

Isoform-specific inhibition of voltage-sensitive Ca^{2+} channels by protein kinase C in adrenal chromaffin cells

Cristina M. Sena^a, Rosa M. Santos^{a,b}, Nick B. Standen^c, Michael R. Boarder^c,
Luís M. Rosário^{a,b}

^aCenter for Neuroscience and Cell Biology, University of Coimbra, 3001-401 Coimbra, Portugal

^bDepartment of Biochemistry, Faculty of Sciences and Technology, University of Coimbra, P.O. Box 3126, 3001-401 Coimbra, Portugal

^cDepartment of Cell Physiology and Pharmacology, School of Medicine, University of Leicester, Leicester LE1 9HN, UK

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Abstract Selective protein kinase C (PKC) activators and inhibitors were used to investigate the involvement of specific PKC isoforms in the modulation of voltage-sensitive Ca^{2+} channels (VSCCs) in bovine adrenal chromaffin cells. Exposure to the phorbol ester phorbol-12,13-dibutyrate (PDBu) inhibited the Ca^{2+} currents elicited by depolarizing voltage steps. This inhibition was occluded by the PKC-specific inhibitor Ro 31-8220 but remained unaffected by Gö 6976, a selective inhibitor of conventional PKC isoforms. PDBu treatment caused the translocation of PKC- α and - ϵ isoforms from cytosol to membranes. PKC- ι and - ζ showed no signs of translocation. It is concluded that VSCCs are specifically inhibited by the activation of PKC- ϵ in chromaffin cells. This may be relevant to the action of phospholipase-linked receptors involved in the control of Ca^{2+} influx, both in catecholaminergic cells and other cell types. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Protein kinase C; Voltage-sensitive Ca^{2+} channel; Adrenal chromaffin cell; Phorbol-12,13-dibutyrate; Ro 31-8220; Gö 6976

1. Introduction

The protein kinase C (PKC) superfamily comprises at least 12 isoforms: the conventional (cPKC) isoforms (Ca^{2+} - and diacylglycerol/phorbol ester-sensitive forms α , β I, β II and γ), the novel (nPKC) isoforms (Ca^{2+} -insensitive and diacylglycerol/phorbol ester-sensitive forms ϵ , η , δ and θ), the atypical (aPKC) isoforms (Ca^{2+} - and diacylglycerol/phorbol ester-insensitive forms ζ and λ/ι) and the recently discovered PKC-related kinases [1]. The PKC isoforms differ in their structure, cofactor requirements, substrate specificity and tissue expression with specific subcellular distribution [2]. These features, combined with the finding that more than one PKC isoform is usually expressed in a single cell type, have led to the notion that each member of the PKC superfamily plays a specific role in the processing of physiological and pathological responses to extracellular stimuli.

Abbreviations: PKC, protein kinase C; PDBu, phorbol-12,13-dibutyrate; PMA, phorbol 12-myristate 13-acetate; VSCC, voltage-sensitive Ca^{2+} channel; I_{Ca} , Ca^{2+} currents; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid

Voltage-sensitive Ca^{2+} channels (VSCCs) are prone to modulation by PKC. Indeed, PKC activation has been shown to either enhance or inhibit depolarization-evoked Ca^{2+} currents (I_{Ca}) depending on the cell type. Specifically, we and others have previously demonstrated that the PKC activator phorbol 12-myristate 13-acetate (PMA) inhibits depolarization-evoked Ca^{2+} influx in catecholamine-secreting adrenal chromaffin cells [3–5], and that L-type VSCCs appear to be the major target for this inhibition [6]. Using a combination of patch-clamp and PKC translocation studies, we have now determined the PKC isoform specificity of phorbol ester-mediated inhibition of voltage-activated calcium currents in chromaffin cells.

2. Materials and methods

Bovine adrenal medulla cells were isolated, purified and cultured essentially as described [6,7]. Experiments were performed between days 2 and 5 of culture.

The whole-cell patch-clamp technique was used to record I_{Ca} , essentially as reported [8]. Patch pipets were filled with a solution containing (in mM): 110 CsCl, 20 TEACl, 14 EGTA (ethylene glycol bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid), 5 MgATP, 0.2 Na_2GTP and 20 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; pH 7.2, adjusted with CsOH). Currents were recorded using an Axopatch 200A voltage-clamp amplifier and pCLAMP software (version 5.5; Axon Instruments, Foster City, CA, USA). Data were acquired at 10–20 kHz and filtered at 5 kHz with an 8-pole low-pass Bessel filter. Cells were superfused with different salt solutions (flow rate $< 1.5 \text{ ml min}^{-1}$) using a flow system in which seven lines fed a common outflow placed near the cell [9]. The perfusion salt solution used at the start of each experiment contained (in mM): 125 NaCl, 5.4 KCl, 1 MgCl_2 , 5 CaCl_2 , 10 glucose and 10 HEPES (pH 7.4, adjusted with NaOH). This solution was subsequently replaced by a Na^+ -free solution containing (in mM): 125 choline chloride, 5 CaCl_2 , 1 MgCl_2 , 5.4 CsCl, 10 glucose and 10 HEPES (pH 7.4, adjusted with TEAOH). All the experiments were conducted at room temperature (20–23°C).

For PKC immunodetection, cultured chromaffin cells were washed with Earle's balanced salt solution and maintained in culture for a further 24 h period in heat-inactivated fetal calf serum-free Dulbecco's modified Eagle's/Ham's F-12 medium. The cells were then treated with phorbol-12,13-dibutyrate (PDBu) for the various time periods indicated in Fig. 1. For the assessment of the amounts of soluble and membrane-associated PKC isoforms, cells were subsequently washed, harvested and lysed as described previously [7]. The protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes as described [7]. For immunoblotting, monoclonal antibodies raised against the different PKC isoforms (α , β , γ , δ , ϵ , ζ , θ , η , ι , λ and μ) were used and immunoreactive proteins detected using an enhanced chemiluminescence detection system. Immunoblotting and densitometric analysis were performed as described previously [7].

For PKC activity determination, cultured chromaffin cells were washed, harvested and scraped into ice-cold homogenization buffer as described previously [7]. The cells were lysed in a Teflon pestle tissue grinder and centrifuged for 10 min at $14000\times g$ to remove insoluble cellular components (4°C). PKC activity was assessed by a fluorescence assay (Colorimetric PKC Assay Kit, SpinZyme Format; Pierce, Rockford, IL, USA), based on affinity separation of the phosphorylated form of a protein substrate (pseudosubstrate peptide), covalently attached to a fluorescent label. The reaction buffer contained excess Ca^{2+} and phosphatidyl-L-serine, thus allowing for detection of both conventional and novel PKC isoforms.

Values are expressed as mean \pm S.E.M. throughout the text and figures. The statistical significance of differences between mean values was assessed by either one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons test or paired Student's *t*-test, as appropriate.

Gö 6976 and PMA were from Calbiochem (Nottingham, UK). RO 31-8220 was a kind gift of Dr. G. Lawton (Roche Products Ltd, Welwyn, Herts, UK). PDBu and all other reagents were from either Sigma (St. Louis, MO, USA) or Merck (Darmstadt, Germany). Reagents for the immunodetection of PKC were as previously described [10] with the exception of monoclonal antibodies against PKC isoforms (Transduction Laboratories, Exeter, UK).

3. Results

We [7] and others [10] have previously reported the presence of PKC- α , - ϵ and - ζ isoforms in bovine chromaffin cells. In the present study we have tested additional PKC antisera and found evidence for another aPKC isoform, PKC- ι . The differential translocation of PKC isoforms from cytosol to membranes in response to treatment with phorbol esters has been widely reported. To understand the effects of several minutes

exposure to PDBu ($1\ \mu\text{M}$) on the detectable isoforms of PKC in chromaffin cells, we investigated its distribution between the cytosolic and membrane fractions using cells cultured in serum-free conditions for 24 h.

Fig. 1 shows that both PKC- α and - ϵ translocated rapidly from cytosol to membranes. Indeed, PKC- α and - ϵ immunoreactivities in the membrane pool reached maximal levels at 10 min of PDBu treatment (not different to membrane PKC- α and - ϵ immunoreactivities at 90 min; $P > 0.05$, assessed by ANOVA). It is also noteworthy that the loss of PKC- ϵ from the cytosol at 10 min of PDBu treatment ($\sim 66\%$) was more profound than the loss of PKC- α ($\sim 42\%$). However, Fig. 1D suggests that the amount of membrane-bound PKC- α at this time point exceeds largely that of membrane-bound PKC- ϵ . This is presumably a consequence of cytosolic PKC- α being present in larger amounts relative to cytosolic PKC- ϵ . PKC- ι and - ζ showed no significant signs of translocation.

Maximal translocation of PKC- ϵ and - α from cytosol to membranes occurred at approximately 100 nM PDBu ($\text{EC}_{50} = 8$ and 17 nM, respectively; data not shown). We therefore set at 200 nM the PDBu concentration to be used in the electrophysiological experiments.

Whole-cell I_{Ca} in chromaffin cells were recorded using Ca^{2+} (5 mM) in the extracellular solution as the charge carrier. Fig. 2A depicts the typical appearance of I_{Ca} recorded under control conditions and in the continued presence (10 min) of PDBu (200 nM). Control I_{Ca} reached a peak (-375 ± 29 pA, \pm S.E.M.; $n = 12$ cells) in less than 8 ms and was partially inactivated thereafter.

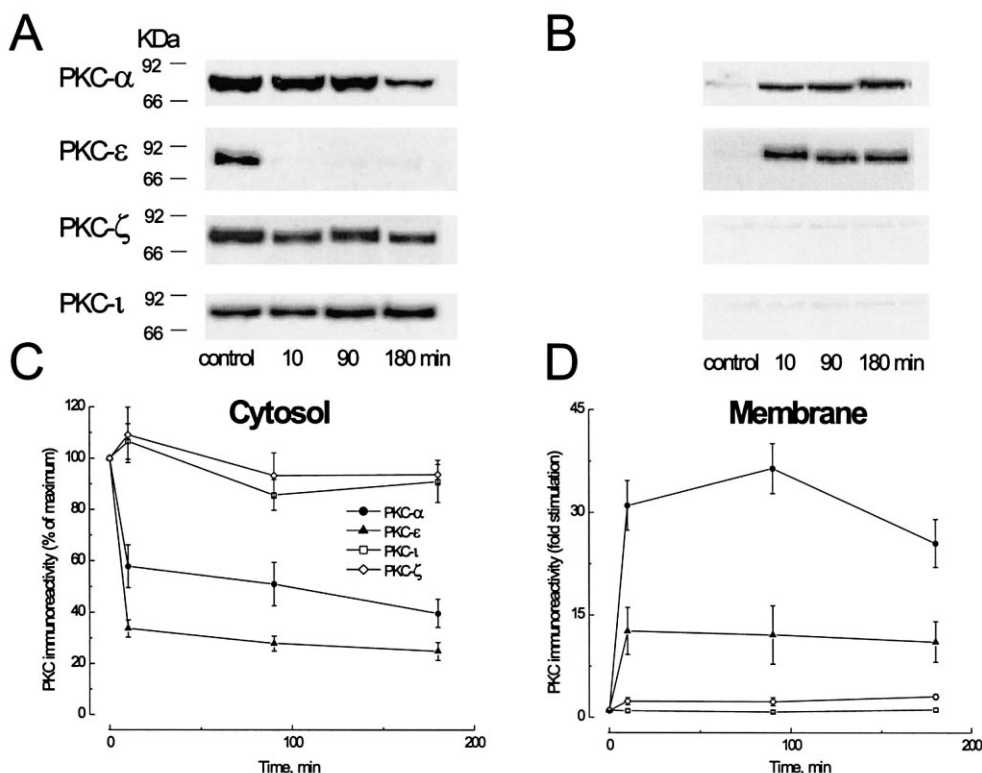


Fig. 1. Translocation of PKC isoforms from cytosol to membranes in response to PDBu. A, B: Effect of exposure to $1\ \mu\text{M}$ PDBu for between 10 and 180 min on levels of PKC- α , - ϵ , - ζ and - ι immunoreactivity on Western blots of cytosolic (A) and membrane (B) fractions from chromaffin cells (autoradiographs of typical experiments). C, D: Pooled immunoreactivity data, collected by laser densitometry of autoradiographs from six different experiments for cytosolic (C) and membrane (D) fractions. Cytosolic readings were normalized to the untreated control levels expressed as 100%. Membrane-bound readings were normalized to fold over unstimulated controls. Data are mean \pm S.E.M. values.

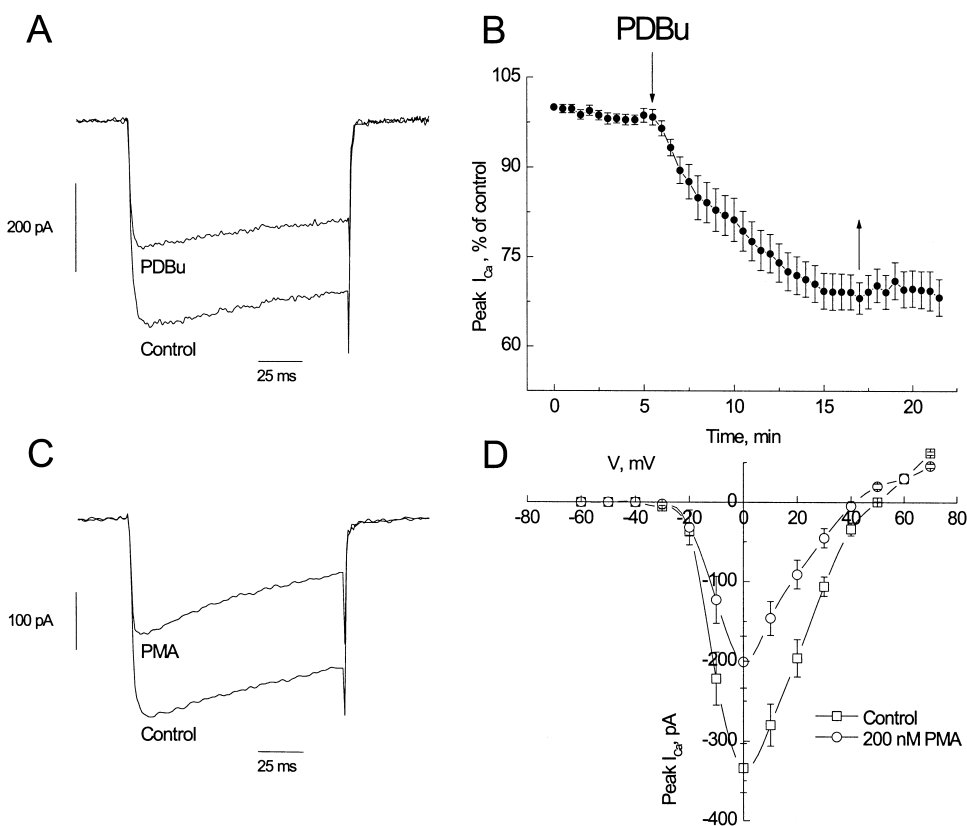


Fig. 2. Inhibitory effects of PKC activators on voltage-activated I_{Ca} . A: Superimposed current traces obtained in the absence ('control') and presence of the phorbol ester PDBu (200 nM, 10 min). Whole-cell I_{Ca} were elicited by stepping membrane potential to 0 mV from a holding potential of -80 mV. B: Time course of the average inhibitory effect of 200 nM PDBu on peak I_{Ca} . Each cell was repetitively stimulated with 125 ms voltage steps every 30 s as in A. PDBu was added to and removed from the perfusion solution as denoted by the arrows. Data are expressed as percentage of peak I_{Ca} recorded in control (6 min prior to PDBu addition). C: Superimposed sample traces obtained in the absence ('control') and presence of the phorbol ester PMA (200 nM, 10 min). Pulse protocol as in A. D: Current-voltage (I - V) curves obtained under control conditions (squares) and throughout PMA exposure after 10 min (circles). I - V curves were constructed by applying 260 ms pulses to various test potentials from a holding potential of -80 mV at 25 s intervals. Data are presented as mean \pm S.E.M. (B: $n=12$ cells; D: $n=5$ cells).

Following stabilization of the I_{Ca} , identical voltage steps were applied to each cell every 30 s. Fig. 2B shows that peak I_{Ca} remained fairly constant for several minutes prior to addition of PDBu. The phorbol ester caused a pronounced, time-dependent reduction in peak I_{Ca} amplitude that appeared to stabilize in less than 10 min (representative trace in Fig. 2A). On average, the inhibition of peak I_{Ca} at this time point amounted to $29 \pm 3\%$ of control (average peak I_{Ca} recorded 6 min prior to PDBu addition). The time necessary to cause 50% of maximal inhibition ($t_{1/2}$) was 4.1 ± 0.5 min ($n=12$ cells). The time course of the PDBu-mediated inhibition of I_{Ca} is therefore similar to that reported in a previous study from our laboratory, using PMA as a pharmacological tool to activate PKC and $[Ca^{2+}]_i$ measurements from single cells as an indirect way of assessing depolarization-evoked I_{Ca} [5]. PDBu did not appear to affect the inactivation properties of I_{Ca} , as shown in Fig. 2A.

The typical effect of another phorbol ester activator of PKC (PMA, 200 nM) on I_{Ca} is depicted in Fig. 2C. PMA reduced peak I_{Ca} (average inhibition at 10 min incubations: $43 \pm 4\%$ of control, $n=12$ cells) with a time course similar to that of PDBu (average $t_{1/2}$: 5.0 ± 0.3 min). I_{Ca} seemed to undergo faster inactivation in the presence of PMA. We have deter-

mined whether the phorbol ester-mediated reduction in peak I_{Ca} might originate from a rightward shift in the voltage dependency of Ca^{2+} channel activity. The I - V curve depicted in Fig. 2D shows that control I_{Ca} did not develop prior to stepping membrane potential to ca. -20 mV, reached a maximum at ca. 0 mV and reversed at around $+50$ mV (average reversal potential: $+46.7 \pm 0.6$ mV, $n=5$ cells). PMA depressed peak I_{Ca} at all voltages in the range $-10/+40$ mV but did not alter its activation mid-point, as determined by fitting the I - V curve data to a modified Boltzmann function: -9.1 ± 0.5 mV versus -9.5 ± 0.6 mV ($n=5$ cells) for control and PMA, respectively. This is in keeping with data from other studies [11].

We have determined whether PKC activation mediates the inhibitory effect of PDBu by pre-incubating the cells with the specific PKC inhibitor Ro 31-8220 [12]. This compound admittedly inhibits most, if not all PKC isoforms known to date. Fig. 3A shows that addition of Ro 31-8220 (10 μ M) caused a significant reduction in peak I_{Ca} . On average this inhibition amounted to $23 \pm 4\%$ (Fig. 3B). Peak I_{Ca} values were nonetheless fully recovered following inhibitor washout and, significantly, exposing Ro 31-8220-pretreated cells to 200 nM PDBu occluded the phorbol ester-mediated inhibition of peak I_{Ca} (inset to Fig. 3A, compare traces a and c). There

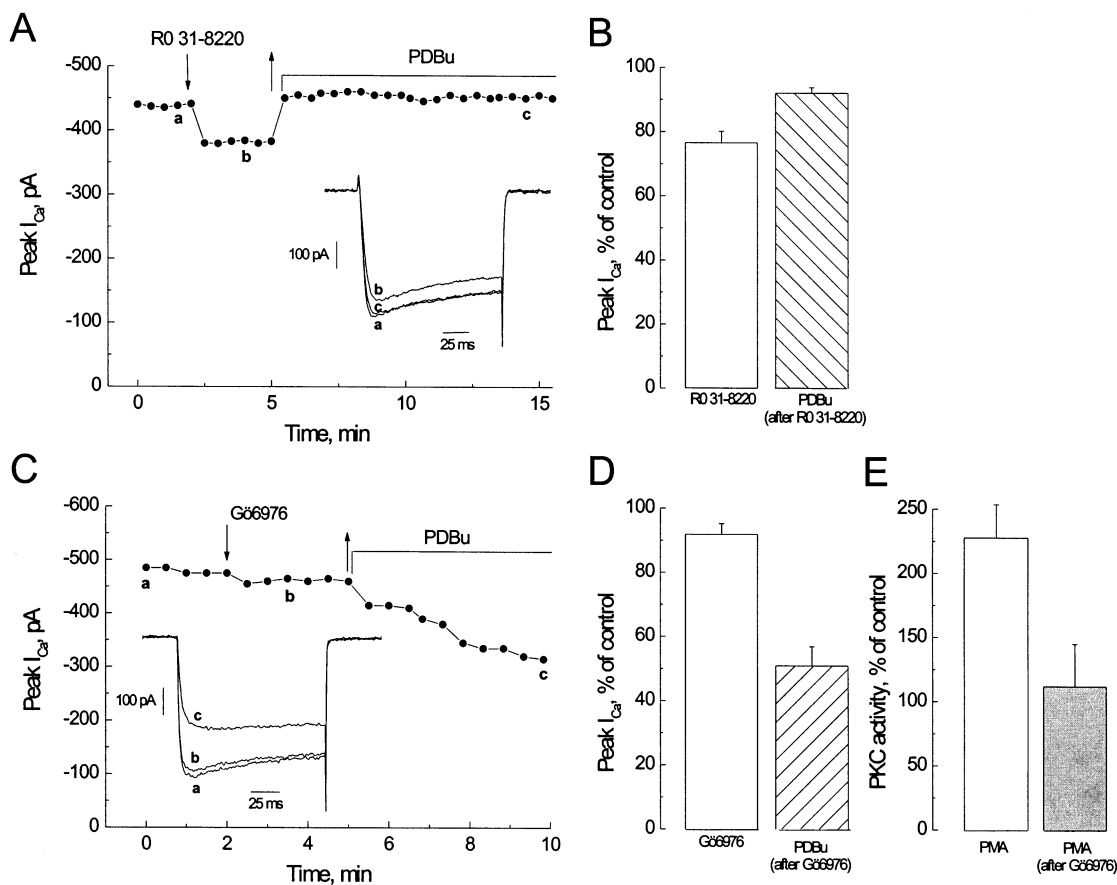


Fig. 3. Differential effects of PKC inhibitors on phorbol ester-induced inhibition of voltage-activated I_{Ca} . A: Effect of PDBu (200 nM) on peak I_{Ca} in a Ro 31-8220-pretreated cell. The cell was repetitively stimulated with 125 ms voltage steps to 0 mV (holding potential -80 mV). Ro 31-8220 (10 μ M) was added to and removed from the perfusion solution as denoted by the arrows. PDBu was applied as indicated by the horizontal bar. Inset: Superimposed current traces obtained in the absence (a) and presence of Ro 31-8220 (b), and after exposure to PDBu (c). B: Average inhibitory effects of Ro 31-8220 alone (left column) and PDBu (200 nM, 10 min) in Ro 31-8220-pretreated cells. Data are expressed as percentage of peak I_{Ca} recorded in control (2 min prior to Ro 31-8220 pretreatment). Data from the experiment depicted in A and six similar experiments. C: Same as in A, except that the PKC inhibitor was Gö 6976 (10 μ M). D: Average inhibitory effects of Gö 6976 alone (left column) and PDBu (200 nM, 10 min) in Gö 6976-pretreated cells. Data are expressed as in B. Data from the experiment depicted in C and three similar experiments. E: Effect of Gö 6976 pretreatment on PKC activity. Cells were stimulated with 200 nM PMA for 5 min both in the absence of Gö 6976 (left column) and after Gö 6976 pretreatment (10 μ M, 3 min). Data are expressed as percentage of PKC activity measured in control (5 min exposures to 0.01% dimethylsulfoxide). Absolute PKC activity in control: 4.7 ± 1.0 mU. PKC activity data were obtained from four experiments in triplicate. Data are presented as mean \pm S.E.M. (B, D and E).

was in some cells a slight but continued I_{Ca} run-down throughout the experiment (see Fig. 2B, left). This may explain why, on average, exposing Ro 31-8220-pretreated cells to PDBu resulted in an apparent 8% inhibition of peak I_{Ca} (Fig. 3B).

In the following experiments we used Gö 6976, a specific inhibitor of cPKC isoforms [13,14]. Gö 6976 concentrations in the μ M range have been shown not to affect nPKC activity in vitro [13], and have been used to differentiate between cPKC and non-cPKC effects in different cell types, including chromaffin cells [15–17]. Pretreatment with 10 μ M Gö 6976 did not impair the PDBu-mediated reduction in peak I_{Ca} , as shown in Fig. 3C (compare traces b and c). Indeed, the average inhibitory effect observed in Gö 6976-pretreated cells amounted to $48 \pm 8\%$ of control (Fig. 3D). This cannot be accounted for by a reversible action of Gö 6976 on PKC activity since the inhibitory effect of PDBu remained in the continued presence of the drug ($n=2$ cells, data not shown; see also [14]). Interestingly, Gö 6976 alone reduced peak I_{Ca} by a modest $9 \pm 3\%$ of control (Fig. 3D) when compared with Ro 31-8220.

We have assessed the effectiveness of Gö 6976 as an inhibitor of phorbol ester-induced PKC activity in chromaffin cells, using an incubation protocol similar to that used for the electrophysiological experiments (exposure to 10 μ M Gö 6976 for 3 min, followed by phorbol ester addition in the absence of the PKC inhibitor). Exposing control cells to 200 nM PMA for 5 min evoked a 2.2-fold increase in PKC activity. This effect was strongly attenuated ($\sim 90\%$) by pretreating the cells with 10 μ M Gö 6976 (Fig. 3E).

4. Discussion

We have shown that, in adrenal chromaffin cells, phorbol ester activators of PKC lead to the inhibition of VSCCs in a seemingly voltage-independent manner. This resembles the inhibitions observed in other cell types [18]. Importantly, our data indicate that PKC-mediated inhibition of VSCCs is isoform-specific in chromaffin cells. Indeed, PDBu inhibition of voltage-activated I_{Ca} is occluded by pretreating the cells with the membrane-permeant bisindolylmaleimide derivative Ro

31-8220. This compound shows little PKC isoform selectivity and inhibits all PKC isoforms tested, including the isoforms expressed in chromaffin cells [12]. In contrast, the PDBu inhibition of I_{Ca} is unaffected by pretreating the cells with the indolocarbazole Gö 6976. This compound discriminates between Ca^{2+} - and non- Ca^{2+} -dependent isoforms of PKC in vitro, inhibiting the cPKC isoforms (including PKC- α), but not the nPKC and aPKC isoforms (including PKC- ϵ) [13,15,19]. Lack of effect of Gö 6976 in the present study cannot be explained by ready washout from the cell membranes at the time of exposure to PDBu, since pretreating the cells with Gö 6976 caused a profound inhibition of phorbol ester-induced PKC activity. The differential action of Ro 31-8220 and Gö 6976, reported in this study, points to PKC- ϵ specifically mediating PDBu inhibition of I_{Ca} , since it is the only nPKC isoform present in chromaffin cells (this work and [7,10,17]). It should be emphasized, in this respect, that PKC- ϵ undergoes ready translocation from cytosol to membranes, and that the time course of this translocation (maximal effect reached in 10 min or less of PDBu treatment) is compatible with the kinetics of the inhibitory effect of PDBu on I_{Ca} ($t_{1/2} \sim 4$ min, maximal inhibition in less than 10 min). This is an important point, since PKC translocation is generally regarded as representing the primary mode of activation of the kinase in mammalian cells [20,21].

One possibility to account for the fact that VSCCs in chromaffin cells are prone to regulation by specific PKC isozymes is that activated PKC might be targeted to discrete sites in the plasma membrane in an isoform-specific fashion [20,22]. According to this model, activated PKC- ϵ in chromaffin cells would be targeted to discrete sites in the close vicinity of VSCCs (most likely of the L-type [6]). In addition, we hypothesize that activated PKC- α might be targeted to membrane sites unrelated to Ca^{2+} influx, thereby meeting alternative regulatory requirements in chromaffin cells. We note in this respect that bradykinin and histamine receptor responses are differentially regulated by PKC isoforms in chromaffin cells [7], suggesting again that PKC- α and - ϵ might be specifically targeted to discrete sites in the plasma membrane in close vicinity to these receptors (or associated G-protein/phospholipase C molecules). Also interestingly, PKC- α and - ϵ appear to be differentially involved in the down-regulation of voltage-sensitive Na^+ channels in chromaffin cells [17]. Other possibilities can nonetheless be invoked to account for the selective regulation of VSCCs by PKC- ϵ in chromaffin cells. For example, cPKC and nPKC isoforms may display some degree of selectivity towards the Ca^{2+} channel phosphorylation sites. To our knowledge, this hypothesis has not yet been addressed conclusively.

The physiological significance of the isoform-specific regulation of VSCCs by PKC-mediated phosphorylation remains unknown. It is however a likely possibility that selected phospholipase-linked receptors might regulate Ca^{2+} influx in a highly directed fashion by a mechanism involving the action of specific PKC isoforms, both in chromaffin cells and other cell types.

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