Modulation of distinct isoforms of L-type calcium channels by G_q-coupled receptors in Xenopus oocytes
Antagonistic effects of Gβγ and protein kinase C

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L-type voltage dependent Ca^{2+} channels (L-VDCC; Ca_{1.2}) are crucial in cardiovascular physiology. In heart and smooth muscle, hormones and transmitters operating via G_{q} enhance L-VDCC currents via essential protein kinase C (PKC) involvement. Heterologous reconstitution studies in Xenopus oocytes suggested that PKC and G_{q}-coupled receptors increased L-VDCC currents only in cardiac long N-terminus (NT) isoforms of α_{1C}, whereas known smooth muscle short-NT isoforms were inhibited by PKC and G_{q} activators. We report a novel regulation of the long-NT α_{1C} isoform by Gβγ. Gβγ inhibited whereas a Gβγ scavenger protein augmented the G_{q}- but not phorbol ester-mediated enhancement of channel activity, suggesting that Gβγ acts upstream from PKC. In vitro binding experiments reveal binding of both Gβγ and PKC to α_{1C}-NT. However, PKC modulation was not altered by mutations of multiple potential phosphorylation sites in the NT, and was attenuated by a mutation of C-terminally located serine S1928. The insertion of exon 9a in intracellular loop 1 rendered the short-NT α_{1C} sensitive to PKC stimulation and to Gβγ scavenging. Our results suggest a complex antagonistic interplay between G_{q}-activated PKC and Gβγ in regulation of L-VDCC, in which multiple cytosolic segments of α_{1C} are involved.

Introduction

L-type voltage-dependent calcium channels (L-VDCC; Ca_{1.2}) play a critical role in excitation-contraction coupling in cardiac, skeletal and smooth muscle.1,3 These channels are known to be modulated by a variety of hormones and transmitters, operating via GPCRs and second messengers, thereby profoundly affecting target tissues.4 A prominent modulatory pathway in the cardiovascular system is the enhancement of L-type Ca^{2+} currents by protein kinase C (PKC). Constitutive activity of PKC may underlie a tonic Ca^{2+} influx via L-VDCC in some smooth muscle cells,3 and activation of PKC is believed to critically participate in the effects of G_{q}-coupled GPCRs and other modulators of Ca_{1.2}. For instance, vasoconstrictors such as angiotensin II and acetylcholine (ACh), operating mainly via G_{q}-coupled GPCRs in smooth muscle, induce release of Ca^{2+} from intracellular stores and enhance L-VDCC currents.4,5 As part of this signaling cascade, protein kinase C (PKC) is activated and was shown to be essential for Ca^{2+} current enhancement (discussed in refs. 5, 8–10). A less prominent inhibitory effect of PKC activators, that occasionally follows the enhancement, has been reported in cardiac and some smooth muscle cells.11–13

The enhancing effect of PKC and G_{q}-activating GPCRs on L-VDCC has been heterologously reconstituted only in Xenopus oocytes,10,14,15 enabling a detailed study of molecular mechanisms of these modulations. Therefore, Xenopus oocytes continued to be the heterologous expression system of choice in the current study.

The Gβγ dimer was also implicated as part of signaling cascades affecting the L-type channel. A complex and incompletely understood synergistic interaction between Gβγ, phosphoinositide 3 (PI3) kinase, PKC and often Src occurs in angiotensin, muscarinic m2 or β-adrenergic receptor-induced enhancements of L-type Ca^{2+} currents in some smooth muscle cells,9,16–18 The N- and C- termini (NT and CT, respectively) of the pore-forming α_{1} subunit of L-VDCC, Ca_{1.2α} (α_{1C}), contain binding sites for Gβγ, and coexpression of Gβγ with the channel in Xenopus oocytes resulted in a dual effect: a tonic Ca^{2+}- and CaM-dependent inhibitory effect, and an enhancement when Ca^{2+} was chelated or when NT and/or CT gating-regulating segments were removed.19 The Gβγ-dependent, Ca^{2+}-independent enhancement is in line with known effects of purified Gβγ on L-VDCC in smooth muscle,10,20,21 yet the physiological role of the inhibitory effect of Gβγ is unknown.

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Neuronal VDCCs are inhibited by GPCRs in processes that are voltage independent (and mediated by several second messengers)\(^\text{22-25}\) and voltage dependent (VD). The VD process is mediated by G\(\beta\gamma\). It is fast, membrane delimited and occurs in members of the Cal2 group.\(^\text{26-28}\) Several intracellular segments of \(\alpha\) subunits of neuronal VDCCs were shown to bind G\(\beta\gamma\); mainly the loop connecting the domains I and II, L1 (see Fig. 1A) and parts of the CT.\(^\text{29-31}\) The NT was clearly identified as another molecular determinant critical for the G\(\beta\gamma\) modulation. It binds L1 in a G\(\beta\gamma\)-dependent manner, thereby rendering the channel sensitive to the G\(\beta\gamma\) inhibition.\(^\text{32-36}\) Furthermore, there is a crosstalk between the G\(\beta\gamma\) and PKC pathways in the modulation of C\(a\)\(_{2,2}\) (N-type) channels. PKC activation was shown to relieve the tonic G\(\beta\gamma\)-mediated inhibition, suggesting a role for PKC phosphorylation of these channels.\(^\text{31,35-37}\) However, no such crosstalk was ever observed or investigated in the L-type channel; L1 of \(\alpha\) does not bind G\(\beta\gamma\).\(^\text{19,29}\)

A crucial factor determining the elaborate PKC signaling paradigm of L-VDCC is which isoform of \(\alpha\) is expressed. We have previously demonstrated the reconstitution of the modulation of L-type calcium channel by PKC and by \(G\)\(_{\alpha}\)-coupled GPCRs in Xenopus oocytes, and found it to be dependent on the isoform of \(\alpha\) used. Only a long-NT (\(\alpha\)\(_{\alpha}\)-LNT; cardiac) isoform was upregulated by these agents, while only a decrease in the current was observed in short NT containing \(\alpha\)\(_{\beta\gamma}\) (\(\alpha\)\(_{\beta\gamma}\)-SNT; smooth muscle/brain type).\(^\text{10,40}\) The exact molecular mechanism underlying the current enhancement was not completely resolved. Moreover, despite the established role of PKC in the regulation of smooth muscle L-VDCCs, only a short NT isoform is known to be expressed in these cells.\(^\text{10,41}\) It was recently shown that the Cav1.2 gene undergoes extensive alternative splicing, often depending, in the smooth muscle, on culturing or pathologic conditions.\(^\text{42}\) Thus, there are two plausible explanations: (1) a unique mechanism which enhances \(\alpha\)\(_{\beta\gamma}\)-SNT in smooth muscle in response to G\(q\) activation must exist; or (2) one of the short-NT isoforms expressed in smooth muscle differs from the short NT \(\alpha\)\(_{\beta\gamma}\) studied so far in a manner that renders it PKC-sensitive.

Here we report the involvement of the G\(\beta\gamma\) dimer in the PKC-mediated, G\(\alpha\)\(_q\)-induced modulation of L-VDCC by opposing the effects of G\(\alpha\)\(_q\)-activating GPCRs but not of \(\beta\)-phorbol myristate acetate (PMA), a potent direct PKC activator. We characterize the effects of activation of the G\(\alpha\)\(_q\)-coupled receptor (m3R) by ACh, as well as involvement of G\(\beta\gamma\) on distinct isoforms of \(\alpha\)\(_{\beta\gamma}\) differing by the length of their NT as well as their L1 loop. Finally, we provide further insight to the mechanism of PKC-induced current enhancement by mutational analysis of putative phosphorylation sites.

### Results

G\(\beta\gamma\) hinders the enhancement of \(I_{\text{Ba}}\) following activation of G\(\alpha\)\(_q\)-coupled receptors. Modulation L-VDCC by ACh was studied in Xenopus oocytes expressing full subunit composition (\(\alpha\)\(_{\beta\gamma}\), \(\beta\)\(_{\alpha\beta}\), \(\delta\)) of the cardiac L-type calcium channel (\(\alpha\)\(_{\beta\gamma}\)-LNT; see Fig. 1A, Ba) and G\(\alpha\)\(_q\)-coupled muscarinic receptors m3R or m1R. Ca\(^{2+}\) channel currents were studied with 40 mM Ba\(^{2+}\) as charge carrier. Typical whole-cell Ba\(^{2+}\) currents (\(I_{\text{Ba}}\)) and a current-voltage (I-V) curve in a representative oocyte expressing \(\alpha\)\(_{\beta\gamma}\)-LNT are shown in Figure 2A. Application of ACh resulted, as previously shown, in upregulation of the current followed by a decline.\(^\text{10}\) Figure 2B illustrates the time course of this effect (“control,” filled circles), and Figure 2C exemplifies typical shapes of \(I_{\text{Ba}}\) recorded at +20 mV before application of ACh (\(t = 0\)) and at the peak of ACh-induced current increase, 5 min after addition of ACh (\(t = 5\)).

We have previously found that the activation of PKC is crucially involved in mediating the increase in \(I_{\text{Ba}}\). However, when the G\(\beta\gamma\) scavenger protein, m-cBARK (a myristoylated C-terminus of \(\beta\)-adrenergic kinase 1), was coexpressed, the ACh-induced increase in \(I_{\text{Ba}}\) was significantly greater than in control. This was observed when either m1R or m3R were expressed to mediate the ACh action via G\(\alpha\)\(_q\) (Fig. 2B, D and E). The effect of m-cBARK was statistically significant, \(p < 0.01\) (summarized in Fig. 2D). Bis-indolylmaleimide (Bis; a specific PKC inhibitor) significantly attenuated the ACh-induced increase in \(I_{\text{Ba}}\), supporting the role of PKC in the current enhancement (see Fig. 2E).\(^\text{10}\) Bis also attenuated the ACh-induced increase in \(I_{\text{Ba}}\) when m-cBARK was coexpressed, but a residual increase could still be

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**Figure 1.** Exon-intron structure of four \(\alpha\) subunit constructs. (A) \(\alpha\)\(_{\beta\gamma}\) protein structure and the location of the protein segment encoded by exon 9a, between exon 9 and 10. (B) Partial exon-intron structure of the four \(\alpha\)\(_{\beta\gamma}\) isoforms used. These differ by the length of the NT (encoded by exon 1a, long-NT; exon 1, short-NT) and L1 (absence or presence of exon 9a).

| Construct | Exon 1 | Exon 2 | Exon 3 | Exon 4 | Exon 5 | Exon 6 | Exon 7 | Exon 8 | Exon 9 | Exon 10 |
|-----------|--------|--------|--------|--------|--------|--------|--------|--------|--------|---------|
| \(\alpha\)\(_{1C}\)-LNT | 1a | 2 | 3 | 4 | 9 | 10 | 11 | 12 |
| \(\alpha\)\(_{1C}\)-LNT+9a | 1a | 2 | 3 | 4 | 9 | 10 | 11 | 12 |
| \(\alpha\)\(_{1C}\)-SNT | | | | | | | | |
| \(\alpha\)\(_{1C}\)-SNT+9a | | | | | | | | | | |
observed (Fig. 2E). The potentiating action of m-cβARK was the first indication of an involvement of Gβγ in the ACh-induced modulation. In order to further substantiate the involvement of the Gβγ dimer, we have coexpressed Gβγ along with the channel and the muscarinic receptor. This completely abolished the ACh-induced increase in I_{Ba} (Fig. 2B–D).

Figure 2. Gβγ negates upregulation of I_{Ba} by G_{q}-coupled muscarinic receptors but not by direct activation of PKC by PMA. (A) a. net Ba^{2+} currents (obtained by subtracting currents remaining after block with 200 μM Cd^{2+}, as described in recorded at 10 mV steps from -70 mV to +50 mV. b. Representative current-voltage curve. Peak amplitudes measured at each voltage step were used. (B) Time course of changes in I_{Ba} in response to ACh in oocytes expressing rabbit long-NT with m3R and m-cβARK or Gβγ. I_{Ba} was measured by 200 ms steps from -80 to 20 mV. After allowing the current to stabilize, ACh was added (as indicated). ACh was washed out after 6 min and I_{Ba} was monitored every 30 sec for additional 5 min. (C) Representative traces depicting I_{Ba} before (t = 0) and 5 min after addition of ACh (t = 5 min). (D) Summary of effects of ACh in oocytes expressed rabbit long-NT α_{1C} and m3R with m-cβARK or Gβγ. Black bars represent the enhanced portion of the modulation measured, in each cell, at the peak of the enhancement. The gray bars represent the declining phase measured 10 min after the addition of ACh or PMA. (E) Summary of the effects of ACh in oocytes expressing rabbit long-NT α_{1C} and m1R. (F) Coexpression of m-cβARK in oocytes expressing α_{1C}ΔN_{2–139} and m1R. a. Summary of the effects of application of PMA to oocytes expressing rabbit long-NT α_{1C} without (control) or with m-cβARK or Gβγ. Black bars represent the enhanced portion of the modulation measured, in each cell, at the peak of the enhancement. The gray bars represent the declining phase measured 10 min after the addition of ACh or PMA. b. Representative traces depicting I_{Ba} before (t = 0) and current recorded at t = 8 min. Statistics: asterisks (*) indicate significant differences from the control group; pound signs (#) indicate significant differences between various groups as indicated by the connecting brackets. All tests were performed by one-way ANOVA (see Experimental Procedures). ** or ##, p < 0.01; ###, p < 0.001. The number of cells tested is indicated on or above the bar.
membrane by biotinylation. No significant changes in m1R expression were noted in oocytes expressing channel alone, with Gβγ, or with m-cβARK (data not shown). Further, the persistence of the ACh-induced decrease in I_{ba} in oocytes coexpressing Gβγ or m-cβARK, does not support a role for changes in plasma membrane receptor levels. While not in the focus of this study, this decrease is a genuine response to the activation of these GPCRs.

To further understand the mode of action of Gβγ in PKC modulation, we activated PKC directly (as opposed to via a receptor) with the phorbol ester PMA and studied the effects of coexpressed Gβγ or m-cβARK. Both were without effect; the increase in I_{ba} by PMA was not significantly different than in the channel expressed alone (Fig. 2G). Compartmentalization of PKC signaling in cardiac T-tubules was previously shown, where PMA had opposing effects on myocytes as compared with receptor activated PKC. Nevertheless, PMA was used here to robustly and directly activate PKC as means to decipher the molecular mechanism underlying PKC modulation and compare those to receptor activated PKC.

Activation of Gq in Xenopus oocytes characteristically results in typical chloride currents. These currents develop since calcium is being released from intracellular stores during the GPCR-Gq-PLC-Ins(1,4,5)P3 activation cascade, and the oocytes contain specific calcium-dependent chloride channels that open consequently. Indeed, activation of m1R by ACh in oocytes expressing only the receptor yielded these typical Cl− currents. Coexpression of Gβγ abolished these Cl− currents, while chelation of Gβγ (by coexpressing m-cβARK) significantly augmented Cl− currents (Fig. 3A and B). Thus, it appears that Gβγ probably acts on an event in the GPCR-Gq signaling pathway that is unrelated to PKC-induced phosphorylation (which is not involved in the activation of Ca2+-dependent Cl channels).

PKC appears to regulate α1C by phosphorylating a C-terminal, but not N-terminal, sites. Although PKC does not phosphorylate the initial 20 or 46 a.a. of long-NT which are crucial for the PKC-induced enhancement of the channel’s current,46 other putative phosphorylation sites exist on α1C, including in proximal and distal NT. We have explored most potential sites of PKC phosphorylation in the NT of the α1C-LNT isoform by mutating Ser or Thr residues to Ala (Fig. S1). The first α1C variant comprised four such mutations proximal to a.a. 75; these mutations also included T27, a site previously reported as potentially phosphorylated by PKC. The second variant comprised five mutations distal to aa. 75. Both mutated channel variants were still potentiated by ACh (Fig. 4A), suggesting that phosphorylation of NT is not involved in PKC-induced phosphorylation of L-VDCC activity. At present we cannot exclude the less likely possibility that one of the remaining non-mutated Ser or Thr in the NT may be a non-conventional PKC phosphorylation site, or

Coexpression of m-cβARK did not alter the modulation by ACh of the NT deletion mutant, α1CΔN2–139 (Fig. 2F). This channel variant lacks the “inhibitory module” segment of the NT (the first 20 a.a.) which is crucial for the modulation of the cardiac-type α1C by ACh and PKC.10,15,43 Both in the absence and presence of m-cβARK, there was only a decline in the current. The decline was quantitatively similar with or without m-cβARK. Thus, m-cβARK affects only the ACh-induced increase and does not operate by reducing or eliminating the decline in I_{ba}. In support, overexpression of Gβγ did not alter the ACh-induced decline in I_{ba} in α1C-SNT (data not shown), indicating that Gβγ operates on the enhancing effect of G_{1α}-activation. To exclude a possible effect of coexpressed Gβγ or m-cβARK on the amount of receptor expressed on the plasma membrane, which may account for the observed changes in modulation, we measured total m1R protein level on the plasma membrane by biotinylation. No significant changes in m1R expression were noted in oocytes expressing channel alone, with Gβγ, or with m-cβARK (data not shown). Further, the persistence of the ACh-induced decrease in I_{ba} in oocytes coexpressing Gβγ or m-cβARK, does not support a role for changes in plasma membrane receptor levels. While not in the focus of this study, this decrease is a genuine response to the activation of these GPCRs.

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that simultaneous mutation of all serines and threonines in the NT might produce a different result.

An important phosphorylation site in α1C is Ser1928 on distal CT. Ser1928 was found to be phosphorylated by protein kinase A (PKA), and also by PKC in vitro. Application of PMA (10 nM) to oocytes expressing the mutated α1C-S1928A with δ and β2b produced a significantly smaller enhancement of I_{Ba} as compared with wt α1C (28% vs. 82%; Fig. 4B). Similarly, the S1928A mutation eliminated the increase in I_{Ba} caused by ACh via m3R, yet left a prominent ACh-induced decrease. This suggests that the C-terminal S1928 may be one of several sites whose phosphorylation by PKC enhances the activity of L-VDCC, whereas NT does not appear to be a target for PKC phosphorylation.

**α1C NT contains a PKC binding site.** Gβγ was previously shown to bind the NT of the L-type channel. A more detailed scan of shorter GST-fused segments of the NT revealed that in vitro synthesized Gβγ binds to the distal GST-fused segments of the NT, NT 95–140 and NT 95–154 (Fig. 5A). This part of NT is encoded by exon 2 and is conserved among all known isoforms of α1C (see Fig. 1). Similar binding experiments with in vitro synthesized PKCα and GST-fused NT sections resulted in a similar binding pattern (Fig. 5B). I_{Ba} upregulation via Gβγ was previously shown to be Ca^{2+} dependent (abolished when Ca^{2+} was strongly chelated) as well as PLC- (hence also diacylglycerol-) dependent. Thus, it appears that a conventional PKC isoform is activated via this cascade. PKCα, a conventional PKC, was shown to be abundant in both cardiac and smooth muscle preparations. This isoform was therefore selected for the in-vitro binding experiments. PKCα bound to GST fused full-length NT (1–154) and to smaller segments: 47–154 and 95–154, but not to the beginning or middle part of NT (segments 1–47, 40–87, 60–120 and 84–120), suggesting that PKC probably binds to the distal part of NT close to the plasma membrane boundary (Fig. 5B and C). These results are at odds with the report that only CT but not NT binds PKCα, but the autoradiography used here for the detection of bound PKCα is a more sensitive method than the western blotting used in the above work. In all, our data support the existence of a macromolecular signaling complex between α1C and PKCα as proposed by Yang et al. where NT may be an anchoring site for PKC.

**To inquire whether Gβγ attenuates the effect of PKC by directly competing for interaction with the NT, binding**
The binding of PKC to NT.

α of 1.5 μ experiments with PKC α carinic receptors still failed to enhance I Ba via the previously manner. that are affected by ACh and PKC, possibly in a G βγ-dependent activation system used, i.e., Xenopus oocytes vs. HEK 293 cells, the difference is not negligible and may require further investigation. Inactivation kinetics of IBa were measured using a 10 sec long test pulse (from -10 mV to +40 mV) and were found to be similar as compared with α 2b/HK variants (Fig. S4 Table S1). It should be noted that the half-activation voltage (V 0) of α 2c-SNT, +9a was shifted to hyperpolarized potentials by ~3 mV. Although such differences may be attributed to the heterologous expression system used, i.e., Xenopus oocytes vs. HEK 293 cells, the difference is not negligible and may require further investigation. Inactivation kinetics of I Ba were measured using a 10 sec long test pulse (from -10 mV to +40 mV) and were found to be similar in all four α 2c variants as compared with α 2c-SNT, when expressed without β 2c. Similar observations were made by Liao et al. in HEK cells, however the shift was much more pronounced, by ~11 mV. Although such differences may be attributed to the heterologous expression system used, i.e., Xenopus oocytes vs. HEK 293 cells, the difference is not negligible and may require further investigation. Inactivation kinetics of I Ba were measured using a 10 sec long test pulse (from -10 mV to +40 mV) and were found to be similar in all four α 2c variants as compared with α 2c-SNT, when expressed without β 2c. Similar observations were made by Liao et al. in HEK cells, however the shift was much more pronounced, by ~11 mV. Although such differences may be attributed to the heterologous expression system used, i.e., Xenopus oocytes vs. HEK 293 cells, the difference is not negligible and may require further investigation. Inactivation kinetics of I Ba were measured using a 10 sec long test pulse (from -10 mV to +40 mV) and were found to be similar in all four α 2c variants as compared with α 2c-SNT, when expressed without β 2c. Similar observations were made by Liao et al. in HEK cells, however the shift was much more pronounced, by ~11 mV. Although such differences may be attributed to the heterologous expression system used, i.e., Xenopus oocytes vs. HEK 293 cells, the difference is not negligible and may require further investigation. Inactivation kinetics of I Ba were measured using a 10 sec long test pulse (from -10 mV to +40 mV) and were found to be similar in all four α 2c variants as compared with α 2c-SNT, when expressed without β 2c. Similar observations were made by Liao et al. in HEK cells, however the shift was much more pronounced, by ~11 mV. Although such differences may be attributed to the heterologous expression system used, i.e., Xenopus oocytes vs. HEK 293 cells, the difference is not negligible and may require further investigation.
the ER to the plasma membrane or in the regulation of maximal open probability.\textsuperscript{43}

Enhanced effect of PKC and ACh on long-L1 (+9a) isoforms. In order to study the effect of PKC on the long-L1 isoforms, we expressed all four isoforms in Xenopus oocytes and measured Ba\textsuperscript{2+} currents at +20 mV before and during application of PMA (10 nM). PMA induced a greater increase in the current in the two long-loop isoforms (+9a); the difference reached statistical significance only in the α\textsubscript{1C}-SNT isoforms (Fig. 7A). The enhancement of I\textsubscript{Ba} in α\textsubscript{1C}-SNT,+9a was not much above basal levels, but remember that normally only a decrease is observed in α\textsubscript{1C}-SNT. The increase in I\textsubscript{Ba} is actually underestimated since it overlaps the decrease. Moreover, the physiological relevance of the decrease in I\textsubscript{Ba} induced by activation of G\textsubscript{q} seen in Xenopus oocytes is not clear, it may be a "side effect" in this model system.\textsuperscript{10} These results strengthen the role of exon 9a in α\textsubscript{1C}-SNT,+9a as participating in the enhancing effect of PKC observed in native smooth muscle tissue.

Following the partial restoration of the increase in I\textsubscript{Ba} by PMA in the α\textsubscript{1C}-SNT,+9a isoform, we set out to examine the effects of ACh, via m3R, in all four isoforms. For the most part, the two long-L1 (+9a) isoforms demonstrated similar responses to application of ACh as their controls; i.e., an increase in I\textsubscript{Ba} in α\textsubscript{1C}-LNT,+9a isoform, and only a decrease in α\textsubscript{1C}-SNT,+9a isoform (Fig. 7B and C). Nevertheless, when m-βARK was coexpressed, in the α\textsubscript{1C}-SNT,+9a isoform there was an increase in the current to slightly above basal level. The increase was especially significant when compared with the decrease seen in the control conditions in the previously characterized α\textsubscript{1C}-SNT (Fig. 7C). Again, exon 9a in α\textsubscript{1C}-SNT,+9a is shown to be important for the G\textsubscript{q}-mediated increased current as observed in native tissues. It also points to the differences between direct activation of PKC by PMA and activation of PKC via the G\textsubscript{q} signaling cascade.

Discussion

PKC-regulation of four Ca\textsubscript{1.2α} isoforms, differing in the length of the initial segment of their NT and the presence of an insertion in cytosolic loop 1 (L1) was studied. Utilizing Xenopus oocytes, the only heterologous system in which the enhancement of α\textsubscript{1C} by PKC has been reconstituted,\textsuperscript{10} we identified an α\textsubscript{1C} isoform with a short-NT and long L1 as a candidate PKC-upregulated isoform in the smooth muscle. PKC also plays a crucial role in mediating the regulation of α\textsubscript{1C} by G\textsubscript{q}-activating GPCRs, such as muscarinic m1 and m3 receptors. We further investigated the molecular mechanism of G\textsubscript{q}-mediated and PKC-induced modulation of α\textsubscript{1C}. We found a novel modulation of G\textsubscript{q} effect by G\textsubscript{βγ}; the G\textsubscript{q}-mediated GPCR effects are tonically attenuated by G\textsubscript{βγ}. In contrast, direct activation of PKC by a phorbol ester is insensitive to G\textsubscript{βγ}, suggesting that G\textsubscript{βγ} acts upstream from PKC. Further, we identified the NT as a possible anchoring site for PKC, where it may serve as part of a modulatory scaffold, possibly also involving the distal CT. Despite the crucial importance of the NT for PKC regulation, its phosphorylation does not appear to mediate the effect of PKC. We found that the C-terminal serine 1928, a known target for both PKA and PKC, is a functionally important
α1C and dually regulate the cardiac isoform of this channel. Nonetheless, its role in modulation of Gq-activated PKC and Gβγ in regulation of L-VDCC, in which NT, CT, and L1 are involved.

The inhibitory Gβγ control of L-VDCC observed in oocytes appears to be at odds with the synergistic or consequential modulation of α1C by Gβγ and PKC observed in smooth muscle in response to Gq- or Gq-activating GPCRs such as β-adrenergic and muscarinic m2 receptors. In these cases Gβγ enhances the L-type currents acting indirectly, via complex mechanisms that involve activation of PI3 kinase, PKC, and PKA or Src.17,18,20,21,64

*Site for PKC effect. Our findings imply a complex antagonistic interplay between Gq-activated PKC and Gβγ in regulation of L-VDCC, in which NT, CT, and L1 are involved.

Gβγ and PKC bind to NT of α1C and oppositely regulate the L-VDCC function. Gβγ was previously shown to directly bind to NT and CT of α1C and dually regulate the cardiac isoform of this channel. Nonetheless, its role in modulation of specifically exerted upon Gq-mediated modulation of L-VDCC. It remains to be investigated whether Gq or Gq-mediated regulation of smooth muscle VDCC, when and if successfully reconstituted in a heterologous system, will be modulated differently by Gβγ.

Though not directly phosphorylated by PKC, the initial 20 a.a. segment of α1C-LNT is crucial for the upregulation of channel activity by PMA and Gq-coupled GPCRs. This segment,

![Figure 7. Isomers of α1C containing the L insertion encoded by exon 9a show a greater sensitivity to PKC activation.](image)
missing in \(\alpha_{\text{IC}-\text{SNT}}\), was identified as an important regulatory module that exerts a tonic control over the gating, reducing the channel’s open probability.\textsuperscript{35,43} We have previously proposed that the action of PKC on \(\alpha_{\text{IC}-\text{LNT}}\) relies upon the relief of this inhibitory control, possibly by phosphorylation of the channel elsewhere.\textsuperscript{35,46} Our new data offer new insights into the role of NT in PKC regulation of \(\alpha_{\text{IC}}\). Using direct in vitro protein-protein interaction measurements, we identify the universally conserved part of NT of \(\alpha_{\text{IC}}\), encoded by exon 2, as a binding site for PKC\(\alpha\) (between a.a. 95–154). Thus, the NT physically interacts with PKC\(\alpha\); this may be how PKC is anchored to the channel and how it exerts its action.

How does G\(\beta\gamma\) oppose the effect of G\(q\)-coupled GPCRs, m1R and m3R? A straightforward interpretation of the results of Figures 2 and 3 (no effect of G\(\beta\gamma\) and m-c\(\beta\)ARK on PMA-induced increase in I\(_{\text{Ba}}\)) and the fact that membrane receptor level is non-significantly affected by coexpressed G\(\beta\gamma\) or m-c\(\beta\)ARK, is that G\(\beta\gamma\) acts upstream of PKC, e.g., by obstructing the activation of G\(_{\text{q}}\) or the activation of phospholipase C by G\(_{\alpha}\) -GTP. In support, despite the apparent proximity of N-terminal PKC and G\(\beta\gamma\) binding sites, G\(\beta\gamma\) does not interfere with the binding of PKC to the NT. The new findings explain why in the oocytes PMA usually induces a greater increase in I\(_{\text{Ba}}\) than G\(_{\text{q}}\)-activating GPCRs (e.g., Figs. 2 and 7): free G\(\beta\gamma\) released upon activation of G\(_{\text{q}}\) would be expected to attenuate further activation of G\(_{\text{q}}\) itself or of phospholipase C (a negative feedback mechanism).\textsuperscript{65,66}

S1928 is important for the functional effect of PKC. The decrease in \(\text{Ba}^{2+}\) current via rabbit long-NT channels reported by McHugh et al.\textsuperscript{47} in HEK cells was eliminated by mutating two threonines at positions 27 and 31, and the authors speculated that these two residues are phosphorylated by PKC and that this phosphorylation underlies the observed decrease. In contrast, in oocytes the PMA- and ACh-induced decrease persists in constructs lacking the first 46 a.a. that include the aforementioned threonines.\textsuperscript{10,15} It appears that PMA-induced decreases in I\(_{\text{Ba}}\) in Xenopus oocytes are not mediated by phosphorylation of the NT.

Whatever the mechanism of decrease in I\(_{\text{Ba}}\), the main and the most important effect of PKC on cardiac and smooth muscle L-VDCCs is the enhancement of the current (see Introduction). The NT plays a crucial role in relaying the effect of PKC to channel gating in long NT isoforms of \(\alpha_{\text{IC}}\), and also contains a PKC-anchoring module as discussed above. However, none of the putative PKC phosphorylation sites in the NT appear to participate in the PKC-induced enhancement of I\(_{\text{Ba}}\). In contrast, we find that PKC effect is greatly reduced by mutation of the major PKA phosphorylation site in the distal CT, S1928. This is in line with phosphorylation of this residue by PKC in vitro.\textsuperscript{35} Furthermore, this part of the channel is an autoinhibitory module which is normally truncated in cardiac cells but remains attached to the C-terminus due to non-covalent binding.\textsuperscript{66,67} The participation of both NT and CT in PKC regulation supports the hypothesis\textsuperscript{19} that channel gating is tightly regulated by interactions between NT and CT (“NT-CT scaffold”), possibly together with L1 (as corroborated by PKC modulation of \(\alpha_{\text{IC}-\text{SNT},9a}\); PKC, G\(\beta\gamma\) and other regulators such as calmodulin act on L-VDCC by conformationally modulating this scaffold.

A short-NT, long-L1 \(\alpha_{\text{IC}-\text{SNT}}\) isoform is a candidate L-VDCC positively regulated by PKC in smooth muscle. Previously studied rabbit and human short NT \(\alpha_{\text{IC}}\) were not upregulated by PKC in heterologous expression systems,\textsuperscript{10,15} yet a short NT isoform is the one expressed in smooth muscle.\textsuperscript{68,69} Both in human and rabbit, an \(\alpha_{\text{IC}-\text{SNT},9a}\), possibly together with L1 (as corroborated by PKC modulation of \(\alpha_{\text{IC}-\text{SNT},9a}\); PKC, G\(\beta\gamma\) and other regulators such as calmodulin act on L-VDCC by conformationally modulating this scaffold.

We have inserted exon 9a to the wt rabbit \(\alpha_{\text{IC}-\text{LNT}}\) and \(\alpha_{\text{IC}-\text{SNT}}\) (that we used throughout our study). Remarkably, there was a notable increase in the current to above basal levels in \(\alpha_{\text{IC}-\text{SNT},9a}\), following PMA application. Further, when both m3R and m-c\(\beta\)ARK were expressed, following application of ACh an increase in the current to above baseline levels was observed in oocytes expressing the \(\alpha_{\text{IC}-\text{SNT},9a}\). The differences in treatments that induce current enhancement in the \(\alpha_{\text{IC}-\text{SNT},9a}\) isoform (application of PMA vs. ACh) probably reside in the way PKC is activated/Application of PMA results in a massive mobilization of PKC to the plasma membrane.\textsuperscript{71} Nonetheless, these findings clearly distinguish this isoform from the wt \(\alpha_{\text{IC}-\text{SNT}}\) isoform in terms of its PKC and G\(q\)-induced modulation. Aorta and bladder contain the short NT and, as we find, either long or short L1 (with or without exon 9a-encoded part). These are not the only isoforms of the channel present in smooth muscle, since it undergoes robust alternative splicing resulting in vast diversity in response to culturing conditions and the surrounding stimuli in vivo. Thus, it is plausible that other isoforms may be upregulated by PKC in smooth muscle; yet up to date only the one containing exon 9a behaved as such. \(\alpha_{\text{IC}-\text{SNT}}\) is not upregulated by PMA or ACh unless it contains exon 9a, as we have shown here.

Materials and Methods

Oocyte culture. All the experiments were performed in accordance with the Tel Aviv University Institutional Animal Care and Use Committee (permits no. 11-99-47 and 11-05-064). Xenopus laevis frogs were maintained and operated, and oocytes were collected, defolliculated, and injected with RNA as described.\textsuperscript{72} Female frogs, maintained at 20 ± 2°C on an 11 h light/13 h dark cycle, were anesthetized in a 0.15% solution of procaine methanesulfonate (MS222), and portions of ovary were removed through a small incision on the abdomen. The incision was sutured, and the animal was returned to a separate tank until it had fully recovered from the anesthesia, and afterwards was returned to a large tank where, together with the other postoperative animals, it was allowed to recover for at least 4 weeks until the next surgery. The animals did not show any signs of postoperative distress.

Oocytes were injected with equal amounts (by weight; 2.5 ng or 1 ng) of the mRNAs of CaV1.2 isoforms (original long-NT isoform: accession number X15539) or its mutants with \(\alpha_{\text{I}}\delta\) (accession number M21948), with or without \(\beta_{\text{ab}}\) (previously
referred to as rabbit heart β1α; accession number X64297), with or without 1 ng of m1R or m3R, and incubated for 3–5 d at 20–22°C in NDE96 solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 2.5 mM Na pyruvate, 50 μg/ml gentamycin, 5 mM HEPES, pH 7.5).

**cDNA constructs and mRNA.** cDNAs of αCI, αI/δ and β1β were as prepared.33 The rabbit heart αIC mutants used here were prepared in our laboratory as described.15 Rabbit αIC short-NT was as prepared,35 resulting in an exchange of exon 1a of αIC-LNT with exon 1 of αIC-SNT. Rat m1R cDNA is in pGEM2. Rat m3R and rabbit PKCα are in pGEM-HJ. m-cARK is in myr-pGEM-HE.65 The RNAs were prepared using a standard procedure previously described, which ensures capping of the 5’ end of the RNA and preferential inclusion of non-capped GTP in the rest of the RNA.32

**Electrophysiology.** Whole cell currents were recorded using the Gene Clamp 500 amplifier (Axon Instruments) using the two-electrode voltage clamp technique in a solution containing 40 mM Ba(OH)₂, 50 mM NaOH, 2 mM KOH and 5 mM HEPES, titrated to pH 7.5 with methanesulfonic acid.32 Stock solution of ACh (1 M) was stored in 10–20 μl aliquots at -20°C and added to the recoding solution at a final concentration of 10 μM. Ba²⁺ currents were measured by 200 ms steps to +20 mV from a holding potential of -80 mV, every 30 sec. Bis-indolyilmelide (Bis) was dissolved in water to 5 mM and stored in aliquots in -20°C. Oocytes were injected with 50 nl of 50 mM BAPTA or EGTA, 30 min or 2–4 h before measurement. Stock solution of β-phorbol-12-myristate 13-acetate (PMA; 10 mM) were prepared in DMSO and stored, protected from light, at -80°C. PMA at a final concentration of 10 nM was added to Ba²⁺ solution. In most experiments, all oocytes were injected with 25 nl of 50 mM BAPTA or EGTA, 30 min or 2–4 h before measurement, respectively, unless otherwise stated. All organic reagents were purchased from Sigma.

Current-voltage relations of the Iᵥm were obtained by stepping the membrane potential from the holding potential (-80 mV) to various voltages in 10 mV steps. Voltage pulses were delivered every 10 sec. In each cell, the net Iᵥm was obtained by subtraction of the residual currents recorded with the same protocols after applying 200 μM Cd²⁺. All experiments were performed at room temperature (20–22°C).

I-V curves were fitted to standard Boltzmann equation in the form of $I_{v_m} = G_{max} \frac{(V_{m} - V_V)}{(1 + \exp(-(V_{m} - V_V)/K_V))}$, where $K_V$ is the slope factor, $V_V$ is the voltage that causes half maximal activation, $G_{max}$ is the maximal conductance, $V_m$ is membrane voltage, $I_{v_m}$ is the current measured at the same voltage, and $V_{v_m}$ is the reversal potential of Iᵥm. The obtained parameters of $G_{max}$ and $V_{v_m}$ were then used to calculate fractional conductance at each $V_{m}$, $G/G_{max}$ using the equation: $G/G_{max} = I/((G_{max} \times V_{m} / V_{v_m}))$, where $G$ is the total macroscopic conductance at $V_{m}$. The conductance-voltage (G-V) curves were plotted with the values of $V_{m}$ and $K_V$ obtained from the fit of the I-V curves, using the following form of the Boltzmann equation: $G/G_{max} = 1/(1 + \exp(-(V_{m} - V_{v_m})/K_V))$.

The waveform of decay of Iᵥm was fitted to a two exponential equation by Levenberg-Marquardt method on Clampfit version 9 (Axon Instruments Inc.) or by a least mean square procedure in SigmaPlot in the form: $f(t) = A_{fast}e^{-t/\tau_{fast}} + A_{slow}e^{-t/\tau_{slow}} + C$, where A is the contribution of each kinetic component (fast or slow), $\tau_{fast}$ and $\tau_{slow}$ are the respective time constants, and C is the non-inactivating current.

**RT-PCR analysis.** Five μg of total human heart, bladder and aorta RNA purchased from Ambion, Inc. (catalog no. 7566, lot 110P43B; cat. no. 7990, lot 103P010802046A; cat. no. 6844, lot 053P010802003A, respectively) were reverse-transcribed with SuperScript II reverse transcriptase (Invitrogen) with primer #1 (see text below and Fig. 4A). Each PCR reaction (50 μl) contained 2.4 μl of the product of RT reaction, 1 μl of 10 mM dNTPs, 20–50 pmol of primers, 5 μl of 10 × PCR buffer, 2 μl of Mg²⁺ (2 mM), and 1 μl of Taq DNA polymerase (Promega). PCR was performed under the following conditions: 95°C for 1 min, 49°C for 1 min, and 72°C for 2 min, repeated 35 times. The final elongation was performed at 72°C for 5 min. The PCR products were analyzed on a 2% agarose gel.

The primers used for RT and PCR were: #1, CTT GGA CTT CTG TGA GCC (end of exon 12); #2, GAG AGT TTT CCA AAG AGA (end of exon 9); #3, TGA GCA TGC CCA CCA CTA GTG (beginning of exon 10); #4, CCT CAA TAC ACC GCA GTA (intron between exon 11 and exon 12); #5, CTG AAC TTT GAC TGT GAG (end of exon 11); #6, GAG GCA CTC CGG CGA GCA (beginning of the exon 9a); #7, TCT GTG GAG TGA CTA AAC (end of exon 9a); #8, CTG GAG TAA TTC CTT CTC (intron between exon 9 and exon 9a); #9, GCC AGC ACT GCC CAG AGG (intron between exon 9a and exon 10); #10, CAA AGA GAG GGA GAA GGC (10 nucl. after the beginning of exon 9a); #11, CAC CAG CCA GTA GAA GAC (starting 80 nucl. from the end of exon 12); #12, TGC CCT GCC CCT CCT CTA A (intron between exon 8 and exon 9).

**Interaction between GST fusion proteins and in vitro synthesized Gγδ and PKCα.** The procedures were essentially as described.46 In brief, [³⁵S]Met/Cys-labeled G8 and PKCα were translated on the template of in vitro synthesized RNAs using a rabbit reticulocyte translation kit (Promega). The GST fusion proteins were synthesized and extracted from Escherichia coli using the Amersham Pharmacia Biotech kit. Purified GST fusion proteins (5–10 μg) or purified GST (10 μg) were incubated with 5 μl of the lysate, containing the ³⁵S-labeled proteins in 500 μl of high K⁺ buffer (150 mM KCl, 50 mM Tris, 5 mM MgCl₂, 1 mM EDTA, pH 7.0) with 0.5% CHAPS or 0.01% Lubrol (as indicated), for 2 h at room temperature, with gentle rocking. In some experiments the incubation was done in the presence of purified 1.5 μg Gγδ (kind gift of C. W. Dessauer, University of Texas, Houston). Then 30 μl of glutathione-Sepharose beads (Amersham Pharmacia Biotech) were added, and the mixture was incubated for 30 min at 4°C and washed four times in 1 ml of the same buffer. Following washing, GST fusion proteins were eluted with 30 μl of 20 mM reduced glutathione in elution buffer (120 mM NaCl, 100 mM TRIS-HCl, pH 8). ³⁵S-labeled proteins were analyzed on 12% SDS-polyacrylamide gels. The labeled products were identified and quantified by autoradiography using PhosphorImager (Molecular Dynamics) as described.76

**Statistics and data presentation.** The data are presented as mean ± SEM, n = number of cells tested. To overcome the
problem of batch-to-batch variability in current amplitudes, the results were normalized as follows: in each oocyte, \( I_{\text{voltage}} \) was normalized to the amplitude measured before application of an agonist. These normalized values were averaged across all oocyte batches tested. Comparisons between two groups (e.g., control and receptor expressing groups) were tested for statistically significant differences (p < 0.05 or better) using two-tailed unpaired t-test. Comparisons of amplitudes of \( I_{\text{voltage}} \) at different times in the same group were done using paired t-test. Comparison between several groups was done using one-way analysis of variance (ANOVA) followed by Tukey’s test, using the SigmaStat software (SPSS Corp.).

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental material may be found here: http://www.landesbioscience.com/journals/channels/article/22016/
L-type calcium channel α-phosphorylation sites in the carboxyl terminus of FF, Glossmann H, Striessnig J. Identification of PK-A

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