Protein kinase C activator inhibits voltage-sensitive Ca^{2+} channels and catecholamine secretion in adrenal chromaffin cells

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Abstract **We have investigated the effects of the phorbol ester 12-myristate 13-acetate (PMA) on depolarization-evoked Ca^{2+} influx and catecholamine secretion in bovine adrenal chromaffin cells. PMA (100 nM) strongly inhibited K^+-evoked $[Ca^{2+}]_i$ transients and Mn^{2+} quenching of fura-2 fluorescence. In contrast, 4α-phorbol 12,13-didecanoate, a phorbol ester inactive on protein kinase C (PKC), had no effect. Maximal PMA-mediated inhibition occurred at 5–10 min incubations and were variable from cell to cell, ranging from 25 to 65% of controls. The $[Ca^{2+}]_i$ transients evoked by the L-type Ca^{2+} channel activator Bay K 8644 were strongly inhibited by 100 nM PMA. PMA (0.1–10 μM) inhibited K^+-evoked adrenaline and noradrenaline release by 23–44%. The data indicate that phorbol ester-mediated activation of PKC inhibits voltage-sensitive Ca^{2+} channels in chromaffin cells, leading to a prominent depression of depolarization-evoked catecholamine secretion.**

Key words: Protein kinase C; 12-Myristate 13-acetate (PMA); Cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$); Mn^{2+} influx; Catecholamine secretion; Voltage-sensitive Ca^{2+} channel

1. Introduction

Chromaffin cells are physiologically activated by acetylcholine, which is released from splanchnic nerve terminals following the electrical stimulation of the nerve and acts on two membrane receptors, the nicotinic and muscarinic receptors. While the stimulation of Ca^{2+} influx through voltage-sensitive Ca^{2+} channels is a predominant feature of nicotinic receptor activation [1], the activation of protein kinase C, subsequent to muscarinic receptor-mediated hydrolysis of PIP_2, is thought to play an important role in catecholamine secretion through the phosphorylation of key proteins of the chromaffin cell, leading to the sensitization of the exocytotic apparatus for the action of Ca^{2+}-mobilizing secretagogues [2]. PKC activation is not exclusive of the muscarinic receptor and may in fact be promoted by a range of other phospholipase C-linked membrane receptors present in chromaffin cells.

PKC-mediated phosphorylation is also known to influence voltage-sensitive Ca^{2+} channels in neurons and other cell types [3,4]. While in some cells PKC activation leads to the enhancement of depolarization-evoked Ca^{2+} currents, probably through a shift in the voltage threshold for activation towards more negative membrane potentials, in other cells the stimulation of the kinase causes the inhibition of the Ca^{2+} currents through an as yet unknown mechanism [5,6]. Indirect evidence suggests that PKC activation either has no effect [7] or has opposite effects [8–10] on depolarization-evoked Ca^{2+} fluxes in chromaffin cells depending on the species. PKC activation inhibits voltage-sensitive Ca^{2+} fluxes in the closely related PC-12 pheochromocytoma cell line [11–13].

In the present work we found that the phorbol ester PMA depressed depolarization-evoked catecholamine release and the underlying $[Ca^{2+}]_i$ transients. It also depressed depolarization-evoked Mn^{2+} influx and the $[Ca^{2+}]_i$ transients induced by a specific L-type Ca^{2+} channel activator, suggesting that voltage-sensitive Ca^{2+} channels in chromaffin cells are under negative control by PKC-mediated phosphorylation processes. The latter processes may be essential for the fine tuning of exocytotic secretion by PKC-linked membrane receptors, not only in these cells but possibly also in neurons and other secretory cells.

2. Materials and methods

Adrenal medulla cells were isolated by collagenase digestion of bovine adrenal glands, purified on a Percoll density gradient and maintained in culture in a 1:1 mixture of Dulbecco's modified Eagle's medium/Ham's F-12 medium, essentially as described previously [14,15]. For cuvette fluorescence experiments, cells were kept in suspension culture in bacterial Petri dishes. For microfluorescence experiments, the cells were plated on glass cover-slips coated with poly-L-lysine. For catecholamine release experiments, the cells were plated on 24-well cluster plates.

The salt solution used in cuvette fluorescence experiments had the following composition (mM): 130 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 15 HEPES-Na, pH 7.35. The cells used for the microfluorescence experiments were perifused with a solution containing (in mM) 120 NaCl, 5 KCl, 25 $NaHCO_3$, 2 $CaCl_2$, 1 $MgCl_2$ and 10 glucose (this solution was constantly gased with 95% O_2/5% CO_2 for a final pH of 7.4).

The $[Ca^{2+}]_i$ was recorded from cell suspensions using the Ca^{2+}-sensitive fluorescent probe fura-2 essentially as described previously [16]. The fluorescence was monitored using a computer-assisted spectrofluorometer (Fluoromax, Spex Industries, Edison, NJ, USA), with excitation at 340 nm and emission at 510 nm. The fluorescence intensities were automatically converted into $[Ca^{2+}]_i$ values using the calibration equation for single excitation measurements and taking the dissociation constant of the fura-2/Ca^{2+} complex as 224 nM [17].

Mn^{2+} influx was monitored from chromaffin cell suspensions by subjecting the cells to 50 μM $MnCl_2$ in the absence of external Ca^{2+} while recording the fura-2 fluorescence at the dye isosbestic point (360 nm), as described [18].

The $[Ca^{2+}]_i$ was recorded from single chromaffin cells using a dual-excitation microfluorescence system (Deltascan, PTI, Princeton, NJ, USA) essentially as described previously [19]. The cells under study were placed in a fast perifusion chamber on the stage of an inverted epifluorescence microscope (Nikon Diaphot, Japan). The temperature

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Abbreviations: Cytosolic free Ca^{2+} concentration, $[Ca^{2+}]_i$; Protein kinase C, PKC; phorbol 12-myristate 13-acetate, PMA; 4α-phorbol 12,13-didecanoate, 4αPDD.

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in the chamber was 37°C. Briefly, fura-2 incorporated into the cells was excited at 340 and 380 nm via two monochromators. The fluorescence was detected by a photomultiplier after passing through a band-pass interference filter centered at 510 nm. The data were automatically corrected for background fluorescence and acquired at 10 Hz by a computer. The fluorescence ratio F_{340}/F_{380} was converted into $[Ca^{2+}]_i$ values by an in vitro calibration procedure essentially as previously described [19].

Catecholamine release experiments were carried out as previously described [14,15]. Briefly, small samples of the medium bathing the cells were collected after 10 min incubations with drugs or control medium, followed by additional 5 min incubations with solutions containing 50 mM KCl. The specific adrenaline and noradrenaline content of these samples was assayed by HPLC. Collagenase B was from Boehringer Mannheim. Tissue culture medium and antibiotics were from Biological Industries, Beth Haemek, Israel. PMA, 4α-PDD and Bay K 8644 were from Calbiochem.

3. Results

Fig. 1A,B depicts the effects of K^+ depolarization on the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), recorded from chromaffin cell suspensions. Raising medium K^+ concentration from 5 to 50 mM evoked a sharp $[Ca^{2+}]_i$ increase, followed by a relatively slow relaxation towards a plateau (leftmost trace in Fig. 1A). This $[Ca^{2+}]_i$ rise reflects primarily Ca^{2+} influx through voltage-sensitive Ca^{2+} channels [16].

We have assessed the effect of 100 nM PMA on the depolarization-evoked $[Ca^{2+}]_i$ transients by comparing the size of these transients in the presence and absence of the phorbol ester. Fig. 1A,B shows that short (10 min) incubations with PMA reduced the peak K^+-evoked $[Ca^{2+}]_i$ rise by approximately 40%. In addition, Fig. 1A,B shows that 4αPDD, a PMA analogue inactive on PKC, failed to affect the magnitude of the $[Ca^{2+}]_i$ transients. Thus, the PMA-mediated inhibition is likely to originate from PKC activation, rather than from a possible non-specific action of the phorbol ester. Indeed, a recent immunoblot analysis of the translocation of different PKC isoforms from the cytosol to the membrane pool indicates that PKC-α and -ε are readily incorporated into the chromaffin cell membranes following 10 min exposures to PMA (C.M. Sena and M.R. Boarder, unpublished observations).

To assess the possibility that PMA might depress the depolarization-evoked $[Ca^{2+}]_i$ transients by inhibiting voltage-sensitive Ca^{2+} channels, we have added the Ca^{2+} channel surrogate Mn^{2+} [18,20] to the cells in the absence of external Ca^{2+} while recording fura-2 fluorescence at the dye isosbestic point. The fluorescence baseline was stable in the absence of Mn^{2+} (data not shown) and started to decline upon addition of the divalent cation (leftmost superimposed traces in Fig. 1C). Raising the medium K^+ concentration to 50 mM caused a rapid and pro-

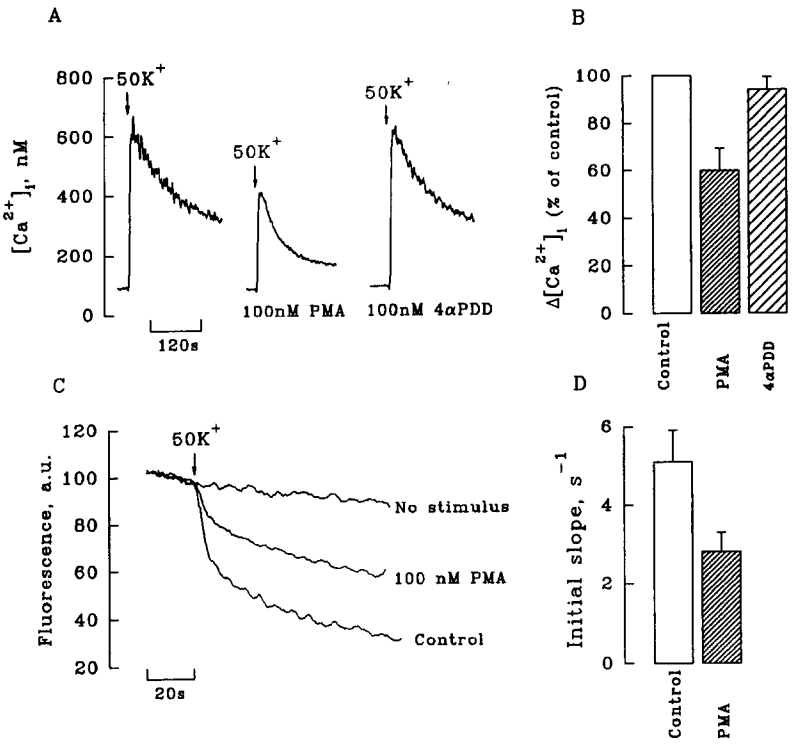

Fig. 1. Inhibition of depolarization-evoked $[Ca^{2+}]_i$ transients and Mn^{2+} influx by the PKC activator PMA. (A) The $[Ca^{2+}]_i$ was recorded from chromaffin cell suspensions. The cells were stimulated with 50 mM KCl (50 K^+) as denoted by the arrows, in the absence (leftmost trace) and in the presence of either PMA (10 min, second trace from left) or 4αPDD (10 min, first trace from right). (B) Average effects of 10 min incubations with 100 nM PMA and 4αPDD on the K^+-evoked $[Ca^{2+}]_i$ transients. The columns labelled 'PMA' and '4αPDD' represent the average peak $[Ca^{2+}]_i$ changes recorded in the presence of PMA and 4αPDD, respectively, normalized to the corresponding control changes. Average control response: 466 ± 52 nM (± S.D., $n = 4$ experiments in duplicate). (C) Superimposed fluorescence traces representing the quenching of fura-2 fluorescence (360 nm excitation) evoked by challenging the cells with 50 mM KCl, in the presence of 50 μM Mn^{2+} and in the absence of external Ca^{2+} (50 K^+, trace labelled 'control'), and the effect of a 10 min incubation with PMA on this quenching (trace labelled '100 nM PMA'). The 'no stimulus' trace depicts an experiment where the cells have not been challenged with high K^+ but were otherwise exposed to Mn^{2+} and 0 Ca^{2+}. (D) Kinetic analysis of the fluorescence quenching experiment depicted in panel C and of three similar experiments. The fluorescence decays, recorded immediately after exposure to 50 mM KCl for 2–4 s periods, were fitted to a linear function (correlation coefficients in the range 0.95–0.99). The columns represent the average of the slopes thus obtained ('control': high K^+ stimulation in the absence of PMA; 'PMA': stimulation in the presence of 100 nM PMA).

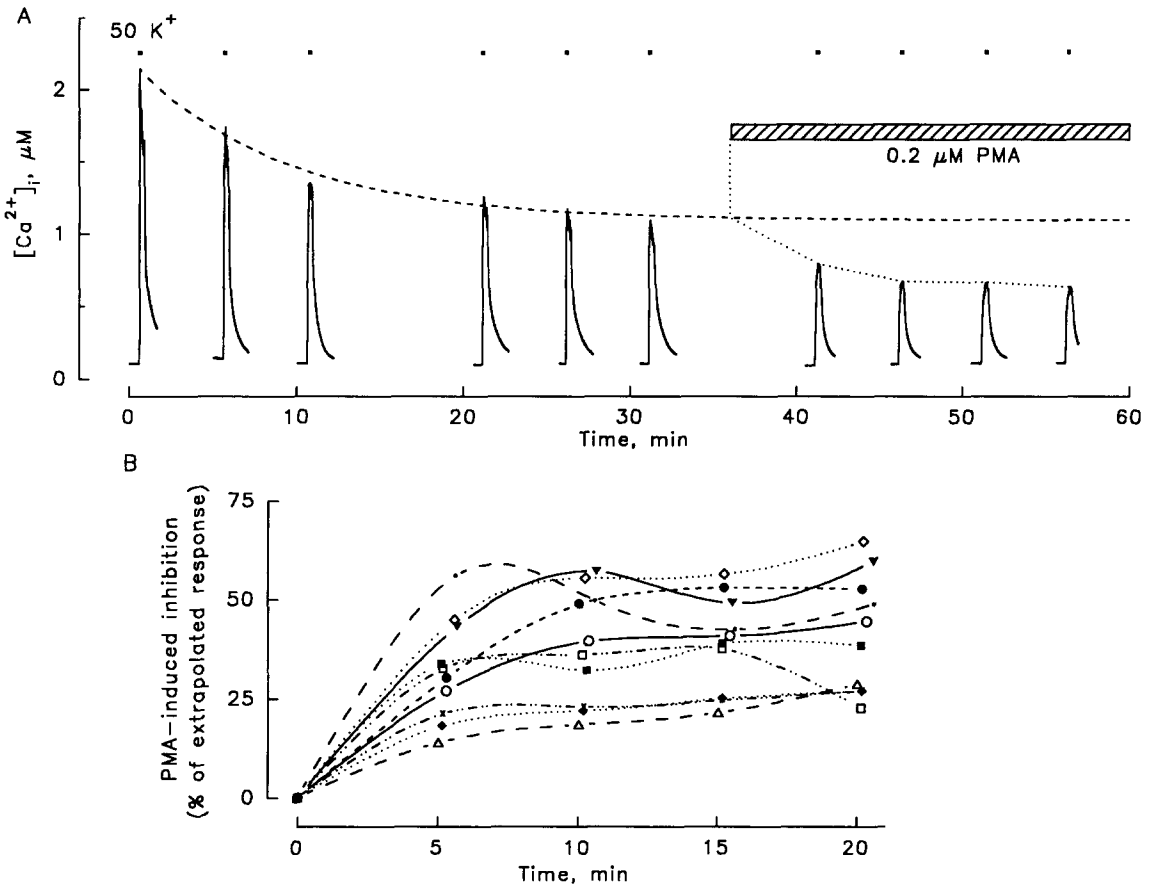

Fig. 2. Time course of the PMA-mediated inhibition of the depolarization-evoked $[Ca^{2+}]_i$ transients. The $[Ca^{2+}]_i$ was recorded from single chromaffin cells. (A) The cell was repeatedly exposed to 50 mM KCl (50 K^+) as denoted by the filled bars, in the absence (six leftmost pulses) and in the presence (remaining pulses) of PMA. The dashed line represents the best fit of the peak $[Ca^{2+}]_i$ values reached during each K^+ pulse to a single exponential decay function (see text). (B) Time course of the PMA-mediated inhibition of the K^+-evoked $[Ca^{2+}]_i$ transients recorded from single chromaffin cells, as assessed from experiments similar to that depicted in panel A by comparing the maximal size of the latter transients to the extrapolated decay function (see text). Time 0 refers to the moment of application of PMA. Each symbol represents an experiment performed on a different cell.

nounced fluorescence decrease (Fig. 1C, trace labelled 'control'). Since Mn^{2+} is a strong quencher of fura-2 fluorescence, this indicates an enhanced access of Mn^{2+} to the cytoplasm. Importantly, the K^+-evoked quenching of fura-2 fluorescence was substantially reduced by 10 min incubations with 100 nM PMA (trace labelled '100 nM PMA'). Since extracellular Mn^{2+} concentration is likely to remain constant throughout the experiment, Mn^{2+} influx through membrane channels can, to a first approximation, be treated as a zero order process. Thus, intracellular accumulation of the divalent cation and the concomitant quenching of fura-2 fluorescence would be expected to proceed linearly immediately after high K^+ stimulation. We have accordingly fitted the early component of the quenching traces to a linear function and assessed the effect of 100 nM PMA on the respective slopes, as depicted in Fig. 1D. It is apparent from this analysis that the phorbol ester decreased the early component of Mn^{2+} influx by 45%, in essential agreement with the $[Ca^{2+}]_i$ data.

We have investigated the time course of the PMA-mediated inhibition of the K^+-evoked $[Ca^{2+}]_i$ transients. To this end, single chromaffin cells were sequentially exposed to brief K^+ pulses, in the absence and presence of 200 nM PMA. The first K^+ pulse (leftmost trace in Fig. 2A) elicited a sharp $[Ca^{2+}]_i$ rise, which was followed by a rapid decay towards a plateau and by a subsequent slow decline throughout the pulse. Repeatedly challenging the cell with similar K^+ pulses led to a pronounced, though slow, decline in the maximal amplitude of the $[Ca^{2+}]_i$ transient. This decline appeared to have occurred essentially at the expenses of the disappearance of the rapid decay phase of the transient (Fig. 2A) and could be fit to a single exponential decay function of the form $F = F_0 + F_1 \exp(-t/\lambda)$, where F_0 and F_1 are constants, t is time and λ represents the time constant of the decay process. (The best fit of this function to the peak $[Ca^{2+}]_i$ values, obtained for successive K^+ pulses, is depicted in Fig. 2A as a dashed line. The λ value obtained from this fit and the average λ computed from similar experiments were 534 and 494 ± 56 s, respectively, ±S.D., $n = 4$ different cells.) Fig. 2A also shows that PMA caused a progressive inhibition of the K^+-evoked $[Ca^{2+}]_i$ transients recorded from single chromaffin cells, as assessed by comparing the maximal size of these transients to the extrapolated decay function.

The analysis of several single cell experiments, similar to that illustrated in Fig. 2A, is depicted in Fig. 2B. Here the PMA-mediated inhibition was expressed as $I = 100\ [F_{ext} - F_{obs}]/F_{ext}$, where F_{obs} is the observed $[Ca^{2+}]_i$ peak for any given K^+ pulse delivered in the presence of PMA and F_{ext} is the extrapolated

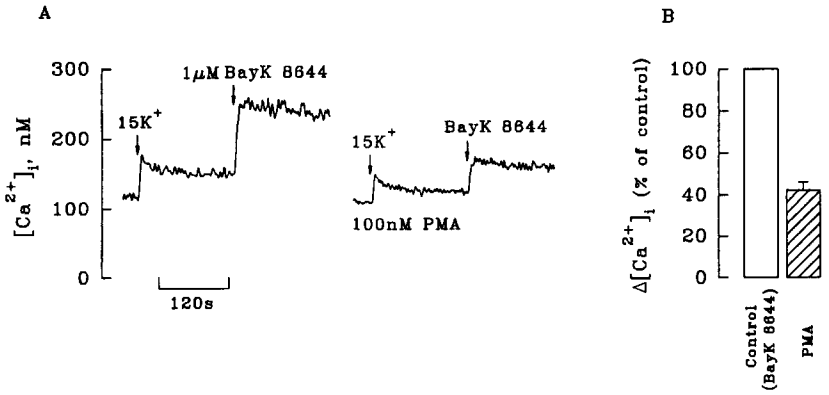

Fig. 3. Inhibition of the Bay K 8644-evoked $[Ca^{2+}]_i$ transients by PMA. (A) Chromaffin cell suspensions were stimulated with 15 mM KCl ($15K^+$) and 1 μM Bay K 8644 as depicted by the arrows, in the absence (left trace) and presence of 100 nM PMA (10 min). (B) Average effects of 10 min incubations with 100 nM PMA on the Bay K 8644-evoked $[Ca^{2+}]_i$ transients. The columns labelled 'control' and 'PMA' represent the normalized average peak $[Ca^{2+}]_i$ changes recorded in the absence and presence of PMA, respectively (15 mM K^+ present).

value of the above decay function for the corresponding pulse. Fig. 2B shows that, for most cells, the PMA-mediated inhibition was near maximal after 10 min incubations with the phorbol ester. Furthermore, Fig. 2B highlights the marked variability of the single cell responses to PMA, with some cells exhibiting relatively small inhibitory effects (less than 25% inhibition at 10 min incubation) and others displaying much larger inhibitions, sometimes amounting to 65% of the control extrapolated response. The phorbol ester vehicle used in the experiments (DMSO) had no detectable effect on the K^+-evoked $[Ca^{2+}]_i$ transients (data not shown).

We have assessed the possibility that exposure to the phorbol ester might lead to inhibition of L-type (dihydropyridine-sensitive) Ca^{2+} channels. To this end, we have examined the effect of 100 nM PMA on the $[Ca^{2+}]_i$ rises brought about by 1 μM Bay K 8644, a specific L-type Ca^{2+} channel activator. In order to enhance the agonistic effect of Bay K 8644 the cells were moderately depolarized by exposure to 15 mM KCl, prior to and throughout application of the dihydropyridine.

Fig. 3A shows that addition of Bay K 8644 to the cells in the presence of 15 mM KCl evoked a rapid $[Ca^{2+}]_i$ rise of approximately 100 nM (average 103 ± 9 nM, $n = 4$ experiments in duplicate). It is also apparent that a 10 min preincubation with PMA reduced the Bay K 8644-evoked $[Ca^{2+}]_i$ rise by approximately 60%. Fig. 3B shows that the average PMA-mediated inhibition of the Bay K 8644 effect was 59 ± 4%.

The effects of 10 min PMA incubations on K^+-evoked adrenaline and noradrenaline release are depicted in Fig. 4. PMA significantly inhibited catecholamine release in the concentration range 0.1–10 μM, the average inhibitions being approximately 23% of the control response at 0.1 μM, 27% at 1 μM and 40% at 10 μM. In contrast, 4αPDD (10 μM) had no effect

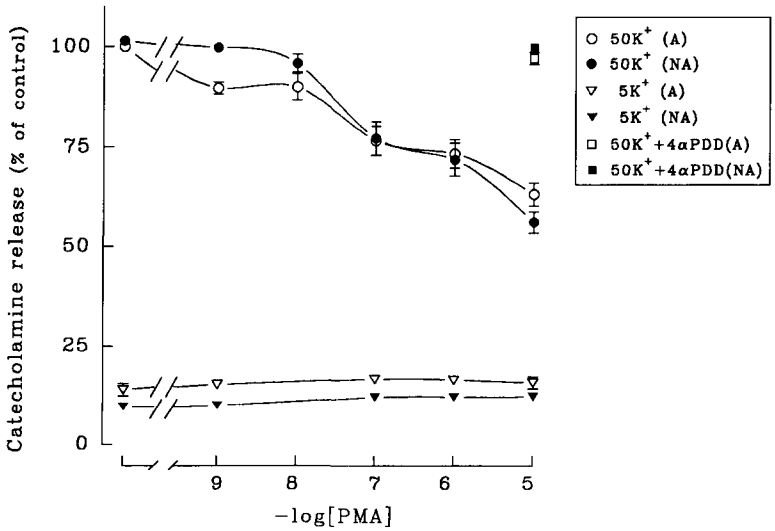

Fig. 4. Dose-dependent inhibition of depolarization-evoked catecholamine release by PMA. K^+-evoked adrenaline (empty circles) and noradrenaline (filled circles) secretion as a function of PMA concentration. Chromaffin cells were exposed to various PMA concentrations for 10 min and stimulated with 50 mM KCl for 5 min, after which the catecholamines released into the medium were assayed by HPLC, as explained in section 2. The secretion is expressed as% of that obtained with KCl in the absence of the drugs. The leftmost points in the plots denote the catecholamine secretion obtained in the absence of PMA (K^+-evoked adrenaline and noradrenaline secretion: 9.1 ± 1.5 and 14.4 ± 2.0% of total cellular content, respectively, $n = 18$ experiments in triplicate). The empty and filled inverted triangles denote basal catecholamine secretion. The empty and full squares denote K^+-evoked adrenaline and noradrenaline secretion, respectively, obtained in the presence of 10 μM 4αPDD.

on K^+-evoked catecholamine secretion. PMA exherted a slight stimulatory effect on basal catecholamine release.

4. Discussion

We have shown that the phorbol ester PMA inhibited depolarization-evoked $[Ca^{2+}]_i$ transients in adrenal chromaffin cells, at concentrations (100–200 nM) known to activate specifically protein kinase C in chromaffin cells and in other cell types. Since the K^+-evoked $[Ca^{2+}]_i$ transients reflect a complex interplay between depolarization-induced Ca^{2+} influx and intracellular Ca^{2+} buffering, the inhibitions invoked by acute PMA exposures can conceivably be caused by inhibition of voltage-sensitive Ca^{2+} channels, by stimulation of Ca^{2+} sequestering and/or extrusion mechanisms, or by both processes. In favour of the former hypothesis, we have shown that PMA inhibited depolarization-evoked Ca^{2+} influx, as assessed by Mn^{2+} quenching of fura-2 fluorescence. Furthermore, we have recently observed that 100 nM PMA causes marginal (less than 10%) inhibitions of the $[Ca^{2+}]_i$ transients brought about by the Ca^{2+} ionophore ionomycin (C.M. Sena and L.M. Rosário, unpublished observations). This stands in contrast to the pronounced inhibitions (40–65%) caused by the phorbol ester on the K^+-evoked $[Ca^{2+}]_i$ transients. Thus, our study indicates that PMA reduced the latter transients in chromaffin cells primarily by inhibiting voltage-sensitive Ca^{2+} channels.

Bovine chromaffin cells express multiple voltage-sensitive Ca^{2+} channels, i.e. the dihydropyridine-sensitive L-type channel, the ω-conotoxin GVIA-sensitive N-type channel and the FTX- and ω-agatoxin IVa-sensitive P-type channel [15,16,21]. The Ca^{2+} currents supported by these channels vary markedly from cell to cell [22], suggesting a highly heterogenous distribution of Ca^{2+} channels among chromaffin cells. While our data suggest that the L-type Ca^{2+} channel is directly or indirectly regulated by the phosphorylation processes triggered by the phorbol ester (the Bay K 8644-evoked $[Ca^{2+}]_i$ transients were strongly inhibited by 100 nM PMA), we cannot rule out the possibility that other channel subtypes might be involved in the PMA effect. Nevertheless, the fact that the extent of the PMA-mediated inhibition of Ca^{2+} influx varies markedly from cell to cell suggests that phosphorylation-sensitive channels are heterogenously distributed among chromaffin cells.

We have shown that PMA inhibits K^+-evoked catecholamine secretion. Although high K^+ depolarization is not the closest mimick of physiological depolarization, K^+-evoked catecholamine secretion is generally considered to reflect mainly the activation of voltage-sensitive Ca^{2+} channels [1]. Other groups have previously shown that phorbol esters enhance both Ca^{2+}-evoked catecholamine secretion from permeabilized cells and ionomycin-evoked secretion from intact cells [22–25]. The simplest model to account for these differences is that PKC activation has antagonistic consequences on the early and late steps of secretion, with the inhibition overcoming the stimulation of the exocytotic machinery that occurs downstream the activation of Ca^{2+} channels. This model raises the possibility that the antagonistic control of the early and late steps of the secretory process might be an essential feature of selected PKC-coupled receptors of the chromaffin cell.

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