Modulation of distinct isoforms of L-type calcium channels by G α q -coupled receptors in Xenopus oocytes

Antagonistic effects of Gβγ and protein kinase C

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L-type voltage dependent Ca2+ channels (L-VDCC; Cav1.2) are crucial in cardiovascular physiology. In heart and smooth muscle, hormones and transmitters operating via G α q enhance L-VDCC currents via essential protein kinase C (PKC) involvement. Heterologous reconstitution studies in Xenopus oocytes suggested that PKC and G α q -coupled receptors increased L-VDCC currents only in cardiac long N-terminus (NT) isoforms of α1C, whereas known smooth muscle short-NT isoforms were inhibited by PKC and G α q activators. We report a novel regulation of the long-NT α1C isofor by Gβγ. Gβγ inhibited whereas a Gβγ scavenger protein augmented the G α 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 α1C-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 α1C sensitive to PKC stimulation and to Gβγ scavenging. Our results suggest a complex antagonistic interplay between G α q -activated PKC and Gβγ in regulation of L-VDCC, in which multiple cytosolic segments of α1C are involved.

Introduction

L-type voltage-dependent calcium channels (L-VDCC; Cav1.2) 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 Ca2+ currents by protein kinase C (PKC). Constitutive activity of PKC may underlie a tonic Ca2+ influx via L-VDCC in some smooth muscle cells,5 and activation of PKC is believed to critically participate in the effects of G α q -coupled GPCRs and other modulators of Cav1.2. For instance, vasoconstrictors such as angiotensin II and acetylcholine (ACh), operating mainly via G α q -coupled GPCRs in smooth muscle, induce release of Ca2+ from intracellular stores and enhance L-VDCC currents.6,7 As part of this signaling cascade, protein kinase C (PKC) is activated and was shown to be essential for Ca2+ 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.8-13

The enhancing effect of PKC and G α 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 Ca2+ currents in some smooth muscle cells.9,16-18 The N- and C- terminus (NT and CT, respectively) of the pore-forming α1 subunit of L-VDCC, Ca1.2α (α1C), contain binding sites for Gβγ, and coexpression of Gβγ with the channel in Xenopus oocytes resulted in a dual effect: a tonic Ca2+- and CaM-dependent inhibitory effect, and an enhancement when Ca2+ was chelated or when NT and/or CT gating-regulating segments were removed.19 The Gβγ-dependent, Ca2+-independent enhancement is in line with known effects of purified Gβγ on L-VDCC in smooth muscle,16,20,21 yet the physiological role of the inhibitory effect of Gβγ is unknown.
Neuronal VDCCs are inhibited by GPCRs in processes that are voltage independent (mediated by several second messengers)\(^{22-25}\) and voltage dependent (VD). The VD process is mediated by Gq}-coupled GPCRs. Typical whole-cell Ba\(^{2+}\) currents (I\(_{Ba}\)) and a current-voltage (I-V) curve in a representative oocyte expressing α\(_{1C}\) protein are shown in Figure 2A. Application of ACh resulted, as previously shown, in upregulation of the current followed by a decline.\(^{10}\) Figure 2B illustrates the time course of this effect (“control,” filled circles), and Figure 2C exemplifies typical shapes of I\(_{Ba}\) and a current-voltage (I-V) curve in a representative oocyte expressing α\(_{1C}\)-LNT are shown in Figure 2A. Application of ACh resulted, as previously shown, in upregulation of the current followed by a decline.\(^{10}\)

**Results**

**Gβγ hinders the enhancement of I\(_{Ba}\) following activation of G\(_q\)-coupled receptors.** Modulation L-VDCC by ACh was studied in Xenopus oocytes expressing full subunit composition (α\(_{1C}\), β\(_{2\alpha}\), α\(_{\delta}\)) of the cardiac L-type calcium channel (α\(_{1C}\)-LNT; see Fig. 1A, Ba) and G\(_q\)-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\(_{Ba}\)) and a current-voltage (I-V) curve in a representative oocyte expressing α\(_{1C}\)-LNT are shown in Figure 2A. Application of ACh resulted, as previously shown, in upregulation of the current followed by a decline.\(^{10}\) Figure 2B illustrates the time course of this effect (“control,” filled circles), and Figure 2C exemplifies typical shapes of I\(_{Ba}\) and a current-voltage (I-V) curve in a representative oocyte expressing α\(_{1C}\)-LNT. The effect of ACh on I\(_{Ba}\) was 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\(_{Ba}\). However, when the Gβγ scavenger protein, m-cBARK (a myristoylated C-terminus of β\(_{2\alpha}\)-adrenergic kinase 1), was coexpressed, the ACh-induced increase in I\(_{Ba}\) was significantly greater than in control. This was observed when either m1R or m3R were expressed to 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\(_{Ba}\) supporting the role of PKC in the current enhancement (see Fig. 2E).\(^{10}\) Bis also attenuated the ACh-induced increase in I\(_{Ba}\) when m-cBARK was coexpressed, but a residual increase could still be observed.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Exon-intron structure of four α\(_{1c}\) constructs. (A) α\(_{1c}\) 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 α\(_{1c}\) 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).

![Figure 2](https://example.com/figure2.png)

**Figure 2.** Exon-intron structure of four α\(_{1c}\) constructs. (A) α\(_{1c}\) 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 α\(_{1c}\) 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).
the Gβγ dimer, we have coexpressed Gβ1γ2 along with the channel and the muscarinic receptor. This completely abolished the ACh-induced increase in IBa (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 i) 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. (F) Summary of the effects of ACh in oocytes expressing rabbit long-NT α_{1C} and m3R. (G) Summary of the effects 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. (G) a. Summary of the effects of application of PMA to oocytes expressing rabbit long-NT α_{1C} with m-cβARK or Gβγ. b. Representative traces of the effects of PMA on initial basal current (t = 0 min) 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; ### or ###, 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.44 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.45 These currents develop since calcium is being released from intracellular stores during the GPCR-Gq-PLC-Ins(1,4,5)P_{3} 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, 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 Ca^{2+}-dependent Cl^- channels).

PKC appears to regulate α_{1C} 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 α_{1C}, including in proximal and distal NT. We have explored most potential sites of PKC phosphorylation in the NT of the α_{1C}-LNT isoform by mutating Ser or Thr residues to Ala (Fig. S1). The first α_{1C} variant comprised four such mutations proximal to a.a. 75; these mutations also included T27, a site previously reported as potentially phosphorylated by PKC.47 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 enhancement 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 that
IBa upregulation via Gq was previously shown to be Ca2+ dependent (abolished when Ca2+ was strongly chelated) as well as PLC- (hence also diacylglycerol-) dependent.10 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.54-56 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,53 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.53 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 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 PKCα, and also by PKC in vitro.58 Application of PMA (10 nM) to oocytes expressing the mutated α1C S1928A with α2δ and β2b produced a significantly smaller enhancement of Iba compared with wt α1C (28% vs. 82%; Fig. 4B). Similarly, the S1928A mutation eliminated the increase in Iba 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.19 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). Iba upregulation via Gq was previously shown to be Ca2+ dependent (abolished when Ca2+ was strongly chelated) as well as PLC- (hence also diacylglycerol-) dependent.10 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.54-56 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,53 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.53 where NT may be an anchoring site for PKC.

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These results suggest that \( G_{\beta \gamma} \) binding of \( \text{PKC}_{\mu} \) experiments with \( \text{PKC}_{\text{carinic receptors}} \) still failed to enhance \( I_{\text{Ba}} \) via the previously manner.

that are affected by ACh and PKC, possibly in a \( G_{\beta \gamma} \) to enhance L-type currents in smooth muscle (see Introduction).

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Despite the use of m-\( \beta \)RK, activation of \( G_{\alpha} \)-coupled muscarinic receptors still failed to enhance \( I_{\text{Ba}} \), via the previously described short-NT isoform, \( \alpha_{1C-SNT} \) (see Fig. 8), and PMA also did not upregulate this isoform (see Fig. 7). Yet, PKC is known to enhance L-type currents in smooth muscle (see Introduction). We envisaged that unique smooth muscle isoforms might exist that are affected by ACh and PKC, possibly in a \( G_{\beta \gamma} \)-dependent manner.

Two isoforms of human \( \alpha_{1C} \) with differences in the cytosolic loop L1 have been described. The corresponding mRNAs differ in the absence or presence of a dispensable 75 nucleotide-long exon 9a, downstream of the DNA segment encoding the AID (\( \alpha_{1C} \) interaction domain with \( \beta \) subunit) (Figs. 1 and 6A; Fig. S2).37 Specific antibodies raised against protein sequences encoded by exon 9a demonstrated its presence in human smooth muscle cells in arteries.38 A detailed study of alternative splicing of human SNT smooth muscle variant also supports the 9a insertion.32 A notable rabbit \( \alpha_{1C} \) smooth muscle variant, referred to as rabbit lung isoform, also contains a 75 nucleotide long insertion in L19 which is absent in the well studied cardiac \( \alpha_{1C-SNT} \) isoform on the channel, we have constructed two chimeras of the rabbit \( \alpha_{1C-LNT} \) and \( \alpha_{1C-SNT} \) into which we inserted the exon 9a sequence into L1 (see Fig. 1B), and compared them to the previously characterized \( \alpha_{1C} \) counterparts (without 9a). Oocytes were injected with equimolar concentrations of RNA corresponding to the \( \alpha_{1C-SNT} \) variant used and auxiliary subunit(s) \( \alpha_{\delta/\theta} \), with or without \( \beta_{2b} \). Currents were measured with Ba\(^2+\) as the charge carrier, at ascending voltage steps from -70 mV to +50 mV. The activation parameters in all four \( \alpha_{1C} \) variants were similar in the absence of \( \beta_{2b} \). As expected, expression of \( \beta_{2b} \) shifted activation to hyperpolarized potentials in all chimeras (Fig. S3, Table S1). It should be noted that the half-activation voltage (V\(_{1/2}\)) of \( \alpha_{1C-SNT} \) +9a was shifted to hyperpolarized potentials by ~3 mV as 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 \( I_{\text{Ba}} \) were measured using a 10 sec long test pulse (from -10 mV to +40 mV) and were found to be similar in all four \( \alpha_{1C} \) variants (Fig. S4, Table S1). It is noteworthy that the increase in \( G_{\alpha_{1C}} \) caused by coexpression of \( \beta_{2b} \) appeared less prominent in +9a than in -9a isoforms, especially in \( \alpha_{1C-SNT} \) (Table S1). While this observation needs further study, it may indicate a novel role for the exon 9a insertion in trafficking from

Figure 5. Both \( G_{\beta \gamma} \) and \( \text{PKC}_{\alpha} \) bind distal segments of \( \alpha_{1C-NT} \). (A) Binding of \([\text{35S}]\)-labeled \( G_{\beta \gamma} \) to different GST-fused fragments of the NT. Immobilized GST fusion proteins were incubated with \( G_{\beta \gamma} \). After washing, \( G_{\beta \gamma} \)-bound proteins were eluted and resolved by SDS-PAGE. \( G_{\beta \gamma} \) was found to bind the distal third of the NT. Similar data was obtained in six more experiments. (B) Binding of \([\text{35S}]\)-labeled \( \text{PKC}_{\alpha} \) to different GST-fused fragments of the NT. \( \text{PKC}_{\alpha} \) was found to bind the distal half of the NT. (C) \([\text{35S}]\)PKC\( \alpha \) binding to segments of the NT was repeated in the presence of 1.5 \( \mu g \) purified \( G_{\beta \gamma} \). The addition of \( G_{\beta \gamma} \) did not alter \( \text{PKC}_{\alpha} \) binding to the NT, suggesting no binding competition for the same site.
the ER to the plasma membrane or in the regulation of maximal open probability.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\(^{2+}\) 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\(_{1c}\)-SNT isoforms (Fig. 7A). The enhancement of I\(_{\text{Ba}}\) in α\(_{1c}\)-SNT,+9a was not much above basal levels, but remember that normally only a decrease is observed in α\(_{1c}\)-SNT. The increase in I\(_{\text{Ba}}\) is actually underestimated since it overlaps the decrease. Moreover, the physiological relevance of the decrease in I\(_{\text{Ba}}\) induced by activation of G\(_ q\) seen in Xenopus oocytes is not clear, it may be a “side effect” in this model system.10 These results strengthen the role of exon 9a in α\(_{1c}\)-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\(_{\text{Ba}}\) by PMA in the α\(_{1c}\)-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\(_{\text{Ba}}\) in α\(_{1c}\)-LNT,+9a isoform, and only a decrease in α\(_{1c}\)-SNT,+9a isoform (Fig. 7B and C). Nevertheless, when m-c\(_{\beta}\)ARK was coexpressed, in the α\(_{1c}\)-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\(_{1c}\)-SNT (Fig. 7C). Again, exon 9a in α\(_{1c}\)-SNT,+9a is shown to be important for the Gq-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\(_ q\) signaling cascade.

**Discussion**

PKC-regulation of four Ca\(_{1.2}\) 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\(_{1c}\) by PKC has been reconstituted,10 we identified an α\(_{1c}\) 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\(_{1c}\) by G\(_ q\)-activating GPCRs, such as muscarinic m1 and m3 receptors. We further investigated the molecular mechanism of G\(_ q\)-mediated and PKC-induced modulation of α\(_{1c}\). We found a novel modulation of G\(_ q\) effect by G\(_{\beta\gamma}\); the G\(_ q\)-mediated GPCR effects are tonically attenuated by G\(_{\beta\gamma}\). In contrast, direct activation of PKC by a phorbol ester is insensitive to G\(_{\beta\gamma}\), suggesting that G\(_{\beta\gamma}\) 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

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**Figure 6.** Exon 9a transcripts found in human RNA samples by RT-PCR. (A) Proposed exon-intron structure and the various primers used. (B) Table summarizing the expected yield of PCR amplicons using the different primers. (C) Representative 2% agarose gels of RT-PCR products. -9a and +9a transcripts were found predominantly in human bladder and aorta RNA, while -9a transcripts alone were predominantly found in human heart RNA.
α1C and dually regulate the cardiac isoform of this channel. Nonetheless, its role in modulation of the L-VDCC function. Gβγ was previously shown to directly bind to NT of α1C-SNT, +9a, while only a decrease was present in α1C-LNT. The inhibitory effect of coexpressed Gβγ on α1c-LNT in Xenopus oocytes is Ca2+- and calmodulin-dependent, and it is replaced by an enhancement under the conditions of Ca2+ chelation.19 In the experiments described in this report, no or mild Ca2+ chelation conditions were used; the calculated intracellular concentration of Ca2+ chelators used (EGTA and BAPTA) was about 2 mM, which may favor the inhibitory action of Gβγ. Other than experimental conditions, different smooth muscle channel isoforms may be modulated by Gq or Gq. The inhibitory effects of Gβγ described here are 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,61 the initial 20 a.a. segment of α1c-LNT is crucial for the upregulation of channel activity by PMA and Gq-coupled GPCRs.30,61 This segment, 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 and PKC bind to NT and CT of α1C and dually regulate the cardiac isoform of this channel. Nonetheless, its role in modulation of this channel remains ambiguous. Here we show that the Gq-mediated upregulation of L-VDCC, reconstituted in Xenopus oocytes, shows a distinct regulation profile by Gβγ that resembles the well-characterized antagonistic regulation of the N-type channels, where Gβγ inhibits whereas PKC enhances channel activity.61 (However, this resemblance does not necessarily imply similarity of molecular mechanisms). When coexpressed with the channel and m3R, Gβγ abolished ACh-induced current enhancement performed by the Gq signaling pathway. Moreover, Gβγ sequestration by m-cβARK further augmented the Gq-mediated increase in I Ba. The latter finding suggests that endogenous free Gβγ, which appears to be relatively high in Xenopus oocytes,62,63 may exert a tonic inhibitory control upon cardiac L-type (α1c-LNT) channels. Taken together, these results propose a role for Gβγ in the modulation of the cardiac L-type calcium channel by Gq-activating GPCRs.

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 These modulations have not been so far heterologously reconstituted, hindering the study of the underlying molecular mechanisms. The controversy between these and our results may be apparent, arising from the experimental conditions used. The electrophysiological experiments in the above studies were usually performed in whole cell mode with 10 mM EGTA in the pipette (strong Ca2+ chelation). The inhibitory effect of coexpressed Gβγ on α1c-LNT in Xenopus oocytes is Ca2+- and calmodulin-dependent, and it is replaced by an enhancement under the conditions of Ca2+ chelation.19
missing in αIC-SNT, was identified as an important regulatory module that exerts a tonic control over the gating, reducing the channel’s open probability.\textsuperscript{15,43} We have previously proposed that the action of PKC on αIC-LNT relies upon the relief of this inhibitory control, possibly by phosphorylation of the channel elsewhere.\textsuperscript{15,46} Our new data offer new insights into the role of NT in PKC regulation of αIC. Using direct in vitro protein-protein interaction measurements, we identify the universally conserved part of NT of αIC encoded by exon 2, as a binding site for PKCα (between a.a. 95–154). Thus, the NT physically interacts with PKCα; this may be how PKC is anchored to the channel and how it exerts its action.

How does Gβγ oppose the effect of Gq-coupled GPCRs, m1R and m3R? A straightforward interpretation of the results of Figures 2 and 3 (no effect of Gβγ and m-cβARK on PMA-induced increase in I_{Ba}) and the fact that membrane receptor level is non-significantly affected by coexpressed Gβγ or m-cβARK, is that Gβγ acts upstream of PKC, e.g., by obstructing the activation of Gq or the activation of phospholipase C by Gq-GTP. In support, despite the apparent proximity of N-terminal PKC and Gβγ binding sites, Gβγ 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_{Ba} than Gq-activating GPCRs (e.g., Figs. 2 and 7): free Gβγ released upon activation of Gq would be expected to attenuate further activation of Gq itself or of phospholipase C (a negative feedback mechanism).\textsuperscript{45}

S1928 is important for the functional effect of PKC. The decrease in Ba\textsuperscript{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_{Ba} in Xenopus oocytes are not mediated by phosphorylation of the NT.

Whatever the mechanism of decrease in I_{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 αIC, 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_{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{33} 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 αIC-SNT,\textsuperscript{9a}) PKC, Gβγ and other regulators such as calmodulin act on L-VDCC by conformationally modulating this scaffold.

A short-NT, long-L1 αIC isoform is a candidate L-VDCC positively regulated by PKC in smooth muscle. Previously studied rabbit and human short NT αIC 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 αIC isoform containing a 75 bp-long insertion in L1, encoded (in humans) by exon 9\textsuperscript{a} or 9a, has been detected.\textsuperscript{98,99} Cardiac isoform of αIC contains a long NT (encoded by exon 1a),\textsuperscript{49} but, according to our RT PCR results, probably lacks exon 9a. This is in line with the findings of Liao et al. who reported lower levels of exon 9a in rat heart than in rat aorta and, using a specific antibody, found this insertion present in human smooth muscle arteries.\textsuperscript{58}

We have inserted exon 9a to the wt rabbit αIC-LNT and αIC-SNT (that we used throughout our study). Remarkably, there was a notable increase in the current to above basal levels in αIC-SNT,\textsuperscript{9a} following PMA application. Further, when both m3R and m-cβARK were expressed, following application of ACh an increase in the current to above baseline levels was observed in oocytes expressing the αIC-SNT,\textsuperscript{9a}. The differences in treatments that induce current enhancement in the αIC-SNT,\textsuperscript{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 αIC-SNT isoform in terms of its PKC and Gq-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. αIC-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). \textit{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 Ca\textsubscript{2}L-1.2\textsubscript{e} isoforms (original long-NT isoform: accession number X15539) or its mutants with αIC\textsubscript{iδ} (accession number M21948), with or without β\textsubscript{2a} (previously
referred to as rabbit heart β1A; 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 α1C, α1D and β1b were as described. The rabbit heart α1C short-NT and β1b were used here to prepare our laboratory as described. Rabbit α1C short-NT was prepared as described, resulting in an exchange of exon 1a of α1C-LNT with exon 1 of α1C-SNT. Rat m1R cDNA is in pGEM2. Rat m3R and rabbit PKCα are in pGEM-HI. m-cαARK is in myr-pGEM-HE. 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.

**Electrophysiology.** Whole cell currents were recorded using the Gene Clamps 500 amplifier (Axon Instruments, Foster City, CA, USA) 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. 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-indolylmaleimide (Bis) was dissolved in water to 5 mM and stored in aliquots in -20°C. Oocytes were injected with 50 nl of BAPTA or EGTA, 20–22°C in NDE96 solution (96 mM NaCl, 2 mM KCl, 1 mM MgCl2, 2.5 mM Na pyruvate, 50 μg/ml gentamycin, 5 mM HEPES, pH 7.5).

Current-voltage relations of the I Ba were obtained by stepping the current measured at the same voltage, and V rev is the reversal potential of I Ba. The obtained parameters of G max and V rev were analyzed on 12% SDS-polyacrylamide gels. The labeled products were identified and quantified by autoradiography using PhosphorImager (Molecular Dynamics) as described.

**RT-PCR analysis.** Five μg of total human heart, bladder and aorta RNA purchased from Ambion, Inc. (catalog no. 7566, lot 110P43B; cat. no. 7790, 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 GCT GTG (beginning of exon 10); #4, CCT CAA TAC ACC GCA GTA (intron between exon 11 and exon 12); #5, CTG AAC TTG GTG (beginning of exon 11); #6, GAG GCA CTC CGG CGG GCA (beginning of the exon 9a); #7, TCT GTG GAG TGA CTA AAC (end of exon 9a); #8, CTG CAG 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 GAC GAC (starting 80 nucl. from the end of exon 12); #12, TGC CCT GCC CCT CTC CTA (intron between exon 8 and exon 9).

**Interaction between GST fusion proteins and in vitro synthesized Gβγ and PKCα.** The procedures were essentially as described. 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 Gβγ (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.

**Statistics and data presentation.** The data are presented as mean ± SEM, n = number of cells tested. To overcome the
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.landembioscience.com/journals/articles/22016/
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