The N terminus of the Cardiac L-type Ca$^{2+}$ Channel $\alpha_{1C}$ Subunit

THE INITIAL SEGMENT IS UBQUITOUS AND CRUCIAL FOR PROTEIN KINASE C MODULATION, BUT IS NOT DIRECTLY PHOSPHORYLATED

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The first 46 amino acids (aa) of the N terminus of the rabbit heart (RH) L-type cardiac Ca$^{2+}$ channel $\alpha_{1C}$ subunit are crucial for the stimulatory action of protein kinase C (PKC) and also hinder channel gating (Shistik, E., Ivanina, T., Blumenstein, Y., and Dascal, N. (1998) J. Biol. Chem. 273, 17901–17909). The mechanism of PKC action and the location of the PKC target site are not known. Moreover, uncertainties in the genomic sequence of the N-terminal region of $\alpha_{1C}$ leave open the question of the presence of RH-type N terminus in L-type channels in mammalian tissues. Here, we demonstrate the presence of $\alpha_{1C}$ protein containing an RH-type initial N-terminal segment in rat heart and brain by using a newly prepared polyclonal antibody. Using deletion mutants of $\alpha_{1C}$ expressed in Xenopus oocytes, we further narrowed down the part of the N terminus crucial for both inhibitory gating and for PKC effect to the first 20 amino acid residues, and we identify the first 5 aa as an important determinant of PKC action and of N-terminal effect on gating. The absence of serines and threonines in the first 5 aa and the absence of phosphorylation by PKC of a glutathione S-transferase-fusion protein containing the initial segment suggest that the effect of PKC does not arise through a direct phosphorylation of this segment. We propose that PKC acts by attenuating the inhibitory action of the N terminus via phosphorylation of a remote site, in the channel or in an auxiliary protein, that interacts with the initial segment of the N terminus.

Voltage-dependent L-type Ca$^{2+}$ channels regulate contraction of cardiac and smooth muscle and excitability and gene expression in the brain (2–4). They consist of three subunits: $\alpha_1$ (main, pore-forming subunit), $\beta$, and $\alpha_2\delta$. The $\alpha_1$ subunits in the heart, smooth muscle, and brain are products of the $\alpha_{1C}$ gene (5). The existence of several cDNA isoforms and the genomic sequence of the $\alpha_{1C}$ DNA suggest the presence of splice variants of RNA and thus of several isoforms of the $\alpha_{1C}$ protein (6–8), but the actual composition of $\alpha_{1C}$ protein isoforms in tissues is still poorly characterized.

The $\alpha_{1C}$ subunit appears to be the main target for modulation by protein kinases A and C (PKA and PKC, respectively), although $\beta$ is also a substrate (9). Both kinases increase the activity of the channel (10–12). PKC has been proposed to mediate the enhancement of L-type Ca$^{2+}$ channels by intracellular ATP (13), angiotensin II (14), glucocorticoids (15), PACAP (16), and arginine-vasopressin (17). After the initial enhancement by PKC-activating phorbol esters, the Ca$^{2+}$ current is often decreased (18, 19), but it is not clear whether the inhibition is phosphorylation-related (20, 21). The dual effect of PKC activators is fully reconstituted in Xenopus oocytes expressing $\alpha_{1C}$ with or without $\alpha_2\delta$ and/or $\beta$; the presence of $\beta$ enhances the action of PKC (21, 22). In the nerve cells, either stimulation (23–26) or inhibition (27–29) of L-type channels by PKC has been reported.

The $\alpha_1$ subunit is composed of four homologous membrane-spanning internal domains, each with six transmembrane $\alpha$-helices and a pore-forming reentrant P loop (30). C and N termini and linkers between domains I-II, II-III, and III-IV are cytosolic. The initial 46 aa of the N terminus of rabbit heart (RH) $\alpha_{1C}$ are crucial for PKC modulation (1). The cytosolic N-terminal part of RH $\alpha_{1C}$ is 154 aa long. Deletion of the first 40 aa or more causes a 5–10-fold increase in the current via RH-type Ca$^{2+}$ channels expressed in Xenopus oocytes (1, 31). This is a result of a change in channel gating because the truncation causes an increase in open probability, without increasing the amount of $\alpha_{1C}$ protein in the plasma membrane (1). These and additional findings led us to propose that the N terminus of $\alpha_{1C}$ acts as an inhibitory gate, and its removal enhances channel activation; PKC increases the current by attenuating the inhibitory action of the N terminus (1). It is not known whether PKC phosphorylates the N terminus.

Despite the importance of the first 46 aa of the RH-type N terminus, its presence in L-type channel proteins in vivo remains uncertain. The only other cDNA of $\alpha_{1C}$ containing a stretch encoding this protein sequence is that cloned from rat aorta and heart (6). $\alpha_{1C}$ cDNAs cloned from rabbit lung, human heart, and rat brain (7, 32–34) do not encode this stretch (see Fig. 2A). It has been proposed that these variations correspond to splice variants of the $\alpha_{1C}$ gene (7), but even this is not certain. The structure of the genomic DNA of human $\alpha_{1C}$ has not been fully resolved in this region; none of the known exons correspond to the RH-type N terminus (8). In contrast, a recent study that utilized an RNase protection assay showed that RNA of the RH-type initial segment is predominant in human heart (35). These discrepancies make it important to clarify whether L-type Ca$^{2+}$ channel isoforms with the RH-type N terminus are common in mammalian tissues.

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§ The abbreviations used are: PKC, protein kinase C; aa, amino acid; GST, glutathione S-transferase; PCR, polymerase chain reaction; PKA, protein kinase A; PMA, 4-b-phorbol 12-myristate 13-acetate; RH, rabbit heart; WT, wild-type; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid.

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α1C Segment Crucial for PKC Effect and for Inhibitory Gating

Here we demonstrate the abundance of the RH-type N terminus in rat heart and brain and map the segment critical for PKC modulation and for inhibition of gating to the very beginning of the N terminus. Our results strongly suggest that PKC effect is not mediated by phosphorylation of this initial segment.

EXPERIMENTAL PROCEDURES

cDNA Constructs and mRNA—cDNAs and RNAs of RH α1C and α2β6 were as described (1). To create α1C N-terminal truncations, PCR amplification with Vent polymerase (New England Biolabs) was performed for α1C aN1–169, and α1C aN1–ΔN3–151, introducing a SalI site followed by an initiation codon (ATG) and then by the original wild-type (WT) α1C sequence starting from the desired base (amino acid numbers correspond to the RH α1C sequence (36)).

The cDNA constructs of the GST fusion proteins of the whole N terminus (N1–169), and of the loop I–II were described previously (1). The cDNAs for N1–154, and N1–125 (encoding the corresponding αC segments) were made by a PCR procedure and inserted into EcoRI and NotI sites of pGEX-4T-1 (Amersham Pharmacia Biotech) and thus linked in-frame to GST. N1–4644A, with serine 44 replaced by alanine, and a cDNA for N-neuronal (N1–124, encoding aa 1–124 of rat brain α1C (37)), were inserted into the same sites of pGEX-4T-1. All PCR products were sequenced at the Tel Aviv University Sequencing Facility. Fusion proteins were generated by transformation into Escherichia coli strain BL21 (Stratagene) followed by induction with 1 mCi isopropyl-thio-b-D-galactopyranoside and affinity purification with GST beads (Amersham Pharmacia Biotech) and elution with 15 or 20 mM reduced glutathione. In the preparation of N1–4644A, protease inhibitors were used: proteinase (10 μg/ml), benzamidine (5 mM), Pefabloc SC (0.2 mM), and EDTA (1 mM). N1–4644A was additionally dialyzed to 0.1 mM ammonium acetate buffer, pH 7.0, aliquoted, and lyophilized. Materials and enzymes for molecular biology were purchased from Roche Molecular Biochemicals, Promega, or MBF Fermentas.

Oocytes and Electrophysiology—Xenopus laevis frogs were maintained and dissected as described (37). Oocytes were injected with equal amounts (by weight) of the mRNAs of α1C, or its mutants, of α2β6, and, in the experiment shown in Fig. 2F, of α2A (2.5 ng for electrophysiological, 5 ng for biochemical experiments) and incubated for 3–5 days at 20–22 °C in NDE96 solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1 mM CaCl2, 2.5 mM Na-pyruvate, 50 μg/ml gentamycin, 5 mM HEPES, pH 7.5). Whole cell currents were recorded using the Gene Clamp 500 amplifier (Axon Instruments, Foster City, CA) using the two-electrode voltage clamp technique 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 (37). Stimulation, data acquisition, and analysis were performed using pCLAMP software (Axon Instruments). Ba2+ currents were measured by a 200-ms step to 20 mV from a holding potential of −80 mV. To study the effect of PMA (10 μM), the voltage pulses were delivered every 10–20 s (see Ref. 1 for details of PMA use).

Antibodies—Card-I and Card-C antibodies were kindly provided by M. M. Hosey (Northwestern University, Chicago, IL) (38). A new antibody (Card-N) was raised against the GST fusion protein of the first 46 aa of the RH-type α1C N terminus was raised in rabbit (since a GST-fusion construct containing the WT 46 aa of RH α1C appeared to degrade in the course of bacterial synthesis, Card-N was actually raised against a more stable fusion protein, N1–4644A, which in serine 44 was mutated to alanine). Card-N was compared with two previously characterized antibodies, Card-I (against residues 812–929 of the II-II′ domain linker) and Card-C (against residues 2156–2169 in the end of the C terminus) (37, 38). Card-N immunoprecipitated the WT RH α1C protein expressed in Xenopus oocytes and metabolically labeled with [35S]methionine/cysteine (Fig. 1A, lane 2), but not the truncated mutant missing the first 46 aa, α1C aN1–ΔN2–46 (Fig. 1A, lane 1). Card-I and Card-C immunoprecipitated both the WT α1C and α1C aN1–ΔN2–46 (Fig. 1A, lane B), the level of expression of the full-length α1C was higher than that of α1C aN1–ΔN2–46 (Fig. 1B), as reported previously (1). Card-C precipitated the WT channel more efficiently than Card-N (Fig. 1A). No [35S]-labeled α1C was detected in oocytes that were not injected with RNA by any of the antibodies (Fig. 1, A and B). The fact that bands of the same size of WT α1C are detected by antibodies directed to the extreme N and C termini and a mid-portion of the channel supports the notion (37) that the oocyte expresses the whole-length protein not truncated at any of its termini. Notably, under the conditions used here, the WT α1C protein runs on SDS-polyacrylamide gels as an ~207-kDa band, as reported previously in the oocytes (1, 37). Because the calculated molecular mass of this protein is ~142 kDa (36), the error (underestimate) of its size is about 35 kDa. The overestimation may result from the established fact that hydrophobic proteins tend to run on SDS-polyacrylamide gels faster than standard, water-soluble molecular mass markers (40).

Western blots of rat ventricular membranes with Card-N revealed a major band at ~207 kDa and a minor band at ~160 kDa; labeling of both bands was completely suppressed in the

RESULT AND DISCUSSION

The RH-type N Terminus Is Present in Rat Heart and Brain—A polyclonal antibody (Card-N) directed against a GST fusion protein of the first 46 aa of the RH-type α1C N terminus was raised in rabbit (since a GST-fusion construct containing the WT 46 aa of RH α1C appeared to degrade in the course of bacterial synthesis, Card-N was actually raised against a more stable fusion protein, N1–4644A, which in serine 44 was mutated to alanine). Card-N was compared with two previously characterized antibodies, Card-I (against residues 812–929 of the II-II′ domain linker) and Card-C (against residues 2156–2169 in the end of the C terminus) (37, 38). Card-N immunoprecipitated the WT RH α1C protein expressed in Xenopus oocytes and metabolically labeled with [35S]methionine/cysteine (Fig. 1A, lane 2), but not the truncated mutant missing the first 46 aa, α1C aN1–ΔN2–46 (Fig. 1A, lane 1). Card-I and Card-C immunoprecipitated both the WT α1C and α1C aN1–ΔN2–46 (Fig. 1A, lane B), the level of expression of the full-length α1C was higher than that of α1C aN1–ΔN2–46 (Fig. 1B), as reported previously (1). Card-C precipitated the WT channel more efficiently than Card-N (Fig. 1A). No [35S]-labeled α1C was detected in oocytes that were not injected with RNA by any of the antibodies (Fig. 1, A and B). The fact that bands of the same size of WT α1C are detected by antibodies directed to the extreme N and C termini and a mid-portion of the channel supports the notion (37) that the oocyte expresses the whole-length protein not truncated at any of its termini. Notably, under the conditions used here, the WT α1C protein runs on SDS-polyacrylamide gels as an ~207-kDa band, as reported previously in the oocytes (1, 37). Because the calculated molecular mass of this protein is ~142 kDa (36), the error (underestimate) of its size is about 35 kDa. The overestimation may result from the established fact that hydrophobic proteins tend to run on SDS-polyacrylamide gels faster than standard, water-soluble molecular mass markers (40).
PKC Modulation—Deletion of the first 46 aa residues of the protein N1–46(S44A). 10 ml of the antibody (dilution 1:1000) were incubated overnight at 4 °C with 80 lanes added, and the antibody/GST fusion protein mixture (lane 1) was coexpressed with α/β. In each lane, immunoprecipitates from 5 oocytes were loaded. C, Western blot of rat ventricular membranes with the Card-N antibody in the absence (lane 2) or presence (lane 1) of the GST-fusion protein N1–46(S44A). 10 ml of the antibody (dilution 1:1000) were incubated overnight at 4 °C with 80 ml of the antibody without N1–46(S44A) (lane 2), were incubated with the nitrocellulose membranes for 2 h at room temperature. D, detecting the Ca2+ channel α1c subunit isoforms in ventricle, brain, and liver by immunoblots with Card-N, Card-I, and Card-C.

The presence of the N1–46(S44A) GST-fusion protein against which the antibody was raised (Fig. 1C). This result confirms the specificity of Card-N and demonstrates, for the first time on the protein level, the presence of an RH-type initial segment in the N terminus of α1C in the rat heart.

Western bios of rat heart (ventricle), brain, and liver were done with all three antibodies (Fig. 1D). Card-C antibody detected an ~207-kDa band in the ventricle. A higher, ~240-kDa band was observed in all tissues. However, we cannot discard the possibility that this labeling is nonspecific because this band was not detected by the other two antibodies. The Card-I and Card-N antibodies detected the ~207-kDa band in ventricle and in the brain, although labeling with Card-I was weak. This may be because of sequence divergence between the rabbit cardiac α1C and isoforms of rat brain channel that show variability in the loop II-III (41). With Card-N, the intensity of this band varied in different bios, and in some instances it was even stronger in the brain than in the ventricle (data not shown). Because brain α1C was not detected by Card-C but detected by Card-N, the very end of the C terminus in a majority of brain α1C protein may be missing or different from the cardiac one. An additional band at ~160 kDa was detected in the liver by Card-I and Card-N but not by Card-C; Card-N detected a similar band in the ventricle. These results support the notion (42) that only a small percentage of the L-type channel in the heart is truncated at the end of its C terminus (see also Ref. 43). The actual size of the truncated protein is probably higher than 160 kDa because of the underestimation of the size by SDS-polyacrylamide gel electrophoresis in our conditions. Because α1C RNA has been found in whole liver but not in hepatocytes (44), the ~160-kDa protein may be present in nonhepatocyte tissues, e.g., in the blood vessels.

The main conclusion of this part of the study is that α1C protein containing the RH-type N terminus is present in rat heart and brain, and the L-type Ca2+ current in these tissues can be expected to be stimulated by PKC. The variability of PKC effects on neuronal L-type channels (23–29) may result from the presence of different isoforms of α1C in different neuronal cell types.

The First Five Amino Acid Residues of α1C. Are Critical for PKC Modulation—Deletion of the first 46 aa residues of the α1C, which are unique to the RH-type N terminus (Fig. 2A), increases the Ca2+ channel current and also eliminates the PKC-induced augmentation (1). The effects of deletions shorter than 40 aa have not been studied (1, 45). To narrow down the

segment crucial for PKC effect, we have prepared three additional deletion mutants: α1C-N2–5, α1C-N2–20, and α1C-N2–25, lacking aa 2–5, 2–20, and 2–25, accordingly. All truncated channels produced whole-cell currents 3–10-fold larger than WT in >5 oocyte batches. For a quantitative comparison, RNAs of WT and of four deletion mutants were prepared on the same day, in parallel, and injected (together with α/β subunit) into oocytes of the same donor. Fig. 2B shows that deletion of the first 20 aa was sufficient to cause a maximal current increase, similar to that produced by the deletion of 46 or 139 aa. Deletion of aa 2–5 also significantly (p < 0.01) increased the current but less well than of 20 or more aa. Because coexpression of the β2A subunit increased WT currents better than those of N-terminal truncation mutants α1C-N2–46 and α1C-N2–39, we proposed that part of β2A-induced enhancement is because of an allosteric hindrance of the N-terminal inhibition of gating (1). Fig. 2F shows that the enhancement of peak currents of α1C-N2–5 and α1C-N2–20 caused by coexpression of β2A is also weaker than that of the WT channels; this was observed at all voltages. Thus, the first 20 aa are crucial for the inhibitory effect of the N terminus on L-type channel gating and on its interaction with β2A, and the first 5 aa constitute an important component.

Fig. 2 shows representative current traces (Fig. 2C and 2E) and time course of the effect of the phorbol ester PMA (Fig. 2D) in oocytes expressing WT, ΔN2–5, or ΔN2–20 α1C (with the α/β subunit). Two oocyte batches in which the WT channel showed high sensitivity to PMA, and the Ca2+ channel current was increased 1.6–4-fold (summarized in Fig. 2E), have been used in these experiments. The removal of aa 2–5 reduced the PMA effect by more than 90%; the remaining increase (13 ± 4.4%; mean ± S.E.) was small but statistically significant (p < 0.02). Channels lacking the first 20 aa were insensitive to PMA (6.4 ± 8.1%). Thus, the first 5 aa are very important, and the first 20 aa are crucial for the PKC effect.

PKC Does Not Phosphorylate the Segment Crucial for Its Physiological Effect—Based on effects of PKC activators and inhibitors, the effect of PMA is expected to result from a PKC-catalyzed phosphorylation (21, 22). Because none of the first 5 aa of α1C are serines or threonines (Fig. 2A), it is not possible that PKC directly phosphorylates this segment. None of the residues in the first 20 aa is a consensus PKC site, but a cryptic site (T10 or S18) might be a target for PKC. Therefore, we have examined in vitro phosphorylation by purified PKC of GST-fusion proteins of segments of the N terminus: N1–46(S44A)}
N1–154 (the whole N terminus), N47–154, and N84–154. As controls, we used GST and the GST fusion proteins of the loop I-II of RHα1C and of the N terminus of the rat brain α1C, N(neuronal)1–124. Fig. 3 shows that N1–154 and N47–154 were strongly phosphorylated; weaker signals were observed in N84–154 and in N(neuronal)1–124. GST alone, N1–46(S44A), and loop I-II were not phosphorylated. Thus, the first 20 aa, contained within the GST fusion protein N1–46(S44A), are not phosphorylated under these conditions, whereas other parts of the N terminus are. The physiological significance of the phosphorylation of these distal parts of the N terminus is unclear at present.

In summary, our results demonstrate the presence of α1C protein isoform(s) containing an RH-type N terminus in rat heart (ventricle) and brain. Further, we have demonstrated that the initial 20 amino acids are crucial both for the inhibitory gating by the N terminus, the allosteric interaction of the N terminus with the β subunit, and for the PKC effect. Removal of the first 5 aa already strongly hampers the inhibitory function of the N terminus and almost fully abolishes the PKC effect. The correlation between the location of residues crucial for these two functions supports the hypothesis (1) that PKC exerts its stimulating action by attenuating the inhibition imposed on the channel by the N terminus.

Determination of the mechanism of PKC action is a challenge for the future. Our data suggest that the effect of PKC is not attained by a direct phosphorylation of the initial 20 aa of α1C.
the N terminus. This conclusion is supported both by the amino acid composition of this segment, especially of the first 5 aa crucial for PKC action, and by the absence of phosphorylation of the GST fusion protein containing the initial segment. What can be the mechanism of PKC action? There are at least two possibilities. PKC may phosphorylate a site at αIC which is remote from the initial N-terminal segment but interacts with it directly or allosterically. If such interaction is permissive for N-terminal effect on gating (inhibition), phosphorylation by PKC may weaken the inhibition. Another possibility is that PKC phosphorylates an auxiliary protein, yet unidentified, which either aids the N-terminal inhibition (and the phosphorylation by PKC) or attenuates the N-terminal inhibition when phosphorylated by PKC.

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