The β_1-subunit is essential for modulation by protein kinase C of an human and a non-human L-type Ca^{2+} channel

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Received 1 November 1995

Abstract **We have investigated in *Xenopus* oocytes the effects of phorbol ester-induced protein kinase C (PKC) stimulation on dihydropyridine (DHP)-insensitive and -sensitive Ca^{2+} channels. DHP-insensitive Ba^{2+} currents (I_{Ba}) were recorded from endogenous channels in non-injected oocytes and in oocytes injected with cRNAs encoding the auxiliary rabbit α_2/δ and β_1 Ca^{2+} channel subunits. A human α_{1C} cRNA, injected alone or in combination with cRNAs of the auxiliary subunits, was used for studying DHP-sensitive I_{Ba}. We found that DHP-insensitive I_{Ba} was increased by 4β-phorbol 12-myristate 13-acetate (PMA), while DHP-sensitive I_{Ba} was decreased. In both cases, the effects depended only on the co-expression of the β_1 subunit.**

Key words: *Xenopus* oocyte; Calcium channel; Protein kinase C

1. Introduction

Voltage-gated Ca^{2+} channels are heteromeric multisubunit protein complexes [1]. The pore-forming α_1 subunit is regularly associated with β and disulfide-linked α_2/δ subunits, while γ subunit has only been found in skeletal muscle [1,2]. When α_1 is co-expressed with auxiliary β and α_2/δ subunits, voltage dependence of activation, current kinetics and amplitudes are markedly affected [3–5]. Both α_1 and β subunits of the Ca^{2+} channel complex bear many putative phosphorylation sites [2,6] which may be involved in the modulation of Ca^{2+} channels by protein kinases [7,8].

When expressed in *Xenopus* oocytes, DHP-sensitive Ba^{2+} currents (I_{Ba}) through the pore-forming rabbit cardiac α_{1C} subunit are transiently upregulated by the phorbol ester PMA [9] which activates PKC. Similar effects could be seen with the α_{1C} subunit alone or in combination with the auxiliary subunits α_2/δ and/or β [9]. In this study, we have used the *Xenopus* oocyte expression system to control the subunit composition of the Ca^{2+} channel complex and compared the effects of PMA on I_{Ba} through a human and a non-human, endogenous voltage-activated Ca^{2+} channels. While I_{Ba} through human Ca^{2+} channels was decreased by PMA, I_{Ba} through non-human channels was increased. By selectively expressing one or several subunits of the Ca^{2+} channel complexes, we could show that both PMA-induced regulations of I_{Ba} depended only on the co-expression of the β_1 subunit. In addition, we failed to find any modulatory effect on I_{Ba} of PKA activators or of paired-pulse facilitation in the human Ca^{2+} channel.

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2. Materials and methods

2.1. Oocyte preparation and cRNAs microinjection

The experimental procedures were described in detail elsewhere [10]. The nucleotide sequence of the α_{1C} cDNA used in the present work has been deposited to the EMBL Data Bank (accession number Z34815). Mutant Ca^{2+} channel $\alpha_{1C(818T)}$ plasmid pHLCC77f ($G^{2777} \rightarrow A$) was prepared by two-mutagenic primers method [11] using the wild-type α_{1C}-encoding plasmid and 30-mer oligonucleotide carrying the desired mismatch (5′-GACGGAGAGTCTCCACCCACCACCAAGATC-3′). Template DNAs for synthesizing rabbit α_2/δ [4] and β_1 [12–13] cRNAs were kindly provided by F. Hofmann and V. Flockerzi (Münich, Germany). cRNAs were synthesized in vitro using the mCAP™ mRNA Capping Kit (Stratagene, La Jolla, CA). Oocytes were microinjected with cRNAs, single or premixed in equimolar ratios. They were dissolved in 5 mM HEPES (pH 6.8) at a concentration of 0.5 ng/nl (50 nl/oocyte). Oocytes were stored for 4–9 days at 18°C in a sterile Barth's medium containing (in mM) NaCl 88, KCl 1, $MgSO_4$ 0.82, $Ca(NO_3)_2$ 0.33, $CaCl_2$ 0.41, $NaHCO_3$ 2.4, HEPES 10, pH 7.5 (NaOH), supplemented with 100 U penicillin/ml and 100 μg streptomycin/ml (Boehringer Mannheim, Rotkreuz, Switzerland). The medium was changed daily.

2.2. Electrophysiological experiments

Oocytes were enzymatically defolliculated (collagenase type IA, 1.5 mg/ml; trypsin inhibitor, type I-S, 1 mg/ml) 1–3 days before the measurements. Voltage-gated currents were recorded by a two-electrode voltage-clamp setup using a Axoclamp 2-A amplifier (Axon Instruments, Burlingame, CA). Glass pipettes (Clark Electromedical Instruments, UK) were filled with a medium containing 3 M KCl or CsCl and had resistances ranged from 0.2 to 1 MΩ. The external bathing solution contained (in mM): $Ba(OH)_2$ 40, NaOH 50, KOH 1, HEPES 10, pH 7.4 (methanesulfonic acid). Voltage-clamp protocols, Ba^{2+} current (I_{Ba}) records, leak subtraction procedure and analysis were performed using the EPC software (Cambridge Electronics Design, Cambridge, UK) running on a microcomputer (Compaq 286). Membrane currents, filtered at 0.5–1 kHz and sampled at 2–4 kHz, were triggered by 1 s step depolarizations applied from a holding potential (V_h) of −90 mV (frequency of stimulation: 0.033 or 0.05 Hz). In some experiments, oocytes were stimulated by a 100-ms depolarizing pulse. Oocytes were continuously superfused with bathing solution throughout the experiment. Drugs were applied externally by a gravity-driven system perfusing the entire experimental chamber (perfusion rate: ≈10–12 ml/min). All experiments were performed at room temperature (20–22°C).

2.3. Materials

4β-PMA was purchased from Sigma (St Louis, MO), ω-conotoxin GVIA from Bachem Feinchemikalien (Bubendorf, Switzerland), (±)-Bay K 8644 from Research Biochemicals (Natick, MA), 4α-PMA from Paesel (Heidelberg, Germany), forskolin from Fluka Chemie (Buchs, Switzerland) and H-89 from Calbiochem Biochemicals (Lucern, Switzerland). (+)-Isradipine is gift from Dr. R.P. Hof (Sandoz, Basel, Switzerland). Unless otherwise stated, all the chemicals and reagents were purchased from Sigma Chemie (Buchs).

3. Results

3.1. DHP-insensitive and -sensitive Ba^{2+} currents

Oocytes injected only with cRNAs coding for the auxiliary subunits α_2/δ and β_1 from rabbit showed a small I_{Ba} with an

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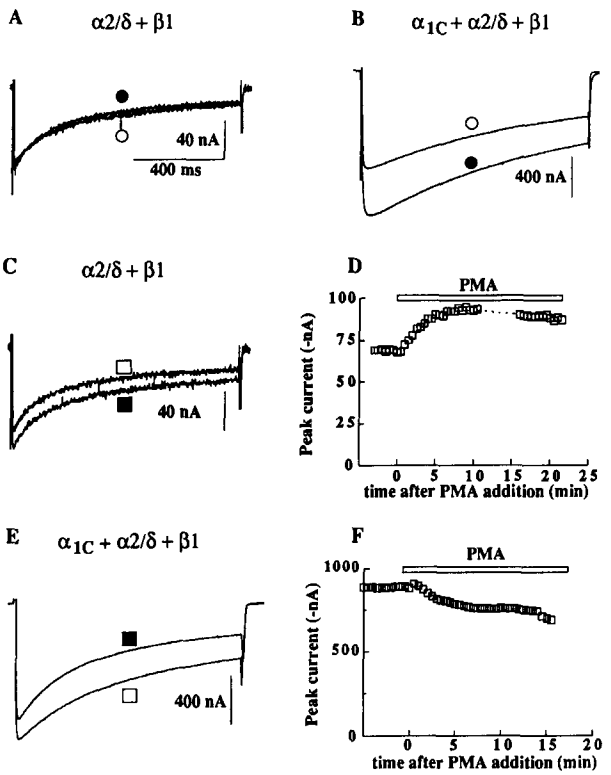

Fig. 1. Effects of the phorbol ester PMA on the non-L-type I_{Ba} and on L-type I_{Ba}. Oocytes were injected with cRNAs coding for the auxiliary α_2/δ and β_1 subunits (A) and or with cRNAs coding for α_{1C}, α_2/δ and β_1 (B). Ba^{2+} currents were elicited by step depolarizations applied from −90 mV to +20 mV for 1s, before (open circles) and after (filled circles) application of 1 μM (A) or 20 nM (B) Bay K 8644. Effects of PMA (10 nM) on the non-L-type (C) and on L-type I_{Ba} (E); voltage clamp records of I_{Ba} obtained before (open squares) and after (filled squares) the addition of PMA are shown. The graphs in D and F show the time courses of changes in amplitudes of the non-L-type I_{Ba} (D) and L-type I_{Ba} (F) during the application of PMA.

average amplitude of 80 ± 10 nA (mean ± S.E.M., n = 29, Fig. 1A) during voltage-clamp pulses to +20 mV from a V_h of −90 mV. Endogenous I_{Ba} was insensitive to the N-type Ca^{2+} channel blocker ω-conotoxin GVIA (not shown) and to DHP antagonists and agonists, such as (+)-isradipine (not shown) and (±)-Bay K 8644 (Fig. 1A), respectively. This DHP-insensitive I_{Ba} will be referred to as a non-L-type I_{Ba} throughout this study.

The injection of all three cRNAs, coding for human α_{1C} and for rabbit α_2/δ and β_1, gave rise to a large I_{Ba} (1290 ± 233 nA, n = 13, Fig. 1B). This current was inhibited by isradipine (not shown) [10] and was increased by (±)-Bay K 8644. It will, therefore, be referred to as an L-type I_{Ba}. Effects of subunit composition on the amplitudes of non-L-type and L-type I_{Ba} are summarized in Table 1.

3.2 Phorbol ester-induced stimulation of Ba^{2+} currents

We have studied the effects of PMA-induced PKC stimulation in oocytes injected with (α_2/δ+β_1) cRNAs and (α_{1C} + α_2/δ + β_1) cRNAs. Fig. 1C shows two representative current traces of the non-L-type I_{Ba} measured in an oocyte injected with (α_2/δ + β_1) cRNAs, before (open square) and 10 min after the addition of PMA (10 nM, filled square). PMA produced an increase in the current amplitude (Fig. 1D) without affecting the voltage dependence of activation of the non-L-type I_{Ba} (not shown). The increase lasted for up to 40 min and reached 126 ± 7% (n = 4, P < 0.01) of the control current when measured 10 min after the application of the phorbol ester. By contrast, PMA (10 nM) inhibited L-type I_{Ba} recorded in oocytes injected with (α_{1C} + α_2/δ + β_1) cRNAs by 22 ± 3% (n = 9, P < 0.05) (Fig. 1E,F), without affecting the voltage dependence of the current (not shown). The inactive analogue of PMA, 4α-PMA (10 nM), had no significant effect on the DHP-sensitive I_{Ba} (increase by 3 ± 1%; n = 3, P > 0.05). Thus, the observed inhibition of L-type I_{Ba} by PMA is most likely due to stimulation of PKC-mediated phosphorylation.

We have tried to find out whether the effects described above are due to a single subunit. Fig. 2 shows traces of the DHP-insensitive current measured in non-injected (A), (α_2/δ)-injected (B) and β_1-injected oocytes (E), before (open squares) and after (filled squares) PMA treatment. PMA caused an upregulation of the non-L-type I_{Ba} only in β_1-injected oocytes by 26 ± 5% (n = 4, P < 0.05) (Fig. 2E), a value similar to the one measured in (α_2/δ + β_1)-injected oocytes (see above). When L-type I_{Ba} was measured in α_{1C}- and in (α_{1C} + α_2/δ)-injected oocytes, it was PMA-insensitive (Fig. 2B,D). Bay K 8644 was applied at the end of each experiment in order to confirm the DHP sensitivity of the current (filled circles). However, when PMA (10 nM) was applied to (α_{1C} + β_1)-injected oocytes, L-type I_{Ba} was inhibited by 37 ± 3% (n = 4, P < 0.01) (Fig. 2F). Our results show that both upregulation of non-L-type I_{Ba} and downregulation of L-type I_{Ba} by PMA depend on the expression of the β_1 subunit.

Our results stand in contrast with a previous report [9]. In that study, I_{Ba} through cloned rabbit cardiac α_{1C} subunits expressed in *Xenopus* oocytes were transiently upregulated by

Table 1
Effects of the auxiliary subunits on the non-L-type I_{Ba} and L-type I_{Ba}

DHP-insensitive I_{Ba} (1)		DHP-sensitive I_{Ba} (2)	
cRNAs injected	Peak current (nA)	cRNAs injected	Peak current (nA)
Non-injected	14 ± 2, n = 21	α_{1C}	56 ± 11, n = 17
α_2/δ	27 ± 2, n = 6	α_{1C} + α_2/δ	18 ± 3, n = 3
β_1	37 ± 10, n = 11	α_{1C} + β_1	606 ± 62, n = 6
α_2/δ + β_1	80 ± 10, n = 29	α_{1C} + α_2/δ + β_1	1290 ± 233, n = 13
		$\alpha_{1C,A818T}$	46 ± 6, n = 3
		$a_{1C,A818T}$ + α_2/δ + β_1	1185 ± 144, n = 8

Values are given as mean±S.E.M., with n, the number of oocytes tested. (1) The DHP-insensitive I_{Ba} activated at potentials more positive than −20 mV and peaked at +10/+20 mV. (2) The DHP-sensitive I_{Ba} measured in α_{1C}- and in (α_{1C} + α_2/δ)-injected oocytes activated at −20/−10 mV and peaked at +20/+30 mV. When measured in (α_{1C} + β_1)- and in (α_{1C} + α_2/δ + β_1)-injected oocytes, L-type I_{Ba} activated at −30/−20 mV and reached maximum amplitudes at +10/+20 mV.

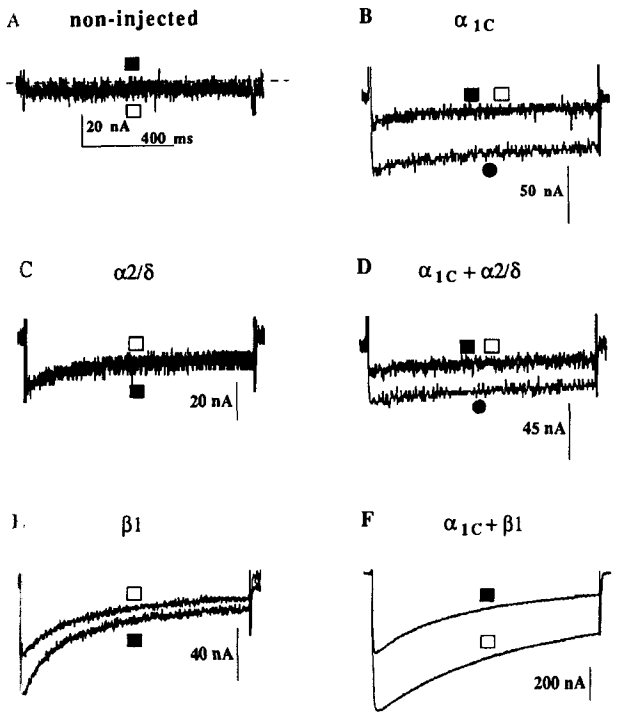

Fig. 2. PMA-induced effects on non-L-type and L-type I_{Ba} depend on the subunit composition. DHP-insensitive currents (A,C,E) and DHP-sensitive currents (B,D,F) were recorded in non-injected oocytes (A) or in oocytes injected with cRNAs coding for α_{1C} (B), α_2/δ (C), $\alpha_{1C} + \alpha_2/\delta$ (D), β_1 (E) or $\alpha_{1C} + \beta_1$ (F). Ba^{2+} currents were recorded before (open squares) and 10 min after (filled squares) the application of 10 nM PMA. In some experiments, Bay K 8644 (20 nM, filled circles) was added at the end of the experiment to confirm the DHP sensitivity of the current.

PMA. Two out of 18 potential phosphorylation sites (Thr^{27} and Thr^{848}) of the rabbit α_{1C} subunit [3] are absent from the human α_{1C} subunit [14]. Thr^{27} is absent due to a different exon encoding the N-terminus of the human α_{1C} and can not be restored by mutation. Therefore, we have prepared a mutant of the human α_{1C} $\alpha_{1C,A818T}$, where Ala was replaced by Thr in position 818, the site equivalent to the rabbit Thr^{848}. After expression in *Xenopus* oocytes, I_{Ba} measured in $\alpha_{1C,A818T}$ and in ($\alpha_{1C,A818T} + \alpha_2/\delta + \beta_1$)-injected oocytes had amplitudes of 46 ± 6 nA ($n = 3$) and 1185 ± 144 nA ($n = 8$, Table 1), respectively. Kinetics and voltage-dependence of activation and inactivation as well as DHP sensitivity of the wild-type and mutant Ca^{2+} channel were identical (not shown). PMA (10 nM) caused a reduction of I_{Ba} in ($\alpha_{1C,A818T} + \alpha_2/\delta + \beta_1$)- injected oocytes by 25 ± 5% ($n = 4$, $P < 0.05$). Similar to oocytes injected with α_{1C} only, I_{Ba} in oocytes injected with $\alpha_{1C,A818T}$ remained insensitive towards PMA ($n = 3$). Thus, the restored PKC phosphorylation site at Thr^{818}, which is equivalent to Thr^{848} of the rabbit α_{1C}, is not involved in the PKC-dependent regulation of L-type α_{1C} Ca^{2+} channels.

3.3. Human L-type Ca^{2+} channel is not modulated by cAMP-dependent protein kinase

cAMP-dependent protein kinase (PKA) activators, like forskolin (1–3 μM, $n = 3$) and 8-Br-cAMP (50–250 μM, $n = 4$), did not change I_{Ba} in α_{1C}- and in ($\alpha_{1C} + \alpha_2/\delta + \beta_1$)-injected ocytes. To check for a constant phosphorylation of the expressed channels [2] we applied H-89, a potent PKA inhibitor. It downregulates L-type α_{1C} I_{Ba} in a time-dependent manner with an IC_{50} of 76 nM [15]. ($\alpha_{1C} + \alpha_2/\delta + \beta_1$)-injected oocytes were repeatedly voltage-clamped from a V_h −90 mV to +20 mV for 1 s until a steady-state was established. H-89 (1–3 μM) was then added and continuously superfused throughout the experiment. After 10 min, I_{Ba} was reduced by only 3–5% ($n = 3$).

3.4. Human L-type Ca^{2+} channel does not show double-pulse facilitation

We have also looked for double-pulse facilitation [16,17] of L-type I_{Ba}. Oocytes injected with ($\alpha_{1C} + \alpha_2/\delta + \beta_1$)-cRNAs were stimulated by test pulses applied from −90 mV to +20 mV for 300 ms, with or without a 500-ms depolarizing prepulse to +60 or +110 mV. The prepulse-test pulse interval was 100 ms. When preceded by a prepulse, the current elicited during the test pulse was smaller than the current measured without prepulse. With depolarizing prepulses to +60 or +110 mV L-type I_{Ba} was reduced by 10 ± 1% ($n = 4$) and 13 ± 1% ($n = 4$), respectively. Increasing the prepulse duration to 800 ms further decreases the test pulse current by 16 ± 2% ($n = 4$) and 17 ± 1% ($n = 4$). Similar experiments carried out in the presence of H-89 (1–3 μM) did not shown current facilitation either. Thus, I_{Ba} facilitation observed by others in rabbit L-type Ca^{2+} channels [16,17] could not be seen in human DHP-sensitive channels.

4. Discussion

The results of this study show that human L-type α_{1C} Ca^{2+} channels have a number of properties different from non-human L-type α_{1C} Ca^{2+} channels reported by others [9,16,17].

When only the human α_{1C} subunit is expressed in *Xenopus* oocytes, PKC stimulation does not affect L-type I_{Ba}. By contrast, when the rabbit α_{1C} subunit is expressed alone, PKC stimulation modulates I_{Ba} in a biphasic manner by initially increasing and subsequently reducing it [9]. Co-expression of the β_1 subunit inhibits the PMA-induced enhancement of rabbit I_{Ba} by 50%, but leaves the reduction of I_{Ba} unaffected [9]. This contrasts with our results were the β_1 subunit is essential for reduction of I_{Ba} through human α_{1C}. There are 16 putative phosphorylation sites which are common for human [14] and rabbit [3] cardiac α_{1C} subunits. Two additional PKC-sensitive phosphorylation sites exist in the rabbit α_{1C} subunit at positions 27 and 848. We hypothesized that these sites, either one or both, determine the different sensitivities to PKC activation between human and rabbit Ca^{2+} channels. The former site (Thr^{27}) is located in the N-terminal end encoded by an exon not found in the human α_{1C} gene [18] and, hence, it can not be mutated. The second additional phosphorylation site of the rabbit α_{1C} (Thr^{848}) is located in the cytoplasmic loop connecting domains IIS6–IIIS1. We have restored this site by mutation and found that the sensitivity towards PKC activation of I_{Ba} through the $\alpha_{1C,A818T}$ mutant was not changed when compared with the wild-type α_{1C}. Thus, it is tempting to speculate that it is the N-terminal phosphorylation site at Thr^{27} that may determine the difference in responses to PKC activation between human and rabbit L-type Ca^{2+} channels. However, so far there is no independent evidence for this notion.

In our experiments, two different types of Ca^{2+} channels are modulated by PKC in opposite ways. The endogenous Ca^{2+} channel in *Xenopus* oocytes, which is upregulated by injection

of the auxiliary subunits α_2/δ and β_1 from rabbit, has not been defined in its molecular structure. However, both the endogenous channel and the human α_{1C} require the expression of the β_1 subunit for the respective modulatory responses to PKC activation. However, β_1 is the main functional target for phosphorylation by this enzyme. Moreover, in agreement with the results by others [19–21], co-expression of the β_1 subunit with an α_{1C} and α_2/δ shifted the activation range of I_{Ba} towards more negative potentials (Table 1, caption).

Although upregulation by PKA of Ca^{2+} or Ba^{2+} currents through L-type Ca^{2+} channels is a prominent feature of this channel type in cardiac cells [7,8], we could not find any effect of PKA activators on the channel expressed in *Xenopus* oocytes. This agrees with recent results by others [22] who showed that expression of L-type Ca^{2+} channels in CHO or HEK cells were not sensitive to PKA. Again in agreement with our results, but in contrast to others [16,17], these authors could not observe double-pulse facilitation in artificially expressed L-type Ca^{2+} channels. The reasons for these discrepancies are not clear.

In conclusion, we have shown that the effects of PKC stimulation on non-L-type and L-type I_{Ba} critically depend on the presence of the cytoplasmic β_1 subunit of the Ca^{2+} channel complex. In addition, rabbit cardiac and human α_{1C} Ca^{2+} channels are differentially modulated by PKC agonists. Furthermore, neither PKA stimulation nor double-pulse facilitation affect human L-type Ca^{2+} channels expressed in *Xenopus* oocytes. Thus, our results demonstrate that in spite of very high amino acid homology with human α_{1C}, important functional properties of non-human α_{1C} subunits can not be extrapolated to the human channel.

Acknowledgements: We thank F. Hofmann and V. Flockerzi (Münich, Germany) for a gift of clones of β_1 and α_2/δ subunits. This work was supported by Swiss National Science Foundation Grant 31–29862.90.

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