitude of contraction. Furthermore, the variable nature of this response suggests that regulatory mechanisms may exist that can titrate the extent of voltage-dependent facilitation.

**Conclusions.** Recombinant  $\alpha_{1C}$  L-type Ca channels expressed in HEK-293 cells can exhibit voltage-dependent facilitation. This facilitation process does not occur when the  $\alpha_{1C}$ -subunit is expressed alone; it is observed only when auxiliary  $\beta$ -subunits are coexpressed. Voltage-dependent facilitation in this system does not require active regulation by PKA, nor does it require activation of heterotrimeric G proteins. We propose that, in the case of cardiac  $\alpha_{1C}$ -type channels expressed with the  $\beta$ -subunit, the L-type Ca channels intrinsically can demonstrate gating behavior responsible for voltage-dependent facilitation. The present study is limited by the fact that other regulatory pathways acting on the expressed L-type Ca channels may be active in HEK-293 cells that are not yet fully appreciated. This is strongly suggested by the heterogeneous response observed in  $\alpha_{1C} + \beta_{1a}$ -expressing cells. Future studies will require careful attention to experi-

mental details, including subunit isoforms employed as a more complete understanding of the regulatory environment of the expression systems.

#### NOTE ADDED IN REVIEW

During the review of this manuscript, another laboratory published their results examining facilitation of L-type Ca channels expressed in HEK-293 cells (15). Dai et al. (15) used the rabbit  $\alpha_{1C-a}$  splice variant, which differs in domain IV S3 from the isoform used in the present study. The  $\beta_{2a}$ -subunit was also different, inasmuch as they used the rabbit heart  $\beta_{2a}$ -subunit, which lacks the amino-terminal palmitoylation site present on the rat  $\beta_{2a}$ -isoform. Nevertheless, they observed voltage-dependent facilitation with  $\alpha_{1C} + \beta_{2a}$  channels with biophysical properties comparable to those described in the present work. They provide additional evidence against a role of cAMP-dependent phosphorylation in voltage-dependent facilitation in this system. Additionally, they demonstrated that coexpression of the  $\alpha_{2\delta}$ -subunit prevented facilitation, which may reconcile why, in at least one previous study in HEK-293 cells, facilitation was not observed (71).

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A preliminary report of these findings has been presented in abstract form (35).

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