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Crucial Role of N Terminus in Function of Cardiac L-type Ca^{2+} Channel and Its Modulation by Protein Kinase C*

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The role of the cytosolic N terminus of the main subunit (α_{1C}) of cardiac L-type voltage-dependent Ca^{2+} channel was studied in *Xenopus* oocyte expression system. Deletion of the initial 46 or 139 amino acids (a.a.) of rabbit heart α_{1C} caused a 5–10-fold increase in the whole cell Ca^{2+} channel current carried by Ba^{2+} (I_{Ba}), as reported previously (Wei, X., Neely, A., Olcese, R., Lang, W., Stefani, E., and Birnbaumer, L. (1996) *Recept. Channels* 4, 205–215). The plasma membrane content of α_{1C} protein, measured immunochemically, was not altered by the 46-a.a. deletion. Patch clamp recordings in the presence of a dihydropyridine agonist showed that this deletion causes a ~10-fold increase in single channel open probability without changing channel density. Thus, the initial segment of the N terminus affects channel gating rather than expression. The increase in I_{Ba} caused by coexpression of the auxiliary β_{2A} subunit was substantially stronger in channels with full-length α_{1C} than in 46- or 139-a.a. truncated mutants, suggesting an interaction between β_{2A} and N terminus. However, only the I–II domain linker of α_{1C}, but not to N or C termini, bound β_{2A} *in vitro*. The well documented increase of I_{Ba} caused by activation of protein kinase C (PKC) was fully eliminated by the 46-a.a. deletion. Thus, the N terminus of α_{1C} plays a crucial role in channel gating and PKC modulation. We propose that PKC and β subunit enhance the activity of the channel in part by relieving an inhibitory control exerted by the N terminus. Since PKC up-regulation of L-type Ca^{2+} channels has been reported in many species, we predict that isoforms of α_{1C} subunits containing the initial N-terminal 46 a.a. similar to those of the rabbit heart α_{1C} are widespread in cardiac and smooth muscle cells.

In the heart, Ca^{2+} current via the voltage-dependent L-type channels (dihydropyridine-sensitive) underlies the plateau of the action potential and provides calcium ions necessary for initiation of cardiac cell contraction (2). Similar channels are found in smooth muscle, where they play a major role in regulation of tonus and contraction (3, 4), and in the nervous system (5, 6). L-type channels are composed of the following three subunits: the main, pore-forming α_{1C}, the cytosolic β2, and the $\alpha_2\delta$ subunit which is mostly extracellular (5, 7–11). α_{1C} contains four homologous membrane domains numbered I–IV, each one with six transmembrane segments and a re-entrant P-loop that forms the pore lining; N- and C-terminal domains and the linkers connecting the domains I–II, II–II, and II–IV are cytosolic (see Ref. 7 for review, and see Fig. 6*A* for a scheme). The C terminus was implicated in Ca^{2+}- and voltage-dependent inactivation (12–15) and modulation by protein kinase A (16–19); linker I–II contains the binding site for the β subunit (20, 21).

Cardiac and smooth muscle L-type channels are tightly regulated by hormonal and neuronal signals via G proteins and protein kinases (22, 23). Protein kinase C (PKC)[1] is one of such regulators; its actions appear to be tissue- and species-specific. PKC activators, such as phorbol esters and diacylglycerols, increase Ca^{2+} channel currents in cardiac and smooth muscle cells of various mammals (24–33), and PKC has been implicated in mediating the stimulation of Ca^{2+} channels by intracellular ATP (34), angiotensin II (26), glucocorticoids (28), pituitary adenylate cyclase-activating polypeptide (33), and arginine-vasopressin (32). PKC up-regulation results from changes in channel gating because it is accompanied by an increase in single channel open probability, P_o (30, 35, 36). In many cases, a biphasic effect of PKC activators has been described, with an increase followed by a later decrease (25, 27, 30), and some preparations such as adult guinea pig heart cells (37, 38) respond to phorbol esters only by a decrease in Ca^{2+} currents, an effect that may not be mediated by PKC (38). The biphasic response to PKC stimulators is fully reconstituted when expression of L-type channels in *Xenopus* oocytes is directed by RNA extracted from rat heart (39, 40) or cRNA of rabbit cardiac α_{1C} subunit (39). Increase of Ca^{2+} channel activity by phorbol esters has also been observed in a mammalian cell line (baby hamster kidney) expressing the rabbit cardiac α_{1C} (36). The potentiation by phorbol esters of Ca^{2+} channels expressed in the oocytes is mediated by PKC because it is mimicked by diacylglycerols and blocked by specific PKC inhibitors (39, 40).

Both α_{1C} and β are substrates for PKC-catalyzed phosphorylation (Ref. 41 and references therein). α_{1C} subunit has been recognized as the target for the Ca^{2+} channel enhancement caused by PKC, since coexpression of the auxiliary subunits was not necessary to reproduce the effect of phorbol esters; on the contrary, coexpression of the β subunit weakened the enhancement suggesting a modulatory effect for this subunit (39). However, it is not known which part of α_{1C} is involved in the PKC action. α_{1C} isoforms cloned from rat brain (42) and human heart (43) are not up-regulated by PKC (43, 44), suggesting that the site of PKC action lies in one of the variable regions. More specifically, Bouron *et al.* (43) proposed that phosphorylation of the initial segment of the N terminus of the rabbit

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[1] The abbreviations used are: PKC, protein kinase C; a.a., amino acid; GST, glutathione *S*-transferase; I_{Ba}, Ba^{2+} current via the voltage-dependent Ca^{2+} channels; PMA, 4β-phorbol 12-myristate 13-acetate; PAGE, polyacrylamide gel electrophoresis; WT, wild type; PCR, polymerase chain reaction.

heart isoform may account for PKC potentiation, but this hypothesis has not been tested. It was unclear how this part of α_{1C} can affect the function of the channel, because in a recent publication Wei *et al.* (1) reported that deletion of up to 120 initial N-terminal amino acids strongly increased the whole cell Ca^{2+} channel current and, proportionally, the total gating charge movement but did not affect the voltage dependence of the charge movement or of the macroscopic current activation. It has been proposed (1) that the N-terminal deletion causes an increase in the amount of functional channels (hence the increase in total gating charge movement) but does not affect channel function.

In the beginning of this study we set out to test which part of α_{1C} accounts for the PKC-induced enhancement of the rabbit heart L-type channel, using deletion and single-site mutagenesis and expression in *Xenopus* oocytes. We found that, as predicted by Bouron *et al.* (43), deletion of the first 46 a.a. (which are thought to be unique to the rabbit heart isoform) eliminates the PKC-induced enhancement. To understand whether and how the N terminus affects the function of the channel, we have undertaken a more elaborate study of the properties of N-terminal deletion mutants and GST fusion proteins. Immunochemical and single channel measurements demonstrated that N-terminal deletions do not increase channel expression but rather enhance activation on single channel level. We find evidence for an interplay between N terminus, PKC, and the β subunit, although we could not detect any direct binding between N terminus and β. Our results point to the possibility that potentiation of L-type Ca^{2+} channels by PKC, and part of the enhancement caused by the β subunit, may result from attenuation of a tonic inhibitory control exerted by the N terminus. Furthermore, since the enhancing effect of PKC on L-type Ca^{2+} channels is widespread among mammalian species, our data suggest that α_{1C} isoforms with N termini of the "rabbit heart" type must also be widespread.

EXPERIMENTAL PROCEDURES

DNA Constructs and mRNA—cDNAs of rabbit heart α1C (pCAH), rabbit heart β2A, and skeletal muscle α2/δ subunits were prepared and used as described previously (45, 46). The cDNAs of the following mutants of α_{1C} were made within the original pCAH construct: ΔN_{88-139}; st1665; S533I; S1575A (16). A PCR-based approach was designed for engineering all the other constructs used in this work. In an attempt to improve the expression of channels composed of α_{1C} alone, we subcloned the coding sequence of α_{1C} into a high expression vector, pGEM-SB, derived from pGEM-HE which contains a 50-base 5′-untranslated region and a 300-base 3′-untranslated region from *Xenopus* α-globin (47). pGEM-SB was produced by a standard PCR procedure used to extend the polylinker which now includes the following restriction sites: *Sma*I, *Bam*HI, *Sal*I, *Cla*I, *Bst*EII, *Eco*RI, *Xba*I, and *Hin*dIII. By using standard PCR procedures, a *Sal*I site was created immediately preceding the initial ATG of α_{1C}, and a *Hin*dIII site was created following the termination codon. After a series of intermediate subcloning and ligation steps, the coding sequence of α_{1C} was inserted between *Sal*I and *Hin*dIII sites of pGEM-SB. The resulting cDNA was termed α_{1C}-SB; for synthesis of sense RNA with T7 polymerase, it was linearized with *Nhe*I. This procedure did not significantly improve the currents directed by the expression of α_{1C} RNA alone, as compared with pCAH, but provided a convenient tool for the creation of N-terminal deletion mutants. The cDNA of the neuronal α_{1C} isoform, rbC-II, was kindly provided by T. P. Snutch (University of British Columbia; see Ref. 42); this DNA was injected directly into the nuclei.

To create α_{1C} N-terminal truncations, PCR amplification with Vent polymerase (New England Biolabs) was performed using 100 ng of WT α_{1C} cDNA as template for 25 cycles of 30 s at 95 °C, 1 min at 55 °C, and 2 min at 72 °C. For each deletion mutant unique forward and reverse primers were used, with a forward primer creating a *Sal*I site followed by an initiation codon and then by the original WT α_{1C} sequence starting from the desired base. For ΔN_{2-46}, the forward primer was 5′-AT GTC GAC TAA ACC ATG G^{330}GT TCC AAC TAT GG-3′. The reverse primer was 5′-GCT AAG GCC ACA CAA TTG GC-3′, which is complementary to nucleotides 687–706 and includes an endogenous unique *Mfe*I site. The restriction sites *Sal*I and *Mfe*I are underlined, respectively. To create ΔN_{2-139}, the forward primer was 5′-TAG CCG GTC GAC ATG A^{609}AG AAC CCC ATC CGG A-3′. Reverse primer sequence was 5′-AG CTC AAT TTT CTC CTC CTT GGC CTC-3′, complementary to nucleotides 2673–2698 and is close but downstream from the endogenous unique *Eco*RI site (the superscript number in the oligonucleotide sequence indicates the corresponding position in the rabbit cardiac α_{1C} sequence) (48). The amplified fragments were used to replace the corresponding fragment in α_{1C}-SB DNA. The net result was the deletion from the second amino acid residue through the number indicated in the name of construct.

DNAs of α_{1C} fragments designed to create glutathione *S*-transferase (GST) fusion proteins were constructed using a similar PCR strategy, with primers containing the desired restriction sites. These fragments were cloned into pGEX-4T-1 (Amersham Pharmacia Biotech). The N-terminal cDNA fragment (N, encoding a.a. 1–154) and three C-terminal cDNA fragments (C, encoding a.a. 1505–2171, C_1, encoding a.a. 1664–1845, and C_2, encoding a.a. 1821–2171) were inserted into *Eco*RI and *Not*I restriction sites of pGEX-4T-1. GST-L_{I-II}, encoding a.a. 438–550, was inserted into *Eco*RI and *Xho*I sites of pGEX-4T-1. The C_1 fragment was additionally subcloned into pGEM-HE vector at the same restriction sites, and two more N-terminal fragments, N_{1-139} and N_{88-139}, were created by a similar PCR procedure and also inserted into pGEM-HE between *Eco*RI and *Hin*dIII for *in vitro* transcription and expression in oocytes. All PCR products were sequenced at the Tel Aviv University Sequencing Facility. Capped mRNAs were synthesized *in vitro* using the suitable RNA polymerases, as described (49). When WT and one of the mutant channels have been compared in electrophysiological experiments, care was taken always to use RNAs derived from the same cDNA vector. Materials and enzymes for molecular biology were purchased from Boehringer-Mannheim, Promega, or MBI Fermentas.

Oocyte Culture and Electrophysiology—Xenopus laevis frogs were maintained and operated, and oocytes were collected, defolliculated, and injected with RNA as described (49, 50). In each experiment, oocytes were injected with equal amounts (by weight) of the mRNAs of the various channel subunits in the desired combinations and with RNAs of additional proteins as detailed in the figure legends. Oocytes were incubated at 20–22 °C in ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5) supplemented with 1 mM $CaCl_2$, 2.5 mM sodium pyruvate, and 50 μg/ml gentamycin. For patch clamp experiments, the vitelline membrane was removed with fine forceps after ~5 min incubation in the bathing solution, as described (49). Whole cell currents were recorded using two-electrode voltage clamp as described (45), 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. Usually, the concentrations of α_{1C} RNA of WT and N-terminal deletion mutants were not equal but chosen in such a way that the amplitudes of the expressed currents were below 3 μA, to avoid artifacts introduced by series resistance and by Ba^{2+}-activated Cl^- currents when larger currents are measured (51). Net Ba^{2+} currents were obtained by a standard leak subtraction procedure or, when α_{1C} subunit alone was expressed, by subtraction of currents measured after inhibiting all Ca^{2+} channel currents by 100 μM Cd^{2+}. Absence of contribution of the endogenous currents of the oocyte was verified by inhibiting I_{Ba} with 10 μM nifedipine. Single channel recordings were done in the cell-attached mode as described (50), using Axopatch 200 amplifier (Axon Instruments, Foster City, CA). Pipettes contained 110 mM $BaCl_2$, 10 mM HEPES/NaOH, pH 7.5. The oocytes were bathed in a solution containing 130 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES/KOH, pH 7.5. Currents were filtered at 2 kHz (4-pole Bessel) and sampled at 10 or 5 kHz. Voltage steps from −80 to 10 mV lasting 140 or 280 ms were delivered every 1 or 2 s. Leak and capacitative currents were subtracted from the traces using blank sweeps during the analysis session. Data acquisition and analysis were done with pCLAMP (Axon Instruments, Foster City, CA).

Immunochemistry—This was performed as described (50, 52). Oocytes were injected with mRNAs and incubated in NDE solution containing 0.5 mCi/ml [^{35}S]methionine/cysteine (Amersham Pharmacia Biotech) for 3–4 days at 22 °C. Plasma membranes together with the vitelline membranes (extracellular collagen-like matrix) were removed manually with fine forceps after a 5–15-min incubation in a low osmolarity solution. The remainder of the cell (internal fraction) was processed separately. 10–30 plasma membranes and 10 internal fractions were solubilized in 100 μl of buffer (4% SDS, 10 mM EDTA, 50 mM Tris, pH 7.5, 1 mM phenylmethanesulfonyl fluoride, 1 mM pepstatin, and 1 mM 1,10-phenanthroline) and heated to 100 °C for 2 min. Following the addition of 100 μl of H_2O and 800 μl of the immunoprecipitation buffer (190 mM NaCl, 6 mM EDTA, 50 mM Tris, pH 7.5, and 2.5% Triton X-100),

homogenates were centrifuged for 10 min at 1000 × *g* at 4 °C. The supernatant was incubated for 16 h with the Card-I polyclonal antibody (53), incubated for 1 h at 4 °C with protein A-Sepharose, and pelleted. Immunoprecipitates were washed 3 times with immunowash buffer (150 mM NaCl, 6 mM EDTA, 50 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, and 0.02% SDS). Samples were boiled in SDS-gel loading buffer and electrophoresed on 3–8% SDS-polyacrylamide gradient gel together with standard molecular mass markers (45–205 kDa). Gels were dried and placed in PhosphorImager (Molecular Dynamics) cassette for up to 3 days. The protein bands of the image were estimated quantitatively using the software ImageQuant, as described (50, 54).

Binding of the GST Fusion Proteins to ^{35}S-Labeled Proteins—This was done essentially as described (21). [^{35}S]Met/Cys-labeled β_{2A} was translated on the template of *in vitro* synthesized RNA using a translation rabbit reticulocyte kit (Promega) according to manufacturer's instructions. The fusion proteins were synthesized and extracted from *Escherichia coli* according to pGEX-4T-1 manufacturer's instructions (Amersham Pharmacia Biotech). The protein concentration was estimated using the Bio-Rad protein assay kit (Munchen, Germany). Purified GST fusion proteins (5–10 μg) or purified GST (~10 μg) were incubated with 5 μl of the lysate containing the ^{35}S-labeled β_{2A} in 500 μl of phosphate-buffered saline with 0.05% Tween 20, for 2 h at room temperature, with gentle rocking. Then the GST fusion protein was immobilized on glutathione-Sepharose beads (Amersham Pharmacia Biotech; 30-μl beads were added) for 30 min at 4 °C and washed four times in 1 ml of phosphate-buffered saline with 0.05% Tween 20. (In some experiments, the ^{35}S-labeled proteins were incubated with GST fusion proteins already immobilized on the glutathione-Sepharose beads.) Following washing, GST fusion proteins were eluted with 20 mM reduced glutathione in elution buffer (120 mM NaCl, 100 mM Tris-HCl, pH 8, 30 μl) and analyzed by SDS-PAGE.

Data Presentation and Statistical Analysis—The results are always presented as means ± S.E. Multiple group comparisons have been done by one-way analysis of variance test followed by Dunnett's test. Two-group comparisons were done using Student's *t* test.

RESULTS

N-terminal Deletions Increase Ca^{2+} Channel Current but Not Protein Expression—To study the role of the first 46 amino acids of the N terminus, we created a deletion mutant of α_{1C} in which these amino acids, except the initial methionine, have been deleted ($\alpha_{1C}\Delta N_{2-46}$; see Fig. 6 for a scheme of the channel to help localize the deletions). Ba^{2+} currents via the expressed Ca^{2+} channels were measured using the two-electrode voltage clamp technique. The subunit composition of the channels used in this study was varied according to the specific questions asked. The expression of wild-type (WT) α_{1C} subunit alone was rarely employed because it resulted in very small currents, usually below 20 nA (*cf.* Ref. 45). In most cases, $\alpha_1\alpha_2\delta$ combination was used, because the β subunit was found to interfere or interact with the modulatory effects of N-terminal deletions and of PKC (see below).

In agreement with Wei *et al.* (1), the whole cell Ca^{2+} channel currents carried by Ba^{2+} (I_{Ba}) via channels containing the mutant $\alpha_{1C}\Delta N_{2-46}$ subunit were 5–10-fold larger than with the wild-type α_{1C} in all subunit combinations tested as follows: α_{1C} alone, $\alpha_1\alpha_2\delta$, or $\alpha_1\alpha_2\delta\beta$ (*e.g.* Fig. 1*A*). Even when the oocytes were injected with twice as much WT α_{1C} RNA, the ΔN_{2-46} mutant still gave ~4-fold larger currents (Fig. 1*B*). The kinetics of the current (Fig. 1*A*; see also Figs. 3 and 5) and the voltage dependence of activation were not altered; the latter is demonstrated by the similarity of the normalized current-voltage (I–V) curves (Fig. 1*C*). An additional deletion mutant missing most of the N terminus, $\alpha_{1C}\Delta N_{2-139}$, increased the current about ~10-fold compared with WT (Fig. 1*D*) and did not shift the I–V curve (data not shown). However, we noticed differences in voltage-dependent inactivation of $\alpha_{1C}\Delta N_{2-139}$ and the WT channels. In the $\alpha_1\alpha_2\delta$ composition, when compared with the WT, the steady-state inactivation curve of the mutant was shifted to more positive potentials; the slope of the curve was increased, and the proportion of non-inactivating current was reduced (Fig. 1*E*). Coexpression of β_{2A} subunit shifted the

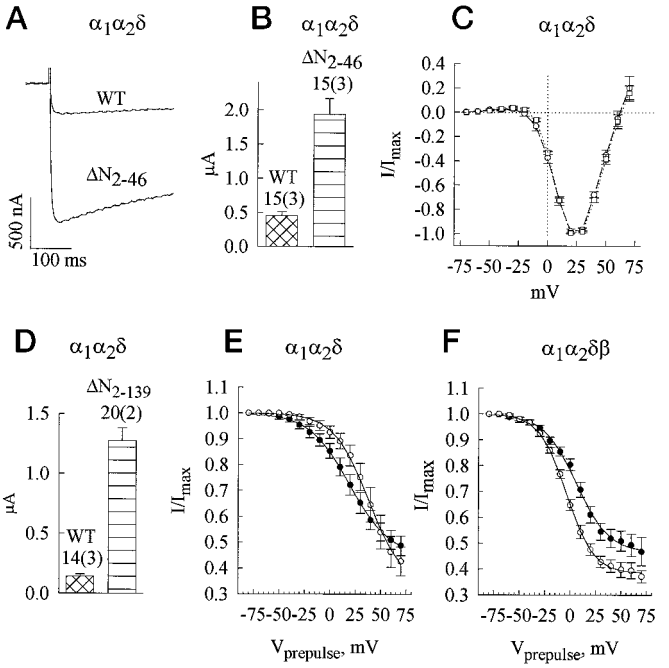

FIG. 1. **Effects of N-terminal deletions on whole cell Ca^{2+} channel current and on voltage dependence of activation and steady state inactivation of I_{Ba}.** *A*, Ca^{2+} channel currents from representative *X. laevis* oocytes of one batch, injected with RNAs of α_{1C} of WT or ΔN_{2-46} deletion mutant, in combination with $\alpha_2\delta$ subunit. Currents were elicited by test pulses to +20 mV from a holding potential of −80 mV. *B*, mean currents of in groups of oocytes injected as explained in *A*, obtained from three batches of oocytes. 5 ng of α_{1C}WT and 2.5 ng of $\alpha_{1C}\Delta N_{2-46}$ cRNAs were injected per oocyte, with an equal amount of $\alpha_2\delta$ RNA. Currents were measured 6–8 days after RNA injection. *Numbers above bars* indicate the number of cells assayed; *numbers in parentheses* indicate the number of donors (oocyte batches). *C*, normalized I–V curves recorded in oocytes of one batch. Currents elicited at each test potential were normalized to the maximal amplitude in the same oocyte. Averaged currents (mean ± S.E.) are plotted as a function of test potential. α_{1C}WT + $\alpha_2\delta$, ○, n = 5; $\alpha_{1C}\Delta N_{2-46}$+ $\alpha_2\delta$, □, n = 6. *D*, Ca^{2+} channel currents measured 3 days after the injection of equal amounts (2.5 ng per oocyte) of cRNAs of α_{1C}WT or $\alpha_{1C}\Delta N_{2-139}$, in combination with $\alpha_2\delta$. *E* and *F*, averaged steady-state inactivation curves in $\alpha_1\alpha_2\delta$ (*E*) or $\alpha_1\alpha_2\delta\beta$ (*F*) channels containing either WT (●) or ΔN_{2-139} (○) α_{1C}. The currents were examined with two-pulse protocol as follows: a 3-s prepulse to different voltages (starting from −80 mV, with 10 mV increments) followed by a test pulse to +20 mV. Data were obtained from two different batches of oocytes including at least five cells in each group. Results are represented as means ± S.E. The averaged data were fitted to the Bolzmann equation: $I_{Ba}/I_{max} = f + 1/\{1 + \exp((V_{prepulse} - V_{½})/K_i)\}$, where I_{max} is the current obtained by the step from −80 to 20 mV, $V_{½}$ is the half-inactivation voltage, K_i is a slope factor, and f is the non-inactivating fraction. The *solid lines* were drawn with the following values (WT is given first, ΔN_{2-139} second): in *E*, K_i was 19.6 mV in WT and 17 mV in ΔN_{2-139}; $V_{½}$, 19 and 38 mV; f, 0.45 and 0.33; in *F*, K_i was 15.9 mV in WT and 13.2 mV in ΔN_{2-139}; $V_{½}$, 6 and −3.6 mV; f, 0.47 and 0.38.

inactivation curve of the WT type channel to negative potentials and increased the slope (compare data shown by *solid circles* in Fig. 1, *E* and *F*), as reported previously (45, 55, 56). In the full subunit composition, the deletion of the 139-a.a. again caused a decrease in the non-inactivating fraction and an increase in the slope. However, unlike in the α_1 $\alpha_2\delta$ channels, in the $\alpha_1\alpha_2\delta\beta$ channels the 139-a.a. deletion caused a leftward shift in the curve. The inactivation kinetics were unaffected. With 3-min long depolarizing pulses to +30 mV, after an initial decay the $\alpha_1\alpha_2/\delta$ channels' current reached a steady-state level (after ~2.4 min) of 61 ± 2% of peak in WT (n = 10) and 57 ± 3% of peak in ΔN_{2-139}. Although the functional significance of the changes in voltage dependence of inactivation is unclear, they indicate that the deletion of N-terminal amino acids may affect gating of the channel. Since the presence of the β subunit

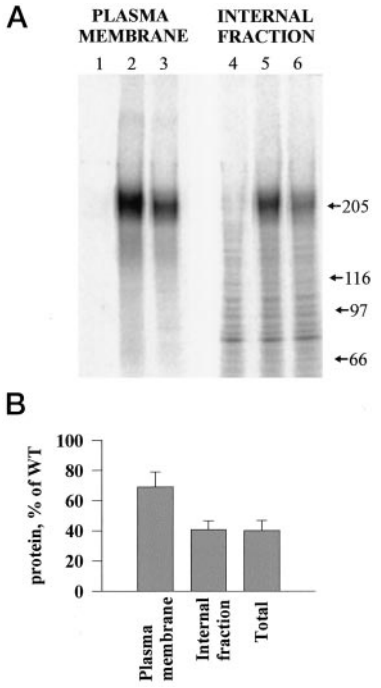

FIG. 2. **Comparison of the expression level of the WT and ΔN_{2-46} α_{1C} protein in plasma membrane and internal fractions of *Xenopus* oocytes.** *A*, digitized PhosphorImager scan of SDS-PAGE analysis of the α_{1C} and $\alpha_{1C}\Delta N_{2-46}$ proteins (coexpressed with $\alpha_2\delta$ subunit) in oocytes metabolically labeled with [^{35}S]Met/Cys. Radiolabeled proteins were immunoprecipitated from plasma membranes (*lanes 1–3*) and internal fractions (*lanes 4–6*) separately. In each lane, immunoprecipitates from 30 plasma membranes and 10 internal fractions were loaded. *Lanes 1* and *4* represent immunoprecipitates from native oocytes that have not been injected with cRNAs. *B*, relative amounts of the $\alpha_{1C}\Delta N_{2-46}$ detected in plasma membrane and internal fraction, calculated as percent of the expressed α_{1C}WT protein in the same batch of oocytes, recalculated per single oocyte. Total protein is the sum of both fractions. Band intensities were measured using PhosphorImager. Data were averaged from three different batches of oocytes and presented as means ± S.E.

modified the effect of the N-terminal deletion, a cross-talk between N terminus and the β subunit is possible.

The increase in whole cell Ca^{2+} channel currents by the N-terminal deletions might be due to an increase in the amount of α_{1C} protein in the plasma membrane. *Xenopus* oocytes present a convenient experimental system to examine this question, since a very clean preparation of the plasma membrane can be obtained by mechanical separation from the rest of the cell ("internal fraction," Refs. 52 and 57). Newly synthesized proteins are metabolically labeled with ^{35}S by incubating the oocytes in [^{35}S]methionine/cysteine for 3–4 days following the RNA injection, immunoprecipitated, and subjected to SDS-PAGE, and the relative amount of protein is quantified using an imaging procedure (50, 52). This allows high precision measurements of changes in the content of protein in whole oocytes and in plasma membrane (50, 52, 54).

The channels were expressed in the $\alpha_1\alpha_2\delta$ composition. α_1 subunit was immunoprecipitated (50) with the Card-I antibody directed against part of the II–III linker (53) and analyzed as explained above. Fig. 2*A* illustrates the results of a representative experiment that demonstrated comparable expression of channels containing either WT or ΔN_{2-46} α_{1C} in the internal fraction (*right panel, lanes 2* and *3*) and in the plasma membrane (Fig. 2*A, left panel*, compare *lanes 5* and *6*). Oocytes uninjected with RNA gave no signal (Fig. 2*A, lanes 1* and *4*). Fig. 2*B* summarizes the results of the quantitative analysis of the experiments in all three oocyte batches tested. It can be seen that the total cellular amount of ΔN_{2-46} α_{1C} was only about half that of the WT, whereas the amounts of WT and ΔN_{2-46} α_{1C} in the plasma membrane were roughly equal (the ~30% reduction in the mutant protein was not statistically significant). These data suggest that the vast increase in the whole cell Ca^{2+} channel current caused by the 46-a.a. deletion is not caused by an increase in the level of expression of the channel protein.

The N-terminal Deletions Modify Channel Gating—If the N-terminal deletion does not alter the amount of channels in the plasma membrane, then the increase in whole cell current must result from an increase in the activity of each channel (which can be measured using the patch clamp technique). In other words, the open probability of a single channel must be higher in ΔN_{2-46} α_{1C} than in the WT α_{1C}, whereas the number of channels in patches of similar sizes must be comparable. To address this question, an accurate estimate of the amount of channels in the patch (N) is imperative (58). Unfortunately, P_o of the L-type channels is low (<1%), making such estimate extremely difficult. However, P_o increases substantially in the presence of dihydropyridine agonists such as (−)-BayK 8644 (reviewed in Ref. 8). In *Xenopus* oocytes expressing L-type Ca^{2+} channels, in the presence of this drug, N can be reliably estimated from the number of overlapping openings in a long series of depolarizing voltage steps, provided that N <3 (50, 59). Before recording single channel activity, we have verified that (−)-BayK 8644 increases the whole cell I_{Ba} via WT and ΔN_{2-46} channels by the same factor at all voltages (Fig. 3. To avoid possible series resistance errors, the amounts of WT and mutant RNAs were adjusted to produce currents of similar amplitudes.). Thus, in the presence of (−)-BayK 8644, the differences between the WT and ΔN_{2-46} channels appear to be preserved.

Single channel recordings were performed in cell-attached configuration with 110 mM Ba^{2+} in the pipette, and in the presence of 1 or 2 μM (−)-BayK 8644 in the bath. Ca^{2+} channels ($\alpha_1\alpha_2\delta$ composition) were activated by depolarizing pulses from −80 to +10 mV. Our first observation was a similarity of the number of channels in membrane patches in oocytes expressing WT or ΔN_{2-46} α_{1C}. For instance, with 0.6 or 1.2 ng of RNA of each subunit per oocyte, and with pipettes of similar resistances (3.5–4.5 megaohms), the average number of channels in a patch was 1.3 ± 0.5 (n = 11) in WT and 1.1 ± 0.3 (n = 21) in ΔN_{2-46} $\alpha_1\alpha_2\delta$ channels.

Fig. 4*A* exemplifies records of channel activity in oocytes expressing WT or ΔN_{2-46} channels (n = 2 in both cases). It appears that the mutant channels spend more time in the open state than the WT ones. Indeed, as shown in Fig. 4*B*, P_o was ~10-fold higher for the ΔN_{2-46} channels (WT, 10 patches; ΔN_{2-46}, 8 patches; $p < 0.01$). Open time distribution was fitted by two exponents with time constants (τ_1 and τ_2) of about 0.4 and 2 ms for both channel types (Fig. 4, *C* and *D*, and Table I), but the fraction of time contributed by the longer openings (f_2) was significantly higher in the ΔN_{2-46} than in WT channels (Table I). The increase in the proportion of long open times may at least partially account for the total increase in P_o caused by the N-terminal deletion. By visual examination, another prominent difference was the prevalence of very long bursts of channel activity in the mutant channels; such bursts were rare in the WT channels. A rigorous burst analysis will require one-channel recordings which were rare in this study. Whatever the main factor contributing to the increase in P_o, it is evident that the N-terminal 46-a.a. deletion causes a major change in the gating properties of the cardiac L-type Ca^{2+} channel, at least in the presence of (−)-BayK 8644. However, we must add a reservation: in a few oocytes where P_o was measured both before and after the addition of (−)-BayK 8644, the increase in P_o was much stronger than in whole cell recordings, ranging

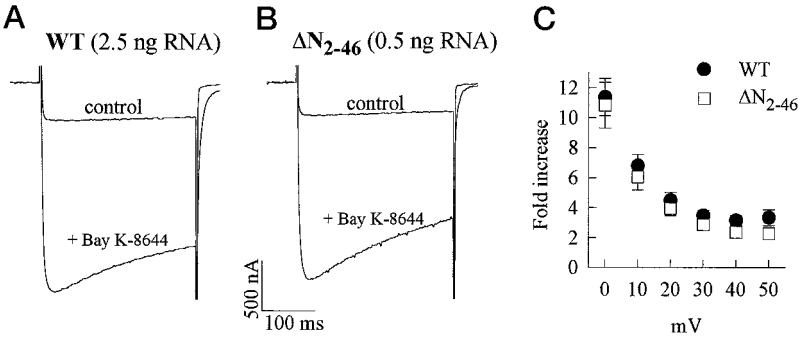

FIG. 3. **(−)-BayK 8644-induced enhancement of I_{Ba} in oocytes expressing WT or ΔN_{2-46} α_{1C} in combination with $\alpha_2\delta$.** In the control group, WT α_{1C} and α_2/δ RNAs were injected at 2.5 ng/oocyte; in the test group, α_{1C} ΔN_{2-46} and α_2/δ RNAs were injected at 0.5 ng/oocyte. *A* and *B* show the typical effect of (−)-BayK 8644 on the currents elicited by depolarization step from −80 mV to +10 mV. *C* summarizes the increase in I_{Ba} induced by application of 1 μM (−)-BayK 8644 at different voltages. Data were averaged from three batches of oocytes (n >15 in all groups). ●, WT; □, ΔN_{2-46}.

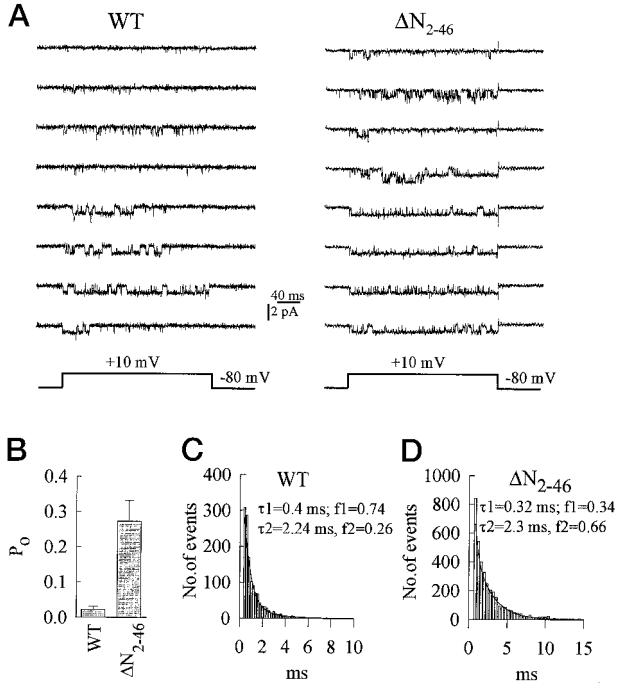

FIG. 4. **Comparison of the single channel behavior of WT and ΔN_{2-46} α_{1C} (coexpressed with $\alpha_2\delta$) in presence of 1 μM (−)-BayK 8644.** *A*, representative consecutive traces of WT and mutant channel activity at 10 mV. *B*, averaged P_o from 10 patches for WT and 8 patches for ΔN_{2-46} channels. *C* and *D*, typical open time histograms. For each of the channels, both brief and long open events were present. The *smooth curves* represent best fits with two exponentials. The values of the time constants (τ_1 and τ_2) and the fractions of total open time spent in each of the open states (f_1 and f_2) are shown in the *insets*.

TABLE I
Parameters of single channel activity of WT and ΔN_{2-46} $\alpha_{1C}\alpha_2$ channels

To calculate P_o (the single channel open probability), the number of channels was determined from the maximal number of overlapping openings. Only patches with no more than three channels have been analyzed. Open time histograms of the first level openings were fitted with two exponents. τ_1 and τ_2 are the short and the long time constants, and f_1 and f_2 are the fraction of total open time contributed by openings corresponding to τ_1 or τ_2, respectively.

	WT; n = 10	ΔN_{2-46}; n = 8
P_o	0.02 ± 0.01	0.27 ± 0.06
τ_1	0.45 ± 0.08	0.41 ± 0.06
f_1	74.3 ± 11.63	37 ± 8[a]
τ_2	2.46 ± 0.44	2.37 ± 0.3
f_2	25.67 ± 11.64	63 ± 8.1[a]

[a] Significantly different from WT ($p < 0.01$).

from 5- to 180-fold. The reason for this phenomenon is unknown, but it warrants caution in extrapolating the findings obtained in the presence of this drug to the characteristics of naïve channels.

To account for the above observations and for the finding that removal of proximal N terminus increases gating charge movement (1), we put forward a working hypothesis: the N terminus hinders activation (*e.g.* by obstructing the movement of the voltage sensor), therefore its deletion improves activation. It is notable that coexpression of the β subunit also improves activation and alters gating charge movement (60, 61), although the details differ (see "Discussion"). Therefore, we assumed that the N terminus and the β subunit may affect a common mechanism and thus they may interact with each other (as also suggested by the changes in voltage-dependent inactivation; see above). This was tested by studying the effect of coexpression of the β subunit with channels containing either WT or one of the deletion mutants of α_{1C} (ΔN_{2-46} or ΔN_{2-139}). Coexpression of β_{2A} with $\alpha_1\alpha_2\delta$ increased the whole cell I_{Ba} both in WT (Fig. 5*A, a*) and in ΔN_{2-139} (Fig. 5*A, b*), but the increase caused by coexpression of β_{2A} was significantly ($p < 0.01$) smaller in ΔN_{2-139} than in WT, at all voltages (Fig. 5*B*). The results with ΔN_{2-46} were similar (in this case, we tested the effect of coexpression of β_{2A} on channels composed of α_{1C} alone).

To probe for a possible physical interaction between the β subunit and the N terminus of α_{1C}, we have measured *in vitro* binding of GST fusion proteins corresponding to some of the intracellular parts of α_{1C}, to β_{2A} synthesized in reticulocyte lysate and labeled with [^{35}S]methionine/cysteine (see Ref. 20). The following GST fusion proteins were used: GST-N, corresponding to amino acids (a.a.) 1–154, *i.e.* the whole-length N terminus; GST-L_{I-II}, corresponding to most of the intracellular linker between domains I and II (a.a. 438–550); and GST-C_1 (a.a. 1664–1845) and GST-C_2 (a.a. 1821–2171), corresponding to two parts of the C terminus. The scheme of the α_{1C} subunit in Fig. 6*A* illustrates the positions of the different pieces. As expected (20, 21), β_{2A} bound to GST-L_{I-II}, but we could not detect binding to any one of the other fusion proteins tested (Fig. 6*B*). (Note that the amounts of all GST fusion proteins loaded on the gel were similar, as demonstrated in Fig. 6*C*.)

If the N terminus obstructs activation, then artificial proteins corresponding to fragments of N terminus may be expected to reduce I_{Ba}. We constructed DNAs encoding proteins corresponding to N-terminal a.a. 1–139 of α_{1C} (N_{1-139}), N-terminal a.a. 88–139 (N_{88-139}), and C-terminal a.a. 1664–1845 ($C_{1664-1845}$; denoted as *C* in Fig. 7). The corresponding RNAs directed the expression of proteins of correct size in reticulocyte

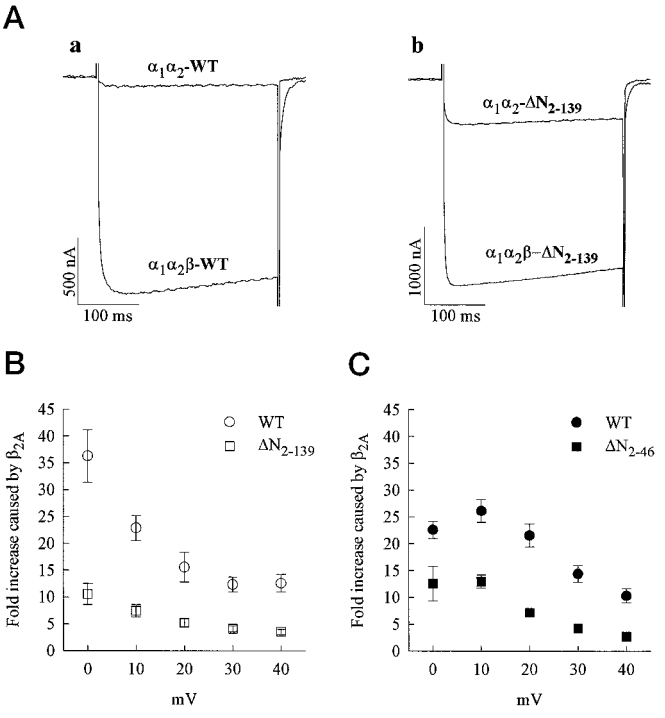

FIG. 5. **The effect of coexpression of the β_{2A} subunit on the amplitude of currents via channels containing α_{1C} of WT, ΔN_{2-139}, and ΔN_{2-46} type.** *A*, currents recorded in representative oocytes of one batch by depolarization from −80 to 20 mV. The oocytes were injected with the subunit combinations indicated near the traces. *a*, RNAs of all subunits were injected at 2.5 ng/oocyte. *b*, RNAs of all subunits were injected at 1 ng/oocyte. *B,* summary of the effects of β_{2A} coexpression on channels containing WT (○) and ΔN_{2-139} (□) α_{1C} (2 batches; 10 oocytes in each group). In all groups, $\alpha_2\delta$ was also expressed. In each oocyte I_{Ba} was expressed as percent of the mean amplitude of the current in the control group of oocytes from the same donor. These normalized values were averaged across all oocyte batches tested. Data are shown as mean ± S.E. *C,* summary of the effects of β_{2A} coexpression on channels with WT (●) and ΔN_{2-46} (■) α_{1C} (two batches; $n = 18$ in each group). In these experiments, $\alpha_2\delta$ was not expressed; thus, channels composed of α_1 alone *versus* $\alpha_1\beta$ were tested. Averaging of data was done as explained in *B*.

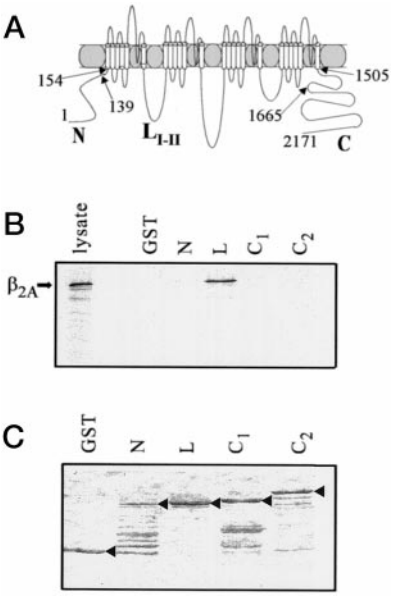

FIG. 6. **Interaction of *in vitro* synthesized β_{2A} with GST fusion proteins of α_{1C} fragments.** *A*, schematic presentation of the α_{1C} subunit of the L-type Ca^{2+} channel (rabbit heart isoform numbering). *B,* interaction of β_{2A} with GST fusion proteins corresponding to parts of α_{1C}. The proteins were separated by SDS-15% PAGE and monitored using PhosphorImager. *N* denotes GST-N (the whole N terminus); *L* denotes GST-L_{I-II} (a.a. 438–550); C_2 denotes GST-C_2 (a.a. 1821–2171(end); C_1 denotes GST-C_1 (a.a. 1664–1845). Similar results were obtained in two additional experiments. *C*, Coomassie Blue staining of the gel shown in *B*. *Arrowheads* indicate the predicted positions of the fusion proteins.

lysate (data not shown). Coexpression of RNAs encoding N_{1-138} and N_{88-139} proteins with channels containing a truncated α_{1C} (either ΔN_{2-46} or ΔN_{2-139}) reduced I_{Ba}, whereas $C_{1665-1845}$ was without effect (Fig. 7). The reduction was stronger when the channels contained the ΔN_{2-139} truncation than ΔN_{2-46}, possibly because in the ΔN_{2-46} α_{1C} the presence of the remaining part of N terminus hindered the access of the exogenous proteins to a target site.

The First 46 Amino Acids Are Essential for PKC-induced Increase in I_{Ba}—Fig. 8*A* shows diaries of representative experiments in which the PKC activator, PMA, was added to the extracellular solution at $t = 0$. In agreement with our previous report (39), PMA caused a biphasic change in I_{Ba}, an increase within several minutes was followed by a later decrease; the increase was stronger in $\alpha_1\alpha_2\delta$ channels than in any combination containing β_{2A}. Fig. 8*B* summarizes the measurements done in two oocyte batches in which we examined the differences in response to PMA among channels of $\alpha_1\alpha_2\delta$, $\alpha_1\beta$, and $\alpha_1\alpha_2\delta\beta$ composition (increase in I_{Ba} 15 min after PMA addition is shown). Clearly, coexpression of β_{2A} significantly reduced the extent of current potentiation caused by PMA.

The C terminus of α_{1C} contains a large number of putative PKC and protein kinase A phosphorylation sites. To examine whether these sites play a role in PKC modulation, we expressed channels based on a truncation mutant, $\alpha_{1C(st1665)}$, in which part of the C terminus beyond a.a. 1665 is missing (16). Full subunit combination, $\alpha_1\alpha_2\delta\beta$, was tested, because the $\alpha_{1C(st1665)}$ mutant usually gave rather small currents when expressed without β. Fig. 8*B* (*right column*) shows that the effect of PMA was not altered by this truncation (compare with the results obtained with WT $\alpha_1\alpha_2\delta\beta$).

In the following experiments, $\alpha_1\alpha_2\delta$ combination was used to allow a better visualization of PMA-induced enhancement of I_{Ba}. The effects of PMA varied among oocyte batches; therefore, mutant and WT channels were always compared in the same batch(es) of oocytes. Fig. 8*C* summarizes the results of this series of experiments and shows that the deletion of the first 46 N-terminal amino acids completely eliminated the PMA-induced increase in I_{Ba}, leaving the reduction phase intact (a representative experiment diary is shown in Fig. 8*A, triangles*). A rat brain α_{1C} isoform with a variant N terminus (see below) did not show an increase in I_{Ba} in response to PMA, in agreement with a previous report (44). The PMA effect remained intact in all other mutants tested, among them α_{1C}S533I (a putative PKC site in linker I–II), α_{1C}S1575A (a C-terminal site preceding the st1665 truncation), and an N-terminal deletion ΔN_{88-139}.

Fig. 8*D* compares a.a. sequences (deduced from the corresponding cDNA sequences) of the initial N-terminal segment of three most widely tested variants of α_{1C} as follows: rabbit heart α_{1C} (48) used in this study (*RH*); rat brain α_{1C} isoform, rbC-II (*RB*; Ref. 42); and a human heart isoform (*HH*; Ref. 43). The latter two isoforms are not up-regulated by PKC activators, and their N termini vary from that of the *RH* α_{1C}. N termini of two additional isoforms cloned from lung (62) and rat brain (rbC-I; Ref. 42) are identical to that of *RB* in Fig. 8*D*. N termini of all isoforms are essentially identical beyond a.a. 46 (numbering by *RH* α_{1C}). A closer examination shows that there is an additional region between a.a. 6 and 20, in which *RB* and *HH* are identical to each other and also show significant homology (33% identity) to *RH* α_{1C}. The correlation between PMA effects

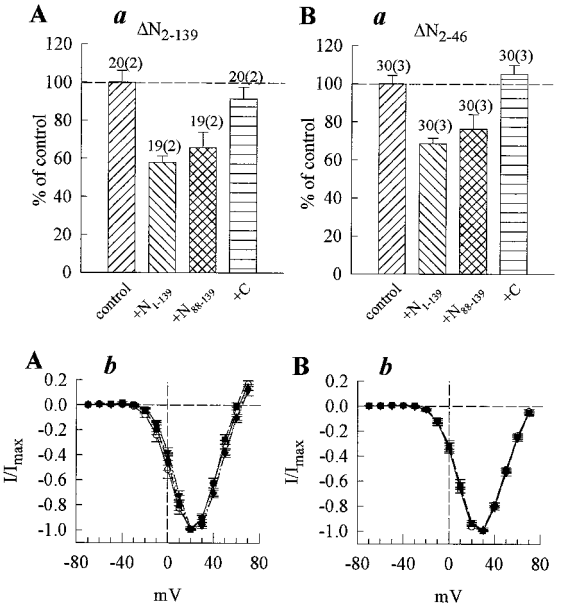

FIG. 7. **Inhibitory effect of coexpression of N-terminal fragments on I_{Ba} via channels containing ΔN_{2-139} (*A*) or ΔN_{2-46} α_{1C} (*B*).** *Top*, summaries of all experiments with ΔN_{2-139} (*A, a*) and ΔN_{2-46} (*B, a*). Channels were expressed in the $\alpha_1\alpha_2\delta$ composition, without (control) or with the addition of RNA of the desired fragment, as indicated *below* the *bars*. In each oocyte, I_{Ba} was expressed as percent of the mean amplitude of the current in the control group of oocytes from the same donor. These normalized values were averaged across all oocyte batches tested. Data represent the means ± S.E. *Numbers above bars* indicate the number of cells assayed, and *numbers in parentheses* indicate the number of donors (oocyte batches). The decrease in I_{Ba} caused by both N-terminal fragments tested was statistically significant in all cases ($p < 0.05$). *Bottom*, normalized I–V curves recorded in the oocytes of the same groups. ○, control; ■, N_{1-139}; ▲, N_{88-139}; ▼, *C*.

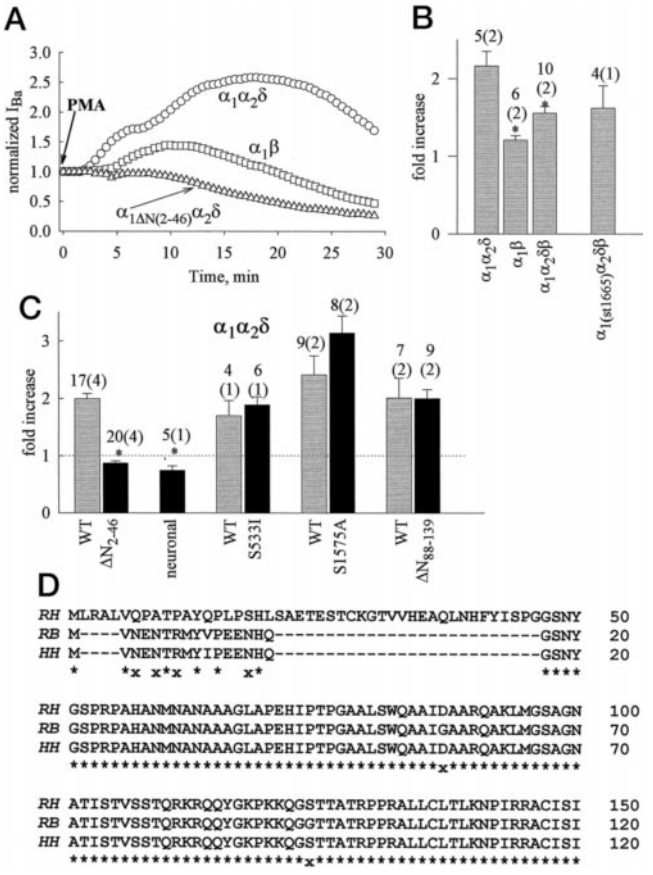

FIG. 8. **Importance of the β subunit and of the unique N-terminal part of α_{1C} in modulation of the channel activity by β-PMA-induced stimulation of PKC.** *A*, changes in I_{Ba} induced by 10 nM β-PMA as a function of the time in representative oocytes injected with the indicated RNAs. 200-ms test pulses from a holding potential of −80 mV to a test potential of +20 mV were applied every 30 s. Peak current amplitudes were normalized to the current in control conditions, whose stabilization was verified for at least 5 min before PMA application. *B*, attenuation of the β-PMA effect by coexpressed β_{2A} subunit. The *right bar* shows the effect of β-PMA on channels containing the $\alpha_{1C(st1665)}$ truncation mutant. In each oocyte, I_{Ba} was expressed as percent of the current amplitude before application of β-PMA. These normalized values were averaged across all oocytes in the batches tested. *Numbers above bars* indicate the number of cells assayed, and *numbers in parentheses* indicate the number of batches. *Asterisks* indicate statistically significant difference ($p < 0.05$) from WT $\alpha_1\alpha_2\delta$, obtained by two-tailed *t*-test. *C*, summary of the effects of β-PMA on different α_1 mutants compared with the WT. The bar denoted *neuronal* corresponds to the neuronal rbC-II α_{1C} isoform. Data analysis and presentation as in *B*. *Asterisks* indicate statistically significant difference ($p < 0.05$) from WT examined in the same batches of oocytes. *D*, alignment of the N-terminal sequences of three isoforms of L-type Ca^{2+} channel α_{1C} subunits (see definitions in the text). *Asterisks* indicate identity in all three sequences, *x* indicates identity in two out of three sequences.

(or their absence) and the primary structure of the initial α_{1C} N-terminal segments of these isoforms supports the idea that the unique initial 46 amino acids of *RH* α_{1C} are essential for PKC modulation.

DISCUSSION

N Terminus Modulates L-type Channel Gating—Our results demonstrate the functional importance of the N terminus of α_{1C} subunit in L-type Ca^{2+} channel function and modulation. Deletion of the initial 46 amino acids of the N terminus, which are unique to rabbit heart isoform, increases the whole cell Ca^{2+} channel current (see also Ref. 1) but does not increase the expression of the channel, as testified by the unchanged plasma membrane content of α_{1C} protein monitored by an immunochemical method, and similar density of functional channels detected by patch clamp methodology. Our data strongly suggest that this deletion alters the gating of the channel. First of all, it enhances the activity of single Ca^{2+} channels as testified by the ~10-fold increase in P_o. This change in channel gating alone is sufficient to account for the increase in whole cell Ca^{2+} channel current caused by this and probably by the other deletions tested (a.a. 2–139). An alteration of channel gating by the N terminus is further supported by differences in voltage dependence of inactivation in WT and ΔN_{2-139} channels, and by a decrease in whole cell current amplitude by coexpression of proteins corresponding to N-terminal a.a. 1–139 or 88–139, but not by a C-terminal protein. The results of the latter experiments imply that, in addition to the first 46 amino acids, other parts of the N terminus participate in its effect; however, a more detailed study will be necessary to scrutinize this hypothesis. We propose that, in L-type channels containing the rabbit heart isoform of α_{1C}, the N terminus imposes a tonic inhibitory control which is relieved in the truncation mutants tested. This mechanism is, to some extent, similar to that proposed to explain the increase in Ca^{2+} channel current caused by C-terminal deletions and by protein kinase A phosphorylation (12, 13).

In expression studies, changes in total gating charge movement (Q_{max}) caused by coexpression of Ca^{2+} channels β or $\alpha_2\delta$ subunits (60, 61, 63, 64) usually correlate well with the amount of α_{1C} protein detected in the membrane by immunochemical methods (50). How can our results be accommodated with the fact that deletions of initial 40–120 a.a. of α_{1C} increase Q_{max} without changing its voltage dependence (1)? We claim that, in general, a change in Q_{max} does not necessarily report a change in the number of functional channels. In various voltage-dependent channels, Q_{max} can be altered by drugs, toxins, or by

fatty acids. For instance, Q_{max} in Na^+ channels is decreased by Anthopleurin-A toxin (65), fatty acids (66), and lidocaine (67); in L-type Ca^{2+} channels, Q_{max} is decreased by dihydropyridines (68, 69). Thus, the N terminus might decrease Q_{max} by directly or allosterically interfering with the movement of the voltage sensor of the channel; removal of the N terminus would increase Q_{max}.

β Subunit and PKC Interact with the N Terminus—Our data suggest a cross-talk between the N terminus of α_{1C} and the β_{2A} subunit. It appears that the presence of the β subunit attenuates the inhibitory effect of the N terminus. This is supported by the following observations. (i) Coexpression of β_{2A} enhances the whole cell currents more efficiently when the N terminus is intact than when a.a. 1–46 or 1–139 are removed. It is possible that part of the β-induced channel enhancement is due to a weakening of the inhibitory effect of the N terminus; this explains why, in the absence of the latter, the augmentation caused by β subunit is less pronounced. (ii) β subunit counteracts the effect of PKC which is mediated via an interaction with the N terminus (see below). A cross-talk between β_{2A} and the N terminus of α_{1C} is also supported by the observation that ΔN_{2-139} deletion-induced changes in voltage-dependent inactivation properties are different in the absence and presence of β_{2A}. The interaction does not appear to be a direct one, since β_{2A} does not bind a GST fusion protein of the first 154 a.a. (Of the GST fusion proteins tested, the only β_{2A}-binding protein was that of the I–II domain linker, which contains a β_{2A}-binding site conserved in all known α_1 subunits (20, 21, 70, 71); the results of Fig. 6 suggest that, unlike α_{1E} (71), α_{1C} does not seem to have a C-terminal β subunit binding site.) Thus, β subunit interacts with the N terminus allosterically ("at distance"). The mechanism is unclear and seems to involve the voltage sensing machinery. It would be an oversimplification to assume that the action of β subunit is mechanistically analogous to removal of the N terminus, because of the differences in the effects of β-coexpression and of N-terminal deletions on charge movement; the former alters the voltage dependence of current activation without changing Q_{max}, and the latter increases Q_{max} (60, 61, 70). In this respect, the enhancement of channel activity by C- and N-terminal deletions is also mechanistically different, since C-terminal truncations do not affect Q_{max} and have been proposed to improve the coupling between voltage sensor movement and pore opening (12).

We have identified the initial 46 a.a. of the α_{1C} as a site indispensable for the potentiating action of PKC on the channel, since the removal of this segment fully eliminates the current increase caused by PKC activation. The decrease caused by PMA must be mediated by an action on another site. It is not clear whether the enhancing effect of PKC is caused by a direct phosphorylation of one of the amino acid residues in this region of the channel. Theoretically, phosphorylation may occur on another part of α_{1C} or even at an unknown protein (present in the oocytes) that modulates the channel via an interaction with the N terminus. Identification of the site of phosphorylation remains an important challenge for the future.

The biophysical mechanism by which PKC enhances the activity of the channel is unknown; one possibility is that it weakens the inhibitory control exerted by the N terminus. This assumption is in line with the observation that the PKC-induced increase in open probability of the channel is accompanied by an increase in the proportion of long openings (30, 36), like the N-terminal deletion (Table I). It is also compatible with the fact that PKC-elicited increase in Ca^{2+} channel current is attenuated by the β subunit; according to our hypothesis, the inhibition imposed by the N terminus is already weakened when β is present, and there is less room for a further improvement of channel activity (an occlusion mechanism). A cross-talk between the β subunit and PKC has also been proposed for the neuronal α_{1B} (N-type) channels (44, 72). However, the details of the proposed interaction differ significantly; in the N-type channel, PKC phosphorylates the I–II domain linker and thus counteracts an inhibitory effect of the G protein βγ subunit (Gβγ) which binds to the same loop; the Ca^{2+} channel β subunit also binds to the same loop, reducing the inhibition caused by Gβγ and thus occluding the PKC effect (72). In the L-type channel, no modulation by Gβγ has been reported; I–II linker is the site of β subunit binding but it is not phosphorylated by PKC[2]; β appears to interact with the PKC target site allosterically rather than sterically.

α_{1C} Subunits with Rabbit Heart-type N Terminus Should Be Widespread—Alternative splicing products of α_{1C} are found in various tissues; they have been proposed to play an important role in generating a diversity of electrophysiological properties (14, 42, 62, 73, 75). Alternative splicing was also proposed to take place in the N terminus, and "rabbit heart," "rat brain," and "lung" cDNAs have been assumed to be splice variants of the same gene product (42, 62). Recently, the genomic structure of human L-type Ca^{2+} channel has been characterized and shown to contain at least 44 invariant and 6 alternative exons (74). The unique stretch between Ser^{21} and Gly^{47} present in rabbit heart isoform (*RH* α_{1C} numbering; Fig. 8*D*) appears to be missing from the human genomic α_{1C} RNA (74), casting doubt on the biological relevance of the isoform cloned from rabbit heart. In fact, however, the details of splicing at the beginning of the human α_{1C} N terminus are unclear. The incomplete sequencing of putative intron 1 that separates between exon 1 ending with Gln^{16} (α_{1C} *HH* numbering; Fig. 8*D*) and exon 2 starting with Gly^{17} (corresponds to Gly^{47} of rabbit heart α_{1C}) left open the possibility of an additional exon(s) in this region (74). In view of the commonality of PKC-induced enhancement of Ca^{2+} channel activity in heart and smooth muscle cells of many mammalian species, it is probable that an additional variable exon encoding an N-terminal sequence similar or identical to the rabbit heart isoform may exist. The presence of an isoform containing this sequence in a particular cell type may be predictive of an enhancing PKC effect, and vice versa.

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