Channels 6:6, 426–437; November/December 2012; © 2012 Landes Bioscience

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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Keywords: calcium channel, protein kinase C, Gβγ, isoform

L-type voltage dependent Ca^{2+} channels (L-VDCCs; $Ca_v1.2$) 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; $Ca_V1.2$) 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,[5] and activation of PKC is believed to critically participate in the effects of G_q-coupled GPCRs and other modulators of $Ca_v1.2$. 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.[6,7] 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, $Ca_v1.2\alpha$ (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,[16,20,21] yet the physiological role of the inhibitory effect of Gβγ is unknown.

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Submitted: 07/16/12; Revised: 08/29/12; Accepted: 08/29/12
http://dx.doi.org/10.4161/chan.22016

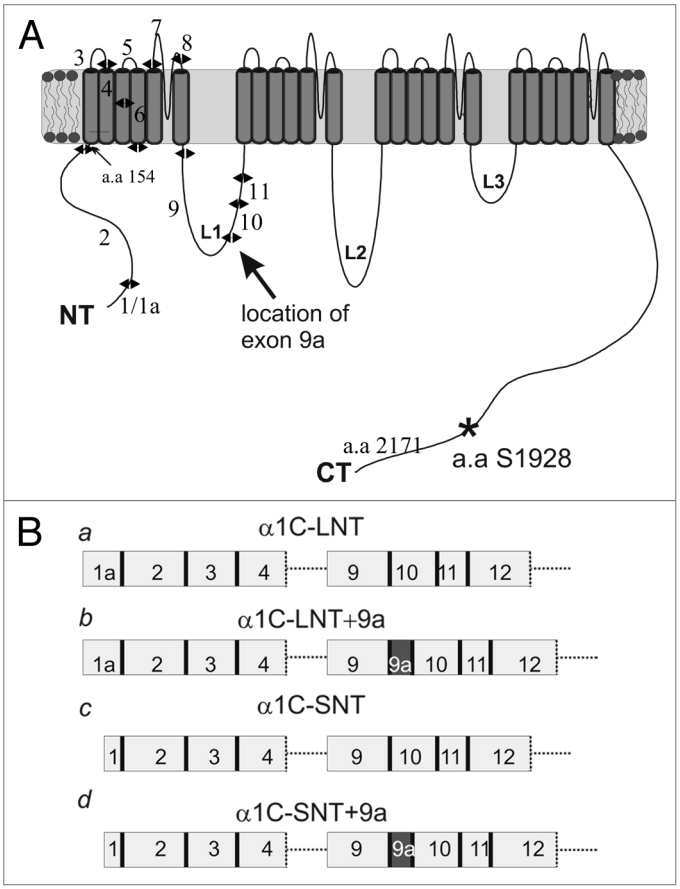

Figure 1. Exon-intron structure of four α_{1C} constructs. (**A**) α_{1C} protein structure and the location of the protein segment encoded by exon 9a, between exon 9 and 10. (**B**) Partial exon-intron structure of the four α_{1C} isoforms used. These differ by the length of the NT (encoded by exon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

Neuronal VDCCs are inhibited by GPCRs in processes that are voltage independent (and mediated by several second messengers)[22-25] and voltage dependent (VD). The VD process is mediated by Gβγ. It is fast, membrane delimited and occurs in members of the Ca_v2 group.[26-28] Several intracellular segments of α_1 subunits of neuronal VDCCs were shown to bind Gβγ; mainly the loop connecting the domains I and II, L1 (see **Fig. 1A**) and parts of the CT.[29-31] The NT was clearly identified as another molecular determinant critical for the Gβγ modulation. It binds L1 in a Gβγ-dependent manner, thereby rendering the channel sensitive to the Gβγ inhibition.[32-36] Furthermore, there is a crosstalk between the Gβγ and PKC pathways in the modulation of $Ca_v2.2$ (N-type) channels. PKC activation was shown to relieve the tonic Gβγ-mediated inhibition, suggesting a role for PKC phosphorylation of these channels.[31,37-39] However, no such crosstalk was ever observed or investigated in the L-type channel; L1 of α_{1C} does not bind Gβγ.[19,29]

A crucial factor determining the elaborate PKC signaling paradigm of L-VDCC is which isoform of α_{1C} is expressed. We have previously demonstrated the reconstitution of the modulation of L-type calcium channel by PKC and by G_q-coupled GPCRs in Xenopus oocytes, and found it to be dependent on the isoform of α_{1C} used. Only a long-NT (α_{1C}-LNT; cardiac) isoform was upregulated by these agents, while only a decrease in the current was observed in short NT containing α_{1C} (α_{1C}-SNT; smooth muscle/brain type).[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.[40,41] It was recently shown that the Cav1.2 gene undergoes extensive alternative splicing, often depending, in the smooth muscle, on culturing or pathologic conditions.[42] Thus, there are two plausible explanations: (1) a unique mechanism which enhances α_{1C}-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 α_{1C} studied so far in a manner that renders it PKC-sensitive.

Here we report the involvement of the Gβγ dimer in the PKC-mediated, G_q-induced modulation of L-VDCC by opposing the effects of G_q-activating GPCRs but not of β-phorbol myristate acetate (PMA), a potent direct PKC activator. We characterize the effects of activation of the G_q-coupled receptor (m3R) by ACh, as well as involvement of Gβγ, on distinct isoforms of α_{1C}, 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

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_{2b}, $\alpha_2\delta$) 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} 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-cβARK (a myristoylated C-terminus of β-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 mediate the ACh action via G_q (**Fig. 2B, D and E**). The effect of m-cβARK 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-cβARK was coexpressed, but a residual increase could still be

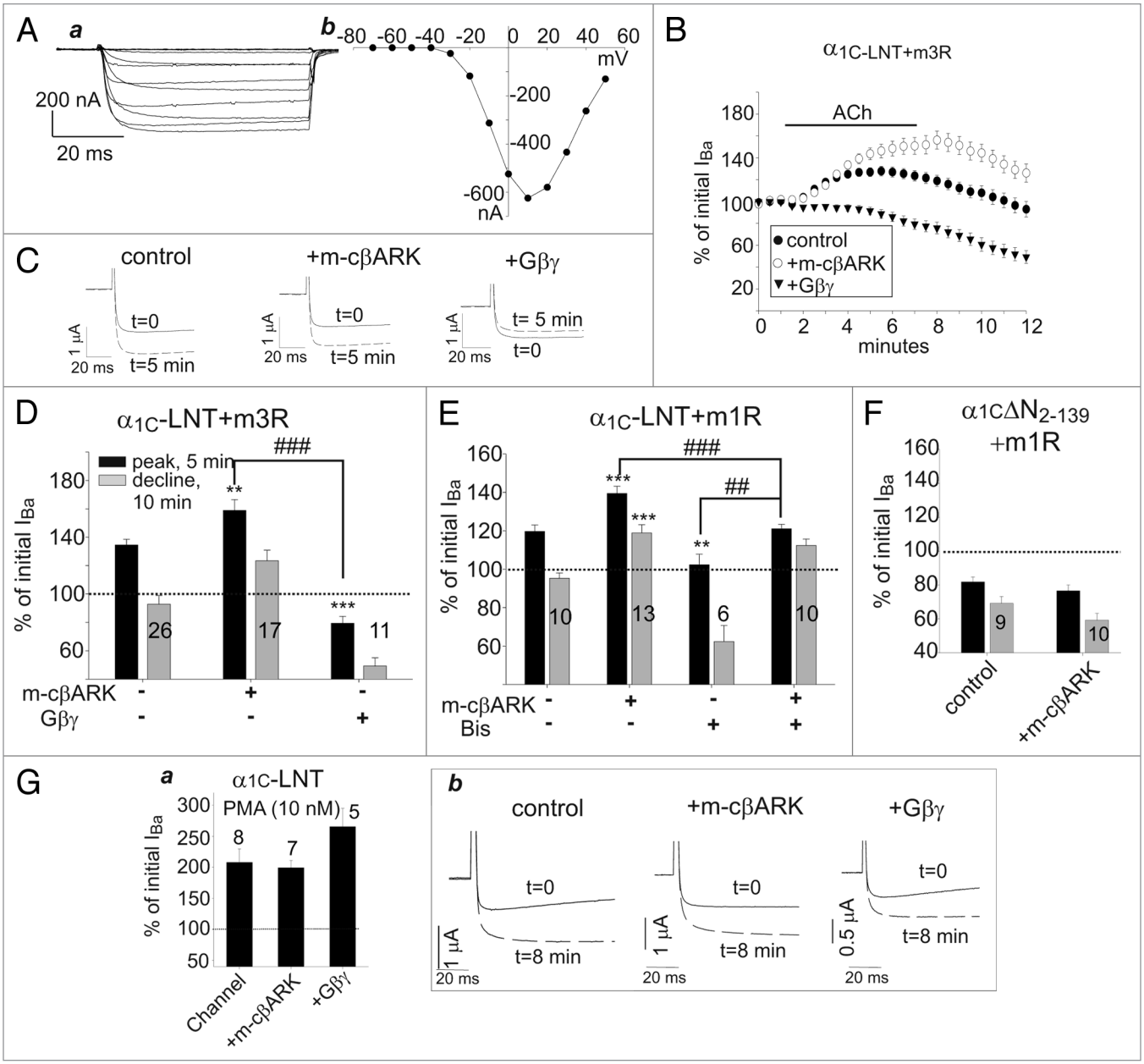

Figure 2. Gβγ negates upregulation of I_{Ba} by G_q-coupled muscarinic receptors but not by direct activation of PKC by PMA. (**A**) a. net Ba^{2+} currents (obtained by subtracting currents remaining after block with 200 μM Cd^{2+}, as described in[43] recorded at 10 mV steps from -70 mV to +50 mV. 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 $\alpha_{1C}\Delta N_{2-139}$ was without effect. 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. 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 ### $p < 0.001$; ###, $p < 0.001$. The number of cells tested is indicated on or above the bar.

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 $G\beta_1\gamma_2$ along with the channel and the muscarinic receptor. This completely abolished the ACh-induced increase in I_{Ba} (**Fig. 2B–D**).

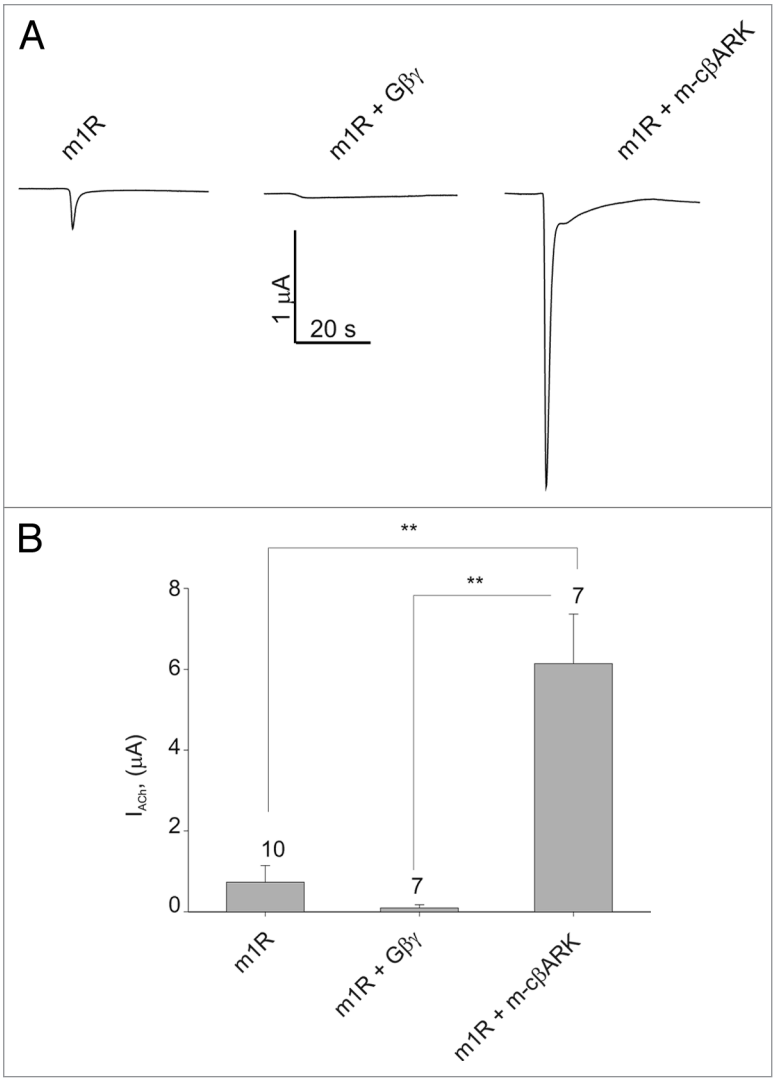

Figure 3. Gβγ abolishes typical chloride currents following Gq-mediated PKC activation. (**A**) Representative traces of ACh-induced chloride currents in Xenopus oocytes expressing m1R alone, with Gβγ or m-cβARK. (**B**) Summary of chloride currents magnitude in oocytes expressing m1R alone, with Gβγ or m-cβARK. **, $p < 0.01$ by t-test.

Coexpression of m-cβARK did not alter the modulation by ACh of the NT deletion mutant, $\alpha_{1C}\Delta N_{2-139}$ (**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 α_{1C} by ACh and PKC.[10,15,40,43] 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 α_{1C}-SNT (data not shown), indicating that Gβγ operates on the enhancing effect of G_q-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.

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-G_q-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 and B**). Thus, it appears that Gβγ probably acts on an event in the GPCR-G_q 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

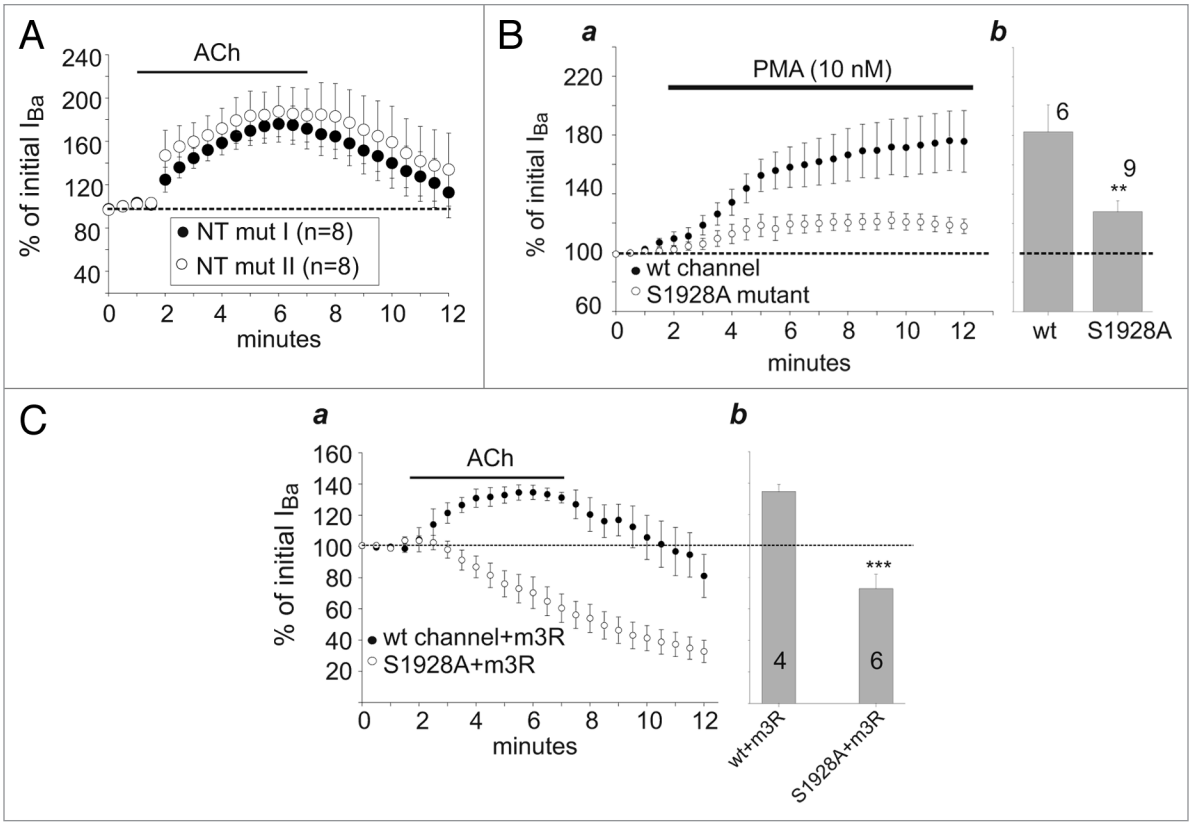

Figure 4. The S1928A mutation in α_{1C} is involved in modulation by PKC. (**A**) Ser/Thr mutations in NT do not affect upregulation of I_{Ba} by ACh. Oocytes were injected with wt α_{1C} or NT mutants 1 or 2 (see **Fig. S1**), NT mut I or NT mut II, $\alpha_2\delta$, β_{2b} and m3R. ACh was applied following current stabilization. The upregulation induced by ACh was not prevented by the mutations of putative PKC phosphorylation sites. (**B**) The effect of S1928A mutation of α_{1C}-LNT on the effect of PMA on I_{Ba}. a. Time-course of the experiments in oocytes expressing wt (closed circles) or S1928A (open circles) α_{1C} and $\alpha_2\delta$, β_{2b}. b. Summary of the enhancement of I_{Ba} caused by PMA, at t = 10 min. The current was significantly less augmented in the S1928A mutant as compared with wt. **, $p < 0.01$ by t-test. (**C**) The effect of S1928A mutation of α_{1C}-LNT on the effect of ACh on I_{Ba}. a. Time-course of the experiments in oocytes co-expressing m3R and wt (closed circles) or S1928A (open circles) α_{1C} and $\alpha_2\delta$, β_{2b}. b. Summary of the effect of Ach on I_{Ba}, at t = 5 min. The current was significantly less augmented in the S1928A mutant as compared with wt. ***, $p < 0.001$ by t-test.

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

An important phosphorylation site in α_{1C} is Ser1928 on distal CT. Ser1928 was found to be phosphorylated by protein kinase A (PKA), and[48-51] also by PKC in vitro.[52,53] Application of PMA (10 nM) to oocytes expressing the mutated α_{1C}S1928A with $\alpha_2\delta$ and β_{2b} produced a significantly smaller enhancement of I_{Ba} compared with wt α_{1C} (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.

α_{1C} 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 $G\beta_1\gamma_2$ 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 α_{1C} (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 G_q was previously shown to be Ca^{2+} dependent (abolished when Ca^{2+} 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 α_{1C} 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

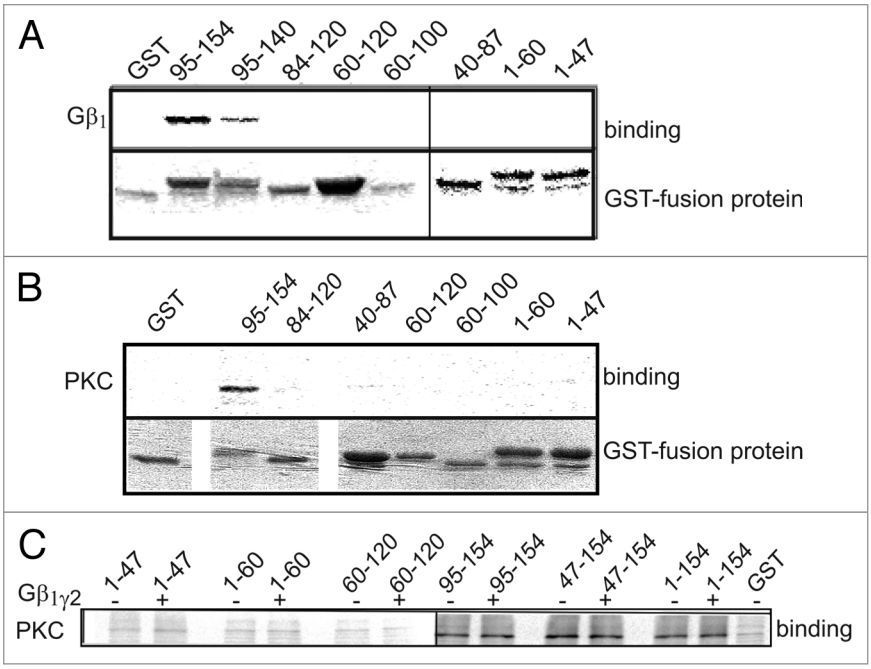

Figure 5. Both Gβγ and PKCα bind distal segments of α_{1C}-NT. (**A**) Binding of [^{35}S]-labeled $G\beta_1\gamma_2$ to different GST-fused fragments of the NT. Immobilized GST fusion proteins were incubated with $G\beta_1\gamma_2$. After washing, $G\beta_1\gamma_2$-bound proteins were eluted and resolved by SDS-PAGE. $G\beta_1\gamma_2$ was found to bind the distal third of the NT. Similar data was obtained in six more experiments. (**B**) Binding of [^{35}S]-labeled PKCα to different GST-fused fragments of the NT. PKCα was found to bind the distal half of the NT. (**C**) [^{35}S]PKCα binding to segments of the NT was repeated in the presence of 1.5 μg purified $G\beta_1\gamma_2$. It did not alter PKC binding to the NT, suggesting no binding competition for the same site.

experiments with PKCα and NT were repeated in the presence of 1.5 μg purified Gβγ. The addition of Gβγ did not alter the binding of PKCα to GST-fused segments of the NT (**Fig. 5C**). These results suggest that Gβγ does not interfere directly with the binding of PKC to NT.

Searching for PKC-regulated short-NT α_{1C} isoforms. Despite the use of m-cβARK, activation of G_q-coupled muscarinic receptors still failed to enhance I_{Ba} via the previously described short-NT isoform, α_{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 Gβγ-dependent manner.

Two isoforms of human α_{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 (α_{1C} interaction domain with β subunit) (**Figs. 1 and 6A**; **Fig. S2**).[57] Specific antibodies raised against protein sequences encoded by exon 9a demonstrated its presence in human smooth muscle cells in arteries.[58] A detailed study of alternative splicing of human SNT smooth muscle variant also supports the 9a insertion.[42] A notable rabbit α_{1C} smooth muscle variant, referred to as rabbit lung isoform, also contains a 75 nucleotide long insertion in L1[59] which is absent in the well studied cardiac α_{1C}-LNT isoform originally cloned form a heart cDNA library.[60] The a.a. composition of both short L1 ("-9a isoform") and long L1 ("+9a isoform," with the insertion encoded by the 75 nucleotides) is highly homologous among human and rabbit α_{1C} (**Fig. S2**). Since L1 is intimately involved in PKC regulation in the N-type channels,[31] we hypothesized that L1 isoforms of L-type channel may also differ in PKC regulation.

We further investigated the presence of exon 9a-encoded insertion in human tissues that express α_{1C}-using RT-PCR on human RNA samples. We were able to detect exon 9a in human heart, bladder and aorta (**Fig. 6**). Interestingly, both +9a and -9a isoforms were detected mainly in human bladder and aorta, indicating the existence of both transcripts, while the human heart RNA contained mostly the -9a isoform of the channel. There was a weak band corresponding to +9a isoform when using primers 2+5 on human heart RNA, but the main and stronger band was -9a, and the +9a band was missing in a separate experiment using the same set of primers. Furthermore, primers 11+2 yielded only -9a transcripts in the human heart RNA (**Fig. 6C**, "2+5," "11+2"). We can thus conclude that human heart RNA mainly contains the -9a transcript.

In order to study the effects of 9a insertion on the channel, we have constructed two chimeras of the rabbit α_{1C}-LNT and α_{1C}-SNT into which we inserted the exon 9a sequence into L1 (see **Fig. 1B**), and compared them to the previously characterized α_{1C} counterparts (without 9a). Oocytes were injected with equimolar concentrations of RNA corresponding to the α_{1C} variant used and auxiliary subunit(s) α_2/δ, with or without β_{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 α_{1C} variants were similar in the absence of β_{2b}. As expected, expression of β_{2b} shifted activation to hyperpolarized potentials in all chimeras (**Fig. S3**, **Table S1**). It should be noted that the half-activation voltage (V_a) of α_{1C}-SNT, +9a was shifted to hyperpolarized potentials by ~3 mV as compared with α_{1C}-SNT, when expressed without β_{2b}. Similar observations were made by Liao et al. in HEK cells, however the shift was much more pronounced, by ~11 mV.[58] 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_{1C} variants (**Fig. S4**, **Table S1**). It is noteworthy that the increase in G_{max} caused by coexpression of β_{2b} appeared less prominent in +9a than in -9a isoforms, especially in α_{1C}-SNT (**Table S1**). While this observation needs further study, it may indicate a novel role for the exon 9a insertion in trafficking from

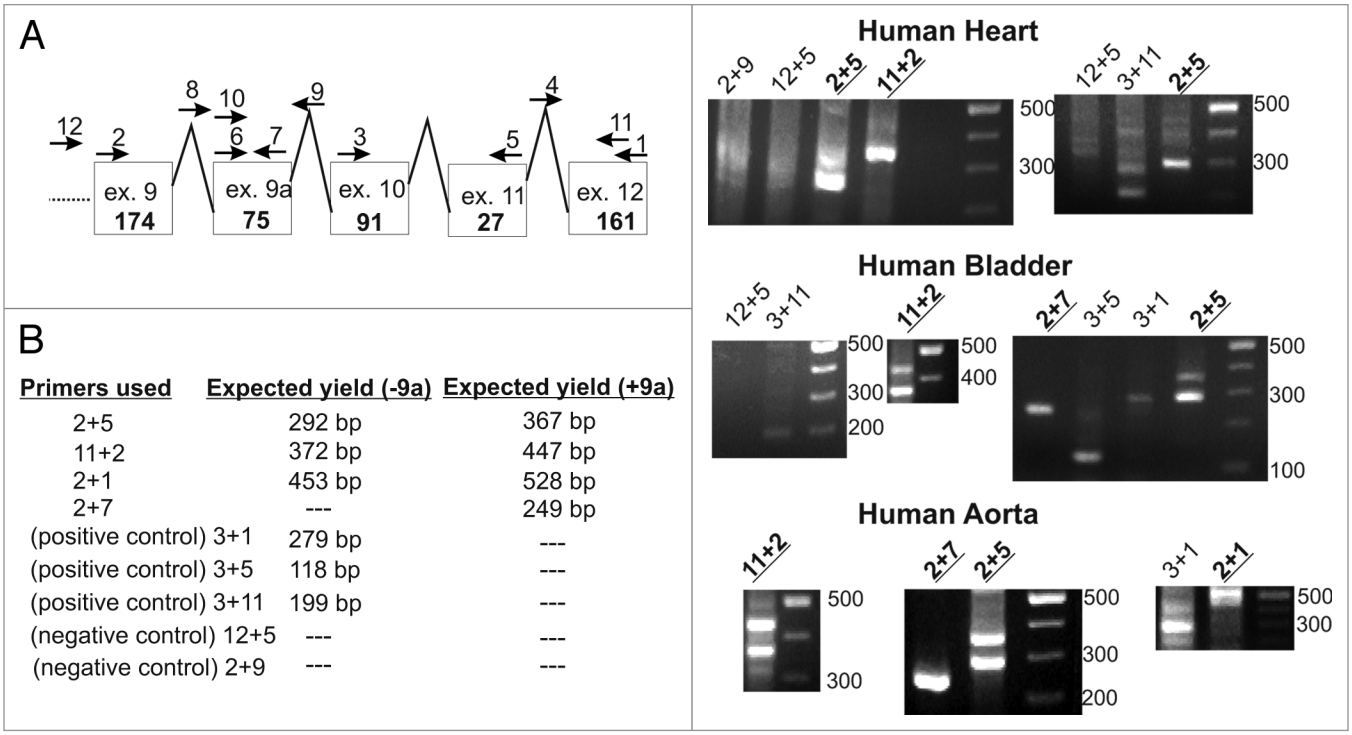

Figure 6. Exon 9a transcripts found in human RNA samples by RT-PCR. (**A**) Proposed exon-intron structure and the various primers used. (**B**) Table summarizing the expected yield of PCR amplicons using the different primers. (**C**) Representative 2% agarose gels of RT-PCR products. -9a and +9a transcripts were found predominantly in human bladder and aorta RNA, while -9a transcripts alone were predominantly found in human heart RNA.

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_{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_{Ba} is actually underestimated since it overlaps the decrease. Moreover, the physiological relevance of the decrease in I_{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_{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_{Ba} in α_{1C}-LNT,+9a isoform, and only a decrease in α_{1C}-SNT,+9a isoform (**Fig. 7B and C**). Nevertheless, when m-cβARK 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 $Ca_v1.2\alpha$ 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 Gβγ; the G_q-mediated GPCR effects are tonically attenuated by Gβγ. In contrast, direct activation of PKC by a phorbol ester is insensitive to Gβγ, suggesting that Gβγ 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

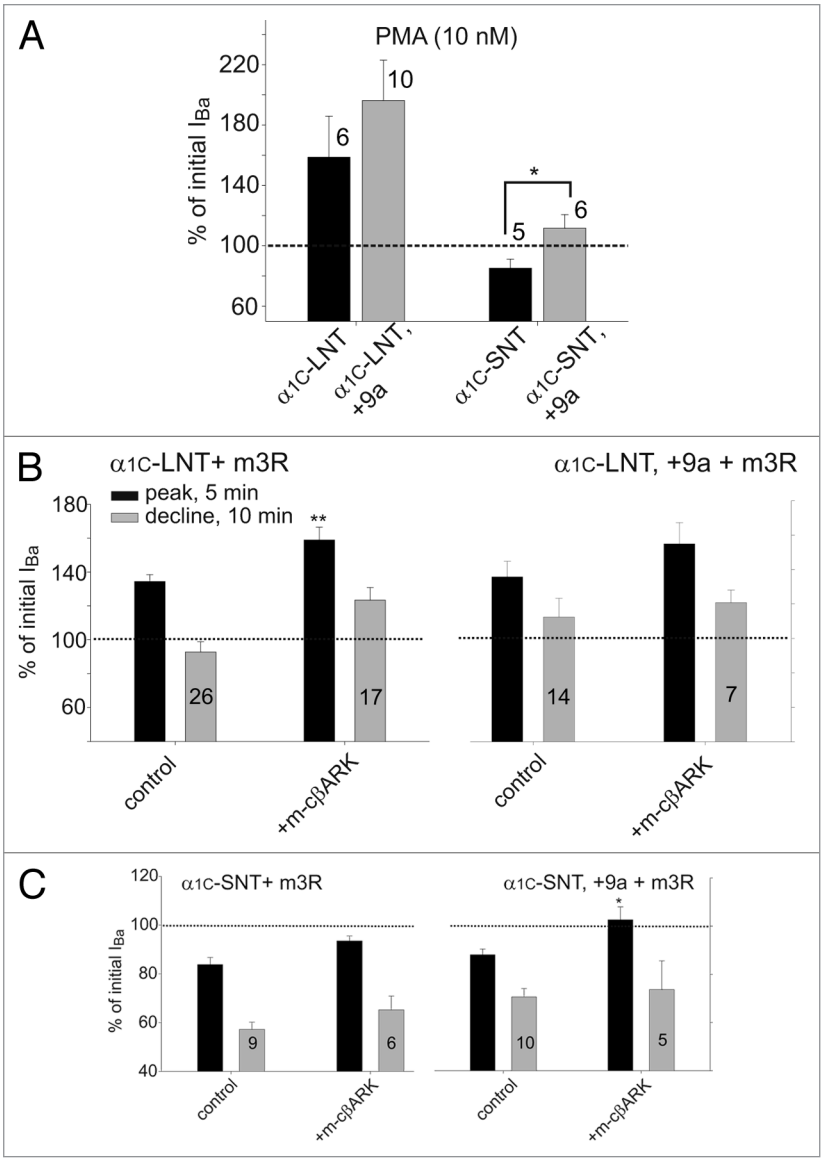

Figure 7. Isoforms of α_{1C} containing the L1 insertion encoded by exon 9a show a greater sensitivity to PKC activation. (**A**) I_{Ba} was recorded at +20 mV in oocytes expressing an α_{1C} isoform (as indicated), $\alpha_2\delta$, β_{2b} and PKCα. Following stabilization of the current, PMA (10 nM) was applied for 12–15 min. Bars depict the maximal increase in I_{Ba} evoked by PMA. *, $p < 0.05$ by t-test. (**B–C**) Oocytes were injected with α_{1C}, $\alpha_2\delta$, β_{2b} and m3R with or without m-cβARK. I_{Ba} was recorded at +20 mV every 30 sec. Following current stabilization, ACh applied for 6 min and further monitored for additional 5 min after ACh washout. Black bars represent the enhanced portion of the current; gray bars represent the declining phase of the current. (**B**) Coexpression of m-cβARK enhanced the increase in I_{Ba} to a similar extent in both long-NT isoform. (**C**) Coexpression of m-cβARK enhanced I_{Ba} in both short NT isoforms. I_{Ba} increased to slightly above baseline levels in oocytes coexpressing m-cβARK and α_{1C}-SNT, +9a, while only a decrease was present in α_{1C}-SNT oocytes. *, $p < 0.05$; **, $p < 0.01$ by t-test (as compared with channel alone without m-cβARK).

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

Gβγ and PKC bind to NT of α_{1C} and oppositely regulate the L-VDCC function. Gβγ was previously shown to directly bind to NT and CT of α_{1C} and dually regulate the cardiac isoform of this channel.[19] Nonetheless, its role in modulation of this channel remains ambiguous. Here we show that the G_q-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 G_q signaling pathway. Moreover, Gβγ sequestration by m-cβARK further augmented the G_q-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 (α_{1C}-LNT) channels. Taken together, these results propose a role for Gβγ in the modulation of the cardiac L-type calcium channel by G_q-activating GPCRs.

The inhibitory Gβγ control of L-VDCC observed in oocytes appears to be at odds with the synergistic or consequential modulation of α_{1C} by Gβγ and PKC observed in smooth muscle in response to G_s- or G_i-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 Ca^{2+} chelation). The inhibitory effect of coexpressed Gβγ on α_{1C}-LNT in Xenopus oocytes is Ca^{2+}- and calmodulin-dependent, and it is replaced by an enhancement under the conditions of Ca^{2+} chelation.[19] In the experiments described in this report, no or mild Ca^{2+} chelation conditions were used; the calculated intracellular concentration of Ca^{2+} 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 G_s or G_i. The inhibitory effects of Gβγ described here are specifically exerted upon G_q-mediated modulation of L-VDCC. It remains to be investigated whether G_s or G_i 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,[46] the initial 20 a.a. segment of α_{1C}-LNT is crucial for the upregulation of channel activity by PMA and G_q-coupled GPCRs.[10,46] This segment,

missing in α_{1C}-SNT, was identified as an important regulatory module that exerts a tonic control over the gating, reducing the channel's open probability.[15,43] We have previously proposed that the action of PKC on α_{1C}-LNT relies upon the relief of this inhibitory control, possibly by phosphorylation of the channel elsewhere.[15,46] Our new data offer new insights into the role of NT in PKC regulation of α_{1C}. Using direct in vitro protein-protein interaction measurements, we identify the universally conserved part of NT of α_{1C}, 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 G_q-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 coespressed Gβγ or m-cβARK, is that Gβγ acts upstream of PKC, e.g., by obstructing the activation of G_q, or the activation of phospholipase C by $G\alpha_q$-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 G_q-activating GPCRs (e.g., **Figs. 2 and 7**): free Gβγ released upon activation of G_q would be expected to attenuate further activation of G_q itself or of phospholipase C (a negative feedback mechanism).[65]

S1928 is important for the functional effect of PKC. The decrease in Ba^{2+} current via rabbit long-NT channels reported by McHugh et al.[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.[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 α_{1C}, 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.[53] 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.[66,67] The participation of both NT and CT in PKC regulation supports the hypothesis[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 α_{1C}-SNT,+9a); PKC, Gβγ and other regulators such as calmodulin act on L-VDCC by conformationally modulating this scaffold.

A short-NT, long-L1 α_{1C} isoform is a candidate L-VDCC positively regulated by PKC in smooth muscle. Previously studied rabbit and human short NT α_{1C} were not upregulated by PKC in heterologous expression systems,[10,15] yet a short NT isoform is the one expressed in smooth muscle.[68,69] Both in human and rabbit, an α_{1C} isoform containing a 75 bp- long insertion in L1, encoded (in humans) by exon 9* or 9a, has been detected.[58,70] Cardiac isoform of α_{1C} contains a long NT (encoded by exon 1a),[40] 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.[58]

We have inserted exon 9a to the wt rabbit α_{1C}-LNT and α_{1C}-SNT (that we used throughout our study). Remarkably, there was a notable increase in the current to above basal levels in α_{1C}-SNT,+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 α_{1C}-SNT,+9a. The differences in treatments that induce current enhancement in the α_{1C}-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.[71] Nonetheless, these findings clearly distinguish this isoform from the wt α_{1C}-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. α_{1C}-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.[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 postoperational animals, it was allowed to recover for at least 4 weeks until the next surgery. The animals did not show any signs of postoperational distress.

Oocytes were injected with equal amounts (by weight; 2.5 ng or 1 ng) of the mRNAs of $Ca_V1.2\alpha$ isoforms (original long-NT isoform: accession number X15539) or its mutants with α_2/δ (accession number M21948), with or without β_{2b} (previously

referred to as rabbit heart β_{2A}; 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 $MgCl_2$, 1 mM $CaCl_2$, 2.5 mM Na pyruvate, 50 μg/ml gentamycine, 5 mM HEPES, pH 7.5).

cDNA constructs and mRNA. cDNAs of α_{1C}, α_2/δ and β_{2b} were as described.[73] The rabbit heart α_{1C} mutants used here were prepared in our laboratory as described.[15] Rabbit α_{1C} short-NT was prepared as described,[43] resulting in an exchange of exon 1a of α_{1C}-LNT with exon 1 of α_{1C}-SNT. Rat m1R cDNA is in pGEM2. Rat m3R and rabbit PKCα are in pGEM-HJ. m-cβARK is in myr-pGEM-HE.[63,74] 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.[72]

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.[75] 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. Ba^{2+} 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 300 μM Bis and additionally incubated in the presence of 5 μM Bis for 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 Ba^{2+} 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 I_{Ba} 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 I_{Ba} was obtained by subtraction of the residual currents recorded with the same protocols after applying 200 μM Cd^{2+}. All experiments were performed at room temperature (20–22°C).

I-V curves were fitted to standard Boltzmann equation in the form of $I_{Ba} = G_{max}(V_m-V_{rev})/(1+\exp(-(V_m-V_a)/K_a)))$, where K_a is the slope factor, V_a is the voltage that causes half maximal activation, G_{max} is the maximal conductance, V_m is membrane voltage, I_{Ba} is 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 then used to calculate fractional conductance at each V_m, G/G_{max}, using the equation: $G/G_{max} = I/(G_{max}(V_m-V_{rev}))$, where G is the total macroscopic conductance at V_m. The conductance-voltage (G-V) curves were plotted with the values of V_a and K_a obtained from the fit of the I-V curves, using the following form of the Boltzmann equation: $G/G_{max} = 1/(1+\exp((-(V_m-V_a)/K_a)))$.

The waveform of decay of I_{Ba} 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), τ_{fast} and τ_{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. 7966, 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 Mg^{2+} (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 GTG (beginning of exon 10); #4, CCT CAA TAC ACC GCA GTA (intron between exon 11 and exon 12); #5, CTG AAC TTT GAC TTG GAG (end 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 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 CTC A (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.[46] In brief, [^{35}S]Met/Cys-labeled $G\beta_1$ 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 ^{35}S-labeled proteins in 500 μl of high K^+ buffer (150 mM KCl, 50 mM Tris, 5 mM $MgCl_2$, 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). ^{35}S-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_{Ba} 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_{Ba} 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.).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This paper was supported by the German-Israel Foundation (grant 930-220.2/2006). We thank Dr Carmen W. Dessauer (University of Texas, Houston) for kindly providing us with purified Gβγ. We thank W.A. Catterall (University of Washington, Seattle) for insightful scientific discussions.

Supplemental Material

Supplemental material may be found here:
http://www.landesbioscience.com/journals/channels/article/22016/

References

1. Hughes AD. Calcium channels in vascular smooth muscle cells. J Vasc Res 1995; 32:353-70; PMID:8562808.
2. Reuter H. Calcium channel modulation by neurotransmitters, enzymes and drugs. Nature 1983; 301:569-74; PMID:6131381; http://dx.doi.org/10.1038/301569a0.
3. Xiong Z, Sperelakis N. Regulation of L-type calcium channels of vascular smooth muscle cells. J Mol Cell Cardiol 1995; 27:75-91; PMID:7760390; http://dx.doi.org/10.1016/S0022-2828(08)80009-0.
4. Catterall WA. Structure and regulation of voltage-gated Ca^{2+} channels. Annu Rev Cell Dev Biol 2000; 16:521-55; PMID:11031246; http://dx.doi.org/10.1146/annurev.cellbio.16.1.521.
5. Navedo MF, Amberg GC, Votaw VS, Santana LF. Constitutively active L-type Ca^{2+} channels. Proc Natl Acad Sci U S A 2005; 102:11112-7; PMID:16040810; http://dx.doi.org/10.1073/pnas.0500360102.
6. Huster M, Frei E, Hofmann F, Wegener JW. A complex of Ca(V)1.2/PKC is involved in muscarinic signaling in smooth muscle. FASEB J 2010; 24:2651-9; PMID:20371628; http://dx.doi.org/10.1096/fj.09-149856.
7. Wang Y, Yan T, Wang Q, Wang W, Xu J, Wu X, et al. PKC-dependent extracellular signal-regulated kinase 1/2 pathway is involved in the inhibition of Ib on AngiotensinII-induced proliferation of vascular smooth muscle cells. Biochem Biophys Res Commun 2008; 375:151-5; PMID:18687307; http://dx.doi.org/10.1016/j.bbrc.2008.07.137.
8. Fish RD, Sperti G, Colucci WS, Clapham DE. Phorbol ester increases the dihydropyridine-sensitive calcium conductance in a vascular smooth muscle cell line. Circ Res 1988; 62:1049-54; PMID:2452033; http://dx.doi.org/10.1161/01.RES.62.5.1049.
9. Callaghan B, Zhong J, Keef KD. Signaling pathway underlying stimulation of L-type Ca2+ channels in rabbit portal vein myocytes by recombinant Gbetagamma subunits. Am J Physiol Heart Circ Physiol 2006; 291:H2541-6; PMID:16877561; http://dx.doi.org/10.1152/ajpheart.00420.2006.
10. Weiss S, Doan T, Bernstein KE, Dascal N. Modulation of cardiac Ca2+ channel by Gq-activating neurotransmitters reconstituted in Xenopus oocytes. J Biol Chem 2004; 279:12503-10; PMID:14722109; http://dx.doi.org/10.1074/jbc.M310196200.
11. Lacerda AE, Rampe D, Brown AM. Effects of protein kinase C activators on cardiac Ca2+ channels. Nature 1988; 335:249-51; PMID:2457814; http://dx.doi.org/10.1038/335249a0.
12. Tseng GN, Boyden PA. Different effects of intracellular Ca and protein kinase C on cardiac T and L Ca currents. Am J Physiol 1991; 261:H364-79; PMID:1652211.
13. Boixel C, Tessier S, Pansard Y, Lang-Lazdunski L, Mercadier JJ, Hatem SN. Tyrosine kinase and protein kinase C regulate L-type Ca(2+) current cooperatively in human atrial myocytes. Am J Physiol Heart Circ Physiol 2000; 278:H670-6; PMID:10666101.
14. Singer-Lahat D, Gershon E, Lotan I, Hullin R, Biel M, Flockerzi V, et al. Modulation of cardiac Ca^{2+} channels in *Xenopus* oocytes by protein kinase C. FEBS Lett 1992; 306:113-8; PMID:1321730; http://dx.doi.org/10.1016/0014-5793(92)80980-U.
15. Shistik E, Ivanina T, Blumenstein Y, Dascal N. Crucial role of N terminus in function of cardiac L-type Ca^{2+} channel and its modulation by protein kinase C. J Biol Chem 1998; 273:17901-9; PMID:9651396; http://dx.doi.org/10.1074/jbc.273.28.17901.
16. Viard P, Exner T, Maier U, Mironneau J, Nürnberg B, Macrez N. Gbetagamma dimers stimulate vascular L-type Ca2+ channels via phosphoinositide 3-kinase. FASEB J 1999; 13:685-94; PMID:10094929.
17. Quignard JF, Mironneau J, Carricaburu V, Fournier B, Babich A, Nurnberg B, et al. Phosphoinositide 3-kinase γ mediates angiotensin II-induced stimulation of L-type calcium channels in vascular myocytes. J Biol Chem 2001; 276:32545-51; PMID:11443116; http://dx.doi.org/10.1074/jbc.M102582200.
18. Callaghan B, Koh SD, Keef KD. Muscarinic M2 receptor stimulation of $Ca_v1.2b$ requires phosphatidylinositol 3-kinase, protein kinase C, and c-Src. Circ Res 2004; 94:626-33; PMID:14739158; http://dx.doi.org/10.1161/01.RES.0000118248.17466.B7.
19. Ivanina T, Blumenstein Y, Shistik E, Barzilai R, Dascal N. Modulation of L-type Ca2+ channels by gbeta gamma and calmodulin via interactions with N and C termini of alpha 1C. J Biol Chem 2000; 275:39846-54; PMID:10995757; http://dx.doi.org/10.1074/jbc.M005881200.
20. Zhong J, Dessauer CW, Keef KD, Hume JR. Regulation of L-type Ca2+ channels in rabbit portal vein by G protein alphas and betagamma subunits. J Physiol 1999; 517:109-20; PMID:10226153; http://dx.doi.org/10.1111/j.1469-7793.1999.0109z.x.
21. Zhong J, Hume JR, Keef KD. β-Adrenergic receptor stimulation of L-type Ca2+ channels in rabbit portal vein myocytes involves both alphas and betagamma G protein subunits. J Physiol 2001; 531:105-15; PMID:11179395; http://dx.doi.org/10.1111/j.1469-7793.2001.0105j.x.
22. Bernheim L, Beech DJ, Hille B. A diffusible second messenger mediates one of the pathways coupling receptors to calcium channels in rat sympathetic neurons. Neuron 1991; 6:859-67; PMID:1647174; http://dx.doi.org/10.1016/0896-6273(91)90226-P.
23. Diversé-Pierluissi M, Dunlap K. Distinct, convergent second messenger pathways modulate neuronal calcium currents. Neuron 1993; 10:753-60; PMID:8097399; http://dx.doi.org/10.1016/0896-6273(93)90175-Q.
24. Diversé-Pierluissi M, Goldsmith PK, Dunlap K. Transmitter-mediated inhibition of N-type calcium channels in sensory neurons involves multiple GTP-binding proteins and subunits. Neuron 1995; 14:191-200; PMID:7826637; http://dx.doi.org/10.1016/0896-6273(95)90254-6.
25. Shapiro MS, Wollmuth LP, Hille B. Modulation of Ca2+ channels by PTX-sensitive G-proteins is blocked by N-ethylmaleimide in rat sympathetic neurons. J Neurosci 1994; 14:7109-16; PMID:7525895.
26. Dolphin AC. Mechanisms of modulation of voltage-dependent calcium channels by G proteins. J Physiol 1998; 506:3-11; PMID:9481669; http://dx.doi.org/10.1111/j.1469-7793.1998.003bx.x.
27. Ikeda SR, Dunlap K. Voltage-dependent modulation of N-type calcium channels: role of G protein subunits. Adv Second Messenger Phosphoprotein Res 1999; 33:131-51; PMID:10218117; http://dx.doi.org/10.1016/S1040-7952(99)80008-1.
28. Zamponi GW, Snutch TP. Modulation of voltage-dependent calcium channels by G proteins. Curr Opin Neurobiol 1998; 8:351-6; PMID:9687363; http://dx.doi.org/10.1016/S0959-4388(98)80060-3.
29. De Waard M, Liu H, Walker D, Scott VE, Gurnett CA, Campbell KP. Direct binding of G-protein betagamma complex to voltage-dependent calcium channels. Nature 1997; 385:446-50; PMID:9009193; http://dx.doi.org/10.1038/385446a0.
30. Qin N, Platano D, Olcese R, Stefani E, Birnbaumer L. Direct interaction of gbetagamma with a C-terminal gbetagamma-binding domain of the Ca2+ channel alpha1 subunit is responsible for channel inhibition by G protein-coupled receptors. Proc Natl Acad Sci U S A 1997; 94:8866-71; PMID:9238069; http://dx.doi.org/10.1073/pnas.94.16.8866.
31. Zamponi GW, Bourinet E, Nelson D, Nargeot J, Snutch TP. Crosstalk between G proteins and protein kinase C mediated by the calcium channel alpha1 subunit. Nature 1997; 385:442-6; PMID:9009192; http://dx.doi.org/10.1038/385442a0.
32. Agler HL, Evans J, Tay LH, Anderson MJ, Colecraft HM, Yue DT. G protein-gated inhibitory module of N-type (ca(v)2.2) ca2+ channels. Neuron 2005; 46:891-904; PMID:15953418; http://dx.doi.org/10.1016/j.neuron.2005.05.011.
33. Cantí C, Page KM, Stephens GJ, Dolphin AC. Identification of residues in the N terminus of alpha1B critical for inhibition of the voltage-dependent calcium channel by Gbeta gamma. J Neurosci 1999; 19:6855-64; PMID:10436043.
34. Page KM, Cantí C, Stephens GJ, Berrow NS, Dolphin AC. Identification of the amino terminus of neuronal Ca2+ channel alpha1 subunits alpha1B and alpha1E as an essential determinant of G-protein modulation. J Neurosci 1998; 18:4815-24; PMID:9634547.
35. Simen AA, Miller RJ. Involvement of regions in domain I in the opioid receptor sensitivity of alpha1B Ca(2+) channels. Mol Pharmacol 2000; 57:1064-74; PMID:10779393.

36. Zhang JF, Ellinor PT, Aldrich RW, Tsien RW. Multiple structural elements in voltage-dependent Ca^{2+} channels support their inhibition by G proteins. Neuron 1996; 17:991-1003; PMID:8938130; http://dx.doi.org/10.1016/S0896-6273(00)80229-9.
37. Swartz KJ. Modulation of Ca2+ channels by protein kinase C in rat central and peripheral neurons: disruption of G protein-mediated inhibition. Neuron 1993; 11:305-20; PMID:8102534; http://dx.doi.org/10.1016/0896-6273(93)90186-U.
38. Barrett CF, Rittenhouse AR. Modulation of N-type calcium channel activity by G-proteins and protein kinase C. J Gen Physiol 2000; 115:277-86; PMID:10694257; http://dx.doi.org/10.1085/jgp.115.3.277.
39. Zhu Y, Ikeda SR. Modulation of Ca(2+)-channel currents by protein kinase C in adult rat sympathetic neurons. J Neurophysiol 1994; 72:1549-60; PMID:7823085.
40. Blumenstein Y, Kanevsky N, Sahar G, Barzilai R, Ivanina T, Dascal N. A novel long N-terminal isoform of human L-type Ca^{2+} channel is up-regulated by protein kinase C. J Biol Chem 2002; 277:3419-23; PMID:11741969; http://dx.doi.org/10.1074/jbc.C100642200.
41. Dai B, Saada N, Echetebu C, Dettbarn C, Palade P. A new promoter for α1C subunit of human L-type cardiac calcium channel $Ca_{(v)}1.2$. Biochem Biophys Res Commun 2002; 296:429-33; PMID:12163037; http://dx.doi.org/10.1016/S0006-291X(02)00894-X.
42. Tiwari S, Zhang Y, Heller J, Abernethy DR, Soldatov NM. Atherosclerosis-related molecular alteration of the human CaV1.2 calcium channel alpha1C subunit. Proc Natl Acad Sci U S A 2006; 103:17024-9; PMID:17071743; http://dx.doi.org/10.1073/pnas.0606539103.
43. Kanevsky N, Dascal N. Regulation of maximal open probability is a separable function of Ca(v)beta subunit in L-type Ca2+ channel, dependent on NH2 terminus of alpha1C (Ca(v)1.2alpha). J Gen Physiol 2006; 128:15-36; PMID:16801381; http://dx.doi.org/10.1085/jgp.200609485.
44. Robu VG, Pfeiffer ES, Robia SL, Balijepalli RC, Pi Y, Kamp TJ, et al. Localization of functional endothelin receptor signaling complexes in cardiac transverse tubules. J Biol Chem 2003; 278:48154-61; PMID:12972433; http://dx.doi.org/10.1074/jbc.M304396200.
45. Dascal N, Cohen S. Further characterization of the slow muscarinic responses in Xenopus oocytes. Pflugers Arch 1987; 409:512-20; PMID:2442712; http://dx.doi.org/10.1007/BF00583809.
46. Shistik E, Keren-Raifman T, Idelson GH, Blumenstein Y, Dascal N, Ivanina T. The N terminus of the cardiac L-type Ca(2+) channel alpha(1C) subunit. The initial segment is ubiquitous and crucial for protein kinase C modulation, but is not directly phosphorylated. J Biol Chem 1999; 274:31145-9; PMID:10531304; http://dx.doi.org/10.1074/jbc.274.44.31145.
47. McHugh D, Sharp EM, Scheuer T, Catterall WA. Inhibition of cardiac L-type calcium channels by protein kinase C phosphorylation of two sites in the N-terminal domain. Proc Natl Acad Sci U S A 2000; 97:12334-8; PMID:11035786; http://dx.doi.org/10.1073/pnas.210384297.
48. Perets T, Blumenstein Y, Shistik E, Lotan I, Dascal N. A potential site of functional modulation by protein kinase A in the cardiac Ca2+ channel alpha 1C subunit. FEBS Lett 1996; 384:189-92; PMID:8612821; http://dx.doi.org/10.1016/0014-5793(96)00303-1.
49. Mitterdorfer J, Froschmayr M, Grabner M, Moebius FF, Glossmann H, Striessnig J. Identification of PK-A phosphorylation sites in the carboxyl terminus of L-type calcium channel α 1 subunits. Biochemistry 1996; 35:9400-6; PMID:8755718; http://dx.doi.org/10.1021/bi960683o.
50. De Jongh KS, Murphy BJ, Colvin AA, Hell JW, Takahashi M, Catterall WA. Specific phosphorylation of a site in the full-length form of the α 1 subunit of the cardiac L-type calcium channel by adenosine 3',5'-cyclic monophosphate-dependent protein kinase. Biochemistry 1996; 35:10392-402; PMID:8756695; http://dx.doi.org/10.1021/bi953023c.
51. Gao T, Yatani A, Dell'Acqua ML, Sako H, Green SA, Dascal N, et al. cAMP-dependent regulation of cardiac L-type Ca2+ channels requires membrane targeting of PKA and phosphorylation of channel subunits. Neuron 1997; 19:185-96; PMID:9247274; http://dx.doi.org/10.1016/S0896-6273(00)80358-X.
52. Yang L, Doshi D, Morrow J, Katchman A, Chen X, Marx SO. Protein kinase C isoforms differentially phosphorylate Ca(v)1.2 alpha(1c). Biochemistry 2009; 48:6674-83; PMID:19527072; http://dx.doi.org/10.1021/bi900322a.
53. Yang L, Liu G, Zakharov SI, Morrow JP, Rybin VO, Steinberg SF, et al. Ser1928 is a common site for Cav1.2 phosphorylation by protein kinase C isoforms. J Biol Chem 2005; 280:207-14; PMID:15509562.
54. Park WS, Son YK, Kim N, Youm JB, Warda M, Ko JH, et al. Direct modulation of Ca(2+)-activated K(+) current by H-89 in rabbit coronary arterial smooth muscle cells. Vascul Pharmacol 2007; 46:105-13; PMID:17052962; http://dx.doi.org/10.1016/j.vph.2006.08.413.
55. Simonis G, Briem SK, Schoen SP, Bock M, Marquetant R, Strasser RH. Protein kinase C in the human heart: differential regulation of the isoforms in aortic stenosis or dilated cardiomyopathy. Mol Cell Biochem 2007; 305:103-11; PMID:17594058; http://dx.doi.org/10.1007/s11010-007-9533-3.
56. Wang Y, Wang W, Wang Q, Wu J, Xu J, Wu X. [Ca2+]i and PKC-alpha are involved in the inhibitory effects of Ib, a novel nonpeptide AngiotensinII subtype AT1 receptor antagonist, on AngiotensinII-induced vascular contraction in vitro. Biochem Biophys Res Commun 2007; 364:118-23; PMID:17931598; http://dx.doi.org/10.1016/j.bbrc.2007.09.101.
57. Tang ZZ, Liang MC, Lu S, Yu D, Yu CY, Yue DT, et al. Transcript scanning reveals novel and extensive splice variations in human l-type voltage-gated calcium channel, Cav1.2 alpha1 subunit. J Biol Chem 2004; 279:44335-43; PMID:15299022; http://dx.doi.org/10.1074/jbc.M407023200.
58. Liao P, Yu D, Lu S, Tang Z, Liang MC, Zeng S, et al. Smooth muscle-selective alternatively spliced exon generates functional variation in Cav1.2 calcium channels. J Biol Chem 2004; 279:50329-35; PMID:15381693; http://dx.doi.org/10.1074/jbc.M409436200.
59. Biel M, Ruth P, Bosse E, Hullin R, Stühmer W, Flockerzi V, et al. Primary structure and functional expression of a high voltage activated calcium channel from rabbit lung. FEBS Lett 1990; 269:409-12; PMID:2169433; http://dx.doi.org/10.1016/0014-5793(90)81205-3.
60. Mikami A, Imoto K, Tanabe T, Niidome T, Mori Y, Takeshima H, et al. Primary structure and functional expression of the cardiac dihydropyridine-sensitive calcium channel. Nature 1989; 340:230-3; PMID:2474130; http://dx.doi.org/10.1038/340230a0.
61. Zamponi GW. Determinants of G protein inhibition of presynaptic calcium channels. Cell Biochem Biophys 2001; 34:79-94; PMID:11394442; http://dx.doi.org/10.1385/CBB:34:1:79.
62. Lutz LB, Kim BE, Jahani D, Hammes SR. G protein β γ subunits inhibit nongenomic progesterone-induced signaling and maturation in Xenopus laevis oocytes. Evidence for a release of inhibition mechanism for cell cycle progression. J Biol Chem 2000; 275:41512-20; PMID:11018039; http://dx.doi.org/10.1074/jbc.M006757200.
63. Rishal I, Porozov Y, Yakubovich D, Varon D, Dascal NG. Gbetagamma-dependent and Gbetagamma-independent basal activity of G protein-activated K+ channels. J Biol Chem 2005; 280:16685-94; PMID:15728579; http://dx.doi.org/10.1074/jbc.M412196200.
64. Viard P, Macrez N, Mironneau C, Mironneau J. Involvement of both G protein alphas and β γ subunits in β-adrenergic stimulation of vascular L-type Ca(2+) channels. Br J Pharmacol 2001; 132:669-76; PMID:11159719; http://dx.doi.org/10.1038/sj.bjp.0703864.
65. Bonacci TM, Ghosh M, Malik S, Smrcka AV. Regulatory interactions between the amino terminus of G-protein betagamma subunits and the catalytic domain of phospholipase Cbeta2. J Biol Chem 2005; 280:10174-81; PMID:15611108; http://dx.doi.org/10.1074/jbc.M412514200.
66. Gao T, Cuadra AE, Ma H, Bunemann M, Gerhardstein BL, Cheng T, et al. C-terminal fragments of the α 1C (CaV1.2) subunit associate with and regulate L-type calcium channels containing C-terminal-truncated α 1C subunits. J Biol Chem 2001; 276:21089-97; PMID:11274161; http://dx.doi.org/10.1074/jbc.M008000200.
67. Hulme JT, Yarov-Yarovoy V, Lin TW, Scheuer T, Catterall WA. Autoinhibitory control of the CaV1.2 channel by its proteolytically processed distal C-terminal domain. J Physiol 2006; 576:87-102; PMID:16809371; http://dx.doi.org/10.1113/jphysiol.2006.111799.
68. Pang L, Koren G, Wang Z, Nattel S. Tissue-specific expression of two human $Ca_{(v)}1.2$ isoforms under the control of distinct 5' flanking regulatory elements. FEBS Lett 2003; 546:349-54; PMID:12832067; http://dx.doi.org/10.1016/S0014-5793(03)00629-X.
69. Saada NI, Carrillo ED, Dai B, Wang WZ, Dettbarn C, Sanchez J, et al. Expression of multiple $Ca_V1.2$ transcripts in rat tissues mediated by different promoters. Cell Calcium 2005; 37:301-9; PMID:15755491; http://dx.doi.org/10.1016/j.ceca.2004.11.003.
70. Biel M, Hullin R, Freundner S, Singer D, Dascal N, Flockerzi V, et al. Tissue-specific expression of high-voltage-activated dihydropyridine-sensitive L-type calcium channels. Eur J Biochem 1991; 200:81-8; PMID:1652442; http://dx.doi.org/10.1111/j.1432-1033.1991.tb21051.x.
71. Nishizuka Y. Studies and perspectives of protein kinase C. Science 1986; 233:305-12; PMID:3014651; http://dx.doi.org/10.1126/science.3014651.
72. Dascal N, Lotan I. Expression of exogenous ion channels and neurotransmitter receptors in RNA-injected *Xenopus* oocytes. Protocols in Molecular Neurobiology, Chapter 13: Humana Press, Totowa, NJ, 1992:205-25.
73. Singer D, Biel M, Lotan I, Flockerzi V, Hofmann F, Dascal N. The roles of the subunits in the function of the calcium channel. Science 1991; 253:1553-7; PMID:1716787; http://dx.doi.org/10.1126/science.1716787.
74. Jing J, Chikvashvili D, Singer-Lahat D, Thornhill WB, Reuveny E, Lotan I. Fast inactivation of a brain K+ channel composed of Kv1.1 and Kvbeta1.1 subunits modulated by G protein beta gamma subunits. EMBO J 1999; 18:1245-56; PMID:10064591; http://dx.doi.org/10.1093/emboj/18.5.1245.
75. Shistik E, Ivanina T, Puri T, Hosey M, Dascal N. Ca2+ current enhancement by alpha 2/delta and beta subunits in Xenopus oocytes: contribution of changes in channel gating and alpha 1 protein level. J Physiol 1995; 489:55-62; PMID:8583415.
76. Ivanina T, Perets T, Thornhill WB, Levin G, Dascal N, Lotan I. Phosphorylation by protein kinase A of RCK1 K^+ channels expressed in *Xenopus* oocytes. Biochemistry 1994; 33:8786-92; PMID:8038169; http://dx.doi.org/10.1021/bi00195a021.