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Role of protein kinase C in cholinergic stimulation of lacrimal gland protein secretion

Driss Zoukhri*, Robin R. Hodges, Deanna M. Dicker, Darlene A. Dartt

Schepens Eye Research Institute and Harvard Medical School, Boston, MA 02114, USA

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Abstract

The purpose of this study was to determine the role of protein kinase C (PKC) isozymes in carbachol-induced protein secretion in the lacrimal gland. Three isoforms of PKC are present in rat lacrimal gland: PKC-α, -δ and -ε. Carbachol translocated PKC-ε during 5 s incubation. Pretreatment with PdBu for 0 to 4 h down-regulated PKC-α by 31% at 20 min, PKC-ε by 36% at 2 h, and PKC-δ by 37% at 4 h. A 2 h phorbol ester treatment inhibited carbachol-induced secretion completely at 1 min and partially at 5, and 20 min, but did not alter the carbachol-induced increase in the intracellular [Ca^{2+}]. We conclude that PKC-α and -ε, but not PKC-δ, are implicated in cholinergic agonist-induced protein secretion in rat lacrimal gland.

Key words: Carbachol; Exocrine gland; Protein kinase C; Phorbol ester; Signal transduction; Exocytosis

1. Introduction

In most secretory tissues studied, cholinergic agonists act by producing 1,4,5-inositol trisphosphate (IP_3) and diacylglycerol from phosphatidylinositol bisphosphate [1]. IP_3 subsequently causes the release of intracellular Ca^{2+} (Ca^{2+}_i) to stimulate secretion, whereas diacylglycerol activates PKC to stimulate secretion [1,2]. In the lacrimal gland, cholinergic agonists are effective stimuli of protein secretion, Ca^{2+}-dependent ion channels, and electrolyte and water secretion [3–5]. In lacrimal gland acini, cholinergic agonists cause a rapid production of IP_3, which mobilizes Ca^{2+}_i, causing its rapid increase [6]. Then IP_3, perhaps with inositol 1,3,4,5-tetrakisphosphate, causes an influx of extracellular Ca^{2+} (Ca^{2+}_o), which sustains a plateau of elevated [Ca^{2+}] . To determine the role of Ca^{2+}, Quin 2 was used to prevent the cholinergic agonist-stimulated increase in Ca^{2+}_i. Chelation of Ca^{2+} completely inhibited the rapid, transient phase of protein secretion and partially (about 60%) inhibited the slower, sustained phase [7]. The rapid phase of cholinergic-stimulated protein secretion appears to be dependent upon Ca^{2+}_i, and the slower phase partially dependent upon influx of Ca^{2+}_o. Sustained protein secretion appears to be dependent upon a different, second mediator. PKC seems a likely candidate for the second mediator because the PKC activator diacylglycerol is produced concomitantly with IP_3.

PKC was originally described as a Ca^{2+} and phospholipid-dependent kinase activated by diacylglycerol produced by the receptor-mediated breakdown of phosphoinositides [8]. Molecular cloning and biochemical techniques have shown that PKC is a family of closely related enzymes consisting of at least eleven different isozymes. The PKC family has been divided into three categories [9]. A first group termed classical PKCs including PKC- α, -βI, -βII and -γ isozymes have a Ca^{2+} and phospholipid-dependent kinase and phorbol ester binding activities. A second group termed new PKCs including PKC-ε, -δ, -θ, η, and μ [10] isoforms are Ca^{2+}-independent and phospholipid-stimulated kinases. And a third group termed atypical PKCs including PKC-ζ, and -λ isoforms are Ca^{2+} and phospholipid-independent kinases.

The rat lacrimal gland contains three isoforms of PKC: PKC-α, -δ, and -ε [11,12]. There is evidence suggesting that PKC might be involved in the mechanisms regulating protein secretion in the lacrimal gland. First, the 4β isomer, but not the inactive 4α isomer, of phorbol 12,13-dibutyrate (PdBu) and phorbol 12-myristate 13-acetate (PMA), stimulate protein secretion in a time- and concentration-dependent manner [13,14]. Second, the diacylglycerol analog 1-oleoyl 2-acetyl-*sn*-glycerol also stimulates protein secretion [14]. Third, cholinergic agonists cause a transient, although not significant, decrease in cytosolic PKC activity [7]. A direct role, however, for PKC or its individual isozymes in cholinergic activation of lacrimal gland cells has not yet been demonstrated. Furthermore, a recent study [12] suggested that PKC might not be the sole effector of phorbol esters in inducing protein secretion because three PKC inhibitors did not block phorbol ester-induced protein secretion.

In the present study, we used phorbol ester treatment to down-regulate PKC isozymes to determine the relative role of PKC isozymes in mediating protein secretion in response to cholinergic agonists. Our results show that PKC-α is the most sensitive to down-regulation and that

*Corresponding author: Schepens Eye Research Institute, 20 Staniford St., Boston, MA 02114, USA. Fax: (1) (617) 720 1069.

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this isoform along with PKC-ε, but not PKC-δ, seems to play a major role in cholinergic-induced protein secretion in the lacrimal gland.

2. Materials and methods

2.1. Materials

PKC-ε specific polyclonal antibody was a gift from Dr. Peter Parker (Ludwig Institute for Cancer Research, London). Upon commercial availability, polyclonal antibodies to PKC-α, -δ, and -ε were obtained from Calbiochem (San Diego, CA) and Gibco BRL (Grand Island, NY). Fura-2 tetra-acetoxymethyl esters and Pluronic F127 were obtained from Molecular Probes (Eugene, OR); PdBu and PMA from LC Services (Waltham, MA); phosphatidylserine from Avanti Polar Lipids (Pelham, AL); [γ-^{32}P]ATP from ICN Radiochemicals (Irvine, CA); CLSPA collagenase from Worthington Biochemical (Freehold, NJ); Protoblot (Western blot AP system) from Promega (Madison, WI); and Protein Kinase C Enzyme Assay System from Amersham (Arlington Heights, IL).

2.2. Methods

2.2.1. Preparation of lacrimal gland acini and down-regulation of PKC. Both exorbital lacrimal glands were removed from male Wistar rats that had been anesthetized with CO_2 for 1 min and then decapitated. Dispersed acini were isolated by collagenase digestion as described previously [4].

The isolated acini were preincubated with or without PdBu (1 μM) for 0, 20 min, 2 h, and 4 h at 37°C. To determine if PdBu treatment down-regulated PKC, total PKC activity was measured as described in section 2.2.4. in acini preincubated with or without PdBu for 2 h. In addition, peroxidase secretion was measured in acini preincubated with or without PdBu for 2 h. Acini were centrifuged at 50 × g for 30 s and rechallenged with PdBu (1 μM) for 0 or 20 min. Peroxidase secretion was measured as described in section 2.2.2.

To determine loss of specific isozymes with time, acini preincubated with PdBu for 0–4 h were centrifuged at 50 × g for 30 s and homogenized in buffer containing 30 mM Tris-HCl (pH 7.5), 10 mM EGTA, 5 mM EDTA, 1 mM dithiothreitol, and 250 mM sucrose. Amount of each isozyme was determined by electrophoresis and immunoblotting as described below.

2.2.2. Measurement of [Ca^{2+}]$_i$ and peroxidase secretion. Acini were incubated in Krebs–Ringer buffer (KRB) containing 1% bovine serum albumin, 0.5 μM fura-2 tetra-acetoxymethyl ester, 10% Pluronic F127, and 50 μM probenicid for 60 min at 22°C. The cells were then washed, and fluorescence was measured at 22°C. Fluorescence was measured at excitation wavelengths of 340 and 380 nm as previously described [7].

To measure peroxidase secretion, acini were incubated for 0 to 20 min at a final volume of 1 ml in KRB buffer containing agonists. To terminate incubation, acini were centrifuged. Peroxidase secretion was measured in duplicate in the supernatant, using a modification of the method of Herzog and Fahimi [15]. The total amount of cellular protein in each tube was measured using the method of Bradford.

2.2.3. Protein kinase C assay. Acini preincubated with or without PdBu were centrifuged at 50 × g for 30 s. The pellet was homogenized at 0°C with a Dounce glass homogenizer in buffer containing 20 mM Tris-HCl (pH 7.5), 2 mM dithiothreitol, 1 μg/ml phosphoramidone, 0.01% leupeptin, 0.28 μM phenylmethylsulfonyl fluoride, 5 mM EDTA, and 10 mM EGTA. The homogenate was stored in 50% glycerol overnight at −20°C. PKC activity was assayed using the Protein Kinase C Enzyme Assay System and by determining ^{32}P incorporation into a synthetic octapeptide NH_2-Arg-Lys-Arg-Thr-Leu-Arg-Arg-Leu, which is phosphorylated by all PKC isozymes. Phosphorylation, initiated by the addition of a 25 μl sample, was carried out for 15 min at 25°C and stopped by the addition of ice-cold 10% TCA. PKC activity was calculated as the difference of ^{32}P incorporation in the presence and absence of phosphatidylserine and PMA. Each sample was assayed in duplicate and standardized to the total cellular protein measured by the method of Bradford.

2.2.4. Electrophoresis and immunoblotting. Cytosol and membrane fractions were prepared by centrifugation of homogenate at 100,000 × g for 60 min. Proteins in homogenate, cytosol, and membranes were separated by SDS-PAGE and transferred to immobilon membranes. All values were standardized to the amount of protein measured by the method of Bradford. After blocking non-specific binding sites, the membranes were incubated with antiserum (1:400 to 1:1,000 dilutions) for 30–60 min. Immunoreactive bands were visualized using alkaline phosphatase. Nonspecific binding was determined by omitting the primary antibody or by preincubating with antibody blocking peptides. Rat brain was homogenized and used as a positive control. The specificity of the PKC-ε antibody supplied by Dr. Parker has been published [16]. The amount of each PKC isozyme present was determined by densitometric scanning using a Computing Densitometer (Model 300A, Molecular Dynamics, Sunnyvale, CA) or an Image Analyzer (Biological Detection Systems, Pittsburgh, PA).

3. Results

3.1. Effect of cholinergic agonists on PKC-ε

Upon activation, PKC has been found to translocate from the cytosol to the plasma membrane [17]. Thus, translocation of PKC may serve as an indicator of its activation upon agonist stimulation. To determine if cholinergic agonists activate PKC, we investigated translocation of the Ca^{2+}-independent isozyme PKC-ε. Acini were incubated with 1 mM carbachol, and the amount of PKC-ε in cytosol and membrane fractions was measured by immunoblotting. The basal PKC-ε amount in control acini cytosol was 30.75 ± 16.87 units/μg protein (n = 4) (Fig. 1) and in control acini membrane was 14.75 ± 4.90 units/μg protein (Fig. 1). A 5 s carbachol stimulation significantly decreased the cytosolic PKC-ε to 14.28 ± 6.74 units/μg protein (n = 3; $P < 0.05$) and increased membrane-bound PKC-ε to 25.82 ± 8.25 units/μg protein ($P < 0.01$) (Fig. 1). These results suggest

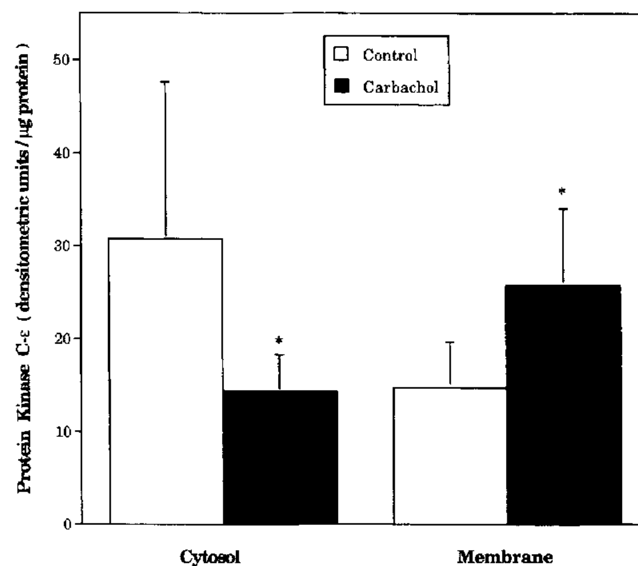

Fig. 1. Effect of carbachol on the intracellular distribution of PKC-ε. The amount of PKC-ε in cytosol and membrane fractions of lacrimal gland acini before (open bars) and after (solid bars) stimulation with 10^{-3} M carbachol for 5 s was measured by immunoblotting as described in section 2. Values are means ± S.E. (n = 3–4). *Significant difference from no added carbachol.

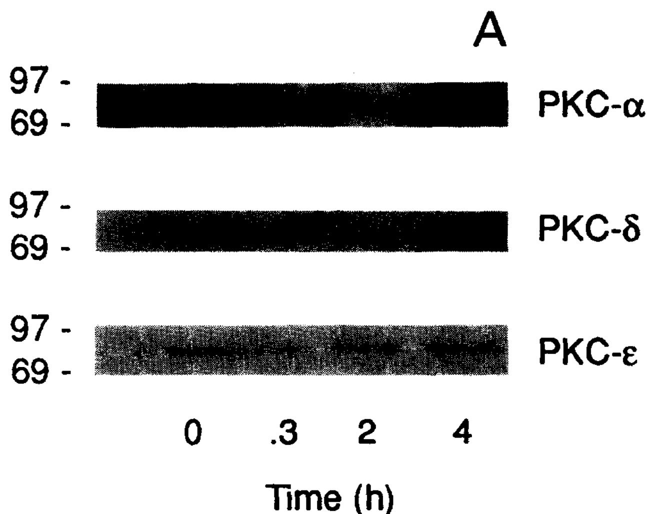

Fig. 2. Effect of PdBu treatment on protein kinase C isozymes. (A) Immunoblot of PKC-α, -δ, and -ε in lacrimal gland acini that had been untreated (0 h) or treated with PdBu for 20 min (0.3 h), 2 or 4 h. Acini were homogenized, polypeptides separated, and PKC isozymes detected as described in section 2.

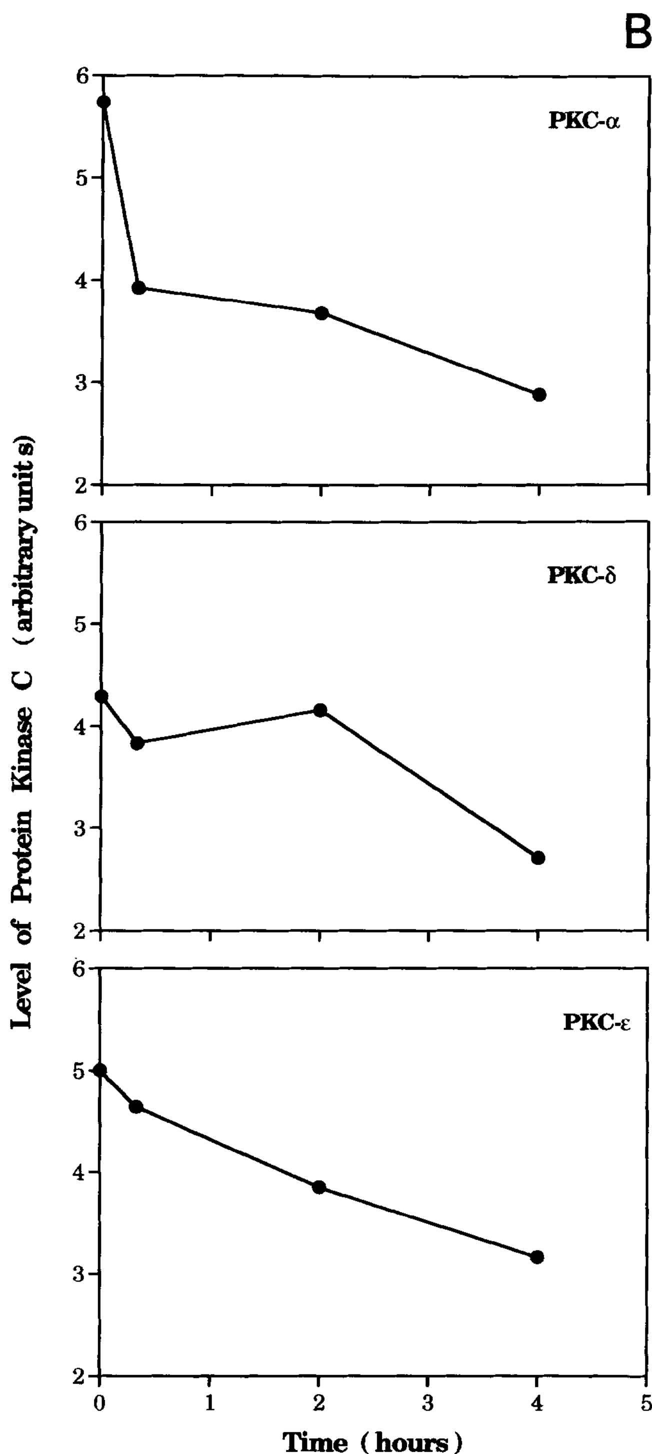

Fig. 2 (B) Data from 6 separate experiments as those shown in (A) are represented.

that at least one isoform of PKC present in the lacrimal gland, PKC-ε, might be activated by cholinergic agonists.

3.2. Protein kinase C activity in PdBu-treated acini

Long-term treatment of cells with phorbol esters results in a loss of PKC activity. This is known as down-regulation of PKC and has been used to determine the role of this enzyme in different biological processes [18]. Acini were treated for 2 h with PdBu at a concentration that caused maximum protein secretion (1 μM) . Total PKC activity was determined in PdBu-treated and untreated acini. In PBDu-treated acini, the total PKC activity in homogenate decreased by 80% to 59.7 pmol ^{32}P/min/mg protein from 294.8 pmol ^{32}P/min/mg protein in untreated acini (Table 1). A second method to determine the efficiency of PKC down-regulation was to measure peroxidase secretion in PdBu-treated and untreated acini. In untreated acini, a 20 min incubation with PdBu (1 μM) stimulated secretion to 12.85 ± 3.04 units/mg protein (Table 1). In PdBu-treated acini, peroxidase secretion was significantly inhibited by 82% to 2.38 ± 2.20 units (Table 1). A 3 h treatment with PdBu did not have any further effect (data not shown). These results demonstrate that lacrimal gland PKC can be effectively down-regulated by phorbol ester treatment.

Table 1
Effect of 2 h PdBu (1 μM) treatment on PKC activity and on 1 μM PdBu-induced peroxidase secretion

	PKC activity (pmol ^{32}P/min/mg protein)	Peroxidase secretion (units/min/mg protein)
Control	294.8 (2)	12.85 ± 3.04 (4)
Treated	59.7 (2)	2.38 ± 2.20 (4)*

Values are means ± S.E.; (n), no. of experiments.
*$P < 0.05$.

3.3. Effect of PdBu treatment on carbachol-induced peroxidase secretion

To determine the role of PKC in cholinergic agonist-

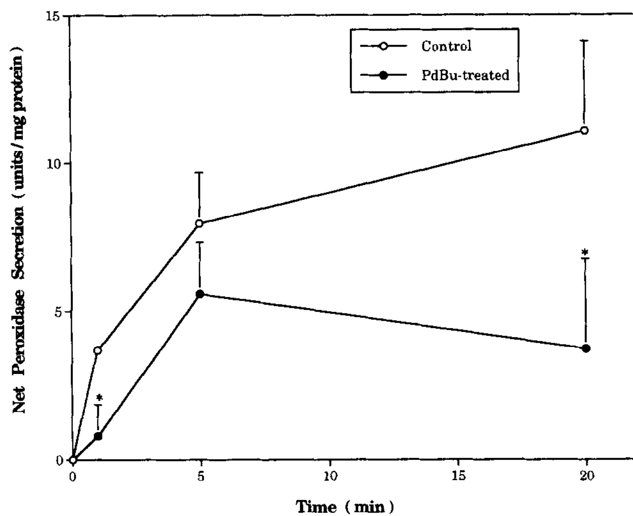

Fig. 3. Effect of PdBu treatment on carbachol-induced peroxidase secretion. Lacrimal gland acini were pretreated for 2 h with 1 μM PdBu, then incubated with 10^{-5} M carbachol. Peroxidase secretion was measured as described in section 2. open circles, control, filled circles, PdBu treated. values are means ± S.E. (n = 4). *Significant difference from control.

stimulated peroxidase secretion, acini with and without a 2 h PdBu treatment were stimulated for 0–20 min with carbachol at a concentration that was maximal for peroxidase secretion (10^{-5} M) . In untreated cells, carbachol caused a rapid increase in peroxidase secretion from 0 to 1 min and a slower, sustained secretion at 5 and 20 min (Fig. 3). A 2 h PdBu treatment significantly ($P < 0.05$) inhibited carbachol-induced secretion at 1 min by 79% ± 29%, by 35% ± 13% at 5 min, but the effect was not significant; and significantly ($P < 0.05$) inhibited secretion by 66% ± 20% at 20 min. Basal secretion in PdBu-treated acini was not significantly different from basal secretion in untreated acini at any time (data not shown).

These results show that PKC plays a major role in the carbachol-induced lacrimal gland protein secretion.

3.4. Effect of PdBu treatment on PKC isozymes

Three PKC isoforms are present in rat lacrimal gland: PKC-α, -δ, and -ε ([12] and Fig. 2A). Each isozyme was present as a single polypeptide with the same molecular weight in both lacrimal gland acini and brain, except that in one experiment PKC-δ was present as a doublet. In order to determine whether all three isoforms are equally down-regulated by PdBu treatment, lacrimal gland acini were treated with 1 μM PdBu for 0, 20 min, 2 h, and 4 h and the amount of each PKC isoform was quantified by immunoblotting.

In cells treated with PdBu for 0 min, all three PKC isozymes were present. With a 20 min PdBu treatment, PKC-α decreased by 31%, but PKC-δ and -ε decreased by only 11% and 7%, respectively (Fig. 2B). With a 2 h PdBu treatment PKC-α decreased by 36%, PKC-ε decreased by 23%, but PKC-δ decreased by only 3%. With a 4 h PdBu treatment PKC-α, -δ, and -ε decreased by 50%, 37% and 37%, respectively. These results show that PKC-α is the most sensitive to down regulation while PKC-δ is the least sensitive. PKC-ε has an intermediate sensitivity. Moreover, taken together with the results in section 3.3., they suggest that PKC-α and -ε, but not PKC-δ, are implicated in the secretory process.

3.5. Effect of PdBu treatment on $[Ca^{2+}]_i$

In the lacrimal gland acini, carbachol induce an increase in $[Ca^{2+}]_i$ [6]. To determine if the 2 h PdBu treatment decreased cholinergic agonist-stimulated secretion by altering $[Ca^{2+}]_i$, the carbachol-induced increase in $[Ca^{2+}]_i$ was measured in untreated and PdBu-treated acini. Basal $[Ca^{2+}]_i$ in untreated cells was 98.4 ± 19.9 nM (n = 4) and in PdBu-treated cells was 78.0 ± 13.1 nM; the difference is not significant. In untreated cells, a concentration of carbachol (10^{-3} M) that was maximal for increasing Ca^{2+} caused a rapid increase in the $[Ca^{2+}]_i$, which decreased to a plateau value maintained for 16 min (n = 4) (Fig. 4). In PdBu-treated acini, the carbachol-induced Ca^{2+} response was not significantly different from untreated cells at any time measured.

These results rule out the possibility that phorbol ester treatment of lacrimal gland acini alters the cholinergic-induced increase in $[Ca^{2+}]_i$, and demonstrate that inhibition of carbachol-induced secretion is solely due to down-regulation of PKC by PdBu treatment.

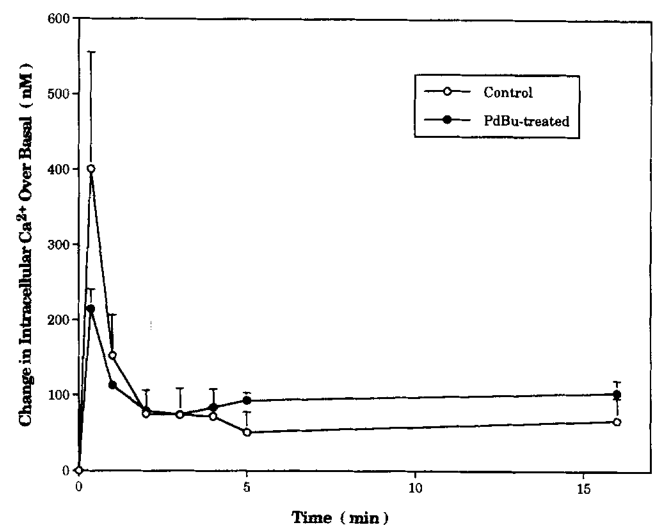

Fig. 4. Effect of PdBu treatment on intracellular $[Ca^{2+}]$. Lacrimal gland acini were pretreated for 2 h with 1 μM PdBu, then incubated with 10^{-3} M carbachol. $[Ca^{2+}]$ was measured as described in section 2. Values are means ± S.E. (n = 4). Data are presented as change in $[Ca^{2+}]_i$ over basal value. No significant difference was seen between PdBu treatment (filled circles) and no treatment (open circles).

4. Discussion

Rat lacrimal gland protein secretion is mainly under the control of cholinergic muscarinic receptors [3,4]. Activation of these receptors leads to the production of two second messengers through the hydrolysis of phosphatidylinositol 4,5-bisphosphate by a phospholipase C [6,19]: IP_3 and DAG. To cause secretion, IP_3 liberates Ca^{2+} from intracellular stores and DAG activates PKC [1,2]. The role of Ca^{2+} in the control of protein secretion in the lacrimal gland has been well documented [20–22]. PKC may play a role in this process as well. Mauduit et al. [13] showed that PMA stimulates newly synthesized protein secretion in the lacrimal gland. Dartt et al. [14] showed that PdBu and OAG, a DAG analog, stimulate peroxidase secretion. Zoukhri et al. [12] showed that both PMA and PdBu stimulate newly synthesized protein secretion in the lacrimal gland. In contrast, three DAG analogs did not stimulate newly synthesized protein secretion and several inhibitors of PKC could not block the phorbol ester-induced secretion. Zoukhri et al. concluded from that study that PKC might not be the only effector of phorbol ester-induced protein secretion in the lacrimal gland [12].

The discrepancy between these studies might be due to the fact that wo different markers of protein secretion were used. In the Dartt et al. study, peroxidase secretion, i.e. one single protein as a marker of protein secretion was used. In the Zoukhri et al. report, tritiated protein secretion, i.e. newly synthesized proteins was employed. It is likely that the mechanisms involved in the control of peroxidase secretion versus newly synthesized protein secretion are different since newly formed granules are preferentially liberated upon stimulation.

Concerning the role of PKC in receptor-mediated protein secretion in the lacrimal gland, Dartt et al. showed that cholinergic agonists cause a transient, although not significant, decrease in cytosolic PKC activity [7]. This suggested that PKC might be involved in cholinergic-induced peroxidase secretion. In the present study, we show that cholinergic agonists induce a rapid (5 s) translocation of PKC-ε from the cytosol into the membrane fraction (Fig. 1). Translocation of PKC has been described in a variety of tissues and cell lines and has been considered as a primary step to PKC activation [2].

Long-term treatment of cells with phorbol esters has been shown to partially, or completely deplete cellular PKC [23]. This phenomenon is known as desensitization or down-regulation of PKC and has been widely used to study the role of PKC in the control of different processes [18]. In the present study, we used PdBu to down-regulate PKC. We found that a 2 h treatment with phorbol esters resulted in an 80% decrease in PKC activity (Table 1). Moreover, the decrease in PKC activity in PdBu-treated acini correlated well with the decrease in PdBu-induced peroxidase secretion in these cells (Table 1). This decreased secretion might be due to depletion of peroxidase from acini with the 2 h PdBu treatment. This is unlikely because phenylephrine, an α_1-adrenergic agonist, still causes peroxidase secretion in PdBu treated acini [24]. Moreover, PdBu treatment increased phenylephrine-induced peroxidase secretion [24]. These results strongly suggest that PKC activity is effectively down-regulated by phorbol ester treatment and are in agreement with Tan and Marty [25] who reported that a 2–4 h treatment of lacrimal gland acinar cells with PMA down-regulated PKC.

We then used a 2 h PdBu treatment to study the role of PKC in cholinergic-induced peroxidase secretion. We found that such a treatment results in a decrease of carbachol-induced protein secretion measured at three time points (79% decrease at 1 min, 35% at 5 min, and 66% at 20 min). These results suggest that PKC appears to play a major role in cholinergic-induced protein secretion.

Activation of lacrimal gland cholinergic receptors leads to an increase in cytosolic [Ca^{2+}] [6]. In order to verify that phorbol ester treatment did not affect the increase in [Ca^{2+}] induced by cholinergic agonists, we compared the effect of carbachol on [Ca^{2+}] in both untreated and PdBu-treated acini. Our results show that the response to carbachol in control acini was indistinguishable from that in treated acini (Fig. 4). We can then conclude that the decrease in carbachol-induced secretion in phorbol ester-treated acini is not due to altered response in [Ca^{2+}] but only to down-regulation of PKC.

As there are three isoforms of PKC present in the lacrimal gland: PKC-α, -δ, and -ε ([12] and Fig. 2A), we conducted experiments to determine if all three isoforms were equally sensitive to down-regulation. Using specific antibodies against these isoforms, we found that PKC-α is very sensitive to proteolysis as 31% of the protein was lost 20 min after application of PdBu. At the same time-point, PKC-δ and -ε were only slightly decreased (11 and 7%, respectively) (Fig. 2B). A 2 h PdBu treatment, a time point that resulted in effective decrease of PKC activity (80%) and peroxidase secretion (82%), PKC-α was down by 36%, PKC-ε by 23%, whereas PKC-δ was decreased by only 3% (Fig. 2B). These results suggest that PKC-α and -ε, but not PKC-δ, are implicated in the cholinergic-induced peroxidase secretion in the lacrimal gland.

We conclude that PKC-α , -δ, and -ε present in the lacrimal gland are unequally down-regulated by phorbol esters with PKC-α being the most sensitive and PKC-δ being the least sensitive, and that PKC-α and -ε, but not -δ, play a role in cholinergic-induced protein secretion.

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