Volume 2, Issue 3 (1994) ISSN: 1060-6823 RCHAE4 2(3) 175–254 (1994)

RECEPTORS & CHANNELS

THE INTERNATIONAL JOURNAL OF RECEPTORS, CHANNELS AND TRANSPORTERS

SIGNAL TRANSDUCTION BY GM-CSF AND IL-3 RECEPTORS
SAKAMOTO K. M., MIGNACCA R. C. AND J. C. GASSON

EXPRESSION OF CLONED INWARD RECTIFIER POTASSIUM CHANNELS
C. T. BOND, M. PESSIA, X-M. XIA, A. LAGRUTTA, M. P. KAVANAUGH AND J. P. ADELMAN

SHAB GENE EXPRESSION IN IDENTIFIED CELLS
D. J. BARO, C. L. COLE, A. R. ZARRIN, S. HUGHES AND R. M. HARRIS-WARRICK

SYNTHESIS OF A GAT-3-SELECTIVE INHIBITOR
LAURENCE A. BORDEN, T. G. MURALIDHAR, KELLI E. SMITH, THERESA A. BRANCHEK, CHARLES GLUCHOWSKI AND RICHARD L. WEINSHANK

cAMP AND CARDIAC Ca^2-CHANNEL IN OOCYTES
DAFNA SINGER-LAHAT, ILANA LOTAN, MARTIN BIEL, VEIT FLOCKERZI, FRANZ HOFMANN AND NATHAN DASCAL

GABA CHANNEL ACTIVATION DOMAINS
JAHANSHAH AMIN AND DAVID S. WEISS

ROLE OF AN INVARIANT CYSTEINE IN Kv2.1 K^+ CHANNEL
ROGER D. ZÜHLKE, HUI-JUAN ZHANG AND ROLF H. JOHO

MYOMETRIAL Na CHANNEL mRNA
M. B. BOYLE AND L. A. M. HESLIP

harwood academic publishers
Published in Switzerland

CONTENTS

Page

REVIEW
SIGNAL TRANSDUCTION BY GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR AND INTERLEUKIN-3 RECEPTORS 175
K.M. SAKAMOTO, R.C. MIGNACCA AND J.C. GASSON*

REVIEW
CLONING AND EXPRESSION OF A FAMILY OF INWARD RECTIFIER POTASSIUM CHANNELS 183
*C. T. BOND, M. PESSIA, X-M. XIA, A. LAGRUTTA, M. P. KAVANAUGH AND J. P. ADELMAN**

REVIEW
SHAB GENE EXPRESSION IN IDENTIFIED NEURONS OF THE PYLORIC NETWORK IN THE LOBSTER STOMATOGASTRIC GANGLION 193
D.J. BARO, C.L. COLE, A.R. ZARRIN, S. HUGHES AND R.M. HARRIS-WARRICK*

CLONING OF THE HUMAN HOMOLOGUE OF THE GABA TRANSPORTER GAT-3 AND IDENTIFICATION OF A NOVEL INHIBITOR WITH SELECTIVITY FOR THIS SITE 207
LAURENCE A. BORDEN, T.G. MURALI DHAR, KELLI E. SMITH, THERESA A. BRANCHEK, CHARLES GLUCHOWSKI, AND RICHARD L. WEINSHANK*

CARDIAC CALCIUM CHANNELS EXPRESSED IN *XENOPUS* OOCYTES ARE MODULATED BY DEPHOSPHORYLATION BUT NOT BY cAMP-DEPENDENT PHOSPHORYLATION 215
*DAFNA SINGER-LAHAT, ILANA LOTAN, MARTIN BIEL, VEIT FLOCKERZI, FRANZ HOFMANN AND NATHAN DASCAL**

HOMOMERIC ρ_1 GABA CHANNELS: ACTIVATION PROPERTIES AND DOMAINS 227
*JAHANSHAH AMIN AND DAVID S. WEISS**

ROLE OF AN INVARIANT CYSTEINE IN GATING AND ION PERMEATION OF THE VOLTAGE-SENSITIVE K^+ CHANNEL Kv2.1 237
*ROGER D. ZÜHLKE, HUI-JUAN ZHANG AND ROLF H. JOHO**

VOLTAGE-DEPENDENT Na+ CHANNEL mRNA EXPRESSION IN PREGNANT MYOMETRIUM 249
M.B. BOYLE AND L.A.M. HESLIP*

*Authors to whom correspondence and reprint requests should be addressed are indicated by an asterisk

Receptors and Channels, 1994, Vol. 2, pp. 215-226
Reprints available directly from the publisher
Photocopying permitted by license only

Cardiac Calcium Channels Expressed in *Xenopus* Oocytes are Modulated by Dephosphorylation but not by cAMP-Dependent Phosphorylation

DAFNA SINGER-LAHAT[†‡], ILANA LOTAN[†], MARTIN BIEL[#], VEIT FLOCKERZI[#], FRANZ HOFMANN[#], and NATHAN DASCAL[†*]

[†]Department of Physiology and Pharmacology, Sackler School of Medicine, Tel Aviv University, Ramat Aviv 69978, Israel, and [#]Institut für Pharmacologie und Toxicologie, Technischen Universität München, 80802 München 40, Germany. [‡]Present address: Laboratory of Cell Biology, NIMH, National Institutes of Health, Building 36, Bethesda, MD 20892, U.S.A.

(Received 28 October 1993; Revised 13 April 1994)

Enhancement of cardiac L-type Ca^{2+} channel activity by norepinephrine via phosphorylation by protein kinase A (PKA) underlies the positive inotropic effect of this transmitter and is a classical example of an ion channel modulation. However, it is not clear whether the channel protein itself (and which subunit) is a substrate for PKA. We have expressed various combinations of the cardiac Ca^{2+} channel subunits in *Xenopus* oocytes by injecting subunit mRNAs. Expression of β or $\alpha_2/\delta+\beta$ subunits potentiated the native (endogenous) Ca^{2+} channel currents in the oocyte (similar to T or N but not L-type). This potentiated endogenous current was enhanced by intracellular injection of cAMP or of the catalytic subunit of PKA, and this effect was reversed by the injection of a PKA inhibitor suggesting the presence of basal phosphatase activity. When a cardiac channel of $\alpha_1+\beta$, $\alpha_1+\alpha_2/\delta$ or $\alpha_1+\alpha_2/\delta+\beta$ composition was expressed at levels high enough that the contribution of the endogenous current became negligible, cAMP and PKA failed to increase the Ca^{2+} channel current, whereas PKA inhibitors and the catalytic subunit of protein phosphatase 1 reduced the amplitude of the current. Reduction of the current by PKA inhibitors was observed regardless of the presence of the β subunit, suggesting a major role for the α_1 subunit in this process. These results suggest that, like in the heart, when expressed in *Xenopus* oocytes, the cardiac L-type Ca^{2+} channels are phosphorylated in basal state and dephosphorylation reduces their activity. However, unlike the situation in the heart, the activity of the channel cannot be enhanced by PKA-catalyzed phosphorylation, suggesting that the channel is already fully phosphorylated in its basal state.

KEY WORDS: Calcium channel phosphorylation phosphatase *Xenopus* oocyte expression subunits

INTRODUCTION

Adrenaline (epinephrine) and noradrenaline (norepinephrine) increase the force of contraction of the heart primarily by enhancing the opening of the L-type voltage-dependent Ca^{2+} channels (see Reuter, 1983). Besides its obvious physiological importance, this phenomenon presents a classical example of modulation of a voltage-dependent ion channel by a neurotransmitter or hormone. There is ample evidence suggesting that this modulation occurs mainly via the cAMP-protein kinase A (PKA) pathway (reviews: Reuter 1983, Tsien 1983, Trautwein and Hescheler 1990), although a direct effect of G_s may contribute a fast component to the enhancement (Yatani *et al*, 1987, Yatani and Brown 1989, Shuba *et al*, 1990; but see Hartzell *et al*, 1991). The skeletal muscle (SkM) L-type Ca^{2+} channel is modulated in a similar way (Flockerzi *et al*, 1986; Arreola *et al*, 1987).

It is widely believed that direct phosphorylation of SkM and cardiac Ca^{2+} L-type channels by PKA mediates this modulation. This belief is based mainly on

the results of biochemical studies of SkM Ca^{2+} channel. Cardiac and SkM L-type Ca^{2+} channels consist of a main, pore-forming subunit α_1, and several auxiliary subunits (α_2/δ, β and γ: see Catterall 1988; Campbell *et al*, 1988; Glossmann and Striessnig 1990; Hofmann *et al*, 1993) that modulate the channel's expression and biophysical properties (Mikami *et al*, 1989; Mori *et al*, 1991; Singer *et al*, 1991; Varadi *et al*, 1991; Lacerda *et al*, 1991). The α_1 subunits of heart and SkM are non-identical (though homologous) products of separate genes (Mikami *et al*, 1989). The same applies to β subunits; SkM β is of $\beta 1$ type, whereas the most abundant cardiac β is of the $\beta 2$ type (Ruth *et al*, 1989; Hullin *et al*, 1992; Pragnell *et al*, 1991; Perez-Reyes *et al*, 1992). The γ subunit has not been found in the heart (Jay *et al*, 1990; Bosse *et al*, 1990). The α_1 subunits of the SkM Ca^{2+} channel is a good substrate for PKA phosphorylation; $\beta 1$ is phosphorylated to a lesser extent, and α_2/δ and γ are not PKA substrates; the target serines phosphorylated *in vitro* by PKA on the SkM α_1 subunit have been identified (Nastainszyk *et al*, 1987; Rohrkasten *et al*, 1988; Jahn *et al*, 1988; Mundina-Weilenmann *et al*, 1991; Rotman *et al*, 1992). When the purified channel is reconstituted in artificial membranes, its activity is enhanced by PKA (Flockerzi *et al*, 1986; Nunoki *et al*, 1989). Thus, it is highly probable that, in the skeletal muscle, the enhancing effect of cAMP-elevating agents is mediated by direct phosphorylation of the SkM Ca^{2+} channel α_1 subunit by PKA. However, it is not clear yet to what extent this conclusion applies to the cardiac channel, because:

i) Biochemical studies of cardiac Ca^{2+} channels have been hampered by the low abundance of the protein; the available data suggest that cardiac α_1 purified from the heart is a surprisingly poor substrate for PKA-catalyzed phosphorylation (Chang and Hosey 1988; Schneider and Hofmann 1988).

ii) A cloned smooth muscle Ca^{2+} channel α_1 subunit is a splice variant of the same RNA as the cardiac α_1 and contains all the same putative phosphorylation sites (Biel *et al*, 1990). The smooth muscle L-type Ca^{2+} channel activity is enhanced by noradrenaline, but this effect is not mediated by cAMP, and cAMP does not enhance smooth muscle Ca^{2+} currents (Weiling *et al*, 1992). Although other, yet uncloned, gene products may constitute a significant portion of smooth muscle Ca^{2+} channel population, these finding do call for caution in extrapolating conclusion reached in work with SkM L-type channels to other tissues.

iii) Recent work on PKA modulation of cardiac Ca^{2+} channels in heterologous expression systems yielded conflicting results. Klockner *et al.* (1992) did not observe any cAMP effects on cardiac Ca^{2+} channels expressed in *Xenopus* oocytes unless they also expressed the β subunit and concluded that the enhancing effect of PKA is due to phosphorylation of the β subunit. In contrast, Yoshida *et al.* (1992) and Sculptoreanu et al. (1993) expressed the cardiac Ca^{2+} channel α_1 subunit in a mammalian cell line (CHO) and were able to demonstrate phosphorylation of the expressed protein and the enhancement of the Ca^{2+} current by cAMP analogs and by PKA. These results strongly suggest a major role for α_1 in the effect of PKA on cardiac Ca^{2+} channel; however, they still do not rule out the possibility of involvement of auxiliary proteins (present in heart an/or CHO cells).

It seems, therefore, that it still remains an open question whether the positive inotropic effect of adrenaline is mediated by PKA-catalyzed phosphorylation of the α_1 or β subunit of the cardiac Ca^{2+} channel, or by phosphorylation of another auxiliary protein that is not an integral part of the channel. We studied the effects of PKA, PKA inhibitors, and protein phosphatases in *Xenopus* oocytes expressing various combinations of cardiac Ca^{2+} channel subunits. The activity of native oocyte's Ca^{2+} channels (that resemble T- or N-type and are insensitive of dihydropyridines (DHPs)) was markedly potentiated by the expression of cardiac β subunit, and was upregulated by cAMP and by PKA, suggesting that this pathway is activatable and effective in the oocytes. However, the activity of the expressed cardiac channel, whatever the subunit combination, was not affected by PKA but could be reduced by PKA inhibitors (PKI) and protein phosphatase type 1 (PP1), suggesting that in this particular expression system PKA-catalyzed phosphorylation either does not occur or does not affect the activity of the channel, whereas dephosphorylation does reduce the channel activity as expected. The PKI-induced reduction of channel activity was observed regardless of the presence of the β subunit, suggesting a major role for α_1 in this effect.

RESULTS

The native (endogenous) Ca^{2+} channel activity in the oocytes is potentiated by expression of the cardiac $\beta 2A$ subunit and is modulated by PKA

To avoid activation of Ca^{2+}-dependent Cl^- channels residing in *Xenopus* oocyte membrane (Miledi 1982;

Table 1 Summary of the effects of phosphorylating and dephosphorylating agents on I_{Ba} in oocytes injected with various combinations of cRNAs of Ca^{2+} channel subunits

Subunit	*Amplitude#*						
combination	*nA*	*% change in I_{Ba} caused by:*					
		cAMP	*PKA*	*PP1*	*PP2A*	*PKI*	*vehicle*
β2A	-127±35 (4)	+42±7 (4)					
α_2+β	-194±25 (17)	+93±7 (17)	+41±12 (15)	+13±2 (3)	+5;+21 (2)	+1±2 (3)	
α_1+$\alpha_{2/\delta}$	-100;-165 (2)	-2;+2 (2)				-37±4 (10)	-11±6 (11)
α_1+β	-139±16 (9)	+24±10 (9)	+12±11 (7)	-29;-30 (2)	0;-14 (2)		
α_1+α_2/δ+β	-520±86 (14)	+33±9 (14)		-22±6 (5)	+7±8 (3)		
α_1+α_2/δ+β*	-1024±33 (4)*	+5±2(4)				-37±4 (12)	-2±4 (5)

All data were obtained in 40 mM $[Ba^{2+}]_{out}$ except when denoted by asterisk. β stands for any one of the three subtypes of β subunit used in this study (β1, β2A, β3). Data with all subtypes of β were pooled since no significant differences have been found in effects of cAMP, PP1 and PP2A on channels containing these subunits. The effects of PKI were tested only in combinations containing β2A.
*Recorded in 2 mM $[Ba^{2+}]_{out}$
#The numbers in this column refer only to cells in which the effect of cAMP injection has been tested.

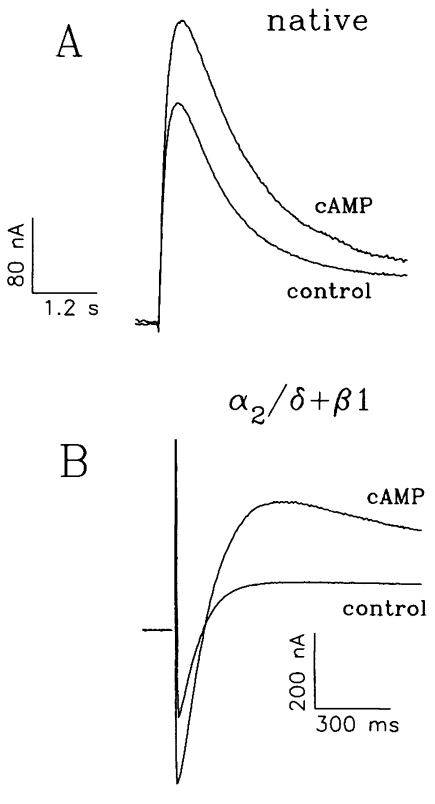

Figure 1 The effect of intracellular injection of 50–80 pmole of cAMP on endogenous (native or potentiated) $I_{Cl(Ca)}$, I_{Ba}, and $I_{Cl(Ba)}$. **(A)** The effect of cAMP on $I_{Cl(Ca)}$ recorded in a native (not injected with any RNA) oocyte in normal ND 96 solution with 1.8 mM Ca^{2+} (same as NDE but without pyruvate and antibiotics; see Methods) by stepping the voltage from -100 to 0 mV. Correction for leak current was made by scaling and digitally subtracting currents evoked by steps from -100 to -50 mV. **(B)** The effect of cAMP on I_{Ba} (inward) and $I_{Cl(Ba)}$ (outward) in an oocyte injected with RNAs of α_2/δ and β1 subunits. The oocyte was not injected with EGTA before the experiment. The current was measured in 40 mM $BaCl_2$ (see Methods) by stepping the voltage from -100 to 20 mV. No leak subtraction was done.

Barish 1983), the recording of macroscopic Ca^{2+} channel current in the oocytes is usually performed in Ca^{2+}-free solutions with Ba^{2+} as the charge carrier (Dascal *et al*, 1986). The endogenous voltage-dependent Ca^{2+} channel in the oocytes is not sensitive to dihydropyridines; the macroscopic Ba^{2+} current (I_{Ba}) rarely exceeds 10–20 nA in 40 mM $[Ba^{2+}]_{out}$ (Dascal *et al*, 1986, 1992; Umbach and Gundersen, 1987) and is practically indistinguishable in 2 mM $[Ba^{2+}]_{out}$ (Lory *et al*, 1990). The native I_{Ba} can be potentiated by the concomitant expression of the auxiliary α_2/δ+β subunits (Singer *et al*, 1991; Williams *et al*, 1992; Table 1), presumably due to an assembly of the newly synthesized auxiliary subunits with the endogenous α_1. We found that expression of the cRNA of the α_2/δ subunit alone did not potentiate the endogenous current (data not shown, see also Mori *et al*, 1991; Singer *et al*, 1991), whereas expression of the β2A subunit alone gave rise to I_{Ba} of ≥100 nA (Table 1) which was insensitive to dihydropyridines (data not shown), suggesting that it represented a potentiated endogenous Ca^{2+} channel.

Intracellular cAMP injection enhances the native I_{Ba} in *Xenopus* oocytes (Dascal *et al*, 1992; Bourinet *et al*, 1992). Expectedly, when recorded in a normal Ca^{2+}-containing ND96 solution, the Ca^{2+} -dependent Cl^- current ($I_{Cl(Ca)}$) of the native oocytes was also increased by 79±18% (n=4) by the injection of 40–80 pmoles of cAMP (Fig. 1A). This enhancement might present a source of artifact if the currents recorded in high Ba^{2+} solutions were contaminated by $I_{Cl(Ca)}$. Fig 1B shows that this is indeed the case when I_{Ba} is measured in a solution containing 40 mM $BaCl_2$ and no Ca^{2+}, in an oocyte injected with α_2/δ+β1 cRNAs. The already potentiated (due to the presence of the auxiliary subunits) native I_{Ba} is further increased by

the injection of cAMP (by 93±7% on the average, see Table 1). However, the inward Ba^{2+} current is followed by a Cl^- (outward) current which is also enhanced by cAMP (termed $I_{Cl(Ba)}$ in the following). This current was strongly inhibited (though not completely abolished) by intracellular injection of EGTA (E. Shistik and N. Dascal, unpublished observations; *cf.* Singer *et al*, 1991), suggesting that Ba^{2+} might activate the Cl^- channel by releasing Ca^{2+} from intracellular stores. To avoid this artifact, further experiments have been done on oocytes injected with EGTA.

Like the native endogenous current, the potentiated I_{Ba} in oocytes expressing the auxiliary subunits (β2A or $\alpha_2/\delta+\beta$) was increased by 40–100% by the intracellular injection of 40–80 pmol cAMP (Fig. 1B, 2A, and Table 1). Endogenous I_{Ba} in oocytes expressing $\alpha_2/\delta+\beta$ subunits was also enhanced by the injection of 0.1–0.4 units of the catalytic subunit of PKA by about 40% (Table 1), suggesting that the cAMP effect was mediated by PKA-catalyzed phosphorylation. In two oocytes, we have injected a PKA-specific protein kinase inhibitor (PKI) peptide after cAMP and observed a clear reduction in I_{Ba}, to a level that was still above the control (Fig. 2A, B). Thus, cAMP activates the endogenous PKA that enhances I_{Ba}, and the endogenous phosphatases dephosphorylate the protein phosphorylated by PKA (possibly the channel itself) once the excessive PKA activity is inhibited. However, the various PKI proteins and peptide used in this study (see Methods; collectively called PKI) had no effect on basal level of I_{Ba} in $\alpha_2/\delta+\beta$ expressing oocytes (Fig. 2C and Table 1). Injection of the catalytic subunits of protein phosphatases 1 and 2A (PP1 and PP2A; see Cohen, 1989) did not decrease but rather slightly increased I_{Ba} by 5–21% (Table 1 and Fig. 2D). Thus, the PKA substrate whose phosphorylation enhances the endogenous I_{Ba} (possibly the native Ca^{2+} channel itself) does not appear to be basally phosphorylated by PKA.

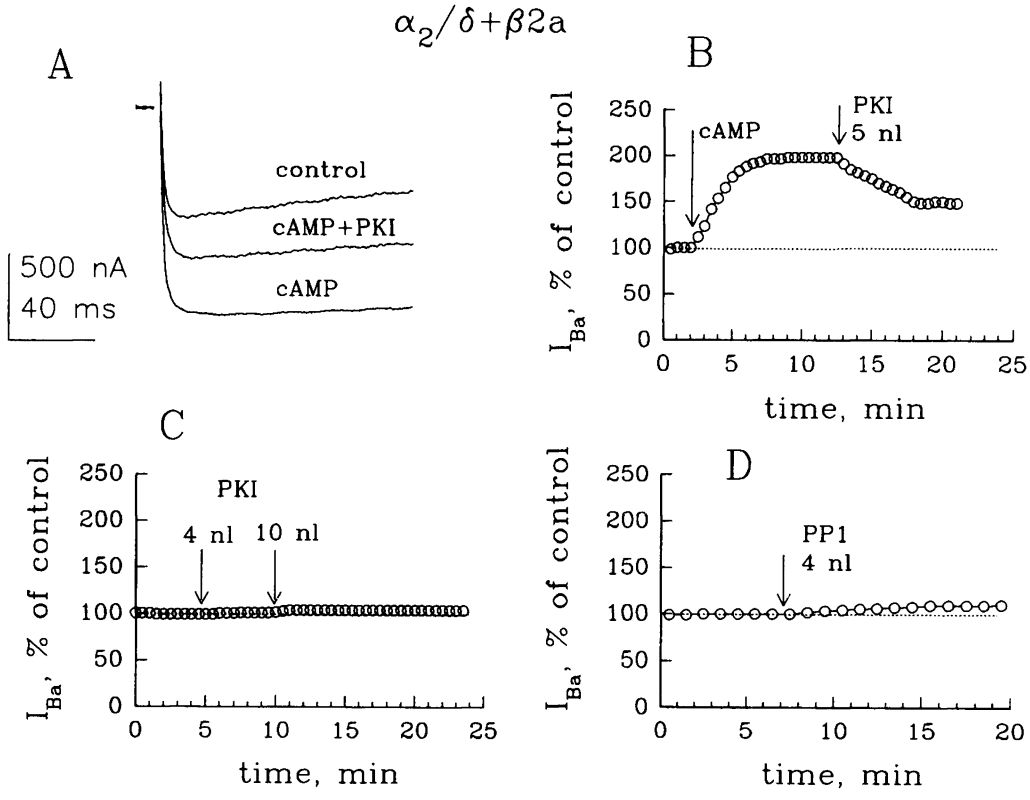

Figure 2 Effects of phosphorylation and dephosphorylation on the potentiated endogenous I_{Ba} in oocytes injected with RNAs of $\alpha_2/\delta+\beta2A$ subunits. Currents were evoked by steps from -80 to 0 mV in the 40 mM $BaCl_2$ solution; all cells were injected with EGTA. **(A)** Effect of cAMP injection, followed by injection of PKI peptide, on I_{Ba}. Calibration: 250 nA, 40 ms. **(B)** The time course of the experiment shown in (A). **(C)** Injection of PKI peptide does not alter I_{Ba}. **(D)** Injection of PP1 does not decrease I_{Ba}.

Ba^{2+} current via cardiac Ca^{2+} channel is not enhanced by PKA activation

In agreement with Klockner *et al* (1992), I_{Ba} in oocytes expressing $\alpha_1+\alpha_2$ subunits was not affected by cAMP injection (Fig. 3A and Table 1). (I_{Ba} in oocytes expressing α_1 alone was too small to study using the intracellular injection methods). In oocytes expressing cardiac α_1 together with a β subunit, I_{Ba} was usually slightly increased (~24% or 12% on the average) by cAMP or PKA injection, respectively (Table 1). However, in many oocyte batches, expression of cardiac $\alpha_1+\beta$ often gave rise to Ba^{2+} currents that were only slightly larger than in oocytes expressing β2A alone, and the observed cAMP-evoked increase might be contributed by the endogenous current (potentiated by the coexpressed β). Indeed, in $\alpha_1+\beta$-expressing cells with large I_{Ba}, the enhancement caused by cAMP was not observed (Fig. 3B).

The test quantitatively for the possible contribution of the endogenous channel to the cAMP-evoked enhancement of I_{Ba}, we used oocytes expressing all subunits found in the heart ($\alpha_1+\alpha_2/\delta+\beta$). Such expression easily gives rise to currents of several μA in 40 mM $[Ba^{2+}]_{out}$ and several hundreds to 2,000 nA in 2 mM $[Ba^{2+}]_{out}$, ensuring that the contribution of the endogenous current to total macroscopic I_{Ba} is minimal. The oocytes were injected with different amounts of cRNAs of α_1, α_2/δ, and β1 or β2A subunits (the ratio of the cRNAs was kept 1:1:1 by weight). When the resulting I_{Ba} was measured 3–5 days later, a phenomenon similar to that observed in oocytes expressing $\alpha_1+\beta$ was observed: small Ba^{2+} currents were enhanced by cAMP injection (Fig. 3C), large currents were unaffected (Fig. 3D). In this series of experiments, in 40 mM $[Ba^{2+}]$, there was a negative correlation between the amplitude of I_{Ba} and the extent of cAMP-induced enhancement (Fig. 3E). Furthermore, with the highest expression levels of cardiac α_1 used in this study, when~1000 nA currents were recorded in 2 mM $[Ba^{2+}]_{out}$, cAMP had practically no effect on I_{Ba} (Table 1, last row). Thus, although when all the data obtained in 40 mM $[Ba^{2+}]_{out}$ are summarized, an average cAMP-induced

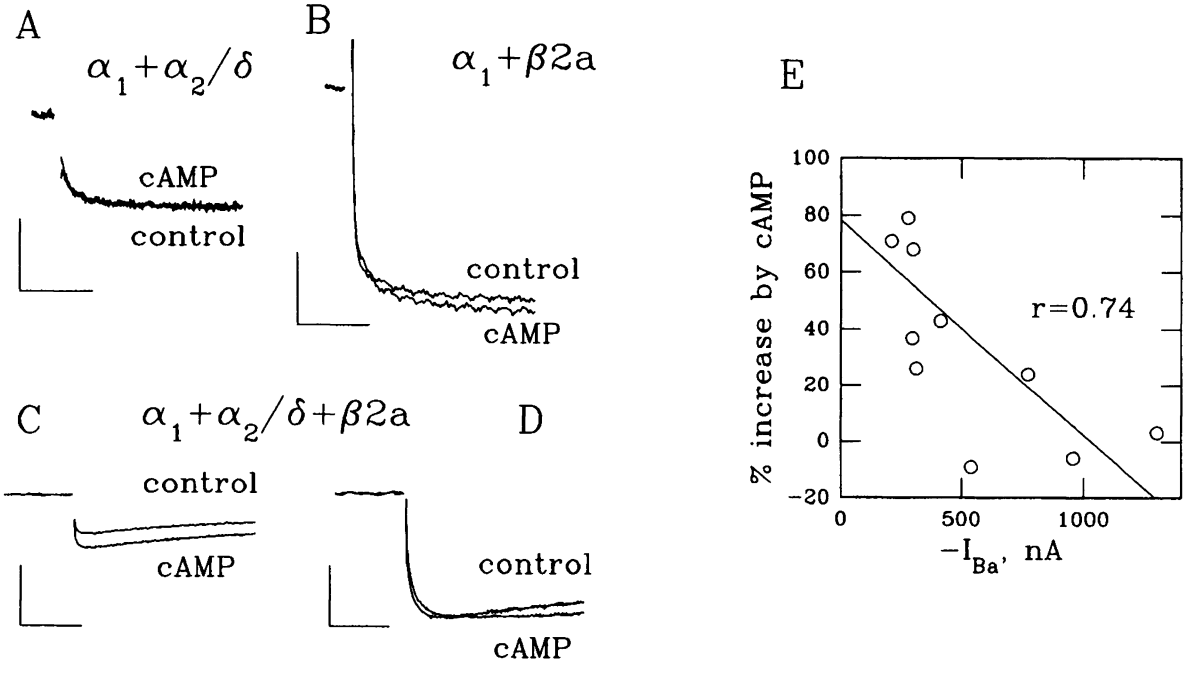

Figure 3 cAMP injection increases I_{Ba} when the β subunit is present, but this may be due to an effect on the endogenous Ca^{2+} channel. The currents were recorded in the 40 mM $BaCl_2$ solution by steps from -80 to 0 mV. Vertical calibration: 75 nA (A, B); 440 nA (C, D). Horizontal calibration: 31 ms (A), 75 ms (B), 128 ms (C, D). **(A)** cAMP does not alter I_{Ba} in an oocyte expressing cardiac α_1 and α_2/δ subunits. **(B)** I_{Ba} in an oocyte with a high level of expression of cardiac $\alpha_1+\beta$2A subunits is not enhanced by cAMP. **(C)** Apparent increase of I_{Ba} in an oocyte injected with cardiac α_1, α_2/δ and β2A subunits' RNA but expressing a small I_{Ba}. **(D)** No effect of cAMP on I_{Ba} in an oocyte injected with cardiac α_1, α_2/δ and β2A subunits' RNA and expressing a large I_{Ba}. **(E)** Inverse correlation between the amplitude of I_{Ba} and the magnitude of cAMP effect in oocytes injected with varying concentrations of RNAs coding for cardiac α_1, α_2/δ and β2A subunits. The straight line is a linear least square fit to the data points; the coefficient of correlation (r) is 0.74.

increase of 33% in I_{Ba} amplitude is apparent, we conclude that it was an artifact caused by the contribution of the endogenous (cAMP-sensitive) current in oocytes with low total macroscopic I_{Ba}. Further experiments on cardiac Ca^{2+} channel of the full composition ($\alpha_1+\alpha_2/\delta+\beta$) were performed in oocytes with high levels of expression of I_{Ba} (>500 nA in 40 mM $[Ba^{2+}]_{out}$). In separate experiments, using oocytes that were not injected with EGTA, we have verified that lack of cAMP effect was not due to the presence of intracellular Ca^{2+} chelator (data not shown).

PKA inhibitors and protein phosphatase 1 reduce the current via the cardiac Ca^{2+} channel by acting mainly on the α1 subunit

Injection of PKI caused a ~37% decrease in the amplitude of I_{Ba} in oocytes expressing the cardiac channel with the complete subunit composition, $\alpha_1+\alpha_2/\delta+\beta$, whereas the injection of vehicle was without a significant effect (Fig. 4A, B and Table 1). Injection of PP1 reduced the current by 22%, whereas PP2A caused no significant changes (Fig. 4C, D, and Table 1). There was no need to test the effect of vehicle in this case, because both phosphatases were dissolved in the same buffer and similar volumes were injected, thus PP2A could serve as a control for PP1 and vice versa. The transient decrease in I_{Ba} observed after the injection of PP2A (Fig. 4D) was interpreted as an injection artifact, since it was also sometimes observed upon injection of cAMP or PKI vehicle. PP1 also reduced I_{Ba} (by~30%) in two oocytes expressing $\alpha_1+\beta$ subunits, whereas PP2A had little effect in other two oocytes (Table 1). These data indicate that, in contrast to the native Ca^{2+} channels, cardiac L-type channels expressed in oocytes (or an associated regulatory protein) are phosphorylated in their basal state, and that dephosphorylation reduces their activity.

To study the possible involvement of the β subunit in the current decrease caused by dephosphorylation, PKI was injected into oocytes expressing only $\alpha_1+\alpha_2$ subunits. These experiments were complicated by the fact that this combination of subunits usually gives rise to relatively small currents (100–200 nA). Although the contribution of the endogenous current in this case is usually negligible, non-specific current changes caused by the injection could introduce an error into the estimate of changes of I_{Ba}. On the average, injection of PKI reduced I_{Ba} in these oocytes by 37±4%, i.e. by the same extent as in channels composed of $\alpha_1+\alpha_2/\delta+\beta$; the injection of vehicle caused a 11±6% reduction in I_{Ba} (Table 1). The difference between these two values is statistically significant ($P<0.01$). In one of the oocyte batches, the Ba^{2+} currents in $\alpha_1+\alpha_2$-expressing oocytes were large and did not show injection artifacts; in these oocytes, injection of the PKI peptide reduced I_{Ba} by 37±5% (Fig. 4E; n=3), whereas the change produced by the injection of the vehicle was 0±2% (Fig. 4F; n=3; $P<0.01$). Thus, PKI was able to reduce the cardiac I_{Ba} regardless of the presence of the β subunit.

DISCUSSION

In this study we examined how the cardiac Ca^{2+} channels expressed in *Xenopus* oocytes are modulated by PKA phosphorylation and by dephosphorylation, and what are the relative roles of α_1 and β subunits in these modulations. The main finding of this study is an unexpected lack of effect of cAMP and PKA on the expressed channel, even when all the known subunits are present. In contrast, the native (endogenous) DHP-insensitive Ca^{2+} channel activity is enhanced by PKA. However, the expressed cardiac Ca^{2+} channel is down-regulated by dephosphorylation by protein phosphatase 1 or by an endogenous phosphatase following inhibition of PKA, regardless of the presence of the β subunit.

Sources of artifact in the study of modulation of Ca^{2+} channels in *Xenopus* oocytes

In agreement with previous reports (Singer *et al*, 1991; Williams *et al*, 1992), we found that the amplitude of the endogenous I_{Ba} (Ba^{2+} current flows via Ca^{2+} channels) is increased by the expression of the β subunit and even more by the simultaneous expression of α_2/δ and β subunits. Expression of the α_2/δ subunit alone does not potentiate the endogenous current. cAMP- or PKA-induced increase of the endogenous I_{Ba} can be mistaken for an increase in Ba^{2+} current flowing via the expressed cardiac channel if the endogenous current constitutes a significant portion of total I_{Ba}. Artifacts of this kind can be minimized by working with oocytes that display high levels of expression of the exogenous Ca^{2+} channels.

An additional artifact arises from the activation of the Ca^{2+}-dependent Cl^- channel by Ba^{2+} influx, probably due to Ba^{2+}-induced increase in intracellular Ca^{2+} concentration. When present, $I_{Cl(Ba)}$ contributes a slow component to the total measured current in high-Ba^{2+} solutions. If the external cation is methanesulfonate, the $I_{Cl(Ba)}$ component has the

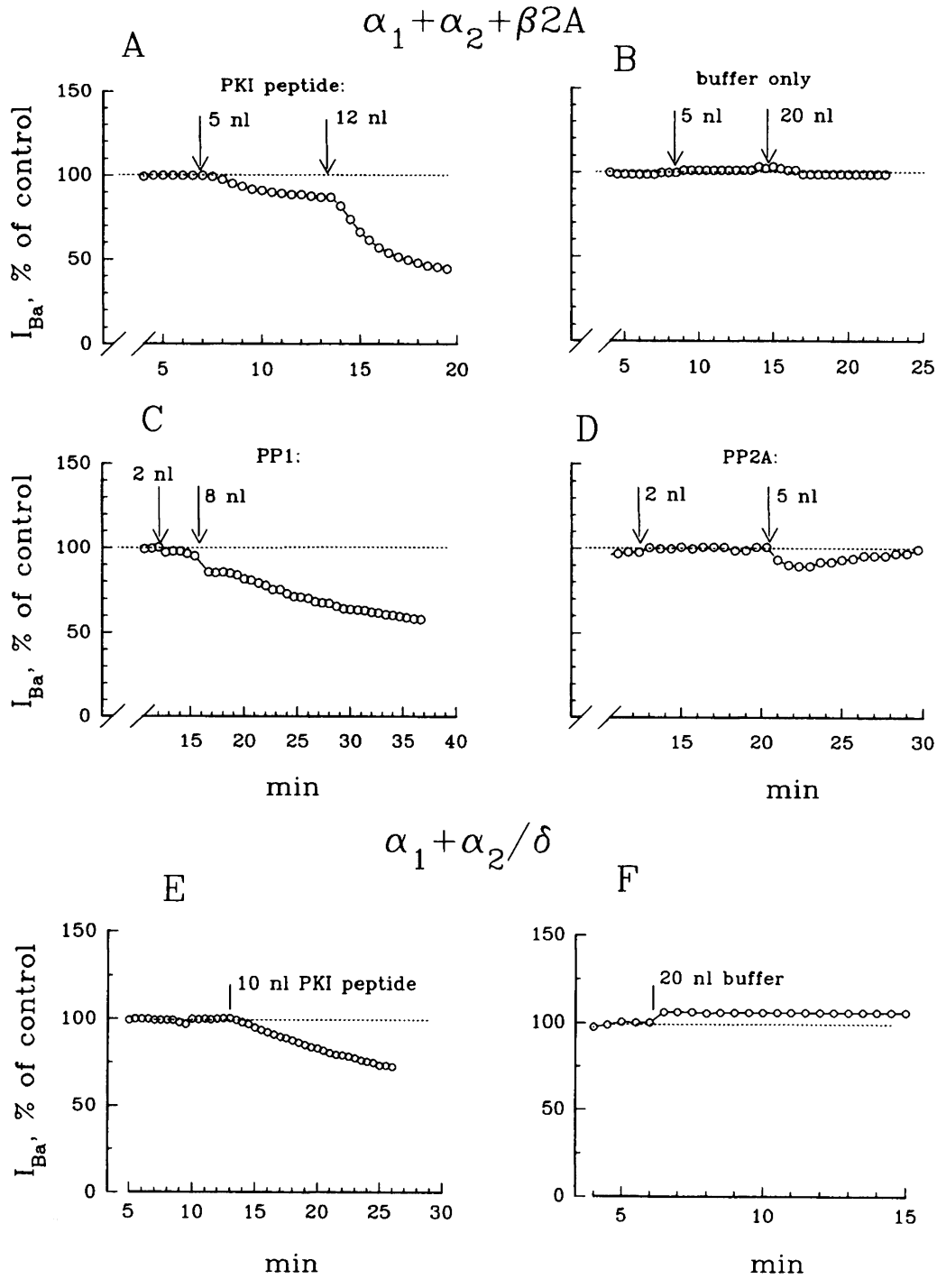

Figure 4 PKI and PPl reduce I_{Ba} in oocytes expressing the cardiac Ca^{2+} channel of the full subunit composition (A-D) and of the $\alpha_1+\alpha_2/\delta$ composition (E, F). **(A)** PKI peptide reduces I_{Ba}; **(B)** the buffer used to dissolve the PKI peptide does not reduce I_{Ba}. Records shown in (A) and (B) were made in oocytes of the same frog, on the same day, in 2 mM $[Ba^{2+}]_{out}$. **(C)** PP1 reduces I_{Ba}; **(D)** PP2A does not significantly affect I_{Ba}. Records in (C) and (D) were made in oocytes of the same batch, on the same day, in 40 mM $[Ba^{2+}]_{out}$. **(E, F)** PKI peptide (E), but not the buffer (F), reduces I_{Ba} in oocytes expressing cardiac $\alpha_1+\alpha_2/\delta$. Records in (E) and (F) were made in oocytes of the same batch, on the same day, in 2 mM $[Ba^{2+}]_{out}$.

same direction as I_{Ba}; any increase in I_{Ba} would also increase $I_{Cl(Ba)}$, leading to overestimation of the actual increase in I_{Ba}. To avoid this artifact, in this study we have used EGTA-injected oocytes and Cl^- as the extracellular anion.

The endogenous Ca^{2+} channels are up-regulated by PKA

cAMP has been shown to enhance the endogenous Ca^{2+} channel current in the oocytes (Dascal *et al*, 1992; Bourinet *et al*, 1992). We have extended this finding and demonstrated that the endogenous current potentiated by coexpression of the auxiliary subunits (β or $\alpha_2/\delta+\beta$) is also enhanced by the injected cAMP or catalytic subunit of PKA, and this effect is partially reversed by a specific peptide inhibitor of PKA. Thus, PKA-catalyzed phosphorylation of the endogenous Ca^{2+} channel or of an associated protein enhances the activity of the channel. It is important to note that these results rule out the possibility that the endogenous PKA in the oocytes is permanently and fully activated. The oocyte's PKA is activatable by cAMP, like in most other cell types, and its activation can be mimicked by the injected exogenous catalytic subunit of PKA. This conclusion is supported by our previous findings concerning modulation of the neuronal Na^{2+} channels expressed in the oocytes (Gershon *et al*, 1992). Furthermore, neither the phosphatases tested by us (PP1 and PP2A) nor protein kinase A inhibitors were able to reduce the endogenous I_{Ba}, suggesting that the channel was not substantially phosphorylated by PKA in its basal state (dephosphorylation of PKA sites might be expected to produce an effect opposite to that of PKA, i.e. to reduce the current). The slight increase in the endogenous I_{Ba} by both phosphatases may be the result of dephosphorylation of sites basally phosphorylated by some other protein kinase(s). The reversal of the cAMP-induced increase in the endogenous I_{Ba} by PKI indicates that some endogenous phosphatase(s) in the oocyte is permanently active and is able of dephosphorylating the protein involved in the regulation of the endogenous Ca^{2+} channel (possibly the channel itself) at the PKA sites(s).

The activity of the cardiac L-channel expressed in the oocytes is not enhanced by PKA

Contrary to expectations and to the results of Klockner *et al* (1992), we did not find evidence for an increase in I_{Ba} in oocytes expressing the cardiac α_1 subunit and any of the tested β subunits (β1, β2A, β3) upon intracellular injection of cAMP or of catalytic subunit of PKA. The increase often observed in oocytes expressing β-containing subunit combinations (either $\alpha_1+\beta$ or $\alpha_1+\alpha_2/\delta+\beta$) with relatively small Ba^{2+} currents (100–400 nA in 40 mM $[Ba^{2+}]_{out}$) could not be reproduced when the level of expression of the cardiac channel was high. We cannot rule out the possibility that at high levels of expression of cardiac Ca^{2+} channels the modulation by PKA becomes impossible for some unknown reason. However, we consider this possibility as highly unlikely: even with high expression levels attainable in the oocytes, the density of the expressed cardiac channels in the membrane certainly does not exceed that in cardiac cells (cf. Sather *et al*, 1993). We have adapted the simplest interpretation for these results: when the level of expression of the cardiac α_1 is low, a significant portion of the auxiliary subunits, especially β, associate with the endogenous α_1, the contribution of the potentiated endogenous current to total I_{Ba} is substantial, hence the observed cAMP effect. The potentiated endogenous current rarely exceeds 300 nA, even when large amounts of RNAs of α_2/δ and β subunits are injected; when cardiac α_1 is also expressed, it is expected to compete with the endogenous α_1 for the auxiliary subunits. Suppose that the total I_{Ba} is 300 nA in 40 mM $[Ba^{2+}]_{out}$, and 100 nA is the contribution of the endogenous current. cAMP may increase the endogenous current to 200 nA, resulting in an overall 33% increase in total I_{Ba}. When more RNA of the Ca^{2+} channel subunits is injected, larger Ba^{2+} currents are observed; if the total I_{Ba} is 4000 nA (this would correspond to about 1000 nA in 2 mM $[Ba^{2+}]_{out}$), and the endogenous current contributes 200 nA, only a 5% increase in total I_{Ba} will occur after cAMP injection. These numeric examples are reasonable and fit very well the observed results, supporting the view that all of the observed cAMP-induced increase in our experiments (and possibly in those of Klockner *et al*, 1992) is due to the change in the endogenous current, while the cardiac channel activity is not affected by cAMP or PKA.

The current via the cardiac L-channel expressed in the oocytes is decreased by dephosphorylation

The activity of the cardiac Ca^{2+} channel is believed to be under a dynamic control of a phosphorylation/dephosphorylation cycle, dephosphorylation be-

ing catalyzed by PP1 and possibly additional phosphatases (Kameyama *et al*, 1986a, b; Hescheler *et al*, 1988). In accord with this, we find that the catalytic subunit of PP1, but not of PP2A, decreases the Ba^{2+} current via the cardiac Ca^{2+} channel expressed in the oocyte. Interestingly, using the same batches and injected amounts of PP1 and PP2A, we have observed opposite effects on a neuronal Na^+ channel expressed in the oocytes: an increase by PP2A, no effect of PP1 (Gershon *et al*, 1992). These observations rule out the possibility that one of the phosphatase batches was inactive. Furthermore, because of the limitations of the injection methodology, the calculated increase of phosphatase activity achieved in these experiments was only 20–60% above the basal level (see Gershon *et al*, 1992). It is quite possible that higher doses of PP1 would reduce I_{Ba} by more than 22–30% observed in the present study. The PP1 effect clearly suggests that, unlike the endogenous Ca^{2+} channel(s), the cardiac L-type Ca^{2+} channel expressed in the oocyte is basally phosphorylated by a protein kinase, and that dephosphorylation by PP1 reduces the channel's activity. In addition, these results support the view (Armstrong and White, 1992; Gershon *et al*, 1992) that modulation of ion channels by dephosphorylation may be phosphatase-specific. However, at present, we cannot rule out the possibility that higher doses of PP2A could also reduce I_{Ba}; further experiments are needed to explore the question of phosphatase specificity in regulation of Ca^{2+} channel function.

The reduction of the cardiac channel activity by PKI supports and extends the conclusions drawn from the phosphatase effects. The finding that PKA-specific inhibitors reduce the L-type Ca^{2+} channel current suggests that the channel is phosphorylated by PKA in its basal state. Once PKA is inhibited by the injected PKI, the dephosphorylation of amino acid residues previously phosphorylated by PKA is probably catalyzed by a constantly active endogenous phosphatase. Thus, at least to some extent, there is a dynamic phosphorylation/dephosphorylation control of the channel activity; however, it is clearly different from that in the heart, where activation of PKA causes a several fold increase in Ca^{2+} current.

The presence of the β subunit is not required for the inhibitory effect of PKI. α_2/δ subunit is not a substrate for PKA (e.g. Hosey and Lazdunsky, 1988). Thus, α_1 is the main target of the putative endogenous phosphatase that dephosphorylates the PKA site(s) causing the reduction of I_{Ba}. This is in line with the suggestion that α_1 is the main target for PKA phosphorylation (Yoshida *et al*, 1992; Sculptoreanu *et al*, 1993). However, on the average, the inhibitory effect of PKI was smaller when the β subunit was "omitted" (a net decrease of 37-11=26%, taking into account the 11% average decrease caused by the injection of the vehicle) than when the channel had the full $\alpha_1+\alpha_2/\delta+\beta$ composition (~37%). Thus, at present we cannot exclude that the β subunit may play a modulatory role in PKA-related dephosphorylation in cardiac Ca^{2+} channels.

Possible explanations and hypotheses

Why is the expressed L-type cardiac Ca^{2+} channel unaffected by PKA? In the following, we discuss several possibilities.

i) The channel expressed in the oocyte may be fully phosphorylated at all PKA sites. This possibility is supported by the inhibitory effects of PP1 and PKI. It is unlikely that a full phosphorylation of the channel would be a result of an excessive activity of PKA: like in other tissues, the PKA in the oocyte can be activated by cAMP and can further exert modulatory effects on voltage-dependent channels (e.g. the endogenous Ca^{2+} channels, the expressed neuronal Na^+ channel). However, the oocyte may be missing a specific phosphatase present in the heart that dephosphorylates PKA sites of the L-type Ca^{2+} channel; in this case the phosphorylation/dephosphorylation equilibrium may be deregulated, and a high level of basal phosphorylation may be expected.

ii) The α_1 subunit of the cardiac L-type Ca^{2+} channel may be incorrectly processed in the oocyte; for instance, it might be truncated, missing the cytoplasmic C terminal portion (rich in putative PKA sites: see Mikami *et al*, 1989). 95% of the SkM α_1 in the native tissue is truncated in this way (De Jongh *et al*, 1991). Part of the cardiac α_1 heterologously expressed in CHO cells is truncated; while the full-length protein (~250 kDa) is phosphorylated by PKA, the truncated form (~200 kDa) is not (Yoshida *et al*, 1992). The hypothesis that the channel expressed in the oocytes had lost the sites "responsible" for the enhancing effect of PKA implies that these sites are different from those "responsible" for the decrease in Ca^{2+} channel current in the oocytes caused by dephosphorylating agents (PKI and PP1).

iii) It is possible that none of the known subunits of the cardiac Ca^{2+} L-type channels is the substrate for PKA-catalyzed phosphorylation that causes the enhancement of the Ca^{2+} current in the heart; an ad-

ditional, yet unidentified, protein (a "missing link") may participate in this effect. In the past, we and others had observed that the cardiac Ca^{2+} channel expressed in oocytes injected with total rat heart RNA was enhanced after cAMP injection (Dascal *et al*, 1986; Lory and Nargeot, 1992). However, in these studies the possible contribution of a potentiated (by the auxiliary subunits) endogenous current might not have been fully appreciated. In recent experiments, we were unable to observe any significant increase in I_{Ba} in oocytes injected with rabbit heart poly(A) RNA; whenever such an increase was observed, a pharmacological test with a dihydropyridine blocker revealed the presence of a substantial dihydropyridine-insensitive current (Singer-Lahat and Dascal, unpublished results). Also, experiments in which the cardiac Ca^{2+} channel subunits' RNAs ($\alpha_1+\alpha_2/\delta+\beta$) were coexpressed with total cardiac RNA in the oocytes, did not yield clear-cut results: in a few oocytes, some cAMP sensitivity was observed, but in a majority of oocytes, I_{Ba} was still insensitive to cAMP (Singer-Lahat and Dascal, unpublished observations). Thus, the "missing link" hypothesis has no experimental support at present.

iv) A yet undetected isoform of the L-type channel α_1 subunit, with a different set of PKA sites and enhanced by PKA phosphorylation, may exist in the heart.

It is clear that the existing knowledge of the mechanism of the physiological effect of PKA on cardiac L-type Ca^{2+} channel, including the present report, is insufficient for a full understanding of PKA modulation of cardiac Ca^{2+} L-type channel. Each of the four possibilities presented above may serve as a working hypothesis for further studies.

METHODS

Xenopus laevis females were obtained from the South African Xenopus Facility (Fish Hoek, South African Republic) or from Xenopus I Inc. (Ann Arbor, MI, USA). The animals were maintained, and oocytes prepared as described (Dascal and Lotan, 1992). Briefly, the frogs were dissected under MS 222 anesthesia, pieces of ovary were removed through incisions in the abdomen which were sutured, and animals were returned to the tanks to be reused later. Oocytes were defolliculated by collagenase (Type IA, Sigma), injected with 50 nl of RNA solution and incubated for 3–7 days at 22°C in a sterile solution (NDE) containing 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes/NaOH (pH=7.6), 2.5 mM Na Pyruvate, 100 μg/ml streptomycin, and 100 U/ml penicillin. In most cases, unless indicated otherwise, the oocytes were injected with 20–50 nl of 4–10 mM K-EGTA solution (pH=7) 4–16 h before the measurement of I_{Ba}.

cDNAs of the various Ca^{2+} channel subunits were from rabbit (see Hullin *et al*, 1992, for details); cRNAs were synthesized *in vitro* as described (Singer *et al*, 1991; Hullin *et al*, 1992), dissolved in water, mixed in the desired combination in equal amounts (by weight), and injected into the oocytes (1–50 ng of each subunit's RNA, depending on the experimental design). Note that the cDNA of the α_2/δ subunit was originally cloned from a skeletal muscle library (Tanabe *et al*, 1987).

Electrophysiological recordings were done as described (Singer *et al*, 1991), using a Dagan 8500 two electrode voltage clamp amplifier, and the pCLAMP software (Axon Instruments, Foster City, CA, USA) for data acquisition and analysis. Two solutions were used: a high-Ba^{2+} solution with 40 mM $BaCl_2$, 50 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM Hepes/NaOH (pH=7.5), and a low-Ba^{2+} solution with 2 mM $BaCl_2$, 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM Hepes (pH=7.5). On a few occasions, a 40 mM Ba methanesulfonate solution was used (Dascal *et al*, 1986) identical to the high-Ba^{2+} solution detailed above, but with methanesulfonate replacing Cl^-. The cells were held at a holding potential of -80 mV in a 500 μl bath constantly perfused with the recording solution; cessation of perfusion usually led to a decrease in I_{Ba}. Stability of the recorded current was verified for at least 5 min before starting any experimental manipulation (e.g. injection of a protein).

Pressure injections into the oocytes were performed while the oocytes were already voltage clamped with one or two injection needles inserted into the cell in addition to the voltage and current microelectrodes, as described elsewhere (e.g. Dascal *et al*, 1984). The substances used for the intracellular injections were obtained and prepared for injection as follows. cAMP, PKI proteins (crude; from rabbit muscle, and type III, from porcine heart), and catalytic subunit of PKA were from Sigma. cAMP was dissolved in water at 10 mM. The PKA catalytic subunit was dissolved in 5 mM dithiotreitol at 40–85 U/ml, stored at 0–4°C, and used no more than 48 h afterwards. In some experiments, the catalytic subunit of PKA prepared as described (Hofmann 1980) was used. A modified protein kinase inhibitor peptide (PKI_{6-24}) was synthesized as described (Fernan-

dez *et al*, 1991). The PKI proteins (12.5–25 μg/μl) and peptide (0.1 μg/μl) were dissolved in the following buffer: 25 mM Tris/HCl (pH=7), 0.05 mM EDTA. The solutions of PKI proteins and the peptide were usually prepared freshly before the experiment; sometimes the solutions were stored at 4°C for no more than 72 h. The catalytic subunits of PP1 and PP2A (a gift from Philip Cohen, University of Dundee, UK) were dissolved at 15 and 3 U/μl, respectively, in a buffer containing 50 mM Tris/HCl (pH=7), 0.1% β-mercaptoethanol, 0.1 mM EGTA, and 50% (v/v) glycerol, and stored at -20°C (see Gershon *et al*, 1992, for additional details).

In many cells, in high-Ba^{2+}, Ca^{2+}-free solutions, intracellular injections caused transient or persistent inward currents and increased the leak current. These artifacts were negligible for cAMP injection, since volumes <5 nl were injected, but became significant when peptides or proteins were injected (usually 5–20 nl). Cells in which intracellular injections caused persistent changes in holding current ("cell deterioration") were discarded. The leak currents were always subtracted. Even then, when I_{Ba} was relatively small (100–200 nA), the injection artifacts and leak changes caused by injection of >5 nl could still cause substantial I_{Ba} measurement errors, as revealed by the injection of similar volumes of vehicle.

The data in the text and in the table are presented as mean±SEM. Tests of statistical significance of differences between mean values have been done using the two-tailed t-test.

ACKNOWLEDGEMENTS

We are grateful to Prof. Philip Cohen (University of Dundee, UK) for providing us with purified protein phosphatases. This work was supported by the Muscular Dystrophy Association, the Israel Basic Research Fund (N.D.), Fond der Chemie, and Deutsche Forschungsgemeinschaft (F.H., V.F.).

REFERENCES

Armstrong DL and White RE (1992) *Trends Neurosci* **15** 403–408
Arreola J Calvo J Garcia MC and Sanchez JA (1987) *J Physiol* **393** 307–330
Barish ME (1983) *J Physiol* **342** 309–325
Biel M Ruth P Bosse E Hullin R Stuhmer W Flockerzi V and Hofmann F (1990) *FEBS Lett* **269** 409–412
Bosse E Regulla S Biel M Ruth P Meyer HE Flockerzi V and Hofmann F (1990) *FEBS Lett* **267** 153–156
Bourinet E Fournier F Lory P Charnet P and Nargeot J (1992) *Pflugers Arch* **421** 247–255
Campbell KP Leung AT and Sharp AH (1988) *Trends Neurosci* **11** 425–430
Catterall WA (1988) Science **242** 50–61
Chang FC and Hosey MM (1988) *J Biol Chem* **263** 18929–18937
Cohen P (1989) *Annu Rev Biochem* **58** 453–508
Dascal N Landau EM and Lass Y (1984) *J Physiol* **352** 551–574
Dascal N Snutch TP Lubbert H Davidson N and Lester HA (1986) *Science* **231** 1147–1150
Dascal N Lotan I Karni E and Gigi A (1992) *J Physiol* **450** 469–490
Dascal N and Lotan I (1992) "Expression of exogenous ion channels and neurotransmitter receptors in RNA-injected *Xenopus* oocytes" in Methods in Molecular Biology v 13: Protocols in Molecular Neurobiology (eds Longstaff A and Revest P) 205–225 Humana Press
De Jongh KS Warner C Colvin AA and Catterall WA (1991) *Proc Natl Acad Sci USA* **88** 10778–10782
Fernandez A Mery J Vandromme M Basset M Cavadore J-C and Lamb NJC (1991) *Exptl Cell Res* **195** 468–477
Flockerzi V Oeken H-J Hofmann F Pelzer D Cavalie A and Trautwein W (1986) *Nature* **323** 66 - 68
Gershon E Weigl L Lotan I Schreibmayer W and Dascal N (1992) *J Neurosci* **12** 3743–3752
Glossmann H and Striessnig J (1990) *Rev Physiol Biochem Pharmacol* **114** 1–105
Hartzell HC Mery P-F Fischmeister R and Szabo G (1991) *Nature* **351** 573–576
Hescheler J Mieskes G Ruegg JC Takai A and Trautwein W (1988) *Pflugers Arch* **412** 248–252
Hosey MM and Lazdunski M (1988) *J Membr Biol* **104** 81–105
Hullin R Freichel M Biel M Singer-Lahat D Dascal N Hofmann F and Flockerzi V (1992) *EMBO J* **11** 885–890
Hofmann F (1980) *J Biol Chem* **255** 1559–1564
Hofmann F Biel M and Flockerzi V (1993) *Annu Rev Neurosci* **17** 399–418
Jahn H Nastainczyk W Rohrkasten A Schneider T and Hofmann F (1988) *Eur J Biochem* **178** 535–542
Jay SD Ellis SB McCue AF Williams ME Vedvick TS Harpold MM and Campbell KP (1990) *Science* **248** 490–492
Kameyama M Hofmann F and Trautwein W (1985) *Pflugers Acrh* **405** 285–293
Kameyama M Hescheler J Hofmann F and Trautwein W (1986a) *Pflugers Arch* **407** 123–128
Kameyama M Hescheler J Mieskes G and Trautwein W (1986b) *Pflugers Arch* **407** 461–463
Klockner U Itagaki K Bodi I and Schwartz A (1992) *Pflugers Arch* **420** 413–415
Lacerda AE Kim HS Ruth P Perez-Reyes E Flockerzi V Hofmann F Birnbaumer L and Brown AM (1991) *Nature* **252** 527–530
Lory P Rassendren FA Richard S Tiaho F and Nargeot J (1990) *J Physiol* **429** 95–112
Lory P and Nargeot J (1992) *Biochem Biophys Res Commun* **182** 1059–1065
Mikami A Imoto K Tanabe T Niidome T Mori Y Takeshima H Narumiya S and Numa S (1989) *Nature* **340** 230–233
Miledi R (1982) *Proc Roy Soc London Ser B* **215** 491–497
Mori Y Friedrich T Kim M-S Mikami A Nakai J Ruth P Bosse E Hofmann F Flockerzi V Furuichi J Mikoshiba K Imoto K Tanabe T and Numa S (1991) *Nature* **350** 398–402
Mundina-Weilenmann C Chang CF Gutierrez LM and Hosey MM (1991) *J Biol Chem* **266** 4067–4073
Nastainczyk W Rohrkasten A Sieber M Rudolph C Schachtele C Marme D and Hofmann F (1977) *Eur J Biochem* **169** 137–142
Nunoki K Florio V and Catterall WA (1989) *Proc Natl Acad Sci USA* **86** 6816–6820
Perez-Reyes E Castellano A Kim HS Bertrand P Baggstrom E Lacerda AE Wei X and Birnbaumer L (1992) *J Biol Chem* **267**

1792–1797
Pragnell M Sakamoto J Jay SD and Campbell KP (1991) *FEBS Lett* **291** 253–258
Reuter H (1983) *Nature* **301** 569–574
Rohrkasten A Meyer HE Nastainczyk W Sieber M and Hofmann F (1988) *J Biol Chem* **263** 15325–15329
Rotman EI DeJongh KS Florio V Lai Y and Catterall WA (1992) *J Biol Chem* **267** 16100–16105
Ruth P Rohrkasten A Biel M Bosse E Regulla S Meyer HE Flockerzi V and Hofmann F (1989) *Science* **245** 1115–1118
Sather WA Tanabe T Zhag J-F Mori Y Adams ME and Tsien RW (1993) *Neuron* **11** 291–303
Shuba YM Hesslinger B Trautwein W McDonald TF and Pelzer D (1990) *J Physiol* **424** 205–228
Singer D Biel M Lotan I Flockerzi V Hofmann F and Dascal N (1991) *Science* **253** 1553–1557
Sculptoreany A Rotman E Takahashi M Scheuer T and Catterall WA (1993) *Proc Natl Acad Sci USA* **90** 10135–10139
Singer-Lahat D Gershon E Lotan I Hullin R Biel M Flockerzi V Hofmann F and Dascal N (1992) *FEBS Lett* **306** 113–118
Tanabe T Takeshima H Mikami A Flockerzi V Takahashi H Kangawa K Kojima M Matsuo H Hirose T and Numa S (1987) *Nature* **328** 313–318
Tiaho F Nargeot J and Richard S (1991) *Pflugers Arch* **419** 596–602
Schneider T and Hofmann F (1988) *Eur J Biochem* **174** 369–375
Trautwein W and Hescheler J (1990) *Annu Rev Physiol* **52** 257–274
Tsien RW (1983) *Annu Rev Physiol* **45** 341–358
Umbach JA and Gundersen CB (1987) *Proc Natl Acad Sci USA* **84** 5464–5468
Varadi G Lory P Schultz D Varadi M and Schwartz A (1991) *Nature* **352** 159–162
Welling A Felbel J Peper K and Hofmann F (1992) *Am J Physiol* **262** L351–L359
Williams ME Feldman DH McCue AF Brenner R Velicelebi G Ellis SB and Harpold MM (1992) *Neuron* **8** 71–84
Yatani A Codina J Imoto Y Reeves JP Burnbaumer L and Brown AM (1987) *Science* **238** 1288–1292
Yatani A and Brown AM (1989) *Science* **245** 71–74
Yoshida A Takahashi M Nishimura S Takeshima H and Kokubun S (1992) *FEBS Lett* **309** 343–349