A potential site of functional modulation by protein kinase A in the cardiac Ca^{2+} channel α_{1C} subunit

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Abstract The well-characterized enhancement of the cardiac Ca^{2+} L-type current by protein kinase A (PKA) is not observed when the corresponding channel is expressed in *Xenopus* oocytes, possibly because it is fully phosphorylated in the basal state. However, the activity of the expressed channel is reduced by PKA inhibitors. Using this paradigm as an assay to search for PKA sites relevant to channel modulation, we have found that mutation of serine 1928 of the α_{1C} subunit to alanine abolishes the modulation of the expressed channel by PKA inhibitors. This effect was independent of the presence of the β subunit. Phosphorylation of serine 1928 of α_{1C} may mediate the modulatory effect of PKA on the cardiac voltage-dependent Ca^{2+} channel.

Key words: Calcium channel; Protein kinase A; Modulation; Mutation; Subunit; *Xenopus* oocyte

1. Introduction

In a vast variety of animal species, the activity of the L-type (dihydropyridine-sensitive) voltage-dependent Ca^{2+} channels in the heart and skeletal muscle (SkM) is strongly enhanced by epinephrine and norepinephrine, mainly via the cAMP-PKA pathway [1–3]. It is widely believed that this modulation is due to phosphorylation of one of the subunits of the channel, catalyzed by PKA [2], and there is good, though not conclusive, evidence for such a direct mechanism in the SkM Ca^{2+} channel. Of the four subunits of this channel (α_{1S}, α_2/δ, β_1 and γ), two (α_{1S} and β_1) are substrates for PKA-catalyzed phosphorylation [4–6], and the channel activity is enhanced by PKA in native cells and after reconstitution of purified channel subunits in artificial membranes [7–9].

In contrast to SkM, the evidence for modulation of the cardiac L-type Ca^{2+} channel by a direct phosphorylation of one of its subunits by PKA is highly controversial. The minimal composition of the cardiac L-type channel is probably $\alpha_{1C} + \alpha_2/\delta + \beta_2$ [10–13]. The main, α_{1C}, subunit of the channel purified from adult cardiac tissue is a poor substrate for PKA [14,15]. A functional demonstration of modulation by PKA of the cardiac Ca^{2+} channel expressed in various cell types also proved difficult and often irreproducible. Several groups reported an enhancement by PKA of currents through cardiac Ca^{2+} channels formed by the α_{1C} subunit alone in CHO and HEK293 cells, although in some cases this increase was rather small or required a previous exposure of the channel to PKA inhibitors [16–18]. However, others [19,20] could not reproduce these results. When expressed in *Xenopus* oocytes, channels composed of cardiac or neuronal α_{1C} or $\alpha_{1C} + \alpha_2/\delta$ are insensitive to PKA or PKA-activating treatments [21–23]. An early report [24] that coexpression of the β subunit with α_{1C} renders the expressed channel sensitive to PKA also could not be confirmed [21–23].

It has been proposed that the failure of PKA to enhance the current via the expressed Ca^{2+} channel in *Xenopus* oocytes may be due to an abnormally high level of its basal phosphorylation [21,22], possibly because of a difference in the balance between phosphorylation and dephosphorylation processes in the heart and in the expression systems. This assumption is supported by the finding that PKA inhibitors reduce the Ca^{2+} current via channels formed by α_{1C} alone or with some or all of the auxiliary subunits in CHO and HEK293 cells and in *Xenopus* oocytes [17,20–22], suggesting that α_{1C} is the main target for this modulation. There is little doubt that the observed decrease in Ca^{2+} channel current is caused by dephosphorylation of PKA sites due to the activity of endogenous protein phosphatases, because the peptide and proteins used to inhibit PKA in these experiments are highly specific toward PKA [21]. If the target sites for dephosphorylation are located in the α_{1C} subunit, their elimination should abolish the effect of PKA inhibitors. Indeed, as reported below, we find that mutation of a single serine residue, Ser-1928, located in the C-terminal part of the α_{1C} subunit, renders the rabbit cardiac L-type Ca^{2+} channel expressed in *Xenopus* oocytes insensitive to PKA inhibitors, independently of the presence of the β_{2A} subunit.

2. Materials and methods

2.1. Oocyte preparation, RNA injection, and α_{1c} mutagenesis

The maintenance and surgery of the frogs, preparation of oocytes, and RNA injection were performed as described elsewhere [25]. RNAs specific for the cardiac α1C (GenBank accession number of cDNA: X15539), β_{2A} and the skeletal muscle α_2/δ subunits were synthesized in vitro using Asp-718-cleaved pCaH, *Not*I-cleaved pCaB2a and *Sal*I-cleaved pCaA2 as templates [12,26]. Capped α_1, α_2/δ and β_{2A} mRNAs were synthesized in vitro using SP6 (for α_1 and α_2/δ) and T7 (for β_{2A}) RNA polymerases, as described [25]. To study the $\alpha_1 + \alpha_2/\delta$ combination, 5 ng of each of the RNAs of α_{1C} and α_2/δ were injected, and the oocytes were incubated for 5–7 days before electrophysiological measurements. To study the $\alpha_1 + \alpha_2/\delta + \beta$ combination, 2.5 ng of each of the RNAs of α_{1C}, α_2/δ and β_{2A} were injected, and the oocytes were incubated for 3–5 days. Mutations S533I, st1665, S1700A and S1928A (see Fig. 2A) were generated by the oligonucleotide-directed in vitro mutagenesis kit (Amersham). In the mutation st1665, a stop codon (TAA) was inserted at the nucleotide position 5184; the resulting cDNA was predicted to encode an α_{1C} deletion mutant lacking all C-terminal amino acids residues starting from K1665. All C-terminal mutations were performed on a cassette created by excising a stretch of α_{1C} cDNA with *Bst*EII and Asp-718, which was subsequently inserted between the corresponding restriction sites of pSupEx-RCK1

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[27]. The mutation S533I was performed on a cassette created by excising a stretch of α_{1C} cDNA with *Mun*I and *Eco*RI which was subsequently inserted between the corresponding restriction sites of pB1-ShH4 [28]. The oligonucleotides were (underlined nucleotides indicate the mutated residue): 5'TGGAAATTCATCCGCTACTGG3' (S533I), 5'CAAGAAGCGCTAAGAGCAAGG3' (st1665), 5'ACGGGCCATCGCCGGAGACCT3' (S1700A), 5'GTCGAAGGGCTGCCTTCCACCTG3' (S1928A). The deletion mutant ΔN_{88-139} was created in two steps. First, a PCR procedure was used to produce a stretch of nucleotides between bases A_{609} and G_{2025} (the latter lies within a unique *Stu*I site), with a forward primer 5'CTCTGCCTCATCGATAAGAACCCCATCCGGA3' (which introduced a *Cla*I site before the position corresponding to the amino acid residue K140), and a reverse primer 5'GACACGAAATAGGCCTGCAG3'. The PCR product was digested with *Cla*I and *Stu*I and subcloned into the cDNA of α_{1C}, replacing the original DNA stretch located between these sites. This procedure eliminated amino acid residues 88 through 139 from the α_{1C} protein. The presence of the designed mutations was confirmed by DNA sequencing as described [27].

2.2. Electrophysiological procedures and data analysis

Whole cell currents were recorded using two-electrode voltage clamp with the Geneclamp 500 amplifier (Axon Instruments, Foster City, CA) as described [12], in a solution containing 40 mM $Ba(OH)_2$, 50 mM NaOH, 2 mM KOH and 5 mM HEPES, titrated to pH 7.5 with methanesulfonic acid. Current-voltage (I-V) relations of the Ba^{2+} current (I_{Ba}) were obtained by stepping the membrane potential from the holding potential (−80 mV) to various voltages in 10 mV steps. Leak currents were subtracted during the analysis session. Data acquisition and analysis were done with pCLAMP (Axon Instruments). A full I-V relation was measured at least in 4 oocytes of each group and, except one experiment (effect of H89 on the channel composed of $\alpha_{1C} + \alpha_2/\delta + \beta_{2A}$; Fig. 3B), each experiment was repeated at least in two different batches of oocytes. To enable comparison between various batches, peak I_{Ba} (measured at +20 mV) in each cell was expressed as percent of the average peak current in the control group of the same oocyte batch, and the normalized currents were averaged across all batches. I_{Ba} in cells of the control groups was normalized in the same way, in order to obtain an estimate of variability of I_{Ba} in these groups. Averaged data are presented as mean ± S.E.M. Whenever two groups were compared, Student's *t*-test was used; for comparison of many groups, non-parametric one-way ANOVA followed by Dunn's paired test was performed.

2.3. Materials

(R_p)-8-Bromoadenosine 3',5' cyclic monophosphorothioate (Rp-cAMPS) and H89 were from Biolog (Bremen, Germany). Rp-cAMPS was dissolved in water at 0.5 M and stored at −20°C. H89 was dissolved in DMSO at 50 mM and stored at −20°C. The final concentration of DMSO in media used for the treatment of the cells with H89 was 0.1%; DMSO alone had no effect on I_{Ba} at this concentration. Materials for molecular biology were from Boehringer-Mannheim and Promega. Other materials were from Sigma.

3. Results

In a previous work [21], we have shown that, in *Xenopus* oocytes, intracellular injection of protein phosphatase 1, or of specific peptide and protein inhibitors of PKA, reduces the current carried by Ba^{2+} (I_{Ba}) via the rabbit cardiac L-type Ca^{2+} channel of various subunit combinations by 30–40% at 0 mV. This tedious and time-consuming procedure proved inefficient for the screening of a large amount of cells that was necessary in order to test a number of mutant α_{1C} channels. Therefore, we have used a procedure in which groups of oocytes were incubated for 60–90 min in the absence (control) or in the presence of cell-permeable PKA inhibitors, Rp-cAMPS (0.5–1 mM) or H-89 (50 μM). An oocyte was then transferred to the regular high-Ba^{2+} recording solution, and I_{Ba} was measured within 5 min. On the average, this treatment reduced the peak I_{Ba} (at +20 mV) through channels of

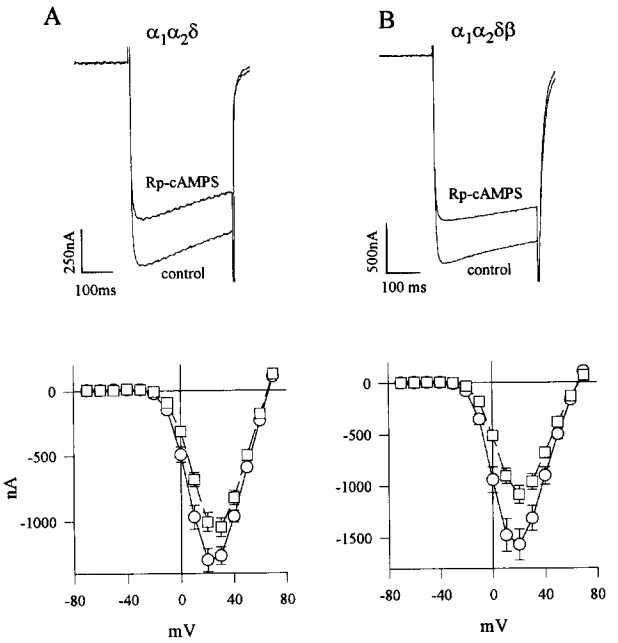

Fig. 1. Treatment by Rp-cAMPS reduces the Ba^{2+} current via cardiac L-type Ca^{2+} channels of $\alpha_{1C}+\alpha_2/\delta$ (A) or $\alpha_{1C} + \alpha_2/\delta + \beta_{2A}$ (B) composition. Upper panels show representative Ba^{2+} currents in oocytes of one batch, measured by voltage steps from −80 to +20 mV. Lower panels show I-V curves averaged from control and Rp-cAMPS-treated oocytes of one batch (n = 6–9 oocytes in each group).

$\alpha_1 + \alpha_2/\delta$ or $\alpha_1 + \alpha_2/\delta + \beta$ composition by about 20% (Fig. 1). The extent of reduction was similar in channels of these two subunit combinations, in accord with our previous findings [21] (see Fig. 3A). The Rp-cAMPS treatment did not cause major changes in the voltage dependence of the Ba^{2+} current (Fig. 1), although a slight (3–5 mV) shift to positive voltages was observed when normalized I-V curves were compared (not shown). Although the extent of reduction of I_{Ba} was relatively small, it was reproducible in all oocyte batches. Note that, due to the shift in I-V curve, the Rp-cAMPS-induced inhibition was more pronounced at negative voltages. For example, in the batch of oocytes shown in Fig. 1, in the $\alpha_1 + \alpha_2/\delta$ combination, the inhibition was 21% at +20 mV, but 35% at 0 mV, comparable to what was observed with intracellular injections. Unfortunately, in some of the mutant channels, the currents were too small to be reliably measured at 0 mV (see below), and the amplitudes of the Ba^{2+} currents were always compared at +20 mV in the following.

Rabbit cardiac α_{1C} protein contains 6 putative 'classical' PKA phosphorylation sites in the cytoplasmic domains [29]. These are S124 located in the N-terminal part, and several serines located in the C-terminal part at the positions 1575, 1627, 1700, 1848, and 1928. In initial experiments, we observed no inhibition by Rp-cAMPS of I_{Ba} via channels composed of α_2/δ, β_{2A}, and a deletion mutant of α_{1C}, α_{1C}(st1665), that lacked all the C-terminal amino acid residues beyond 1664 (data not shown), suggesting that a functionally important PKA site in α_{1C} is located in the C terminus beyond this residue. The Ba^{2+} currents via channels of the α_{1C}(st1665) + α_2/δ composition were too small to be measured reliably, and it was impossible to assess the extent of regulation of the mutant channel in the absence of the β subunit. We have then created a series of mutations of α_{1C} (Fig.

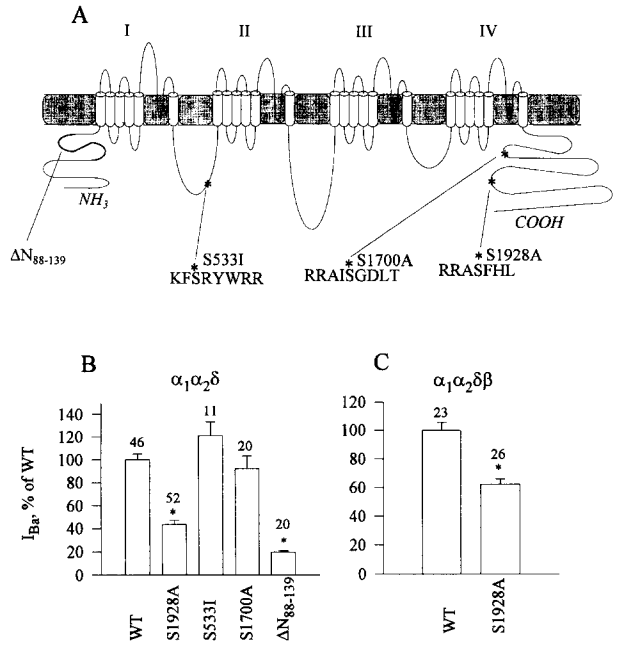

Fig. 2. Expression of α_{1C} mutants in the oocytes. A: Schematic presentation of the α_{1C} subunits and of the mutations made in it. B and C: Average amplitudes of Ba^{2+} currents in WT and mutants channels composed of $\alpha_{1C} + \alpha_2/\delta$ (B) and $\alpha_{1C} + \alpha_2/\delta + \beta_{2A}$ (C). Whole-cell Ba^{2+} currents in these experiments were 729 ± 70 nA ($\alpha_{1C} + \alpha_2/\delta$, $n = 46$) and 2055 ± 142 nA ($\alpha_{1C} + \alpha_2/\delta + \beta_{2A}$, $n = 23$) at +20 mV. The numbers above the bars indicate the numbers of cells tested. Asterisks indicate statistically significant differences ($P < 0.05$) obtained in two-tailed t-tests (C) or in a multiple group comparison (B) as described in section 2.

2A), two of which (single site mutations S1700A and S1928A) eliminated prominent putative PKA phosphorylation sites from the C terminus, one (a ΔN_{88-139} deletion) eliminated an N-terminus PKA site (S124), and one (single site mutation S533I) eliminated a serine that is a putative protein kinase C (but not PKA) site. Channels containing the mutants α_{1C}(S1928A) and $\alpha_{1C}(\Delta N_{88-139})$, in combination with α_2/δ, gave rise to whole-cell Ba^{2+} currents that were significantly smaller than in control channels containing the wild type (WT) α_{1C} (Fig. 2B). A significantly smaller I_{Ba} in channels containing the α_{1C}(S1928A) mutant was also observed in the presence of the β_{2A} subunit (Fig. 2C).

The S1928A mutation completely eliminated the inhibitory effect of Rp-cAMPS on I_{Ba} (Fig. 3A). In all other mutants, there were no significant differences in the extent of inhibition caused by Rp-cAMPS (Fig. 3A). Another PKA inhibitor, H89, which was somewhat more potent than Rp-cAMPS in reducing the WT I_{Ba}, also failed to reduce the current via channels containing the α_{1C}(S1928A) mutant (Fig. 3B). The presence of the β_{2A} subunit did not affect the inhibition of I_{Ba} by Rp-cAMPS or H89, and did not restore their effect on the channel containing the S1928A mutation (Fig. 3A,B).

4. Discussion

The main finding of this study is that the elimination of a single serine residue in the C-terminus of the pore-forming α_{1C} subunit of the L-type Ca^{2+} channel, serine 1928, suppresses the ability of PKA inhibitors to modulate (reduce) the current. This strongly suggests that a direct phosphorylation by PKA of this amino acid residue is involved in modulation of the channel's activity. Since our assay involved the measurement of effects of dephosphorylation (as a result of inhibition of the basal activity of PKA) rather than phosphorylation, it is not clear whether S1928 is the site whose phosphorylation by PKA normally causes the Ca^{2+} current increase in cardiac cells. However, if the hypothesis [17,21,22] that the inability of PKA to enhance the channel's activity is due to an abnormally high level of basal phosphorylation in expression systems is correct, then there is a good chance that S1928 is indeed the functionally important site whose phosphorylation by PKA underlies most of the adrenergic regulation of the cardiac Ca^{2+} channel. It is noteworthy that channels containing the α_{1C}(S1928A) mutant displayed a strongly reduced current amplitude. This is in line with the notion that the WT α_{1C} expressed in the oocytes is normally highly phosphorylated by PKA; α_{1C}(S1928A) cannot be phosphorylated and, therefore, conducts less current.

Serine 1928 is conserved in neuronal and smooth muscle splice variants of α_{1C} [30–34]. It is unclear why the smooth muscle L-type Ca^{2+} currents are not enhanced by PKA [35–38]. It may be hypothesized, by analogy with oocytes and CHO cells, that in smooth muscle cells the channel is fully phosphorylated; another possibility is that a majority of the channels contain a truncated α_{1C} subunit that, due a post-translational modification, loses a part of its C-terminus including the residue analogous to S1928. Posttranslational C-terminus truncation is well documented in the α_{1S} subunit of the skeletal muscle [5,39–41] and has been reported in the cardiac α_{1C} protein [18]. Further studies will be needed to clarify this issue. However, C-terminal truncation of α_{1C} cannot explain the lack of an enhancing effect of PKA on cardiac L-type Ca^{2+} channels expressed in CHO cells and in *Xenopus* oocytes, for several reasons. (1) The α_{1C} protein in the plasma membrane of the oocytes and the membrane fraction of CHO cells is detected by a site-specific antibody directed against the very end of the C-terminus [42,43]. In both cases, a comparable amount of α_{1C} was detected by a different antibody directed against an intracellular loop between repeats II and III, suggesting that most of the expressed protein was not truncated ([43]; T. Ivanina and N. Dascal, unpublished observations). (2) The fact that mutation of S1928 to alanine has a robust effect on the channel modulation by PKA inhibitors suggests that, normally, this residue is present in a significant fraction of the functional WT channels.

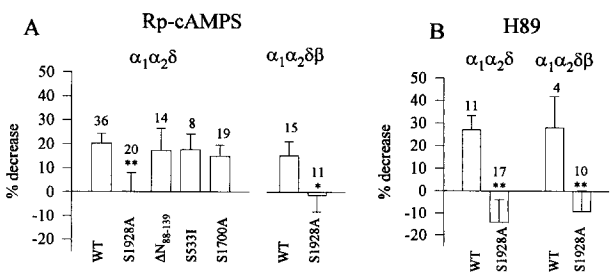

Fig. 3. The effects of PKA inhibitors on Ba^{2+} currents through WT and mutant cardiac Ca^{2+} channels. Ordinate presents the decrease caused in peak I_{Ba} by 0.5–1 mM Rp-cAMPS (A) and 50 μM H89 (B). Double asterisks (**) indicate statistically significant differences ($P < 0.05$) (A, left panel). A single asterisk (*) indicates $P < 0.05$ in a one-tailed but not a two-tailed t-test (A, right panel).

In light of our findings, it is interesting to compare the prominent PKA phosphorylation sites in cardiac α_{1C} and the SkM α_{1S}. The major sites phosphorylated by PKA in the α_{1S} protein in vitro and in vivo are serines 1757 and 1854 in the full-length form [5,44], and serine 687 in the C-terminal truncated form [4,44]. None of these residues are conserved in α_{1C}. Serine 1928 of α_{1C} is not conserved in α_{1S}. One implication of these differences is that the molecular mechanisms of modulation of cardiac and skeletal muscle L-type Ca^{2+} channels by PKA may be dissimilar, in spite of the homology in structure and the analogy in the physiological effects of this modulation.

A cardiac β subunit is a substrate for PKA phosphorylation [15], and it has been proposed that the positive modulation of the cardiac L-type Ca^{2+} channel may be due to phosphorylation of this subunit [10,15]. The presence of β may be necessary for a voltage-dependent facilitation of Ca^{2+} channel current which, in turn, may be modulated by PKA [22]. However, most studies in expression systems do not support a role for the β subunit, since coexpression of β with α_{1C} does not restore the positive modulation of the channel by PKA in *Xenopus* oocytes and in CHO cells and does not significantly alter the reducing effects of PKA inhibitors [20–23]. Our finding that the removal of a single PKA site in α_{1C} completely eliminates the effects of PKA inhibitors, and that the presence of the β_{2A} subunit does not restore these effects, also argues against the involvement of the β subunit in PKA modulation in the expression systems. However, since it appears that the PKA phosphorylation–dephosphorylation equilibrium is different (and possibly unique) in the heart cells, such a role for β there cannot be excluded.

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