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RESEARCH PAPER

Protein kinase C enhances plasma membrane expression of cardiac L-type calcium channel, $Ca_V1.2$

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ABSTRACT

L-type-voltage-dependent Ca^{2+} channels (L-VDCCs; $Ca_V1.2$, α_{1C}), crucial in cardiovascular physiology and pathology, are modulated via activation of G-protein-coupled receptors and subsequently protein kinase C (PKC). Despite extensive study, key aspects of the mechanisms leading to PKC-induced Ca^{2+} current increase are unresolved. A notable residue, Ser1928, located in the distal C-terminus (dCT) of α_{1C} was shown to be phosphorylated by PKC. $Ca_V1.2$ undergoes posttranslational modifications yielding full-length and proteolytically cleaved CT-truncated forms. We have previously shown that, in *Xenopus* oocytes, activation of PKC enhances α_{1C} macroscopic currents. This increase depended on the isoform of α_{1C} expressed. Only isoforms containing the cardiac, long N-terminus (L-NT), were upregulated by PKC. Ser1928 was also crucial for the full effect of PKC. Here we report that, in *Xenopus* oocytes, following PKC activation the amount of α_{1C} protein expressed in the plasma membrane (PM) increases within minutes. The increase in PM content is greater with full-length α_{1C} than in dCT-truncated α_{1C}, and requires Ser1928. The same was observed in HL-1 cells, a mouse atrium cell line natively expressing cardiac α_{1C}, which undergoes the proteolytic cleavage of the dCT, thus providing a native setting for exploring the effects of PKC in cardiomyocytes. Interestingly, activation of PKC preferentially increased the PM levels of full-length, L-NT α_{1C}. Our findings suggest that part of PKC regulation of $Ca_V1.2$ in the heart involves changes in channel's cellular fate. The mechanism of this PKC regulation appears to involve the C-terminus of α_{1C}, possibly corroborating the previously proposed role of NT-CT interactions within α_{1C}.

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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] and in excitability and excitation-transcription coupling in neurons and cardiomyocytes.[4-7] Ca^{2+} influx via these channels following membrane depolarization is the trigger for ryanodine receptor (RyR) activation and massive Ca^{2+} release from the sarcoplasmic reticulum (SR) leading to mechanic contraction.[8-11]

L-VDCCs are multi-subunit protein complexes containing, as a minimum, α_1 (pore forming subunit), and β and $\alpha_2\delta$ as auxiliary subunits. The *CACNA1C* gene encodes α_{1C}, the main subunit of $Ca_V1.2$ – the L-VDCC expressed in cardiac and smooth muscle and in the brain.[12,13] α_{1C} is alternatively spliced in different tissues. Among multiple splice variants, we have previously shown that the cardiac isoform contains exon 1A and is referred to as the "long-NT" (L-NT) isoform, while the smooth muscle isoform contains exon 1 and is referred to as the "short-NT (S-NT) isoform.[14-17] Furthermore, an insertion of another exon, 9a, between exons 9 and 10 in a smooth muscle isoforms was found.[18-20]

Many hormones and transmitters modulate $Ca_V1.2$ channels via activation of G-Protein Coupled Receptors (GPCRs) and second messengers.[21] A prominent modulatory pathway in the cardiovascular system is the enhancement of L-type Ca^{2+} currents by protein kinase C (PKC), via activation of Gq-coupled receptors by angiotensin II (AngII), acetylcholine (ACh), or directly by phorbol esters such as β-phorbol 12-myristate 13-acetate (PMA) [22,23] (reviewed in [24]). As part of this signaling cascade, PKC is activated and was shown to be essential for Ca^{2+} current enhancement (discussed in [22,25-27]). PKC inhibitors block AngII-induced vasoconstriction.[28-31] Heterologous expression and

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reconstitution of the enhancing effects of PKC was so far successful only in *Xenopus* oocytes,[20,22,32,33] while Cav1.2 expression and PKC activation in mammalian (HEK) cells yielded only a current decrease.[34] In our previous studies using *Xenopus* oocytes expression system, we have demonstrated that PKC activation (either directly by PMA or indirectly via a Gq-coupled receptor) of the cardiac (L-NT) isoform (and a distinct smooth muscle isoform containing exon 9a, S-NT+9a) resulted in a macroscopic current increase. No increase in current was observed in smooth muscle (S-NT, -9a) isoforms used, or in L-NT-deletion mutants.[22,23]

In vitro studies, using GST-fused segments of α_{1C}, and *in vivo*, in rat and mouse heart lysates, demonstrated that PKC phosphorylates α_{1C} isoforms. α_{1C} is a substrate for PKC on different residues, most prominently on Ser1928.[35,36] Cardiac and neuronal α_{1C} is post-translationally modified, giving a full-length and a CT-truncated form, ~240 kDa and 210 kDa, respectively.[37-39] The latter is the result of a proteolytic cleavage around amino acid (a.a.) ~1800[40] (heterologous expression yields only the full-length α_{1C} protein). The cleaved distal-CT, dCT, is a ~35 KDa protein. It appears to remain associated with the main α_{1C} subunit, tonically inhibiting it.[41] Ser1928 is located on the distal, truncated, part of the CT.

Initially, Ser1928 was considered to be the main residue phosphorylated by protein kinase A (PKA) following β-adrenergic stimulation, but, until very recently, without a defined function in PKA regulation (reviewed in[42]). Recent studies revealed important roles for phosphorylation of Ser1928 by PKA. First, it disrupts a physical interaction between α_{1C} and β2-AR.[43] Importantly, in smooth muscle and neurons (but not in the heart), phosphorylation of Ser1928 is essential for PKA regulation and has been speculated to also be important for PKC regulation of $Ca_V1.2$.[44,45] Notably, Ser1928 is also significant in PKC modulation of $Ca_V1.2$. A mutation of Ser1928 to alanine significantly reduces the PMA- and Gq- induced increase in $Ca_V1.2$ channels expressed in *Xenopus* oocytes,[23] revealing an important role for this residue in the modulation of $Ca_V1.2$ by PKC.

Despite extensive study, the mechanism by which PKC augments $Ca_V1.2$ current is unclear. We have previously studied the role of PKC in upregulation $Ca_V1.2$ currents in *Xenopus* oocytes. We hypothesize that the increase in current results from enhanced channel expression in the plasma membrane (PM). This work complements all of the previously published electrophysiological data in *Xenopus* oocytes. Here we examine PM expression of $Ca_V1.2$ using two distinct methodologies, and show that activation of PKC augments PM expression of α_{1C} which may, in part, account for increased currents. In addition, we report a similar effect of PKC activation in a more "native" environment using HL-1 cells, which is a murine cardiomyocyte cell line that exhibits typical adult cardiomyocytes characteristics.[46-48]

Results

Increased α_{1C}-L-NT PM expression following PKC activation

We wanted to quantify heterologously expressed α_{1C} PM expression in *Xenopus* oocytes following PKC activation. To do so, we used 2 distinct methods: α_{1C} immunocytochemistry in giant membrane patches (GMP)[52] and surface biotinylation followed by Western blotting. We injected 5 ng/oocyte of RNAs of α_{1C}, β_{2b}, α_2/δ (with or without m3R). Direct activation of PKC by application of 10 nM PMA (n = 90) for 5 minutes resulted in a significant increase of 125.76 ± 12.3% in PM expression of α_{1C} in GMP compared with control (untreated oocytes, n = 88) (Fig. 1Aa). Treatment with DMSO alone (vehicle) did not significantly affect the amount of α_{1C} in the PM (87.7 ± 21.57% of control, n = 5). Supporting evidence was obtained by surface biotinylation experiments. In all 4 experiments, activated PKC increased the amount of α_{1C} in PM. However, the increase in PM expression was less prominent than observed in GMP because of some internalization of biotin in oocytes. There was an increase of 36.08 ± 8.76% ($p < 0.05$) in surface expression of PMA treated oocytes as compared with untreated oocytes (Fig. 1b).

To verify the involvement of PKC in this process, we applied PMA in the presence of the specific PKC inhibitor, Bis-indolylmaleimide (Bis). Oocytes were injected with 50 nl of 300 μM Bis, 2–4 h before the experiment. Bis did not significantly reduce the "basal" expression of α_{1C}, yet significantly reduced the effect of PMA. Application of Bis resulted in a non significant decrease in α_{1C} membrane expression to 85.08 ± 15.14% of untreated oocytes (Fig. 1Ab). Application of PMA in Bis-treated oocytes resulted in 67.3 ± 16.7% membrane expression (n = 11) as compared with oocytes treated with Bis alone (no PMA,

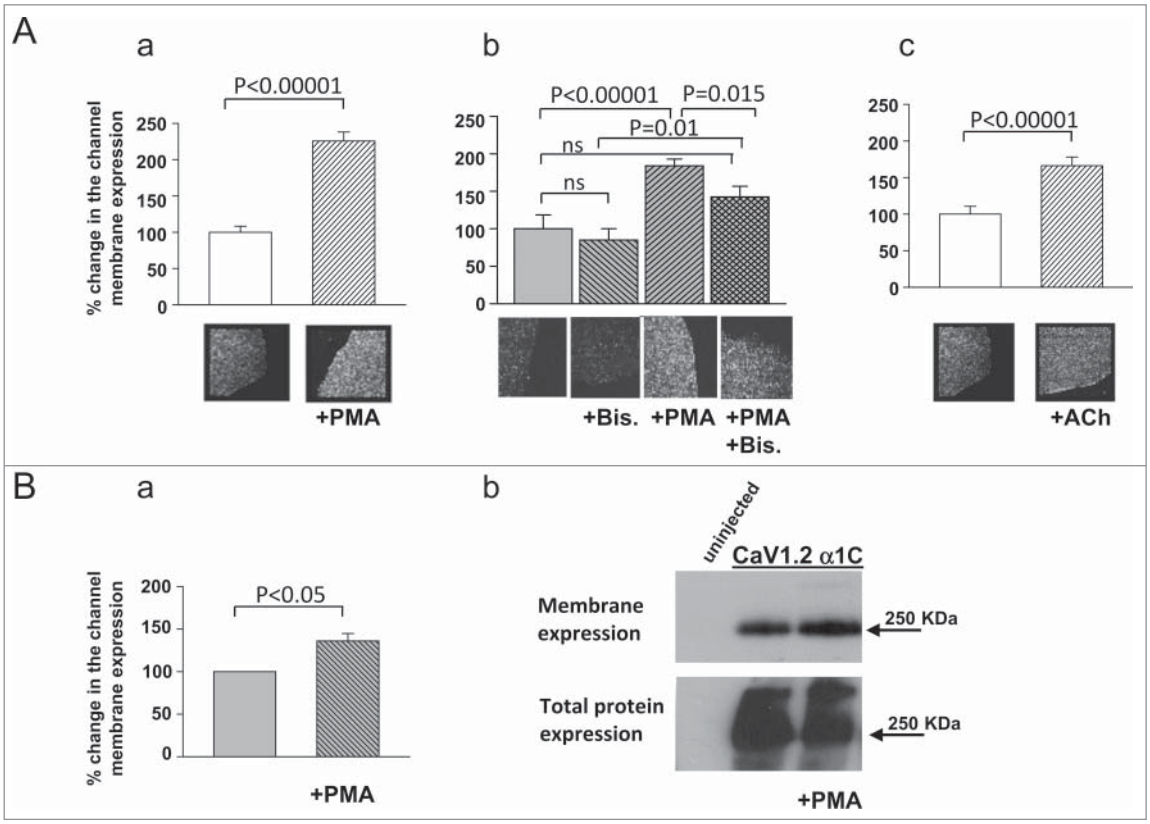

Figure 1. Plasma membrane expression of α_{1C} is upregulated by PKC. (A) Giant oocyte membrane patches stained for α_{1C} before and after PKC activation by PMA (*Aa*: PMA, n = 90; control, n = 88) or by ACh via m3R activation (*Ac:* ACh, n = 36; control n = 27), resulted in increased PM expression following PKC activation. Bis prevented the enhancing effects of PMA (*Ab:* control, n = 9; Bis, n = 8; PMA, n = 12; PMA+Bis, n = 11) (B) α_{1C} content in *Xenopus* oocytes plasma membrane after PKC activation measured by surface biotinylation followed by Western blot. Bars show protein amounts normalized to channel expression without PKC activation.

vn = 8) (Fig. 1Ab), and this difference was statistically significant indicating that Bis did not completely inhibit the effect of PMA. However, a significant difference in the effect of PMA was observed between untreated (only PMA, n = 12) and Bis-treated (Bis and PMA, n = 11) groups: 84.5 ± 9.08% increase vs. 42.34 ± 14.24%, respectively (Fig. 1Ab). Thus, Bis significantly attenuated the effect of PMA. To further substantiate the role of PKC using a physiologic activator, we co-expressed a Gq-coupled receptor (m3R) along with $Ca_V1.2$. Activation of m3R by ACh leads to downstream activation of PKC.[53-55] Application of ACh (10 μM) increased α_{1C} PM content within 8 minutes by 66.14 ± 11.4% (n = 36) compared with untreated oocytes (n = 27) in GMP (Fig. 1Ac).

dCT and Ser1928 are essential for the enhanced PM expression

The role of dCT in this modulation was studied by expressing a dCT-deletion mutant in *Xenopus* oocytes, $\alpha_{1C}\Delta1821$ (with β2b and $\alpha2/\delta$). PMA enhanced PM expression of $\alpha_{1C}\Delta1821$ by 75.02 ± 25.31% (n = 6) as compared with untreated $\alpha_{1C}\Delta1821$ oocytes. The effect of PMA on the dCT-deletion mutant was significant, yet significantly lower than the effect of PMA on full-length α_{1C} in this set of experiments (217.16 ± 53.4% increase, n = 6). These results suggest an important role for dCT in modulation of $Ca_V1.2$ by PKC. To further study the role of dCT, we co-expressed dCT with $\alpha_{1C}\Delta1821$. Interestingly, coexpression of dCT did not restore the full effect of PMA (Fig. 2A)

Ser1928, located in the dCT of α_{1C}, is an important phosphorylation site for PKC,[3536] and for PKA.[39,57-59] We have previously shown that when this residue is mutated to alanine (S1928A), in *Xenopus* oocytes the extent of $Ca_V1.2$ currents enhancement by PMA was greatly attenuated as compared with wt α_{1C}.[23] We expressed wt α_{1C} or S1928A-α_{1C} (with β2b and $\alpha2/\delta$) and quantified PM α_{1C} contents before and after a 5 minute application of PMA, by analyzing GMPs. The

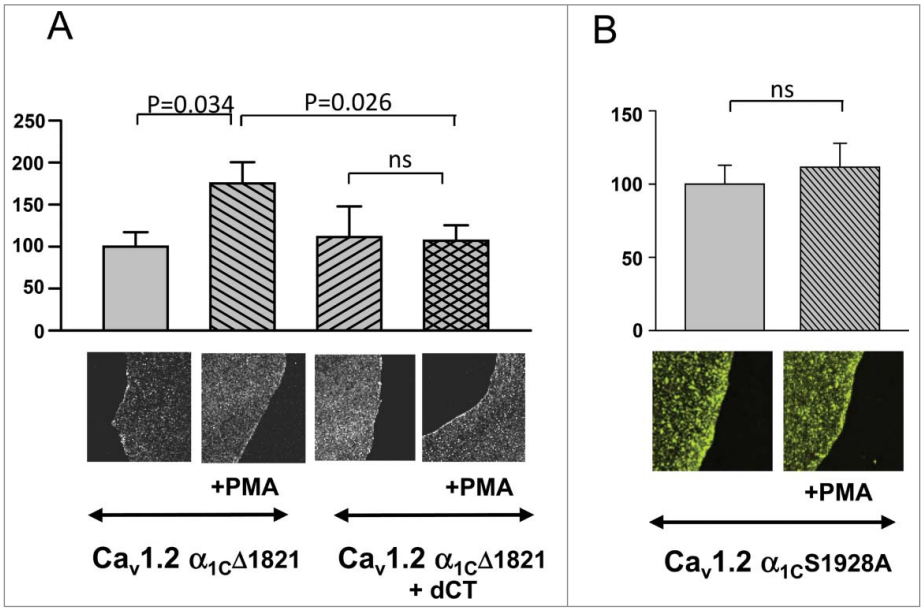

Figure 2. Involvement of dCT and Ser1928 in PKC regulation of α_{1C}. Giant oocyte membrane patches were stained for α_{1C} before and after PKC activation by PMA. Oocytes were injected with 5 ng channel RNA/oocyte. (A) Activation of PKC significantly enhanced PM expression in oocytes expressing $\alpha_{1C}\Delta1821$ (n = 6), but not in oocytes coexpressing $\alpha_{1C}\Delta1821$ and dCT (n = 5). (B) Activation of PKC failed to increase PM expression in oocytes expressing α_{1C} S1928A (n = 28) as compared with control, wt α_{1C} (n = 22).

increase in PM content following PKC activation with PMA, was practically absent in oocytes expressing S1928A- α_{1C} (111.5 ± 16.4% of control, $p > 0.05$; Fig. 2B; compare with the 225% of control for PMA-treated wild-type α_{1C}; Fig. 1A). Thus, PM expression patterns resemble previously reported electrophysiological findings, and emphasize the importance of this specific residue, located in the dCT, in PKC regulation of the full-length cardiac α_{1C}.

PM expression of α_{1C} in HL-1 cells is upregulated by PKC

Prior to studying the effects of PKC on HL-1 cells, we examined α_{1C} expression in these cells. Confocal images of intact HL-1 cells stained with α_{1C} antibody revealed a diffused staining pattern that was not restricted to PM (Fig. 3A). Using Western blotting and immunocytochemistry, we were able to detect robust expression of α_{1C} (Fig. 3), as previously reported by others.[46] Interestingly, we detected 2 distinct bands corresponding to molecular weights of ~250 kDa and ~210 kDa. These bands likely represent the full- length and dCT-truncated α_{1C}, respectively. This is supported by the comparison of the 2 bands detected in HL-1, with the bands of full-length and dCT-truncated α_{1C} separately expressed in Xenopus oocytes and analyzed on the same blot (Fig. 3b). Previous studies have shown that the extent of truncation of α_{1C} varies[39,60,61] (also reviewed in[42]).

Next, we examined the effects of PKC activation on α_{1C} PM expression. PKC was activated by PMA and α_{1C} expression was detected by immunofluorescence and surface biotinylation. The latter method enabled us to distinguish between the full-length and dCT-truncated forms of α_{1C} in the PM. PMA application significantly increased PM content of α_{1C} almost 2-fold, while DMSO did not change the PM expression, as detected by immunofluorescence. Interestingly, confocal images revealed a diffused expression pattern in untreated or DMSO-treated cells. PKC activation seemed to mobilize the channels and resulted in staining apparently much more confined to the PM, with a 84 ± 21% (n = 3) increase in the α_{1C} labeling in the cell periphery (Fig. 4A). Surface biotinylation also demonstrated a 37 ± 8% increase in PM α_{1C} content following activation of PKC by PMA (Fig. 5A). We have further separately analyzed the effect PKC had on the 2 distinct bands, presumably representing the full-length and truncated α_{1C}. The intensity of the upper band was increased by 67 ± 11% ($P < 0.01$), whereas the lower band only by 31 ± 10% ($P < 0.05$) (Fig. 5b). Thus, the PM expression of the full-length α_{1C}, was enhanced by PKC to a greater extent as compared with the non-truncated α_{1C}. These findings highlight the role of the dCT and possibly the

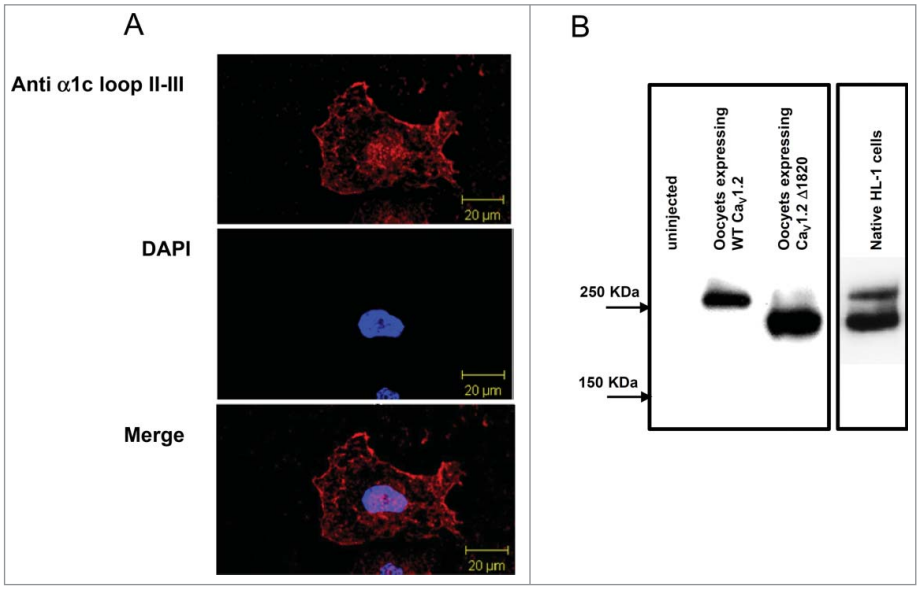

Figure 3. Native HL-1 cells express α_{1C}. (A) Confocal images of HL-1 cells stained with α_{1C} antibody. (B) Western blot of native HL-1 cells, with lysates of oocytes expressing either full-length of dCT-truncated α_{1C} as a reference. HL-1 cells express 2 distinct molecular weights of $\alpha_{1C,}$ probably corresponding to full-length and dCT-truncated channels.

role of Ser1928 located on the dCT. Thus, quite remarkably, $Ca_V1.2$ PM expression in HL-1 cells, which resemble adult cardiomyocytes, is upregulated following PKC activation.

Discussion

$Ca_V1.2$ is the main calcium channel in the heart and smooth muscle, responsible for normal myocyte contraction.[1-3] This channel was shown to be regulated both by PKA and PKC (reviewed in[24,42,62]). PKC was previously shown to enhance L-NT $Ca_V1.2$ currents in *Xenopus* oocytes.[22,23,51,56] Furthermore, Ser1928, located on α_{1C}-CT was identified as a crucial residue for this regulation.[23] Here, we studied the regulation of PM expression of α_{1C} by PKC in *Xenopus* oocytes and HL-1 cells. Despite previous notion that PKC enhances $Ca_V1.2$ currents due to a change in

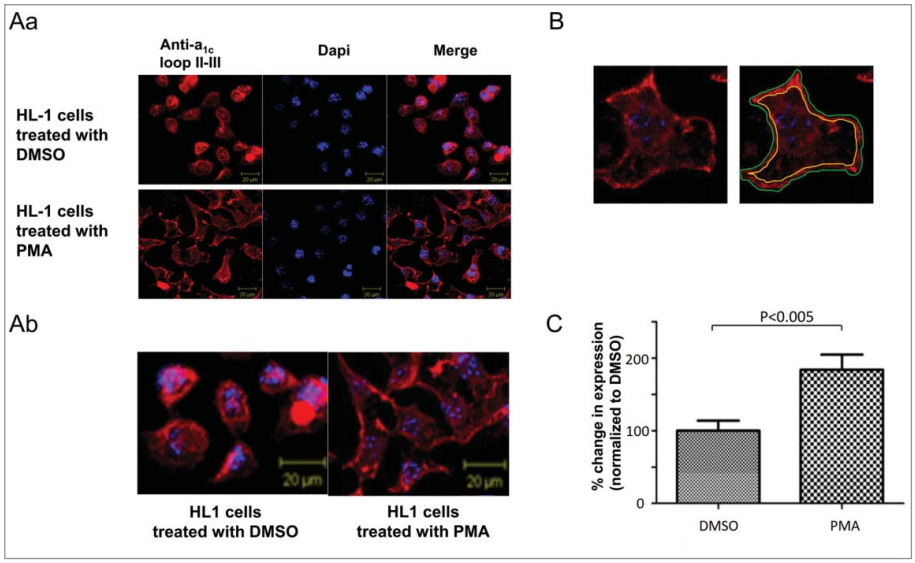

Figure 4. PKC increases α_{1C} expression PM of HL-1 cells. (A) Hl-1 cells were treated with DMSO (vehicle) or PMA and visualized by staining $\alpha_{1C.}$ Staining was much more confined to PM in PMA-treated cells. The lower panel is a zoom on a selected area from the upper panel images. (B) A representative cell depicting how PM content of α_{1C} was quantified. (C) Quantification of PM content of α_{1C} in DMSO vs. PMA treated HL-1 cells.

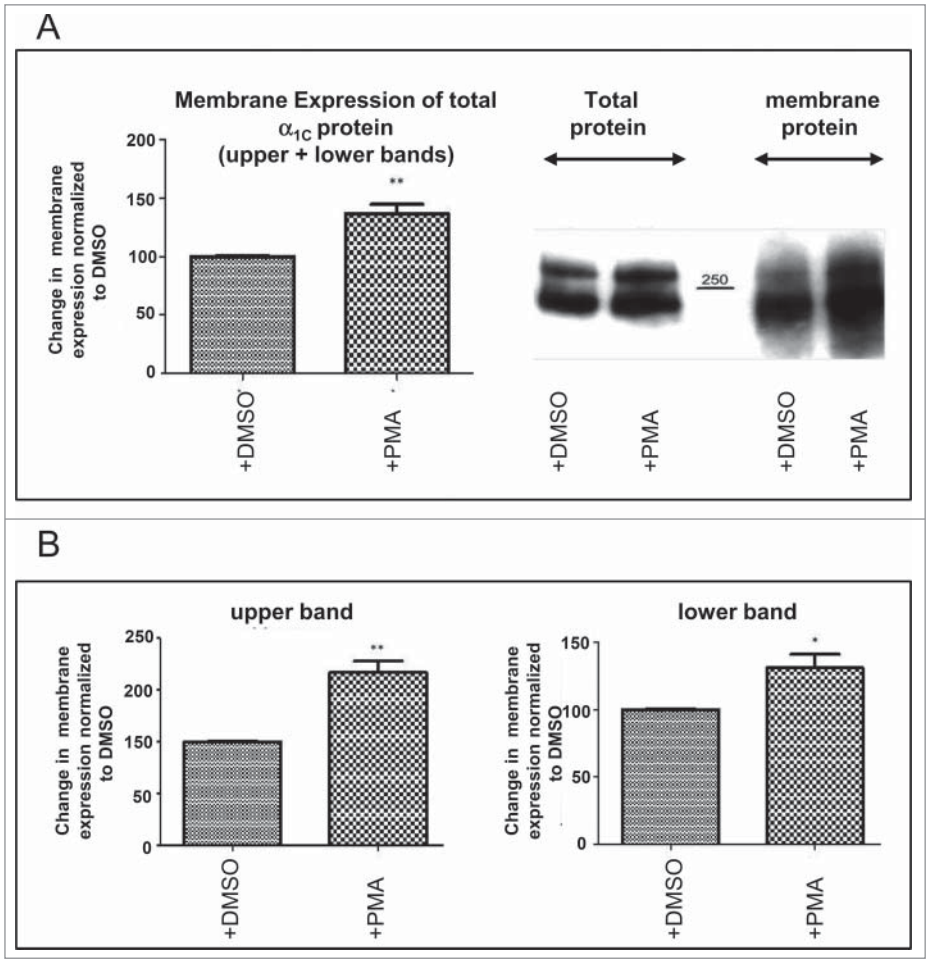

Figure 5. PKC increases the PM level of α_{1C} in HL-1 cells. Quantification of the amount of α_{1C} protein in HL-1 cells PM was done by biotinylation. (A) Quantification of total α_{1C} protein. (B) Quantification of distinct molecular weights of α_{1C}, corresponding to full-length and dCT-truncated α_{1C}.

gating,[63-65] here we report that activation of PKC increases α_{1C} expression in PM, and intact CT is critical for the increased expression.

$Ca_V1.2$ is mobilized to and removed from the PM following various stimuli such as distinct cell signaling, G protein activation, phosphorylation and post-translational modifications (reviewed in[66]). It is well established that $Ca_V1.2$ channels in cardiomyocytes and smooth muscle cells form macromolecular complexes.[63,67] Furthermore, it has been recently demonstrated that dynamin and cortactin co-localize next to the actin cytoskeleton. Activation of L-VDCC by GnRH enhanced Ca^{2+} influx and induced PM remodeling.[68] The extensive trafficking mechanism of α_{1C} to PM and intracellularly is tightly regulated, and PKC may be one such regulator that increases the amount of α_{1C} in PM, thus enhancing macroscopic currents.

HL-1 cells are derived from AT-1 mouse atrial cardiomyocyte tumor lineage, and retain hallmark cardiomyocyte characteristics, including robust $Ca_V1.2$ expression.[46] Immunofluorescent images revealed a diffused staining pattern that changed dramatically upon PKC activation and became much more restricted to the PM. In addition, biotinylation experiments demonstrated increased amount of α_{1C} in PM of HL-1 cells. The increase in α_{1C} content in the PM within several minutes may be attributed to enhanced trafficking to the PM rather than changes in internalization, thus supporting the hypothesis of clustering of $Ca_V1.2$ channels in cardiomyocytes and smooth muscle cells.[63,67] Nevertheless, we still cannot rule out the possibility that PKC activation reduces the rate of internalization of α_{1C}, which can also result in increased PM α_{1C} content.

Interestingly, we were able to detect 2 distinct molecular sizes of α_{1C} corresponding to full-length (~250 kDa) and a dCT-truncated (~210 kDa) α_{1C}.[39,40,69] in HL-1 cells. The extent of truncation is

still disputed and probably differs among species (reviewed in[42]). Ser1928, a residue known to be phosphorylated by PKC,[3536] and crucial for current increase by PKC,[23] is absent in dCT-truncated α_{1C}. However, it has been suggested that the cleaved dCT remains associated with α_{1C} via non-covalent bonds,[3940] and so the role of Ser1928 in the truncated dCT remains to be resolved. The PKC-induced increase in PM content of the lower molecular weight band (dCT-truncated α_{1C}) was lower than full-length α_{1C}, in HL-1 cells. In correlation, PKC-induced enhancement of PM content of a dCT-deletion mutant ($\alpha_{1C\text{-}\Delta1800}$) expressed in oocytes was significantly lower than full-length α_{1C}.

Xenopus oocyte is a very convenient model to study the effects of PKC by expressing various α_{1C} mutants. This is the only heterologous expression system in which macroscopic $Ca_V1.2$ currents are enhanced following PKC activation; the amount of RNA injected for protein expression is accurately calculated and calibrated; the PM can be manually separated from the cell and its protein content can be quantified using specific antibodies. The significance of the distal part of the CT, which contains Ser1928 residue, in increased PM expression following PKC activation was confirmed in this preparation as well. Our present results, in which the effect of PKC on PM localization of $Ca_V1.2$ was very similar in HL-1 cells and in *Xenopus* oocytes, further validate the use of this model system for studies of mechanisms of modulation of the channel by PKC. Activation of PKC by PMA in oocytes was confirmed using a specific PKC inhibitor, Bis, that successfully and significantly reduced the effects of PKC. However, the robust activation of PKC by PMA was not completely blocked by PMA. It is conceivable that PMA affects additional cellular pathways that are not blocked by Bis. Still, when major phosphorylation sites are deleted or mutated (e.g. Ser1928), most of the effect of PKC is not observed, supporting a major involvement of PKC in PMA-induced changes in PM content of $Ca_V1.2$. It is not clear why coexpression of dCT with the truncated α_{1C} did not restore the effect of PMA. It is possible that, when coexpressed with $\alpha_{1C}\Delta1821$, dCT that contains Ser1928 phosphorylation site competes with other phosphorylation sites on $\alpha_{1C}\Delta1821$, thereby eliminating the effect of PKC on $\alpha_{1C}\Delta1821$. Taken together, our results suggest that dCT plays a role in PKC modulation, and Ser1928 is a crucial residue for this modulation.

In conclusion, the modulation of $Ca_V1.2$ by PKC (leading to enhanced macroscopic currents) involves increased α_{1C} expression in PM. The dCT and phosphorylation of Ser1928 are crucial for this effect. As previously proposed the requirement for a L-NT isoform may indicate that a complex NT-CT interaction[70,71] underlies the mechanism of the effect of PKC on α_{1C}.

Materials and methods

Oocyte culture

All the experiments were performed in accordance with the Tel Aviv University Institutional Animal Care and Use Committee (permit no. M-13–002). *Xenopus laevis* frogs were maintained and operated, and oocytes were collected, defolliculated, and injected with RNA as described.[49] 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; 5 ng) of the mRNAs of $Ca_V1.2\alpha$ isoforms (original long-NT isoform: accession no. X15539) or its mutants with α_2/δ (accession number M21948), with or without β_{2b} (accession number X64297), with or without 1 ng of m_3R, 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).

HL-1 cell culture

HL-1 cells were maintained as described in Claycomb *et al.* 1998.[46] Briefly, cells were plated in gelatin/fibronectin-coated culture flasks and maintained in Claycomb medium supplemented with 10% fetal bovine serum, 0.1 mM norepinephrine, 2 mM glutamine (Sigma) and 100 units/ml penicillin-streptomycin (Gibco), at 37°C in a humidified 5% CO_2 environment. The media was replaced every 24–48 hours. The culture reaches confluency within 72 hours.

cDNA constructs and mRNA

cDNAs of α_{1C}, α_2/δ and β_{2b} were as described.[50] The rabbit heart α_{1C} mutants used here were prepared in our laboratory as described.[51] Rat m_3R is in pGEM-HJ. The RNAs were prepared using a standard procedure described previously, which ensures capping of the 5′ end of the RNA and preferential inclusion of non-capped GTP in the rest of the RNA.[49]

Western blotting

Cultured HL-1 cells were washed 3 times in cold phosphate-buffered saline (PBS) to wash away remaining media and scraped with a cell scraper. Homogenization of the cells was done by gentle rotating the cells for 1 hour at 4°C in hypotonic lysis buffer (5 mM TRIS pH 7.4, 1 mM EDTA and Complete protease inhibitor mix (ThermoFisher)). The homogenate was spun at 1000 × g for 10 min at 4°C to separate debris and heavy molecules like DNA. Protein concentrations were determined using BCA protein assay kit (Sigma). Proteins were separated on a 6% SDS gel and transferred onto nitrocellulose membrane (Bio-Rad). Membranes were blocked with 5% nonfat dry milk in TBST (Tris 20 mM, pH 7.6, 120 mM NaCl, 0.1% Tween), then incubated anti-α_{1C} polyclonal antibody (Alomone, Jerusalem). After 3 washes with TBST, they were incubated with a horseradish-peroxidase-labeled anti-rabbit secondary antibody (Jackson Immunoresearch Laboratories). Membranes were washed 3 times with TBST, signals were developed with ECL$^+$ (Amersham Pharmacia Biotech).

Surface biotinylation

HL-1 cells

HL-1 cells were cultured in 100 mm culture dishes (Corning) to confluency. Separate plates were treated with PMA (Alomone Laboratories) dissolved in DMSO to final concentration of 10 nM PMA and 1% v/v DMSO, or 1% v/v DMSO alone (vehicle) in supplemented Claycomb medium for 7 min with gentle rocking. Following treatment, culture medium was aspirated and the plates were washed with cold PBS containing 0.9 mM $CaCl_2$ and 1 mM $MgCl_2$ 3 times to block any endocytosis. Then the dishes were incubated at room temperature in the presence of 1 mg/ml of the membrane-impermeable reagent EZ-Link Sulfo-NHS-LC-biotin (Thermo Scientific) in PBS. The cells were washed 3 times with quenching solution (PBS supplemented with 10 mM glycine (Sigma)) to stop the reaction. Following an additional wash in PBS, the cells were scraped into 1 ml hypotonic lysis buffer (5 mM TRIS pH 7.4, 1 mM EDTA, and complete protease inhibitor cocktail (ThermoFisher)). The scraped cells were collected into pre-chilled Eppendorf tubes and gently agitated on a rotator at 4°C for 1 hour. The lysate was spun at 1000 g for 10 min to sediment debris and heavy molecules like DNA. The supernatant was collected and a fraction kept aside as "total protein." The protein content was determined using the BCA protein estimation kit with bovine serum albumin as standard. PMA and DMSO samples were adjusted to similar protein concentration. The adjusted samples were spun at 40000 g in 4°C for 30 min to enrich the membrane fraction. The supernatant was discarded and the pellet was resuspended in lysis buffer supplemented with protease inhibitor complex. Immobilized Streptavidin-Agarose resin (Thermo Scientific) was added to this suspension, and the reaction mixture was gently rotated at 4°C for 16 h. The resin was spun down at 5000 *g* for 5 min and washed 3 times with ice-cold lysis buffer to remove any non-specific bound proteins. The beads were incubated in sample buffer to elute the "bound protein." The eluted proteins were separated on a 6% SDS gel. Since the total protein content in both samples is equal, we did not use any loading control. Proteins were transferred to nitrocellulose membranes for Western blotting. Band intensity was measured using ImageJ. The results were analyzed by Student's unpaired t-test using Prism software (GraphPad). Statistical significance limit was set at $p < 0.05$.

Oocytes

After mRNA injection, oocytes were incubated at 18°C for 4 d before experiments. Twenty oocytes from each group were washed with 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), and then transferred to biotinylation solution (NDE96 containing 1.5 mg/ml of freshly prepared EZ-Link Sulfo-NHS-LC-biotin). Oocytes were incubated on a rocker at room temperature for 20 min. Fresh biotinylation solution was added for further 20 min incubation. Oocytes were transferred to an ice-cold quenching solution (NDE96 with 50 mM glycine) and incubated for 10 min. Then, oocytes

were washed in NDE96 for 10 min and transferred to a Eppendorf tube containing homogenization buffer (20 mM Hepes pH = 8.0, 5 mM EDTA, 5mM EGTA, 100 mM NaCl, protease inhibitor cocktail (ThermoFisher)). Oocytes were homogenized by pipetting on ice. Homogenates were cleared by centrifugation (700 *g* for 15 min). 1/60 from total volume of the supernatant was removed for measuring the total protein (bound+unbound). The supernatant was mixed with streptavidin bead slurry (ThermoFisher) and incubated on a rocker overnight at 4°C. Beads were separated by centrifugation and washed 3 times with homogenization buffer. Elution of the proteins from the beads was done by adding sample buffer and heating the samples at 65°C for 5 min. The eluted proteins were loaded on 6% SDS-PAGE. Proteins were transferred to nitrocellulose membranes for Western blotting.

HL-1 immunocytochemistry

HL-1 cells were fixed in 4% paraformaldehyde and rinsed in PBS 3 times. The cells were then permeabilized using 0.25% Triton X-100 (sigma) and rinsed in PBS. Cells were blocked in 10% normal donkey serum (Jackson Immunoresearch Laboratories) to eliminate non-specific binding. Cells were then incubated with anti-$Ca_V1.2$-ATTO-488 antibody (Alomone Labs) overnight at 4°C, rinsed in PBS to remove excess antibody and images were taken with a confocal laser scanning microscope (Zeiss 510 META). ImageJ (http://imagej.nih.gov/ij/) was used to quantify the expression of $Ca_V1.2$ in the membrane with and without PMA treatment. Fluorescence of the whole cell was measured in 'integrated density' using the free hand drawing tool. A second measurement was made at about 6–10 pixels inside the first one and this was considered as the fluorescence from cytoplasm. The intensity of whole cell was then subtracted from the intensity of the cytoplasm to give the intensity of the membrane. The intensity obtained for the membrane fraction was later corrected for background, by subtracting separate background measurements. The values obtained were analyzed using unpaired t-test using the Prism software.

Giant membrane patches

Giant excised patches of oocyte membrane were prepared as described.[52] Oocytes were mechanically devitellinized using tweezers in a hypertonic solution (in mM: 6 NaCl, 150 KCl, 4 $MgCl_2$, 10 Hepes, pH 7.6) and transferred onto a coverslip in EGTA-containing ND96 solution (in mM: 96 NaCl, 2 KCl, 1 $MgCl_2$, 5 Hepes, 5 EGTA, pH 7.6) with their animal pole facing the coverslip, for 30 min. The oocytes were then removed with a jet of solution using a Pasteur pipette, leaving a giant membrane patch attached to the coverslip, with the cytosolic part facing the medium, and the extracellular surface facing the coverslip. The coverslip was washed thoroughly with fresh ND96 solution, and fixated using 4% formaldehyde in EGTA-containing ND96 solution for 30 min. Coverslips were mounted on a glass slide. Giant membrane patches were stained with primary antibody anti-α_{1C} polyclonal antibody (Alomone, Jerusalem), followed by incubation with Cy3 fused secondary antibody. Fluorescent labeling was examined with the Zeiss 510 META confocal microscope, using a 63 × oil-immersion objective. Cy3 was excited by 488 nm laser and the intensities were measured at 560–569 nm window in the spectral mode. In each experiment, all oocytes from the different groups were studied using a constant set of imaging parameters. Net fluorescence intensity per unit area was obtained by subtracting an averaged background signal measured in the same way on the coverslip outside the oocytes.

Statistics and data presentation

The data are presented as mean±SEM, n = number of cells tested. Comparisons between 2 groups (e.g., control and PMA treated groups) were tested for statistically significant differences ($P<0.05$ or better) using 2-tailed unpaired *t* – test. Comparison between several groups was done using one-way analysis of variance (ANOVA) followed by Tukey's test, using the SigmaPlot software (SPSS Corp.).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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