The EMBO Journal vol.13 no.11 pp.2502-2507, 1994

Double-pulse facilitation of smooth muscle α_1-subunit Ca^{2+} channels expressed in CHO cells

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Communicated by B.Hamprecht

Frequent strong depolarizations facilitate Ca^{2+} channels in various cell types by shifting their gating behavior towards mode 2, which is characterized by long openings and high probability of being open. In cardiac cells, the same type of gating behavior is potentiated by β-adrenoceptors presumably acting via phosphorylation of a protein identical to or associated with the channel. Voltage-dependent phosphorylation has also been reported to underlie Ca^{2+} channel facilitation in chromaffin adrenal medulla and in skeletal muscle cells. We studied a possible voltage-dependent facilitation of the principal channel forming α_1-subunit of the dihydropyridine-sensitive smooth muscle Ca^{2+} channel. Single channel and whole-cell Ca^{2+} currents were recorded in Chinese hamster ovary cells stably expressing the class C_b Ca^{2+} channel α_1-subunit. Strong depolarizing voltage-clamp steps preceding the test pulse resulted in a 2- to 3-fold increase of the single Ca^{2+} channel activity and induction of mode 2-like gating behavior. Accordingly we observed a significant potentiation of the whole-cell current by ~50%. In contrast to the previous suggestions we found no experimental evidence for involvement of channel phosphorylation by protein kinases (cAMP-dependent protein kinase, protein kinase C and other protein kinases utilizing ATPγS) in the control and facilitated current. The data demonstrate that the L-type Ca^{2+} channel α_1-subunit solely expressed in Chinese hamster ovary cells is subject to a voltage-dependent facilitation but not to phosphorylation. We suggest that this newly identified type of voltage-dependent facilitation of Ca^{2+} channels is due to a direct voltage-dependent conformational change inducing the same type of gating behavior as otherwise induced by phosphorylation.

Key words: Ca^{2+} channel/dihydropyridine sensitivity/facilitation/single channel current/smooth muscle/whole-cell current

Introduction

Voltage-dependent dihydropyridine (DHP)-sensitive (L-type) Ca^{2+} channels are found in virtually all excitable tissues (for review see Tsien *et al.*, 1988; Bean, 1989). In heart and smooth muscle cells they represent the major pathway for voltage-gated Ca^{2+} entry and play a crucial role in excitation-contraction coupling. Frequent depolarizations have been reported to progressively increase L-type Ca^{2+} channel currents in ventricular cardiomyocytes (Lee, 1987). A voltage-dependent facilitation of Ca^{2+} currents has also been observed in bovine chromaffin cells (Fenwick *et al.*, 1982; Artalejo *et al.*, 1991a,b), rat sympathetic and sensory neurons (Ikeda, 1991; Tatebayashi and Ogata, 1992), neuroblastoma cells (Kasai, 1991) and in rat cerebellar granules (Forti and Pietrobon, 1993). For L-type Ca^{2+} channels it has been shown that the depolarizing prepulses induce a shift of the normal Ca^{2+} channel gating behavior (mode 1) to the so-called mode 2 gating, which is characterized by long-lasting openings interrupted by fast closing events (Hess *et al.*, 1984; Pietrobon and Hess, 1990). Since in cardiac cells, a mode 2 gating was also observed under stimulation of β-adrenoceptors (Yue *et al.*, 1990), presumably by acting via a cAMP-dependent phosphorylation of Ca^{2+} channels (Trautwein and Hescheler, 1990), and since in chromaffin cells blockage of phosphatases by okadaic acid enhanced facilitation (Artalejo *et al.*, 1992), it was concluded that the strong depolarizations induce a change of the phosphorylation state of channels and by this induce the facilitation. Such a mechanism was also supported by a recent report by Sculptureanu *et al.* (1993) demonstrating that voltage-dependent facilitation of L-type Ca^{2+} channel activity during tetanic stimulation of skeletal muscle cells required cAMP-dependent protein phosphorylation. Alternatively, a voltage-dependent temporary recovery from tonic inhibition by G-proteins may underlie the voltage-dependent facilitation (Kasai, 1991).

In order to study the phenomenon of facilitation in more detail, we performed experiments on Chinese hamster ovary (CHO) cells permanently transfected with cDNA of the class C_b α_1-subunit (Hofmann *et al.*, 1994) from smooth muscle but lacking the β- and γ-subunits of Ca^{2+} channels (CHOCa9, Bosse *et al.*, 1992). Previously, we demonstrated in CHOCa9 cells that the α_1-subunit cDNA alone is sufficient to express functional DHP-sensitive L-type Ca^{2+} channels (Bosse *et al.*, 1992). Wild-type CHO^- cells lacked a significant Ca^{2+} current. The class C_b α_1-subunit exhibits high homology to the cardiac C_a α_1-subunit sharing all putative phosphorylation sites (for review see Campbell *et al.*, 1988; Catterall *et al.*, 1988; Hofmann *et al.*, 1990; Catterall, 1991; Hullin *et al.*, 1993). However, presumably since CHOCa9 cells are deficient in the β- and γ-subunits of Ca^{2+} channels, we did not find a significant regulation by phosphorylation or G-proteins. The rationale of the present experiments on CHOCa9 cells was to investigate (i)

whether the α_1-subunit alone is facilitated by depolarization and (ii) whether a phosphorylation is involved. We found in single channel as well as in whole cell experiments a facilitation of the Ca^{2+} channel current, which was not altered by stimulation or inhibition of protein kinases. We conclude that facilitation may be due to a direct voltage-dependent conformational change of the smooth muscle α_1-subunit of the Ca^{2+} channel. We also did not find evidence for an involvement of G-proteins in the facilitation. Although the involvement of G-proteins and protein kinases in the receptor-dependent regulation of Ca^{2+} channels is widely proven (for review see Schultz *et al.*, 1990; Trautwein *et al.*, 1990), at present it is not understood which channel subunit is the functional target of these signal transducers.

Results

Single Ca^{2+} channel activity of CHOCa9 cells was measured in the cell-attached mode during repetitive voltage-clamp pulses from −50 mV to −10 mV. The inward deflections were of short duration and exhibited an amplitude of ~1.65 pA (Figure 1A, left). From eight patches we determined the mean open probability (P_o) to 0.006 ± 0.001. Facilitating prepulses to +70 mV markedly increased the Ca^{2+} channel activity (Figure 1A, right) which was best seen in the averaged current traces (Figure 1B). The plot of P_o from 200 consecutive current traces (Figure 1C) indicated that facilitation raised P_o in some sweeps even as high as 0.6 (i.e. 100 times the control P_o) which was never seen under control conditions. In eight cells, the mean P_o was raised to 0.02 ± 0.002, i.e. about 3 times the control value. The increase of P_o was mainly due to the occurrence of long-lasting openings (mode 2-like) during the test pulses but not to a reduction of the number of current traces without activity (blanks). As illustrated by the open time histograms (Figure 2A), the duration of Ca^{2+} channel openings under control conditions never exceeded 35 ms. In contrast, Ca^{2+} channels under facilitation exhibited openings as long as 220 ms (see Figure 2B). The control open time distribution was well fitted with a monoexponential function with a time constant τ_{o1} of 0.38 ms. Facilitation induced an additional slow exponential component with a time constant τ_{o2} (Figure 2, lower part). The estimated values of τ_{o1} and τ_{o2} were 0.32 and 3.9 ms, respectively (n = 8). First latency analysis (n = 8) revealed a facilitation-induced slight decrease of the delay before channels open (Figure 3A and B) which is also reflected in the accelerated Ca^{2+} channel activation observed in the average currents of Figure 1B as well as in the whole cell currents of Figure 4. The closed time distribution under control conditions was well fitted by a biexponential function with time constants τ_{c1} and τ_{c2} of 28.2 and 236.3 ms, respectively (Figure 3C and D). Under facilitation, the short intercalated closure events during long-lasting openings (see Figure 1A) evoked an additional fast exponential component (τ_{c1*}). The corresponding closed time distribution was fitted with time constants of τ_{c1}*, τ_{c1} and τ_{c2} amounting to 1.6, 32.5 and 205.6 ms, respectively.

Whole-cell current recordings in CHOCa9 cells were performed in order to test a possible involvement of protein kinases or G-proteins in Ca^{2+} channel facilitation. As demonstrated in Figure 4 facilitation in control cells induced

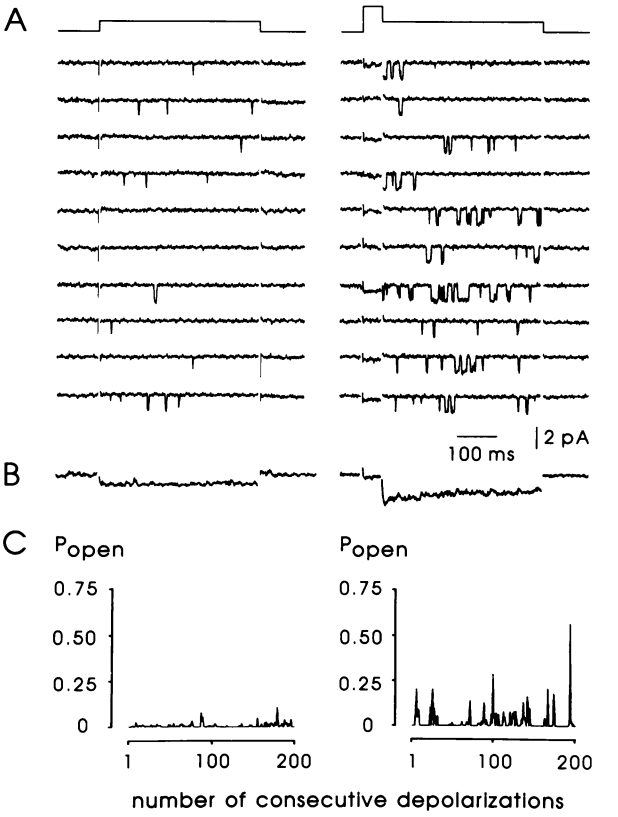

Fig. 1. Effects of depolarizing prepulses on single Ca^{2+} channel currents through L-type α_1-subunits expressed in CHO cells. (**A**) Single Ca^{2+} channel currents were elicited during repetitive 400 ms test pulses to −10 mV from a holding potential of −50 mV. Shown are consecutive current traces recorded in two series of pulse protocols from the same cell: Left: standard test pulses; right: standard test pulses preceded by facilitating prepulses (50 ms long, +70 mV). The pulse protocols are schematically represented on the top of each family of sweeps. The Ca^{2+} channel activity under standard test pulses (left panels) and under facilitation (right panels) was analyzed by averaging current sweeps (**B**) and by plotting the open probability versus the number of consecutive depolarizations (**C**).

a 30−70% increase of the Ca^{2+} channel current. On average, the current increased by a factor I_{pre}/I_{con} (the ratio between facilitated current and control current) of 1.51 ± 0.13 (n = 12). As previously described for cardiac and chromaffin cells (Pietrobon and Hess, 1990; Artalejo *et al.*, 1991a), the facilitated Ca^{2+} channel currents in CHOCa9 cells elicited a faster activation and inactivation time course than control currents. Both the control and the facilitated currents were almost completely blocked under the DHP Ca^{2+} channel antagonist (+)PN 200-110 (2 μM, not shown) suggesting that the same L-type Ca^{2+} channels underly these currents. The intracellularly applied catalytic subunit of the cAMP-dependent protein kinase (PKA) and the specific PKA inhibitor [PKI(6-22) amide] (Trautwein and Hescheler, 1990) were tested for their ability to enhance or to inhibit Ca^{2+} channel current facilitation. In line with previous experiments (Bosse *et al.*, 1992), the catalytic subunit of PKA (7 μM) plus ATPγS (10 mM, resulting in phosphatase-resistant thiophosphorylation) infused for at least 15 min into CHOCa9 cells did not significantly ($P > 0.05$) increase the amplitude of basal Ca^{2+} channel currents (no depolarizing prepulses). The increase never exceeded 20%

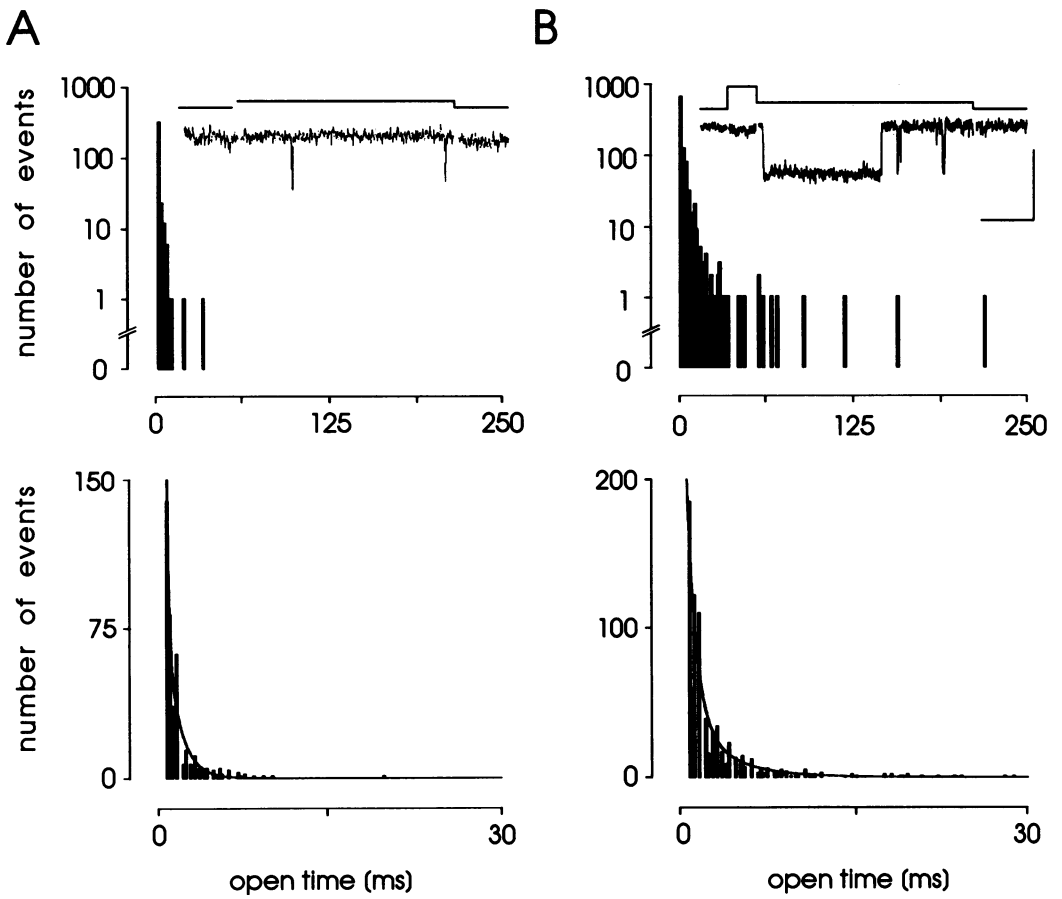

Fig. 2. Open time distributions of currents through the single L-type α_1-subunits recorded under standard test pulses (**A**) and under standard test pulses preceded by facilitating prepulses (**B**). The details of pulse protocols are given in the legend to Figure 1. The open time histograms in the upper part of both panels include all events from eight patches and were collected using a bin width of 10 ms. Note the logarithmic scale of the *y*-axis. The inset in panel B shows an example of an extremely long Ca^{2+} channel opening observed exclusively under facilitating prepulses. The horizontal and vertical scale bars represent 100 ms and 2 pA, respectively. The open time histograms in the lower part are zoomed out from the upper histograms and demonstrate the distribution of open times shorter than 30 ms (bin width of 2 ms, linear scale of *y*-axis). The smooth curves represent best fits using a monoexponential function with a time constant of 0.38 ms (panel A) or a biexponential function with time constants of 0.32 and 3.9 ms (panel B).

(Figure 4A) and was within the normal range of current variation during prolonged recordings. There was also no significant ($P > 0.05$) alteration of the Ca^{2+} channel facilitation under the catalytic subunit of PKA plus ATPγS (Figure 4A, insets). On average, I_{pre}/I_{con} amounted to 1.67 ± 0.14 ($n = 6$) in cells infused with the catalytic subunit of PKA plus ATPγS (Figure 4C). Similar results were obtained if only the catalytic subunit was infused (1.65 ± 0.18, $n = 18$). Furthermore, the intracellularly infused PKI(6-22) amide (5 μM) was without effect on Ca^{2+} channel facilitation (Figure 4C and E). The average I_{pre}/I_{con} was 1.47 ± 0.12 ($n = 6$). Since the Ca^{2+} channel α_1-subunit may be a target for phosphorylation by protein kinase C (PKC) (see Jahn *et al.*, 1988), we examined the possible role of an endogenous PKC in the facilitation of Ca^{2+} channel currents in CHOCa9 cells. Phorbol 12-myristate 13-acetate (500 nM), a direct activator of PKC, had no effect on basal Ca^{2+} channel currents and did not alter the amount of facilitation ($n = 4$, not shown). GDPβS is known to stabilize the α-subunit of heterotrimeric G-proteins in their inactivated form. As demonstrated in Figure 4D, intracellular infusion of GDPβS (200 μM) did not alter Ca^{2+} channel facilitation, I_{pre}/I_{con} being 1.59 ± 0.1 ($n = 8$).

Discussion

Our data demonstrate that the class C_b Ca^{2+} channel α_1-subunit alone, in the absence of the α_2-, δ-, β- and γ-subunits, is subject to a depolarization-induced facilitation. In our hands the facilitation-dependent stimulation amounted to 2- to 3-fold in single channel and 1.5-fold in whole cell experiments. Similar prepulse-induced potentiation of Ca^{2+} currents has been previously reported for chromaffin cells to be 1.6-fold (Artalejo *et al.*, 1991a), for skeletal muscle to be 5- to 10-fold (Sculptureanu *et al.*, 1993) and for cardiac cells to be ~3-fold (Pietrobon and Hess, 1990). In accordance with previous reports, we found in CHOCa9 cells that under facilitation the gating behavior of the sole α_1-subunit switched from mode 1 gating characterized by short openings to mode 2 gating characterized by long-lasting openings intercalated by short closing events (Pietrobon and Hess, 1990; Artalejo *et al.*, 1991b). Obviously, the α_1-subunit alone is capable of producing mode 2 gating.

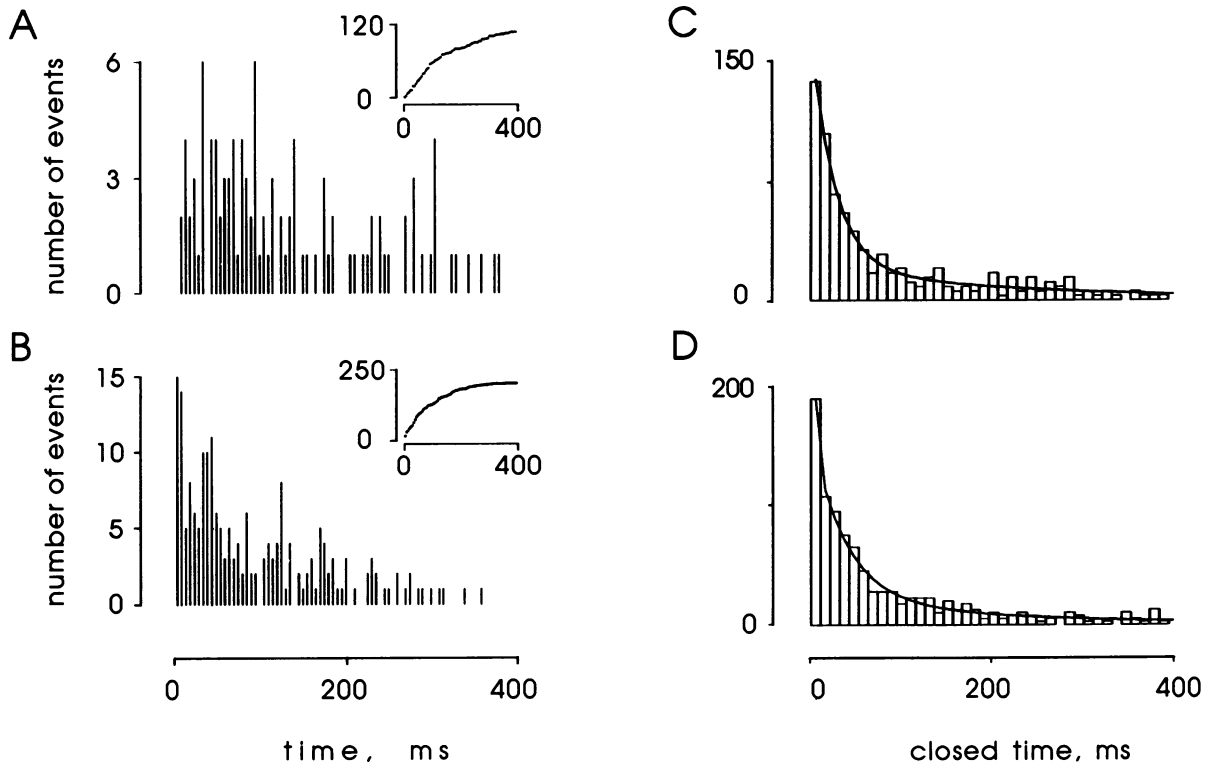

Fig. 3. First latency distributions of currents through single L-type α_1-subunits recorded under standard test pulses (**A**) and under standard test pulses preceded by facilitating prepulses (**B**). The details of pulse protocols are given in the legend to Figure 1, the procedure of determining first latencies is described in Materials and methods. For better visualization, the histograms are also represented as cumulative functions (see insets). Closed time distributions of currents through single L-type α_1-subunits recorded under standard test pulses (**C**) and under standard test pulses preceded by facilitating prepulses (**D**). The smooth curves represent exponential fits of the closed time distributions. Control data are fitted with a biexponential function yielding time constants of 28.2 and 236.3 ms. Under facilitation three exponential curves were required to fit the data. The corresponding time constants amounted to 1.6, 32.5 and 205.6 ms.

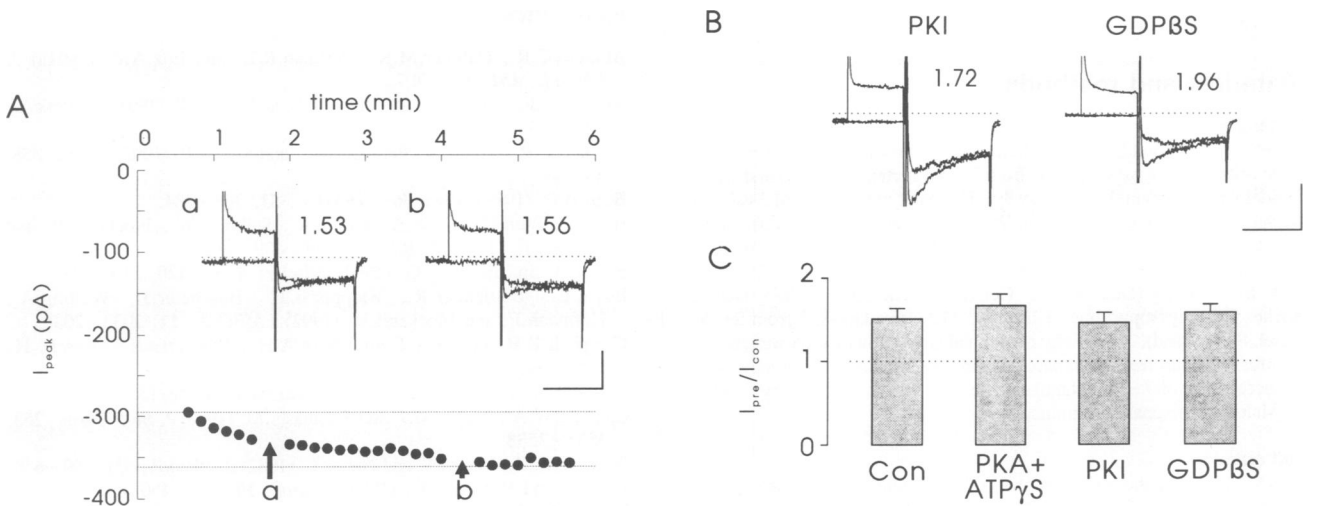

Fig. 4. Effects of phosphorylation and G-proteins on the prepulse-induced facilitation of the whole-cell current through L-type α_1-subunits expressed in CHO cells. Panel **A** illustrates a typical time course of the whole cell peak Ca^{2+} channel current measured at a test potential of +10 mV during intracellular infusion of the catalytic subunit of PKA (7 μM) plus ATPγS (10 mM). At the times indicated by arrows the effect of facilitating prepulses was tested as illustrated by the corresponding insets. The original current traces were recorded using the standard and the double pulse protocol. Voltage-clamp steps to −10 mV were applied from a holding potential of −80 mV. The prepulse potential was +90 mV. Panel **B** illustrates original current traces obtained from cells intracellularly infused with the PKA inhibitor PKI(6-22) amide (5 μM, left), and GDPβS (200 μM, right), respectively. The numbers above the current traces correspond to the determined ratios between facilitated currents and control currents (I_{pre}/I_{con}). The vertical scale bars of panels A and B represent 50 and 100 pA, respectively; the horizontal scale bars represent 50 ms. The diagram in panel **C** summarizes data from all experiments under these conditions. Shown are mean values (±SEM) of I_{pre}/I_{con}. The corresponding experimental conditions are indicated below, Con corresponds to measurements of I_{pre}/I_{con} under control conditions (n = 12), PKA+ATPγS to measurements after intracellular infusion of the catalytic subunit of PKA plus ATPγS (n = 6), PKI to measurements after PKI(6-22) amide (n = 6) and GDPβS to measurements after GDPβS (n = 8). The differences were statistically not significant ($P > 0.05$).

This observation is of interest in respect to a general understanding of how Ca^{2+} channels are modulated. Induction of mode 2 gating has also been reported to underly the β-adrenergic stimulation of the cardiac L-type Ca^{2+} channel, presumably via a cAMP-dependent phosphorylation (Yue *et al.*, 1990), as well as the stimulation by vasopressin of smooth muscle urinary bladder cells (Bonev and Isenberg, 1992). Consequently, it was suggested that the depolarization-induced facilitation is due to a voltage-dependent phosphorylation mimicking the effects of hormonal stimulation of the Ca^{2+} channel. In line with this, Artalejo and coworkers (1992) reported that the phosphatase inhibitor okadaic acid and ATPγS stabilized the facilitation of the Ca^{2+} channel current in chromaffin cells. In skeletal muscle facilitation was shown to be promoted by PKA (Sculptureanu *et al.*, 1993).

We expected that phosphorylation may be also involved in the voltage-dependent facilitation of the L-type current through α_1-subunits solely expressed in CHOCa9 cells. However, our results did not provide any evidence for such a mechanism. Infusion of the catalytic subunit of PKA plus ATPγS, of the PKI(6-22) amide, of phorbol esters and of GDPβS had no effect on facilitation. Therefore, our data suggest that the Ca^{2+} channel facilitation in CHOCa9 cells is primarily due to a voltage-dependent conformational change of the α_1-subunit. Phosphorylation may be required when additional Ca^{2+} channel subunits are coexpressed with the α_1-subunit. Phosphorylation of one of these subunits may then result in a conformational change of the α_1-subunit similar to that induced by strong depolarizing prepulses. Therefore future experiments expressing various subunit compositions of Ca^{2+} channels in CHO cells are necessary to determine the minimum requirement of subunits to reconstitute modulation by facilitation and phosphorylation.

Materials and methods

Materials

Dulbecco's modified Eagle's medium and dialyzed fetal bovine serum and tissue culture dishes were obtained from Gibco (Berlin, Germany) and Falcon (Heidelberg, Germany), respectively. The catalytic subunit of PKA was purchased from Promega (Madison, WI), guanosine 5′-*O*-(2-thiodiphosphate) (GDPβS), adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS), the PKA inhibitor PKI(6-22) amide as well as the phorbol ester, phorbol 12-myristate 13-acetate were from Sigma (Deisenhofen, Germany). The L-type Ca^{2+} channel blocker 1,4-dihydropyridine (+)PN 200-110 was a kind gift from Sandoz (Basel, Switzerland). The activity of the catalytic subunit of PKA was checked by infusing it into freshly prepared murine ventricular cardiomyocytes. At a concentration of 7 μM it stimulated the Ca^{2+} channel current by ~300% (V.Maltsev, personal communication).

Cell culture

cDNA containing the entire protein-coding region of the class C_b Ca^{2+} channel α_1-subunit (Biel *et al.*, 1990) was introduced into the expression vector p91023(B) (Wong *et al.*, 1985) containing the dihydrofolate reductase gene and the plasmid was transfected into CHO cells by electroporation (Bosse *et al.*, 1992). Transfected cells were selected due to their resistance to Dulbecco's modified Eagle's medium supplemented with 10% dialyzed fetal bovine serum (lacking purines, glycine and/or thymidine), streptomycin (30 μg/ml), penicillin (30 units/ml) and non-essential amino acids. For electrophysiological studies cells of the subclone CHOCa9 (Bosse *et al.*, 1992) were cultured on glass slides (5 × 9 mm) at a density of ~10^3/mm^2 for 2–5 days.

Electrophysiology

Ca^{2+} channel currents were recorded in the cell-attached and in the whole cell configuration of the patch-clamp technique (Hamill *et al.*, 1981). For single channel measurements, patch electrodes were filled with a solution containing (in mM): $BaCl_2$ 80, NaCl 50 and HEPES 10 (pH 7.4 at 23°C). The bath solution contained (in mM): potassium aspartate 130, $MgCl_2$ 1, glucose 20, EGTA 10 and HEPES 5 (pH 7.4 with KOH at 23°C). Ca^{2+} channels were activated by 400 ms voltage-clamp steps from a holding potential of −50 mV to a test potential of −10 mV (stimulus frequency 0.1 Hz). For induction of facilitation the test pulse was preceded by a 50 ms depolarizing prepulse to +70 mV. The data were analyzed off-line (sampling rate of 5–10 kHz, eight pole low-pass Bessel filter with a cut-off frequency of 1–2 kHz) using the pClamp 5.5 software (Axon Instruments Inc., Forster City, USA). Only patches with one channel (no visible superimpositions during the full duration of recording) were taken into account for analysis. Data for time distribution analysis were collected from large ensembles of sweeps obtained in eight patches and visualized in histograms (Figures 2 and 3). Events representing tail currents of Ca^{2+} channel openings during the prepulse were excluded from first latency analysis.

For recording of whole-cell Ba^{2+} currents the bath chamber was continuously perfused with a solution containing (in mM): NaCl 125, $BaCl_2$ 10.8, CsCl 5.4, $MgCl_2$ 1.0, glucose 10, HEPES 10 (pH 7.4 at 37°C). The pipette solution contained (in mM): CsCl 120, $MgCl_2$ 3, Mg-ATP 5, EGTA 10, HEPES 5 (pH 7.4 at 37°C) and in some experiments was supplemented with the catalytic subunit of PKA, GDPβs (200 μM), the specific PKA inhibitor PKI(6-22) amide (5 μM), ATPγS (10 mM) or the phorbol ester, phorbol 12-myristate 13-acetate (500 nM). Whole-cell currents were recorded at a test potential of −10 mV, the holding potential being −80 mV. In line with previous reports (Pietrobon and Hess, 1990; Artalejo *et al.*, 1991a,b), Ca^{2+} channel facilitation in CHOCa9 cells became larger with increasing amplitude of depolarizing prepulses. We routinely applied 50 ms prepulses to +90 mV. The significance of effects was tested using the Student's *t*-test; the probability level is given as *P*.

Acknowledgements

We are grateful to I.Reinsch for expert technical assistance and to W.Stamm for skilful engineering. We also thank Drs J.Patlak and G.Schultz for valuable discussion. The study was supported by funds of the Deutsche Forschungsgemeinschaft to J.H., V.F. and F.H.

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Received on November 10, 1993; revised on March 3, 1994

Note added

In a recent publication [Sculptoreanu,A., Rotman,E., Takahashi,M., Scheuer,T. and Catterall,W.A. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 10135–10139], which appeared while this paper was being revised, Sculptoreanu *et al.* described a facilitation of the class C_a α_1 subunit expressed in CHO cells after partial phosphorylation by the catalytic subunit of the PKA. However, using the same voltage-clamp protocol as well as similar external and internal solutions we were not able to reproduce these experiments on our class C_b clone (n = 9).