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Modulation of cardiac Ca^{2+} channels in *Xenopus* oocytes by protein kinase C

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L-Type calcium channel was expressed in *Xenopus laevis* oocytes injected with RNAs coding for different cardiac Ca^{2+} channel subunits, or with total heart RNA. The effects of activation of protein kinase C (PKC) by the phorbol ester PMA (4β-phorbol 12-myristate 13-acetate) were studied. Currents through channels composed of the main (α_1) subunit alone were initially increased and then decreased by PMA. A similar biphasic modulation was observed when the α_1 subunit was expressed in combination with α_2/δ, β and/or γ subunits, and when the channels were expressed following injection of total rat heart RNA. No effects on the voltage dependence of activation were observed. The effects of PMA were blocked by staurosporine, a protein kinase inhibitor. β subunit moderated the enhancement caused by PMA. We conclude that both enhancement and inhibition of cardiac L-type Ca^{2+} currents by PKC are mediated via an effect on the α_1 subunit, while the β subunit may play a mild modulatory role.

Calcium channel; Calcium channel subunit; Protein kinase C; Phorbol ester; *Xenopus* oocyte

1. INTRODUCTION

The dihydropyridine (DHP)-sensitive L-type Ca^{2+} channel carries a major part of Ca^{2+} current in skeletal, smooth and cardiac muscle [1,2]. This channel is one of the main targets of the various physiological modulators in these tissues [1,3–5]. The cardiac Ca^{2+} current is modulated by the protein kinase C (PKC) cascade, which may mediate the physiological effects of angiotensin II and α1-adrenergic agonists in the heart [6,7]. The effect of phorbol esters (PKC activators) such as β-phorbol 12-myristate 13-acetate (PMA) on cardiac L-channel is biphasic: the Ca^{2+} current is initially enhanced but, after several minutes, inhibition of Ca^{2+} current follows [7,8].

The L-type Ca^{2+} channel from skeletal muscle (SKM) consists of 5 subunits [5,9,10]: α_1 (165 kDa); α_2/δ (130/28 kDa), β (55 kDa), and γ (32 kDa). Identical or homologous proteins, except γ, are found in the heart (α_1, α_2/δ, β) [11–18]. The α_1 subunit is the main, pore-forming subunit [5,19,20–23]. It produces functional Ca^{2+} channels when expressed in various expression systems such as oocytes and cell lines [11,24–27]. The roles of other ('auxiliary') subunits are less well understood. Studies in RNA-injected *Xenopus* oocytes have demonstrated that α_2/δ and SKM β (β1) or cardiac β subunits (β2a, β2b, β3) enhance the expressed Ca^{2+} current amplitude [11,24,25,28–33]. The auxiliary subunits also modulate activation and inactivation kinetics and voltage sensitivity of Ca^{2+} channels in expression systems [29–31].

The phosphorylation of which subunit mediates the physiological effect of PKC activators is not clear. The biochemistry of in vitro phosphorylation of L-channel is documented only in skeletal muscle (SKM), where a membrane-associated form of α_1 subunit is efficiently phosphorylated by PKC [34,35]. The β subunit is rich in putative PKC phosphorylation sites [13] and is a substrate for PKC phosphorylation [34–36]. Thus, phosphorylation of either α_1 or β could account for the physiological effect of PKC on cardiac Ca^{2+} current, and it is even theoretically possible that the biphasic effect of PKC activators might be due to PKC action on different subunits.

The present study examines the roles of Ca^{2+} channel subunits in determining the effects of a PKC activator, PMA, on cardiac L-type Ca^{2+} channels expressed in *Xenopus* oocytes. We find that a channel composed of cardiac α_1 subunit alone is modulated by PMA in a biphasic manner (increase followed by a decrease), like in the heart, and that the auxiliary subunits may at most exert moderate modulatory effects.

2. MATERIALS AND METHODS

Frogs were maintained and dissected as described [39,40]. The frogs were dissected under MS-222 anesthesia. Pieces of ovarian lobes were removed via small incisions on the belly; the latter was sutured, and

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the animals were returned to the tanks after recovering from anesthesia (see [40] for details). Oocytes were defolliculated by a 2- to 3-h treatment with 1.5–2 mg/ml collagenase (Type 1A, Sigma) in Ca-free ND96 solution (in mM): 96 NaCl, 2 KCl, 1 $MgCl_2$, 5 HEPES/NaOH, pH 7.5. Clones of rabbit cardiac α_1 [16] and β2a [28] and of SKM α_2/δ, β1 and γ subunits [13,15,29] were used to synthesize cRNAs in vitro and then the cRNA was injected into *Xenopus* oocytes. Equal doses (5 ng/oocyte) of each subunit were injected in different combinations in a total volume of water of 50 nl. Total RNA was extracted from rat (7 days old) hearts using LiCl procedure as described [40] (rats were anesthetized with ether and sacrificed by decapitation). After 2–4 days of incubation at 22°C in ND96 containing 1.8 mM $CaCl_2$ and antibiotics [40], one day before testing the currents, the oocytes were injected with 200 or 400 pmol ethyleneglycerol-bis-(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA; Na salt, pH 7) using standard injection techniques [40]. The currents through the channels were measured using the two-electrode voltage-clamp technique, routinely in a solution containing (in mM): 40 $BaCl_2$, 2 KCl, 60 NaCl, 5 HEPES/NaOH, pH 7.5. When the currents exceeded 2 μA in the high-Ba^{2+} solution, 2 mM $BaCl_2$ solution was used (Ca-free ND96 with the addition of 2 mM $BaCl_2$). Currents in oocytes injected with rat heart RNA were measured in the Ba/NMDG solution (in mM: 40 Ba^{2+}, 60 NMDG, 2 K^+, 5 HEPES/NaOH, pH 7.5) in which Na^+ was substituted by *N*-methyl-D-glucamine (NMDG) and the only anion was methanesulfonate [41]. The two-electrode voltage-clamp data analysis and subtraction procedures were as described [29,42]. After setting the membrane potential at the holding level (−80 mV), stabilization of the Ba^{2+} current (I_{Ba}) was verified by constant monitoring of the current for 10–40 min (I_{Ba} often grew for up to 30 min until reaching a constant value; see [29]). In order to overcome problems of Ba^{2+}-dependent decrease in I_{Ba} amplitude observed in cells with large currents upon frequent repetitive depolarizations, interpulse intervals that allowed a full recovery of I_{Ba} (30–60 s, depending on current amplitude) were used [29]. The membrane capacitance was measured as the integral of the capacitative current evoked by a voltage step from −80 to −70 mV.

4β-phorbol 12-myristate 13-acetate (PMA) and staurosporine were from Sigma, 4α-phorbol 12-myristate 13-acetate (α-PMA) was from LC Services. These substances were dissolved at 10^{-2} M in dimethylsulfoxide (DMSO) and stored in light-protected vials at −20°C. Final concentration was prepared shortly before each experiment.

3. RESULTS

Oocytes injected with RNA of the cardiac α_1 subunit alone or in combination with a mixture of α_2/δ, β, and γ subunit RNA displayed Ba^{2+} currents (Fig. 1) which we have previously shown to be dihydropyridine-sensitive [29]. Oocytes injected with a mixture of α_2/δ and β1 (SKM) or β2a (cardiac) subunit RNA, without the cardiac α_1 subunit, expressed quite large currents (20–100 nA with β1 and 150–300 nA with β2a in a 40 mM Ba^{2+} solution; Fig. 1B). These currents were unaffected by the dihydropyridine agonist (−) Bay K 8644 (1 μM) and the antagonist isradipine (10 μM) (data not shown; cf. [29]) and most probably resulted from the association of the newly synthesized α_2/δ and β subunits with a native DHP-insensitive α_1 subunit already existing in the oocyte [29,33]. In oocytes injected with subunit combinations containing cardiac α_1 I_{Ba} was enhanced by (−) Bay K 8644 and blocked by 80–95% by 10 μM isradipine (data not shown). Therefore, the contribution of the oocyte's native (DHP-insensitive; see [41–45]) current in these cases was minor or negligible. In most oocytes

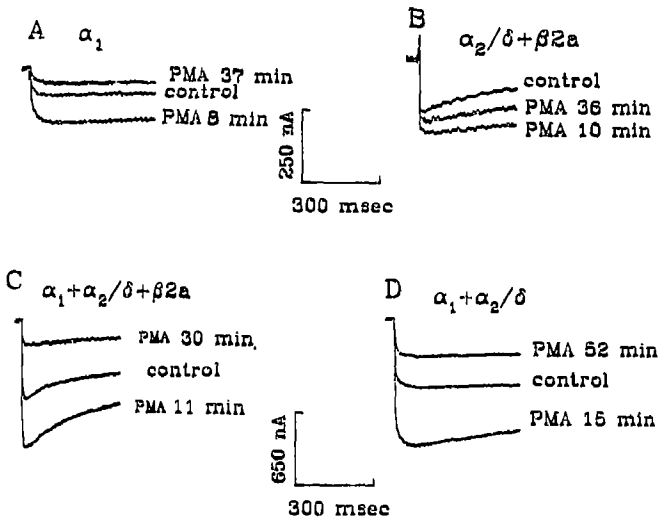

Fig. 1. Examples of I_{Ba} obtained in a 40 mM $BaCl_2$ solution by steps from −100 mV to 0 mV before and after PMA (10 nM) application in oocytes of different groups (subunit combinations) as indicated. During the experiment the holding potential was −80 mV. Net I_{Ba} was obtained by Cd-subtraction procedure (subtraction of currents recorded after addition of 400 μM $CdCl_2$ to the recording solution; see [29].

injected with α_1 subunit RNA alone, I_{Ba} was rather small (5–40 nA), and the experiments were performed in the presence of 0.5 μM (−) Bay K 8644.

Fig. 1 shows typical Ba^{2+} currents in oocytes injected with four different Ca^{2+} channel subunit combinations ('groups'), and illustrates the effect of PMA. The kinetics of the currents were not considerably affected by PMA, whereas the amplitude was dramatically changed. The time course of PMA effect in different groups is exemplified in Fig. 2. In 64 (out of 65 tested) oocytes of all groups, PMA (10 nM) caused a transient increase in I_{Ba}. The current amplitude began to increase 1–2 min after PMA application (or, in a few cells, after a slight decrease that lasted for 1–2 min), and reached a maximum after 7–12 min. None of the auxiliary subunits appeared to play an exclusive role in determining the time course of the enhancement caused by PMA (summarized in Table I). The extent and duration of the increase varied among oocytes of each group. Altogether, I_{Ba} was increased by 131% in the α_1 group and by 142% in the $\alpha_1+\alpha_2/\delta$ group (Table I). This is significantly stronger than in the groups that differ by the addition of the β subunit. Thus, in the $\alpha_1+\beta$1 group the current was increased by 58% ($P<0.02$ compared with α_1); in the $\alpha_1+\alpha_2/\delta+\beta$1 and $\alpha_1+\alpha_2/\delta+\beta$2a groups the current was increased by 58% and 52%, respectively (in both cases $P<0.02$ compared with $\alpha_1+\alpha_2/\delta$; Table I). Thus, the β subunit appears to restrain the enhancing effect of PMA.

Typically, in cardiac α_1-containing groups, the current started to decrease shortly after reaching a maximum and by 30 min after PMA application decreased

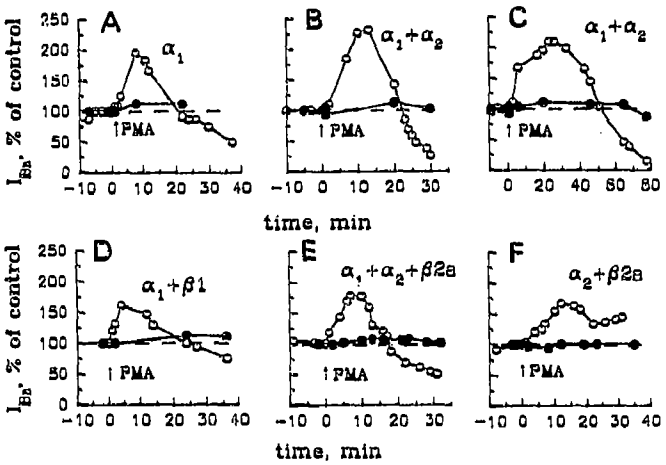

Fig. 2. Examples of the effect of PMA (10 nM) on I_{Ba} and the membrane capacitance as a function of time. The I_{Ba} (open circles) and capacitance (closed circles) values are shown in percents of the control values (the basal current amplitude and the basal membrane capacitance, before PMA application). 0 min indicates the PMA application time. α_2 in this figure stands for α_2/δ.

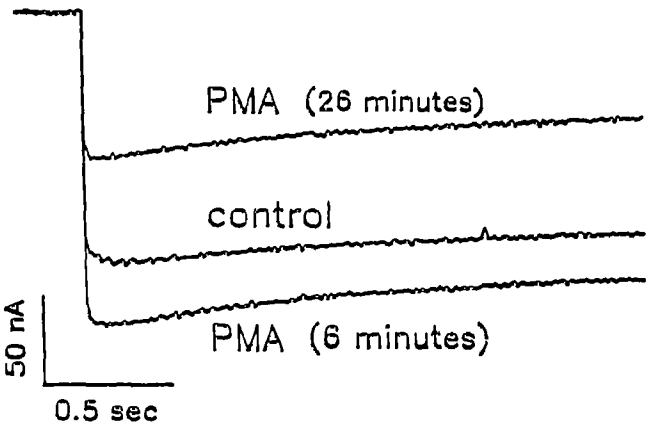

Fig. 3. The effect of PMA (50 nM) on I_{Ba} expressed in oocyte injected with rat heart RNA. Net I_{Ba} was obtained by Cd-subtraction procedure in 40 mM Ba-methanesulfonate solution (see section 2). The holding potential was −50 mV and the current was elicited by steps from −100 mV to 0 mV

below the control level (Fig. 2A,B,D,E). However, in some cells the decrease was delayed, as exemplified in Fig. 2C. In order to verify that the decrease in I_{Ba} by PMA was not a result of an accompanying reduction in the membrane surface area [46], the membrane capacitance was monitored along with the development of PMA effect on I_{Ba}. The capacitance was unchanged or slightly increased (after 10 min in PMA, it was 105±2% of control, n=56; after 30 min in PMA, it still was 105±2% of control, n=39; see Fig. 2), and only later returned to the control level or dropped below it (e.g. Fig. 2C). In most oocytes, even 50 min of PMA applications did not change the membrane capacitance by more than 10% below the control level (cf. [47]). Oocytes in which the capacitance decreased by more than 10% after 30 min were discarded.

Quantification of the inhibitory effect of PMA was done by comparing I_{Ba} 30 min after PMA application in oocytes of different groups, as summarized in Table I (cells with delayed decrease were summarized separately from the 'typical' cells in which the current was decreased below the control level by 30 min). The extent of PMA-induced decrease was similar in all α_1-containing groups except those of the $\alpha_1+\alpha_2/\delta+\gamma$ group (Table I).

PMA caused a similar biphasic effect in oocytes injected with rat heart RNA: a transient increase in I_{Ba} which was followed by a sustained decrease (Fig. 3; n=4). Another phorbol ester, 4β-phorbol dibutyrate, have been previously reported to cause similar effects in heart RNA-injected oocytes [48].

In contrast to groups that contained cardiac α_1, in oocytes that did not contain this subunit (groups $\alpha_2/\delta+\beta1$ and $\alpha_2/\delta+\beta2a$), even a sustained PMA application did not cause any decrease in I_{Ba} below the control level for up to 40 min (Figs. 1B and 2F, and Table I). In addition, we noticed a slowing of the rate of decay of

Table I

Effects of 10 nM PMA on I_{Ba} in oocytes injected with various Ca^{2+} channel subunit combinations (groups)

Group	Maximal increase in I_{Ba} (% above control)	Time to maximal increase (min)	I_{Ba} 30 min after PMA (% of control)	
			Typical cells	Cells with delayed decrease
α_1	131±26 (11.6)	11±1 (11.6)	71± 8 (6.3)	169± 10 (4.3)
$\alpha_1+\alpha_2/\delta$	142±31 (14.6)	13±1 (14.6)	74± 6 (9.5)	109±204 (2.2)
$\alpha_1+\beta1$	58± 6 (6.2)	7±1 (6.2)	65±13 (4.1)	120 (1.1)
$\alpha_1+\alpha_2/\delta+\beta1$	58±10 (18.4)	11±1 (7.4)	56± 6 (7.5)	149± 11 (5.1)
$\alpha_1+\alpha_2/\delta+\beta2a$	52±14 (7.2)	9±1 (7.2)	56± 2 (5)	—
$\alpha_1+\alpha_2/\delta+\gamma$	143±34 (8.3)	11±1 (8.3)	88±13 (6.2)	143± 12 (4.2)
$\alpha_2+\beta1$	100±12 (4.1)	8±1 (4.1)	145± 2 (3.1)	—
$\alpha_2+\beta2a$	55±11 (4.2)	9±1 (4.2)	125± 7 (3.1)	—

The entries are mean ± S.E.M. (number of oocytes, number of frogs). The currents were evoked by steps from −100 to 0 mV. Net I_{Ba} was estimated by a leak subtraction procedure: leak current evoked by a step from −100 to −50 mV was scaled up and subtracted from the total current measured at 0 mV. In most cells of the α_1 group, the experiments were performed in the presence of 0.5 μM (−) Bay K 8644.

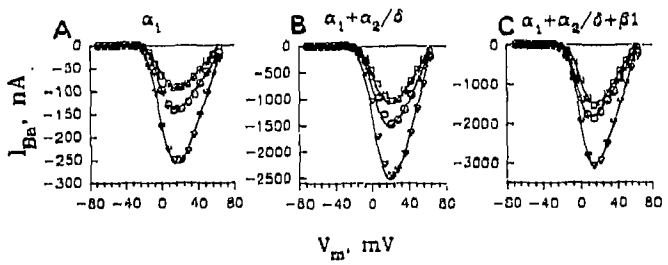

Fig. 4. Current–voltage curves in the presence and absence of PMA. Net I_{Ba} evoked by depolarizing pulses to different voltages were measured by the Cd-subtraction procedure as explained in legend to Fig. 1. The data was fitted to a modified Boltzmann equation as described by Dascal and Lotan [47], assuming two activating gates:

$$I=G_{max}(V_m-V_r)/(1+\exp((V_a-V_m)/K_a))^2,$$

where V_m is the membrane voltage, and G_{max}, V_a, K_a and V_r are as explained in the text. Circles show the control curve (before PMA application); triangles, during the enhancement phase of the current (shortly after PMA application); squares, during the inhibitory phase of the PMA effect. In A, the parameters V_r (mV), G_{max} (μS), V_a (mV) and K_a (mV) were 69, 3.1, −6.7, 8.7 in control; 67, 6.2, −6, 9.7 during the enhancement phase; 63, 2.7, −5, 11 during the inhibition phase. In B, the parameters were 66, 41, 1.2, 10 in control; 65, 70, 1.1, 10.3 during the enhancement phase; 65, 31, 1, 10.6 during the inhibition phase. In C, the parameters were 64, 41, −7.3, 7.2 in control; 63, 73, −4.9, 7.5 during the enhancement phase; 60, 41, −3.9, 7.9 during the inhibition phase.

I_{Ba} by PMA in these groups (e.g. Fig. 1), but this phenomenon was not analyzed further.

To examine the effect of PMA on the voltage dependence of Ca^{2+} channel activation, current–voltage (I–V) curves obtained in individual oocytes were fitted to the Boltzmann equation (Fig. 4) as described previously [41] in control and during the PMA-induced increase and then decrease of I_{Ba}. The description of the activation in terms of the Boltzmann equation was a convenient way to compare the properties of channels before and after PMA application, without bearing on any specific gating mechanism (see [49]). PMA did not cause any changes in the V_r (reversal potential), V_a (half-activation voltage) or K_a (the slope factor, inversely related to the steepness of the activation curve). The only parameter that was changed upon PMA application was G_{max} (maximal macroscopic Ba^{2+} conductance) (Table II).

Table II

Parameters of voltage dependence of activation of I_{Ba} in groups α_1 and $\alpha_1+\alpha_2/\delta$ in the absence and presence of PMA

Group and conditions	V_r	G_{max}	V_a	K_a	n
$\alpha_1+\alpha_2/\delta$ control	65±3	27±4	0.5±2	9±1	7
$\alpha_1+\alpha_2/\delta$-enhancement phase	65±2	50±8	0.9±1	10±1	6
$\alpha_1+\alpha_2/\delta$-inhibitory phase	62±1	17±3	0.9±2	10±1	7
α_1 control	58±3	4±0.2	−3±1	10±1	3
α_1-enhancement phase	60±5	6±0.4	−4±1	10±1	3
α_1-inhibitory phase	56±5	3±0.1	−2±2	11±1	3

The entries are mean ± S.E.M. (number of oocytes). V_r, G_{max}, V_a and K_a are as explained in the text.

Two types of control experiments have been undertaken in order to assure that PMA action on Ba^{2+} currents was via PKC activation. (i) PMA action on I_{Ba} in $\alpha_1+\alpha_2/\delta$ and $\alpha_1+\alpha_2/\delta+\beta1$ groups was tested in the presence of staurosporine (1 μM), a potent protein kinase inhibitor. All measurements were done during a two-day experiment in oocytes of the same frog, in order to avoid the frog-to-frog variability (Table III). Staurosporine attenuated the PMA-induced increase in I_{Ba} by 65% in both $\alpha_1+\alpha_2/\delta$ and $\alpha_1+\alpha_2/\delta+\beta1$ groups (Table III; $P<0.05$), and also weakened by 82% the decrease measured 30 min after PMA application (tested only in the $\alpha_1+\alpha_2/\delta+\beta1$ group) (Table III; $P<0.05$). We note, however, that even in the presence of staurosporine, after 30 min at which the measurements were taken, I_{Ba} continued to decrease at a slower rate in the following 20–30 min, so that the main effect of staurosporine actually was a slowing down of the inhibitory effect of PMA. (ii) 4α-PMA (10 nM), an inactive PMA isomer that does not activate PKC, did not alter I_{Ba} in $\alpha_1+\alpha_2/\delta$ and $\alpha_1+\alpha_2/\delta+\beta1$ groups (Table III).

4. DISCUSSION

In this study we addressed two questions: (i) whether it is possible to study modulation of cardiac Ca^{2+} channels by PKC in *Xenopus* oocytes; (ii) which of the cardiac L-type Ca^{2+} channel subunits is the target for PKC action. The answer to the first question is positive: the cardiac Ca^{2+} channel expressed in the oocytes is modulated by the phorbol ester PMA in the same way as in single canine ventricular and Purkinje cells [7] and in primary cultures of neonatal rat ventricular myocytes [8]. Modulation by PMA was similar in Ca^{2+} channels expressed after the injection of either total heart RNA or the cRNA mixtures of the different channel subunits.

Table III

Verification of specificity of PMA affects with staurosporine and α-PMA

Group	Treatment	I_{Ba} during maximal enhancement (% of control)	I_{Ba} 30 min after PMA application (% of control)
$\alpha_1+\alpha_2/\delta$	PMA	262±41 (4)	
	PMA+staurosporine	158± 9 (4)	
	α-PMA	105± 3 (4)	104+ 5 (4)
$\alpha_1+\alpha_2/\delta+\beta1$	PMA	181±11 (6)	49±12 (3)
	PMA+staurosporine	128±16 (3)	91± 5 (3)
	α-PMA	108±13 (6)	98± 2 (4)

The entries are mean ± S.E.M. (number of oocytes). The effects of staurosporine were tested in oocytes of the same frog and in the same experiment as the control current, to avoid frog-to-frog variability. The effects of α-PMA were tested in a separate experiment.

In both cases PMA caused an increase and then a decrease of the DHP-sensitive current. These effects could not be mimicked by inactive isomer of PMA, α-PMA, and were substantially blocked by a PKC inhibitor, staurosporine. These results suggest that the oocyte can be used for further studies of the molecular mechanisms of Ca^{2+} channel modulation by PKC.

The answer to the second question was obtained in experiments in which channels composed of various subunit combinations were challenged with PMA. The biphasic effect of PMA was observed in oocytes injected with cardiac α_1 alone, and this suggests that the α_1 subunit is the main target of PKC action in the Ca^{2+} channel. This is the main finding of this study. It is not clear whether the effect of PMA is due to a direct phosphorylation of α_1 or via an additional protein. This should be clarified in further studies aimed at finding the exact phosphorylation site or sites of PKC.

The presence of the other auxiliary subunits of the Ca^{2+} channel hardly altered the PMA effect: α_2/δ subunits did not influence it at all, whereas the β subunit reduced the extent of Ba^{2+} current potentiation caused by PMA. Thus, the β subunit may play a modulatory role. A simple scenario may be that β is phosphorylated at one or more of the many putative PKC phosphorylation sites [13,28], and the resulting conformational change affects (reduces) its coupling to α_1, or causes in turn a conformational change in α_1 followed by an alternation in the channel function. Another possible mechanism for such modulation may be a simple competition between α_1 and β for the available PKC molecules, resulting in less phosphorylation of α_1.

The presence of the γ subunit attenuated the inhibitory effect of PMA, suggesting that this subunit may also have a modulatory role. However, since γ subunit has not been found [14,15] in the cardiac Ca^{2+} channel, it is difficult to assign any physiological role to this effect.

PMA did not alter the parameters of the voltage dependence of activation of the cardiac Ca^{2+} channel. The only parameter that was changed was G_{max} (maximal macroscopic conductance) which might result from an increase in the number of Ca^{2+} channels opened, or from an increase in the open time or probability of opening of the channel.

Oocytes injected with $\alpha_2/\delta+\beta$ subunits displayed a DHP-insensitive Ba^{2+} current most probably due to their coupling to the endogenous DHP-insensitive α_1 subunit (cf. [29,33]). When β2a was present, the amplitude of this current was higher than when the channel contained β1. We conclude that β2a subunit links more efficiently to an endogenous α_1 subunit protein to form a functional channel. In oocytes injected with $\alpha_2/\delta+\beta$ (β1 or β2a) subunits cRNA, PMA caused only an increase of I_{Ba}. This finding supports the notion that the pore-forming subunit of these channels is the endogenous α_1 subunit, since PMA has been reported to enhance the oocyte's endogenous I_{Ba} [45]. In addition, it suggests that the decrease of the current by PMA in channels containing the cardiac DHP-sensitive α_1 was a specific effect of PKC on the α_1 subunit rather than a side effect of PKC on internalization of the membrane, which could also result in a decrease in I_{Ba}.

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