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The role of subunit composition on prepulse facilitation of the cardiac L-type calcium channel

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Abstract Facilitation of calcium current by depolarizing prepulses has been observed in many cells including cardiac muscle. The mechanism underlying prepulse facilitation is controversial with respect to the requirements of channel subunits and cAMP kinase. We found that coexpression of the cardiac $\alpha_{1C\text{-}a}$ subunit with the cardiac β_{2a} subunit significantly promotes the facilitation of I_{Ba} by strong depolarizing prepulses. The magnitude of I_{Ba} facilitation depended on the voltage potential of the prepulse and the interval duration between prepulse and test pulse. Prepulse facilitation was not affected by coexpression of AKAP79 and conditions favoring cAMP-dependent phosphorylation. Prepulse facilitation was also observed in cells expressing an $\alpha_{1C\text{-}a}$ subunit which was truncated at residue 1733 removing the cAMP kinase site at Ser-1928. Facilitation was abolished by coexpression of the $\alpha_2\delta$-1 or $\alpha_2\delta$-3 subunit. We conclude that the expressed $\alpha_{1C\text{-}a}$ β_{2a} complex is sufficient to support prepulse facilitation. Facilitation is prevented by coexpression of the $\alpha_2\delta$ subunit.

Key words: L-type calcium channel; Facilitation; Calcium channel subunit composition; cAMP kinase scaffold protein AKAP79; cAMP kinase

1. Introduction

L-type calcium channels play a crucial role in excitation-contraction coupling in cardiac and other muscles. The cardiac Ca^{2+} channel is a complex of several proteins including the pore forming α_1 subunit and the auxiliary β and $\alpha_2\delta$ subunits (reviewed in [1]). The cardiac L-type current (I_{Ca} or I_{Ba}) is upregulated by cAMP-dependent phosphorylation of the α_{1C} subunit or a closely associated protein [2,3]. In addition, I_{Ca} is upregulated by a voltage-dependent mechanism, called facilitation. Facilitation occurs either during a train of repetitive depolarization [4] or after a single strong depolarizing prepulse [5]. It was proposed that a strong depolarizing prepulse drives L-type calcium channels from their normal gating pattern into a mode of gating characterized by long opening and high open probability [5]. The structural mechanism underlying voltage-dependent facilitation is still not clear. Artalejo et al. [6] suggested that facilitation requires cAMP-dependent phosphorylation. However, their findings were refuted by Garcia and Carbonne [7].

I_{Ba} of the expressed α_{1C} subunit shows facilitation, which has been reported to be independent of cAMP-dependent phosphorylation [8–11] or dependent on cAMP-dependent phosphorylation [12,13]. A careful study, which used the α_{1C-a} and α_{1C-b} splice variants stably expressed in CHO and HEK 293 cells and transient expression of α_{1C-b}, cardiac β_{2a} and $\alpha_2\delta$-1, was unable to reproduce a stimulatory effect of cAMP kinase on I_{Ba} [14]. In addition, facilitation was not observed in cells coexpressing the α_{1C-b}, cardiac β_{2a} and $\alpha_2\delta$-1 subunits [14]. These conflicting results were apparently solved, when Gao and coworkers [15] reported that cAMP kinase-dependent stimulation of I_{Ba} required the coexpression of the cAMP kinase-anchoring protein AKAP79, α_{1C-a} and neuronal β_{2a} subunit. AKAP79 anchors the kinase at the plasma membrane. These authors reported further that phosphorylation of Ser-1928 of the α_{1C} subunit was required for cAMP-dependent stimulation of I_{Ba} [15]. The subunit combination used by Gao et al. [15] is interesting, since it was shown that the neuronal β_{2a} subunit is targeted to the plasma membrane by palmitoylation of two amino-terminal cysteines [16] and prevents facilitation of the expressed α_{1C} channel [10,11]. However, the neuronal β_{2a} subunit is not expressed in rabbit cardiac muscle [11] suggesting that the subunit composition used may not represent that found in the cardiac myocyte.

A possible explanation for the discrepant results is that the use of different splice variants of the α_{1C} and β subunit may affect the outcome of the experiments. In line with this consideration is the finding of Qin and colleagues [11], that replacing the two cysteines by serine in the neuronal β_{2a} subunit allows the induction of prepulse facilitation of the α_{1C} subunit. To clarify some of the questions posed above, we have expressed functional cardiac L-type calcium channels in HEK293 cells in different combinations of the truncated α_{1C-at}, and the full-length α_{1C-a} and cardiac β_{2a} (cloned from rabbit heart) with or without $\alpha_2\delta$ and AKAP79.

2. Materials and methods

Sp-5,6-DCI-cBIMPS (cBIMPS) was from Biolog, Hamburg, microcystin, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H89) was from Biomol. Stock solutions of H89 and cBIMPS were dissolved in dimethylsulfoxide (DMSO) and stored at −20°C. The stock solutions were diluted with the pipette or bath solution before the experiment. AKAP79 was cloned from HEK293 cells by a PCR-based approach. The full-length clone was sequenced on both strands to confirm identity with the published sequence [17]. The $\alpha_2\delta$-1 and α_{1C-a} subunits have been described [18] and the $\alpha_2\delta$-3 subunit was recently cloned from mouse brain as described in [19]. The cell line HH8, which stably expresses the α_{1C-a} subunit truncated at the carboxy-terminal residue 1733, has been described as α_{1C-at} [20].

HEK293 cells were transiently transfected with $\alpha_{1C-a}\beta_{2a}$, $\alpha_{1C-a}\beta_{2a}\alpha_2\delta$-1 (or $\alpha_2\delta$-3) or $\alpha_{1C-a}\beta_{2a}$+AKAP79. Cells were seeded in Petri dishes. A cell-containing dish was mounted on an inverted microscope and was constantly perfused with modified Tyrode's solution containing (in mM): NaCl 82, tetraethylammonium chloride (TEA-Cl) 20, $BaCl_2$ 30, CsCl 5, $MgCl_2$ 1, EGTA 0.1, HEPES 5, glucose 10 at pH 7.4 adjusted with NaOH. For external application, a rapid solution exchanger was used. The pipette solution contained

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(in mM): CsCl 102, TEA-Cl 10, EGTA 10, $MgCl_2$ 1, MgATP 3, HEPES 5, pH 7.4 adjusted with CsOH. The pipette had a resistance of 1.5–3 MΩ. Whole cell barium current (I_{Ba}) was measured at room temperature using either an EPC-7 amplifier and pCLAMP software (Axon Instruments) or EPC-9 (HEKA). If not stated otherwise, I_{Ba} was measured during a double pulse protocol from a holding potential of −80 mV and 0.1 Hz. Test pulses to +10 mV were preceded by 100 ms prepulses to +100 mV with a 10 ms interval at −20 mV. Data were plotted and statistically analyzed by ORIGIN software. Data are given as mean ± S.E.M.

3. Results and discussion

3.1. Facilitation of I_{Ba} in cells expressing $\alpha_{1C-a}\beta_{2a}$ subunit

Initially, we screened several cell lines expressing various combinations of calcium channel subunits. Prepulse facilitation was observed regularly in cells expressing the subunit combination $\alpha_{1C-a}\beta_{2a}$ (Fig. 1). Prepulse facilitation was prevented by superfusion of the cells with the dihydropyridine nisoldipine (Fig. 1C). A strong depolarization prepulse to +100 mV markedly increased the amplitude of I_{Ba} for test

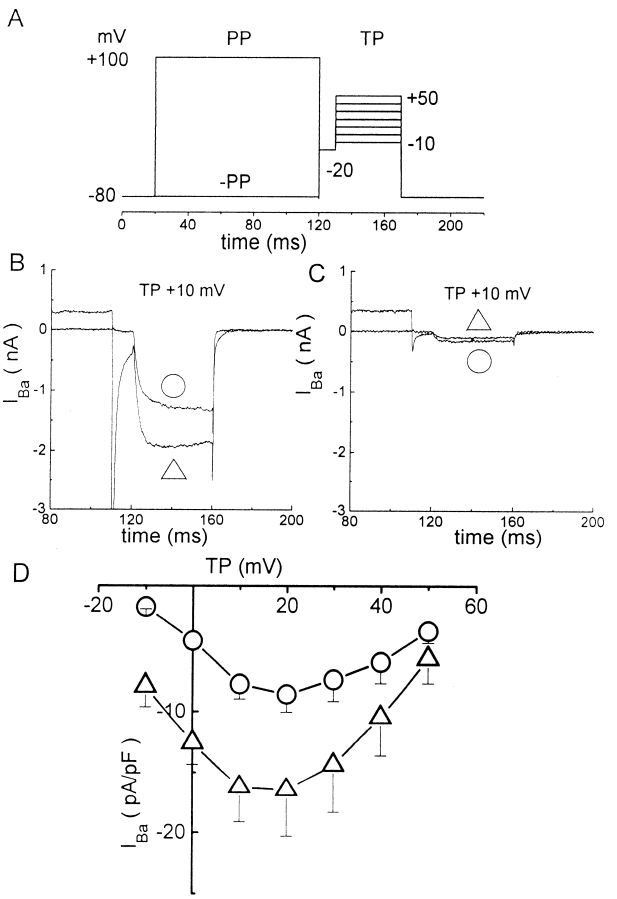

Fig. 1. Facilitation of I_{Ba} in HEK293 cells transfected with the $\alpha_{1C-a}\beta_{2a}$ subunits. A: Pulse protocol; PP, prepulse; −PP, without prepulse; TP, test potential. B: Superimposed current traces of I_{Ba} at TP +10 mV in the absence (circle) or presence (triangle) of a prepulse to +100 mV. C: Superimposed current traces of I_{Ba} at TP +10 mV in the absence (circle) or presence (triangle) of a prepulse to +100 mV after extracellular perfusion of 100 nM nisoldepine for 3 min. D: *I-V* relation of I_{Ba} without (circle) and with (triangle) a prepulse to +100 mV. Values are mean ± S.E.M. ($n = 6$). Note that the prepulse induced an increase in I_{Ba} amplitude, but did not significantly affect the voltage-dependent activation of the Ca^{2+} channel.

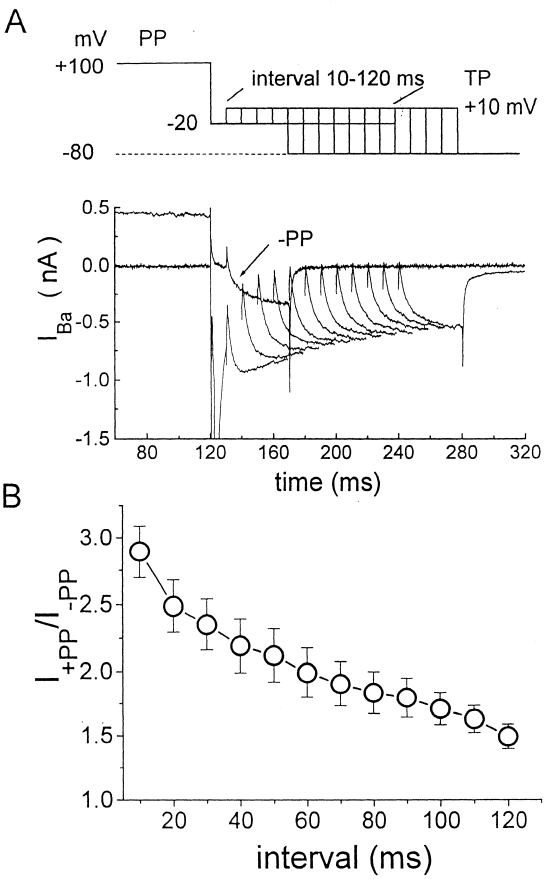

Fig. 2. Dependence of I_{Ba} facilitation on interval duration. A: Superimposed current traces (lower part) and pulse protocol (upper part). I_{Ba} was elicited by a test potential (TP) to +10 mV following a prepulse (PP) to +100 mV with 10–120 ms interval duration at −20 mV between PP and TP. −PP, current trace in the absence of PP. B: Summary of relation between facilitated I_{Ba} and interval duration. Facilitation is expressed as the I_{Ba} ratio (I_{+pp}/I_{-pp}). Values are mean ± S.E.M. ($n = 5$) from cells transfected with the $\alpha_{1C-a}\beta_{2a}$ subunits.

pulses from −10 mV to +30 mV (Fig. 1D). At +20 mV, I_{Ba} was potentiated from 8.6 ± 1.4 pA/pF to 16.5 ± 3.8 pA/pF. The stimulation of I_{Ba} amplitude became smaller for test pulses positive to +30 mV. The *I-V* relations of I_{Ba} was not shifted significantly by the prepulse. The extent of facilitation decreased with the length of the interval between prepulse and test pulse (Fig. 2). A prepulse from −80 mV to +100 mV, followed by an interval of less than 50 ms at −20 mV, facilitated I_{Ba} 2–3-fold. Stimulation decreased to 1.5-fold when the interval duration was longer than 100 ms. The amplitude of the facilitated I_{Ba} depended on the prepulse potential (Fig. 3). Prepulses from −80 mV up to 0 mV did not augment I_{Ba}, whereas I_{Ba} was potentiated by prepulses between 0 mV and 140 mV with an apparent saturation around 140 mV. I_{Ba} was increased nearly 4-fold with prepulse to +140 mV. As observed previously [14], coexpression of the $\alpha_2\delta$-1 or $\alpha_2\delta$-3 subunit together with the $\alpha_{1C-a}\beta_{2a}$ complex prevented prepulse facilitation (Fig. 4). Prepulses from −60 mV to +40 mV decreased I_{Ba}, since the coexpressed $\alpha_2\delta$ subunit shifts the steady-state inactivation curve to more negative membrane potentials [19]. Prepulses from +60 mV to +140 mV increase I_{Ba} compared to the current measured after a prepulse to +40 mV. However, after a prepulse to +140 mV, I_{Ba}

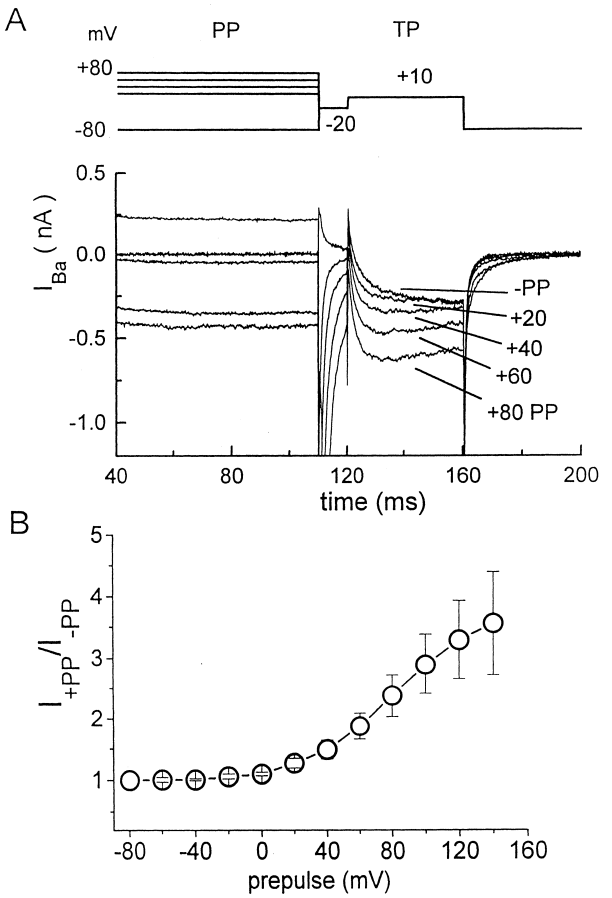

Fig. 3. Dependence of I_{Ba} facilitation on voltage of PP. A: Superimposed current traces (lower part) and pulse protocol (upper part). I_{Ba} was elicited at TP +10 mV following PPs from −80 mV to +100 mV. B: Summary of relation between facilitated I_{Ba} and voltage of PP. Values are mean ± S.E.M. ($n = 8$) from cells transfected with the $\alpha_{1C-a}\beta_{2a}$ subunits.

is still smaller than without prepulse indicating that $\alpha_2\delta$ decreased I_{Ba} amplitude presumably by voltage-dependent inactivation during the prepulse.

3.2. Facilitation of I_{Ba} and cAMP-dependent protein phosphorylation

Several studies reported that prepulse facilitation was augmented in the presence of active cAMP kinase [12,13] and required the cAMP kinase scaffolding protein AKAP79 [15]. AKAP79 is expressed at low levels in HEK293 cells and localizes the type II cAMP kinase holoenzyme to the membrane. We cloned AKAP79 from a cDNA library of HEK293 cells. The deduced sequence of the cloned AKAP79 was identical to that reported by Carr and coworkers [17]. Overexpression of AKAP79 in combination with cBIMPS, a cell-permeable analogue of cAMP which activates preferentially type II cAMP kinase, and a high concentration of okadaic acid (2 μM) to inhibit protein phosphatase I and IIa were used to favor cAMP-dependent phosphorylation of the expressed channel. Inclusion of cBIMPS and okadaic acid in various solutions was without effect on the magnitude of prepulse dependent I_{Ba} facilitation (Fig. 5). Facilitation was also not augmented in cells that coexpressed the α_{1C} β_{2a} and AKAP79 and were incubated in the presence of cBIMPS and okadaic acid. These negative results could be explained if the channel was phosphorylated during the prepulse by endogenously activated cAMP kinase. However, and in agreement with previous results [14], 1 μM H89, a concentration 100-fold above the IC_{50} value for active cAMP kinase, did not attenuate prepulse facilitation.

These experiments provided evidence that prepulse facilitation of the expressed cardiac L-type calcium channel did not require cAMP-dependent phosphorylation. However, the biological significance of these experiment could be questioned, since over 90% of the L-type calcium channel present in cardiac plasma membranes is truncated at residue 1870 [21] and does not contain the cAMP kinase site at Ser-1928 [21,22]. We therefore tested prepulse facilitation in a cell line (HH8) that stably expressed the α_{1C-a} subunit truncated at residue 1733 [20]. Fig. 6 shows the voltage dependence of I_{Ba} facilitation in the HH8 cells. Almost no facilitation was observed after prepulses from −80 mV to +20 mV. Prepulses positive to +20 mV potentiated I_{Ba} in a voltage-dependent manner (Fig. 6B). A prepulse to +120 mV increased mean I_{Ba} 2-fold from -4.1 ± 0.2 pA/pF to -8.9 ± 1.0 pA/pF. This stimulation (1.6 ± 0.1, $n = 9$) at a prepulse to +100 mV is significantly different at $P < 0.05$ from that observed with the $\alpha_{1C-a}\beta_{2a}$ complex (2.9 ± 0.5, $n = 8$), but is almost identical to that reported with the full-length α_{1C-b} subunit [8]. We were unable to demonstrate an involvement of cAMP-dependent phos-

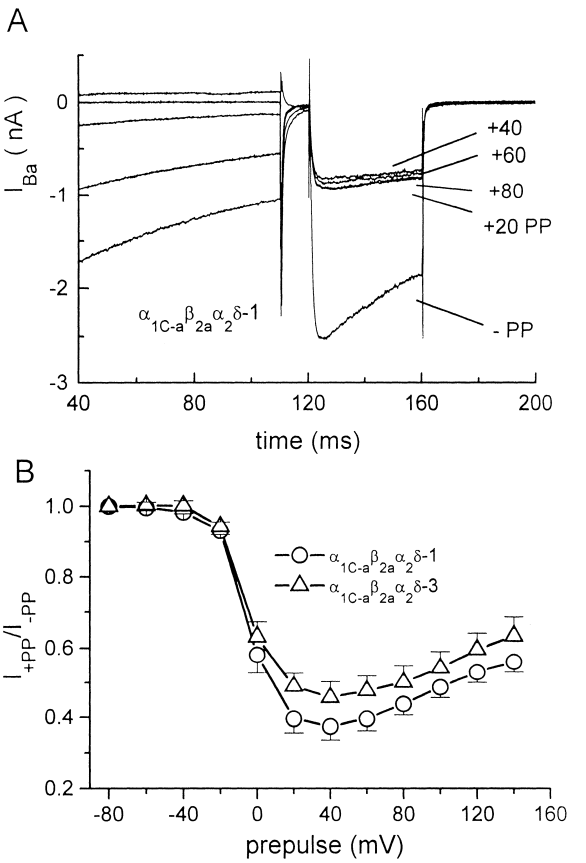

Fig. 4. The $\alpha_2\delta$ subunit inhibits facilitation. A: Representative current trace of I_{Ba} in HEK 293 cells transiently cotransfected with $\alpha_{1C-a}\beta_{2a}\alpha_2\delta$-1 subunits. The same pulse protocool as in Fig. 3. B: Summary of relation between I_{Ba} ratio (I_{+pp}/I_{-pp}) and voltage of PP in cells transfected with $\alpha_{1C-a}\beta_{2a}$ $\alpha_2\delta$-1 (circle, n=9) or $\alpha_{1C-a}\beta_{2a}\alpha_2\delta$-3 (triangle, $n = 7$) subunits.

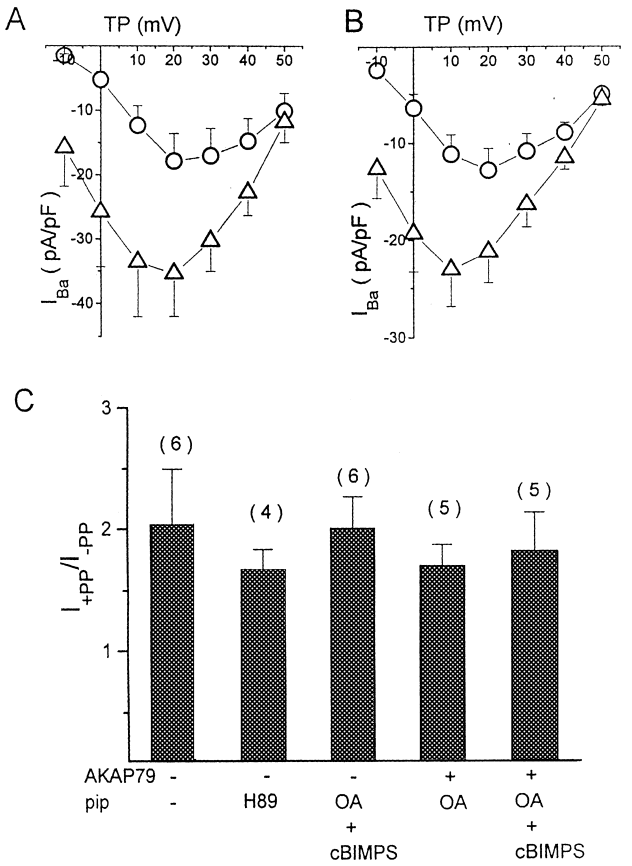

Fig. 5. cAMP-dependent phosphorylation and facilitation. A, B: *I-V* relation of I_{Ba} without (circle) and with (triangle) a PP to +100 mV in the presence of 100 μM cBIMPS and 2 μM ocadaic acid (OA) in the pipette solution. Cell were transfected with $\alpha_{1C-a}\beta_{2a}$ (A, $n=6$) and $\alpha_{1C-a}\beta_{2a}$ plus AKAP79 (B, $n=5$). C: Summary of I_{Ba} facilitation tested at TP +20 mV (I_{+pp}/I_{-pp}). The cAMP kinase inhibitor H89 was 1 μM in the pipette solution (pip). Facilitation was measured after stable inward currents were obtained, usually 5 min after breaking the membrane.

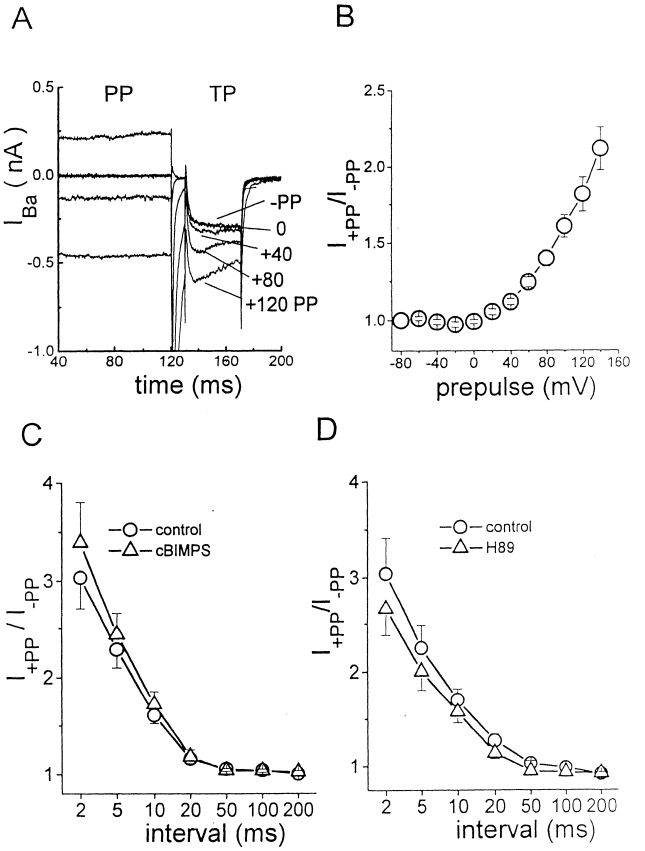

Fig. 6. Facilitation of I_{Ba} of the stable cell line HH8. The stable cell line HH8 expresses an α_{1C-a} subunit truncated at residue 1733 of the carboxy-terminal tail. Representative current trace of I_{Ba} (A) and summary of prepulse facilitation (B, $n=9$). The same pulse protocol as in Fig. 3; TP was +10 mV. C: Facilitation of I_{Ba} (I_{+pp}/I_{-pp}) measured at a TP +10 mV following a +100 mV PP with different intervals of 2–200 ms in the presence of normal perfusion solution (circle) or extracellular solution containing 100 μM cBIMPS (triangle) ($n=7$). D: Facilitation of I_{Ba} (I_{+pp}/I_{-pp}) obtained with normal pipette solution (circle, $n=6$) or with 1 μM H89 in pipette solution (triangle, $n=6$).

phorylation in the facilitation mechanism. Neither the addition of the cAMP kinase activator cBIMPS nor that of the cAMP kinase blocker H89 affected the extent of I_{Ba} facilitation of the truncated channel in HH8 cells (Fig. 6C,D).

This report supports the notion that prepulse facilitation is an important property of L-type calcium channels, which may regulate the influx of calcium ions from the outside into the cytosol of various cells. A major factor regulating facilitation in HEK293 cells is the subunit composition of the calcium channel complex. I_{Ba} was facilitated by a strong depolarizing prepulse in HEK293 cells expressing the α_{1C-b} subunit [8], the α_{1C-at} subunit or the $\alpha_{1C-a}\beta_{2a}$ complex, but not in cells expressing the $\alpha_{1C-a}\beta_{2a}\alpha_2\delta$-1 or the $\alpha_{1C-a}\beta_{2a}\alpha_2\delta$-3 complex. Obviously, the $\alpha_2\delta$ subunits used inhibit facilitation. Our results exclude also that cAMP kinase is involved in the facilitation process of the $\alpha_{1C-a}\beta_{2a}$ complex, since H89 and cAMP did not significantly affect facilitation. In contrast to reports that phosphorylation of serine 1928 of α_{1C-a} mediates the modulatory effect of cAMP kinase on the L-type calcium channel [15,22], I_{Ba} was facilitated in HEK293 cells stably transfected with α_{1C-at}, confirming previous reports [8,14,23] that phosphorylation of the carboxy-terminal serines is not necessary for facilitation. Furthermore, coexpression of AKAP79 and the $\alpha_{1C-a}\beta_{2\alpha}$ complex had no significant influence on facilitation. Although auxiliary subunits play a crucial role in this regulatory mechanism, our results indicate that facilitation is an intrinsic property of the calcium channel α_{1C} subunit.

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