The role of the cytosolic N terminus of the main subunit (α1C) of cardiac L-type voltage-dependent Ca2+ channel was studied in Xenopus oocyte expression system. Deletion of the initial 46 or 139 amino acids (a.a.) of rabbit heart α1C caused a 5-10-fold increase in the whole cell Ca2+ channel current carried by Ba2+ (I_{Ba}), as reported previously (Wei, X., Neely, A., Olcese, R., Lang, W., Stefanelli, E., and Birnbaumer, L. (1996) Recept. Channels 4, 205–215). The plasma membrane content of α1C 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 β2A subunit was substantially stronger in channels with full-length α1C than in 46- or 139-a.a. truncated mutants, suggesting an interaction between β2A and N terminus. However, only the I-II domain linker of α1C, but not to N or C termini, bound β2A 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 α1C 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 Ca2+ channels has been reported in many species, we predict that isoforms of α1C subunits containing the initial N-terminal 46 a.a. similar to those of the rabbit heart α1C are widespread in cardiac and smooth muscle cells.

In the heart, Ca2+ current via the voltage-dependent L-type channels (dihydropyridine-sensitive) underlies the plateau of L-type channel 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 α1C subunit. Increase of Ca2+ channel activity by phorbol esters has also been observed in a mammalian cell line (baby hamster kidney) expressing the rabbit cardiac α1C subunit (39). The potentiation by phorbol esters of Ca2+ channels expressed in the oocytes is mediated by PKC because it is mimicked by diacylglycerols and blocked by specific PKC inhibitors (39, 40).

Both α1C and β are substrates for PKC-catalyzed phosphorylation (Ref. 41 and references therein). α1C subunit has been recognized as the target for the Ca2+ 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 α1C is involved in the PKC action. α1C 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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heart isoform may account for PKC potentiation, but this hypothesis has not been tested. It was unclear how this part of α1C 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 α1C 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. (45), 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 α1C isoforms with N termini of the “rabbit heart” type must also be widespread.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs and mRNAs—cDNAs of rabbit heart α1C (pCAH), rabbit heart β1C, and skeletal muscle α1S (pSAPES), titrated with HEPES, tris(hydroxymethyl)aminomethane, and magnesium chloride, with κ-casein (50 μg/ml) were injected with RNA as described (49, 50).** 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 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, 10 mM HEPES, and 3 mM C$_4$-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 KC$_2$, 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 DCLAMP (Axon Instruments, Foster City, CA).

**Immunocytochemistry—This was performed as described (50, 52). Oocytes were injected with mRNAs and incubated in NDE solution containing 0.5 mM 2-methyl-1-propanol and 25% (w/v) glycine and 50 mM CaCl$_2$ 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 phenylmethylsulfonyl fluoride, 1 mM pepstatin, and 1 mM 1,10-phenanthroline) and heated to 100 °C for 2 min. 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 phenylmethylsulfonyl fluoride, 1 mM pepstatin, and 1 mM 1,10-phenanthroline) and heated to 100 °C for 2 min. 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 phenylmethylsulfonyl 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$_2$O 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 35S-Labeled Proteins—**This was done essentially as described (21). The SMet/Cys-labeled β3a 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 Bie-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 35S-labeled β3a in 500 μl of phosphate-buffered saline with 0.05% Tween 20. (In 53), incubated for 1 h at 4 °C with protein A-Sepharose, and pelleted.

**Results**

**N-terminal Deletions Increase Ca2+ 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 α1C, in which these amino acids, except the initial methionine, have been deleted (α1CΔN2-46; see Fig. 6 for a scheme of the channel to help localize the deletions). Ba2+ currents via the expressed Ca2+ channels were measured using the two-electrode voltage clamp technique. The subunit composition of the channels used was varied according to the specific questions asked. The expression of wild-type (WT) α1C subunit alone was rarely employed because it resulted in very small currents, usually below 20 nA (cf. Ref. 45). In most cases, α1C,αδ 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 Ca2+ channel currents carried by Bi3+ (1mM) via channels containing the mutant α1CΔN2-46 subunit were 5–10-fold larger than with the wild-type α1C in all subunit combinations tested as follows: α1C alone, α1Cα2δ, or α1Cα2δβ (e.g. Fig. 1A). Even when the oocytes were injected with twice as much WT α1C RNA, the ΔN2-46 mutant still gave ~4-fold larger currents (Fig. 1B). The kinetics of the current (Fig. 1A; 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. 1C). An additional deletion mutant missing most of the N terminus, α1CΔN1-139, increased the current about ~10-fold compared with WT (Fig. 1D) and did not shift the I–V curve (data not shown). However, we noticed differences in voltage-dependent inactivation of α1CΔN1-139 and the WT channels. In the α1C,αδ 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. 1E).

Coexpression of β2A subunit shifted the 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 α1C,αδ channels, in α1C,αβδ channels of the 139-a.a. deletion caused a leftward shift of the curve. The inactivation kinetics were unaffected. With 3-mM long depolarizing pulses to +30 mV, after an initial decay the α1C,αδ,β6 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 ΔN2-139. 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
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 \(^{[35S]}\)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. α\(_{1C}\), relative amounts of the α\(_{1C}\)ΔN\(_{2–46}\) 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.

The increase in whole cell Ca\(^{2+}\) channel currents by the N-terminal deletions was significantly higher in the α\(_{1C}\) and ΔN\(_{2–46}\) α\(_{1C}\) in the plasma membrane, 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\(_{Ca}\) 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 (α\(_{1C}\)δ 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 megohms), 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\(_{2–46}\) α\(_{1C}\)δ channels.

Fig. 4A 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. 4B, 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 \(I_{Na}\) in oocytes expressing WT or \(\Delta N_{2–46}\). a, in combination with \(\alpha_2\). In the control group, WT cRNA and \(\alpha_2\) cRNA were injected at 2.5 ng/oocyte; in the test group, \(\alpha_1C\) \(\Delta N_{2–46}\) and \(\alpha_2\) cRNAs 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 \(I_{Na}\) induced by application of 1 \(\mu M\) (−)-BayK 8644 at different voltages. Data were averaged from three batches of oocytes (\(n > 15\) in all groups).

\(\bigcirc\), WT; \(\Box\), \(\Delta N_{2–46}\).

Fig. 4. Comparisons of the single channel behavior of WT and \(\Delta N_{2–46}\). 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 \(\Delta N_{2–46}\) 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 \(\tau_1\) and \(\tau_2\) 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 \(\Delta N_{2–46}\) channels |
|-----------------------------|-------------------|
| \(P_o\)                     | \(\Delta N_{2–46}\) |
| 0.02 ± 0.01                 | 0.27 ± 0.06       |
| 0.45 ± 0.08                 | 0.41 ± 0.06       |
| 74.3 ± 11.63                | 37 ± 8           |
| 2.46 ± 0.44                 | 2.37 ± 0.3       |
| 25.67 ± 11.64               | 63 ± 8.1         |

\(\alpha_1C\) subunit produces a channel that shows a significant increase in \(P_o\) and \(f_1\), but we could not detect binding to any one of the other fusion proteins tested (Fig. 6B). The results with \(\alpha_1C\) were similar (in this case, we tested the effect of coexpression of \(\alpha_2B\) on channels composed of \(\alpha_1C\) alone).

To probe for a possible physical interaction between the \(\beta\) subunit and the N terminus of \(\alpha_1C\), we have measured \(\Delta N_{2–46}\) binding of GST fusion proteins corresponding to some of the intracellular parts of \(\alpha_1C\), to \(\beta_{2A}\) synthesized in reticulocyte lysate and eluted with \(\text{[35S]}\text{methionine/cysteine} \) (see Ref. 20). The following GST fusion proteins were used: GST-N, corresponding to amino acids (a.a.) 1–154, i.e. the whole N terminus; GST-LI-II, corresponding to most of the intracellular linker between domains I and II (a.a. 438–550); and GST-C1 (a.a. 1664–1845) and GST-C2 (a.a. 1821–2171), corresponding to two parts of the C terminus. The scheme of the \(\alpha_1C\) subunit in Fig. 6A illustrates the positions of the different pieces. As expected (20, 21), \(\beta_{2A}\) bound to GST-L1-II, but we could not detect binding to any one of the other fusion proteins tested (Fig. 6B). (Note that the amounts of all GST fusion proteins loaded on the gel were similar, as demonstrated in Fig. 6C.)

If the N terminus obstructs activation, then artificial proteins corresponding to fragments of N terminus may be expected to reduce \(I_{Na}\). We constructed DNAs encoding proteins corresponding to N-terminal a.a. 1–139 of \(\alpha_1C\) (N139), N-terminal a.a. 88–139 (N88139), and C-terminal a.a. 1664–1845 (C1664–1845), denoted as C in Fig. 7. The corresponding RNAs directed the expression of proteins of correct size in reticulocyte 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 native 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 \(\beta\) 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 \(\beta\) 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 \(\beta\) subunit with channels containing either WT or one of the deletion mutants of \(\alpha_1C\) (\(\Delta N_{2–46}\) or \(\Delta N_{2–139}\)). Coexpression of \(\beta_{2A}\) with \(\alpha_1C\beta\) increased the whole cell \(I_{Na}\) both in WT (Fig. 5A, a) and in \(\Delta N_{2–139}\) (Fig. 5A, b), but the increase caused by coexpression of \(\beta_{2A}\) was significantly \((p < 0.01)\) smaller in \(\Delta N_{2–139}\) than in WT, at all voltages (Fig. 5B). The results with \(\Delta N_{2–46}\) were similar (in this case, we tested the effect of coexpression of \(\beta_{2A}\) on channels composed of \(\alpha_1C\) alone).
lysate (data not shown). Coexpression of RNAs encoding N1–138 and N88–139 proteins with channels containing a truncated α1C (either ΔN2–46 or ΔN2–139) reduced I_{Ba} whereas C1665–1845 was without effect (Fig. 7). The reduction was stronger when the channels contained the ΔN2–139 truncation than ΔN2–46 possibly because in the ΔN2–46 α1C 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. 8A shows diaries of representative experiments in which the PKC activator, PMA, was added to the extracellular solution at 1 nM. The oocytes were injected with the subunit combinations indicated near the traces. α, 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 β2A coexpression on currents containing WT (○) and ΔN2–139 (□) α1C (2 batches; 10 oocytes in each group). In all groups, α2Δβ 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 β2A coexpression on channels containing WT (●) and ΔN2–46 (□) α1C (two batches; n = 18 in each group). In these experiments, α2Δβ was not expressed; thus, channels composed of α1C alone versus α1Cβ2A were tested. Averaging of data was done as explained in B.

Full subunit combination, α1Cα2Δβ, was tested, because the α1C(R461665) mutant usually gave rather small currents when expressed without β. Fig. 8B (right column) shows that the effect of PMA was not altered by this truncation (compare with the results obtained with WT α1Cβ2A).

In the following experiments, α1Cα2Δβ 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. 8C 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. 8A, triangles). A rat brain α1C 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 α1C(S533I) (a putative PKC site in linker I–II), α1C(S1575A) (a C-terminal site preceding the S1665 truncation), and an N-terminal deletion ΔN88–139.

Fig. 8D compares a.a. sequences (deduced from the corresponding cDNA sequences) of the initial N-terminal segment of three most widely tested variants of α1C as follows: rabbit heart α1C (48) used in this study (RH); rat brain α1C 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 α1C. N termini of two additional isoforms cloned from lung (62) and rat brain (rBC-I; Ref. 42) are identical to that of RH α1C. The correlation between PMA effects
N Terminal in Cardiac Ca\textsuperscript{2+} Channel Function and Modulation

DISCUSSION

N Terminal Modulates L-type Channel Gating—Our results demonstrate the functional importance of the N terminus of α\textsubscript{1C} subunit in L-type Ca\textsuperscript{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\textsuperscript{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\textsubscript{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\textsuperscript{2+} channels as testified by the −10-fold increase in \( P_{e} \). This change in channel gating alone is sufficient to account for the increase in whole cell Ca\textsuperscript{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\textsubscript{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\textsubscript{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\textsuperscript{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 (\( Q_{\text{max}} \)) caused by coexpression of Ca\textsuperscript{2+} channels β or α\textsubscript{\textbeta} subunits (60, 61, 63, 64) usually correlate well with the amount of α\textsubscript{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\textsubscript{1C} increase \( Q_{\text{max}} \) without changing its voltage dependence (1)? We claim that, in general, a change in \( Q_{\text{max}} \) does not necessarily report a change in the number of functional channels. In various voltage-dependent channels, \( Q_{\text{max}} \) can be altered by drugs, toxins, or by

FIG. 7. Inhibitory effect of coexpression of N-terminal fragments on \( I_{\text{Ba}} \) via channels containing ΔN\textsubscript{2−139} (A) or ΔN\textsubscript{2−46} α\textsubscript{1C} (B). Top, summaries of all experiments with ΔN\textsubscript{2−139} (a, b) and ΔN\textsubscript{2−46} (a, b). Channels were expressed in the α\textsubscript{1C}β\textsubscript{δ} composition, without (control) or with the addition of RNA of the desired fragment, as indicated below the bars. In each oocyte, \( I_{\text{Ba}} \) 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 \( I_{\text{Ba}} \) 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. C, control; □, N\textsubscript{1−139}; Δ, N\textsubscript{1−139}; V, C.

FIG. 8. Importance of the β subunit and of the unique N-terminal part of α\textsubscript{1C} in modulation of the channel activity by β-PMA-induced stimulation of PKC. A, changes in \( I_{\text{Ba}} \) 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\textsubscript{δ} subunit. The right bar shows the effect of β-PMA on channels containing the α\textsubscript{1C}β\textsubscript{δ} truncation mutant. In each oocyte, \( I_{\text{Ba}} \) 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 α\textsubscript{1C}β\textsubscript{δ} obtained by two-tailed t-test. C, summary of the effects of β-PMA on different α\textsubscript{1C} mutants compared with the WT. The bar denoted neuronal corresponds to the neuronal rbc-II α\textsubscript{1C} isofrom. 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\textsuperscript{2+} channel α\textsubscript{1C} subunits (see definitions in the text). Asterisks indicate identity in all three sequences, x indicates identity in two out of three sequences.
fatty acids. For instance, $Q_{\text{max}}$ in Na⁺ channels is decreased by Anthopleurin-A toxin (65), fatty acids (66), and lidocaine (67); in L-type Ca²⁺ channels, $Q_{\text{max}}$ is decreased by dihydropyridines (68, 69). Thus, the N terminus might decrease $Q_{\text{max}}$ by directly or allosterically interfering with the movement of the voltage sensor of the channel; removal of the N terminus would increase $Q_{\text{max}}$.

$\beta$ Subunit and PKC Interact with the N Terminus—Our data suggest a cross-talk between the N terminus of $\alpha_{\text{IC}}$ and the $\beta_{2A}$ subunit. It appears that the presence of the $\beta$ subunit attenuates the inhibitory effect of the N terminus. This is supported by the following observations. (i) Coexpression of $\beta_{2A}$ 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 $\beta$-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 attenuation caused by $\beta$ subunit is less pronounced. (ii) $\beta$ subunit counteracts the effect of PKC which is mediated via an interaction with the N terminus (see below). A cross-talk between $\beta_{2A}$ and the N terminus of $\alpha_{\text{IC}}$ is also supported by the observation that $\Delta N_{\text{L,139}}$ deletion-induced changes in voltage-dependent inactivation properties are different in the absence and presence of $\beta_{2A}$. The interaction does not appear to be a direct one, since $\beta_{2A}$ does not bind a GST fusion protein of the first 154 a.a. (Of the GST fusion proteins tested, the only $\beta_{2A}$-binding protein was that of the I–II domain linker, which contains a $\beta_{2A}$-binding site conserved in all known $\alpha_1$ subunits (20, 21, 70, 71); the results of Fig. 6 suggest that, unlike $\alpha_{1E}$ (71), $\alpha_{\text{IC}}$ does not seem to have a C-terminal $\beta$ subunit binding site.) Thus, $\beta$ 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 $\beta$ subunit is mechanistically analogous to removal of the N terminus, because of the differences in the effects of $\beta$-coexpression and of N-terminal deletions on charge movement; the former alters the voltage dependence of current activation without changing $Q_{\text{max}}$ and the latter increases $Q_{\text{max}}$ (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 $Q_{\text{max}}$ 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 $\alpha_{\text{IC}}$ as a site indispensable for the potentiation effect 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 $\alpha_{\text{IC}}$ 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 Ca²⁺ channel current is attenuated by the $\beta$ subunit; according to our hypothesis, the inhibition imposed by the N terminus is already weakened when $\beta$ is present, and there is less room for a further improvement of channel activity (an occlusion mechanism). A cross-talk between the $\beta$ subunit and PKC has also been proposed for the neuronal $\alpha_{1B}$ (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 $\beta y$ subunit ($\alpha y$) which binds to the same loop; the Ca²⁺ channel $\beta$ subunit also binds to the same loop, reducing the inhibition caused by $\alpha y$ and thus occluding the PKC effect (72). In the L-type channel, no modulation by $\alpha y$ has been reported; I–II linker is the site of $\beta$ subunit binding but it is not phosphorylated by PKC²; $\beta$ appears to interact with the PKC target site allosterically rather than sterically.

References—We thank I. Lotan for many discussions and for a critical reading of the manuscript; M. Hosey for the gift of the Card-I antibody; and T. P. Snutch for the gift of the rbC-II cDNA.

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