J. Membrane Biol. 157, 169–175 (1997)

The Journal of
Membrane
Biology

Immunolocalization of Lacrimal Gland PKC Isoforms. Effect of Phorbol Esters and Cholinergic Agonists on Their Cellular Distribution

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Received: 25 June 1996/Revised: 24 December 1996

Abstract. In previous studies, we showed that lacrimal gland acini express three isoforms of protein kinase C (PKC): PKCα,-δ, and -ϵ. In the present study, we report the identification of two other PKC isoforms, namely PKCμ and -ι/λ. Using immunofluorescence techniques, we showed that these isoforms are differentially located. PKCα and -μ showed the most prominent membrane localization, whereas PKCδ, -ϵ and -ι/λ were mainly cytosolic. Using cell fractionation and western blotting techniques, we showed that the phorbol ester, phorbol 12, 13-dibutyrate (PdBu, 10^{-6} M), translocated all PKC isoforms, except PKCι/λ, from the soluble fraction into the particulate fraction. The effect was maximum at 5 min and persisted at 10 min. PKCϵ was the most responsive to PdBu reaching almost maximal translocation at a PdBu concentration as low as 10^{-9} M. The cholinergic agonist, carbachol (10^{-5} and 10^{-3} M), induced translocation which was transient for PKCδ, and -μ, but persisted for 10 min for PKCϵ. Carbachol did not translocate PKCα and, like PdBu, did not translocate PKCι/λ. We concluded that lacrimal gland PKC isoforms are differentially localized and that they translocate differentially in response to phorbol esters and cholinergic agonists.

Key words: Exocrine gland — Muscarinic — α_1-adrenergic — Translocation — Phorbol esters

Introduction

Lacrimal gland protein secretion is mainly under the control of cholinergic muscarinic receptors (Dartt, 1989; Herman et al., 1978). The lacrimal gland muscarinic receptors, of the M_3 suptype, are coupled to the hydrolysis of phosphatidylinositol 4,5-bisphosphate by phospholipase C (Mauduit, Jammes & Rossignol, 1993), generating two second messenger molecules, inositol 1,4,5-triphosphate (IP_3) and diacyglycerol (DAG) (Berridge, 1987). IP_3 interacts with specific receptors present on the endoplasmic reticulum to liberate Ca^{2+} into the cytosol (Berridge, 1987). The free Ca^{2+} in conjunction with calmodulin will then activate Ca^{2+} and calmodulin-dependent protein kinases. DAG interacts with and activates protein kinase C (PKC) (Newton, 1995; Niedel & Blackshear, 1986; Nishizuka, 1986). Activation of these kinases leads to the phosphorylation of specific substrates thought to trigger lacrimal gland protein secretion.

PKC was originally described as a Ca^{2+} and phospholipid-dependent protein kinase activated by diacylglycerol produced by the receptor-mediated breakdown of phosphoinositides (Kishimoto et al., 1980). 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 (Newton, 1995; Nishizuka, 1995). A first group, termed classical or conventional PKCs (cPKC) including PKCα, -βI, βII and -γ isoforms, have a Ca^{2+} and DAG-dependent kinase and phorbol-ester-binding activities. A second group, termed new or novel PKCs (nPKC) including PKCϵ, -δ, -θ, -η, and -μ (the human homologue of the mouse protein kinase D) (Johannes et al., 1994) isoforms, are Ca^{2+}-independent and DAG-stimulated kinases. A third group termed atypical PKCs (aPKC) including PKCζ, and -ι/λ isoforms, are Ca^{2+} and DAG-independent kinases.

Structurally, PKC has been divided into two domains: a regulatory domain in the amino terminal half and a catalytic domain in the carboxyl terminal half. The regulatory domain of cPKC contains two conserved regions C1 and C2. The C1 region contains two tandem repeats of cysteine-rich motif responsible for DAG and phorbol-ester-binding (Ono et al., 1989). The aPKC isoforms, unlike cPKC and nPKC isoforms, contain only

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one cysteine-rich motif and hence do not bind DAG/phorbol esters. The C2 region, called CalB, interacts with phospholipids in a Ca^{2+}-dependent manner and is implicated in translocation to membranes of cPKC isoforms (Luo et al., 1993). The nPKC and aPKC isoforms lack the C2 region and hence do not need Ca^{2+} for their enzymatic activity. The catalytic domain of PKC contains two additional conserved regions, C3 and C4 responsible for ATP and protein substrate binding, respectively (Newton, 1995).

In previous studies, we showed that lacrimal gland acini express three isoforms of PKC: PKCα, -δ, and -ε (Zoukhri et al., 1994). Using long-term treatment with phorbol esters to downregulate PKC, we showed that PKCα, and -ε, and to a lesser extent PKCδ, play a central role in phorbol-ester- as well as cholinergic-induced protein secretion in the lacrimal gland (Zoukhri et al., 1994). Those findings were recently confirmed using pseudosubstrate-derived inhibitory peptides to selectively inhibit PKC isoforms (Zoukhri et al., 1997).

In the present study, we report the identification of two other PKC isoforms, namely PKCμ and -ι/λ. Using immunofluorescence techniques, we show that PKC isoforms are differentially located in the lacrimal gland. Using western blotting techniques, we show that the phorbol ester, phorbol 12, 13-dibutyrate (PdBu, 10^{-6} M) stimulates the translocation of PKCα, -δ, -ε and -μ, but not PKCι/λ, from the soluble to the particulate fraction. The cholinergic agonist, carbachol (10^{-5} and 10^{-3} M), stimulates the translocation of PKCδ, -ε and -μ, but not PKCα and -ι/λ.

Materials and Methods

Materials

Polyclonal antibodies to PKCα, -δ, -ε, -μ, and -ι/λ and their corresponding control peptides were obtained from Santa Cruz (Santa Cruz, CA); a monoclonal antibody to PKCι/λ was used for western blotting and was from Transduction Laboratories (Lexington, KY). Phorbol esters were from LC Laboratories (Waltham, MA); collagenase CLS III from Worthington Biochemical (Freehold, NJ); enhanced chemiluminescence detection system from Amersham (Arlington Heights, IL) or Pierce (Rockford, IL). All other chemicals were from Sigma (St. Louis, MO).

Immunohistochemistry

All experiments were in accordance with the USDA Animal Welfare Act (1985) and were approved by the Schepens Eye Research Institute Animal Care and Use Committee. Male Wistar rats were anesthetized for 1 min in CO_2, decapitated, and both exorbital lacrimal glands were removed.

Lacrimal glands were fixed in 4% formaldehyde in phosphate buffered saline (PBS, containing in mM: 145 NaCl, 7.3 Na_2HPO_4, and 2.7 NaH_2PO_4 at pH 7.2) for 4 h at 4°C. After cryopreservation overnight in 30% sucrose in PBS, the tissue was frozen in O.C.T. embedding medium. Cryostat sections (6 μm) were placed on gelatin coated slides and air dried for 2 hr. Sections were incubated with the indicated primary antibody diluted in PBS for 1 hr at room temperature. The secondary antibody (1:50), conjugated to FITC, was applied for 1 hr at room temperature. Coverslips were mounted with a medium consisting of glycerol and paraphenylendiamine. Sections were viewed using a Nikon UFXII microscope equipped for epi-illumination. Negative controls included omission of the primary antibody and preabsorption of the primary antibody with the corresponding peptide (10-fold excess and overnight incubation at 4°C) used for immunization.

Preparation of Rat Lacrimal Gland Acini

The lacrimal glands were minced and incubated in Krebs-Ringer bicarbonate buffer (in mM: 119 NaCl, 4.8 KCl, 1 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, and 25 $NaHCO_3$) supplemented with 10 mM Hepes, 5.5 mM glucose (KRB-Hepes), and 0.5% BSA, pH 7.4 containing collagenase (150 U/ml). Lacrimal gland lobules were subjected to gentle pipetting, ten times at regular time intervals, through tips of decreasing diameter. The preparation was then filtered through nylon mesh (150 μm pore size), and the acini pelleted with a 2 min centrifugation at 50 × *g*. The pellet was washed twice by centrifugation (50 × *g*, 2 min) through a 4% BSA solution made in KRB-Hepes buffer. The dispersed acini were allowed to recover for 30 min in 5 ml fresh KRB-Hepes buffer containing 0.5% BSA.

Electrophoresis and Immunoblotting

Lacrimal gland acini were incubated for the indicated period in 1 ml of KRB-Hepes buffer with or without agonists. Reactions were terminated by a brief centrifugation at 500 × *g* and addition of 1 ml ice-cold homogenization buffer (in mM: 30 Tris-HCl, pH 7.5, 10 EGTA, 5 EDTA, 1 dithiothreitol, and 250 sucrose). The cells were then disrupted by sonication. Soluble and particulate fractions were prepared by centrifugation of the homogenate at 100,000 × *g* for 1 hr. Proteins in both fractions were separated by SDS-PAGE (7.5 or 12% acrylamide gels) and transferred to nitrocellulose membranes as previously described (Zoukhri et al., 1994). After blocking nonspecific sites overnight at 4°C in 5% dried milk in buffer containing 10 mM Tris-HCl pH 8.0, 150 mM NaCl, and 0.05% Tween-20 (TBST), the membranes were incubated for 1 hr at room temperature with the primary antibody (1 μg/ml) diluted in TBST. The membranes were washed three times in TBST and incubated with the secondary antibody conjugated to horseradish peroxidase (1:2000 in TBST). Immunoreactive bands were visualized using the enhanced chemiluminescence method according to manufacturer's protocol. The films were digitally scanned using BDS-Image (Biological Detection System, Pittsburgh, PA) and analyzed with National Institutes of Health Image software (version 1.58).

Results

Cellular Location of PKC Isozymes

Using immunofluorescence microscopy, we determined the cellular location of the PKC isoforms present in the lacrimal gland. Using an antibody to PKCα, immunofluorescence was seen on the basolateral and apical membranes of all acini clearly outlining the individual aciniar cells in the acinus (Fig. 1*A*). Immunofluorescence was visible (arrows) near the apical membrane suggesting that PKCα might be associated with the

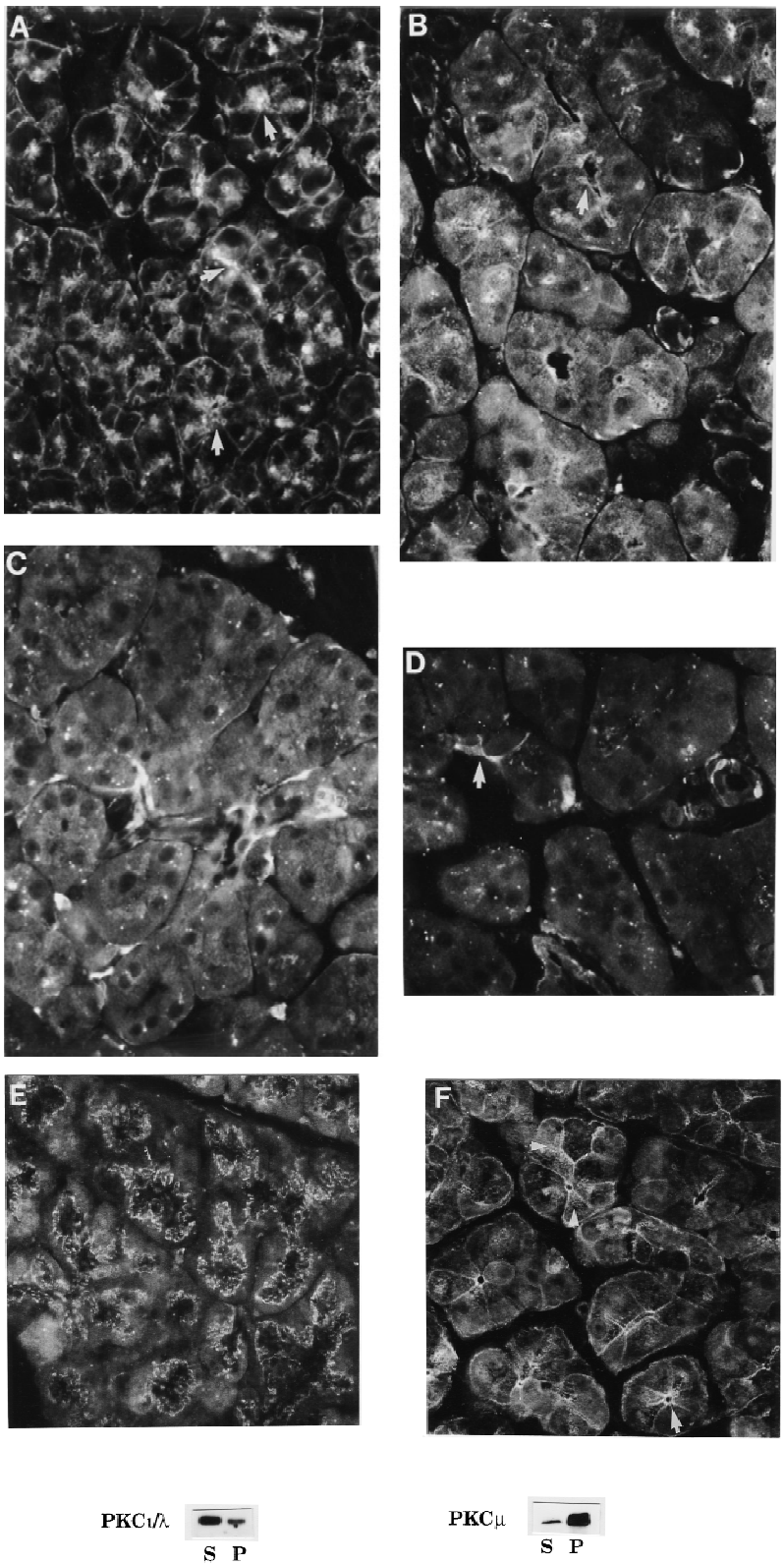

Fig. 1. Immunofluorescence micrographs showing the cellular distribution of: (*A*) PKCα; arrows indicate labeling of apical membranes and possibly secretory granules of lacrimal gland acini (antibody was diluted 1:200 in PBS). (*B*) PKCϵ; arrow indicates labeling of apical membranes and possibly secretory granules and/or trans-golgi network (antibody was diluted 1:100). (*C*) and (*D*) PKCδ; arrow on *D* indicates labeling of a myoepithelial cell (antibody was diluted 1:50). (*E*) PKCι/λ (antibody was diluted 1:400). The blot below shows the amount of PKCι/λ immunoreactivity in the soluble (S) and particulate (P) fractions from unstimulated lacrimal gland acini homogenate. (*F*) PKCμ; arrow indicates labeling of apical membranes and possibly secretory granules, arrowheads indicate an intracellular filamentous network (antibody was diluted 1:400). The blot below shows the amount of PKCμ immunoreactivity in the soluble (S) and particulate (P) fractions from unstimulated lacrimal gland acini homogenate. Similar results were obtained in 2 other experiments. Magnification, ×430.

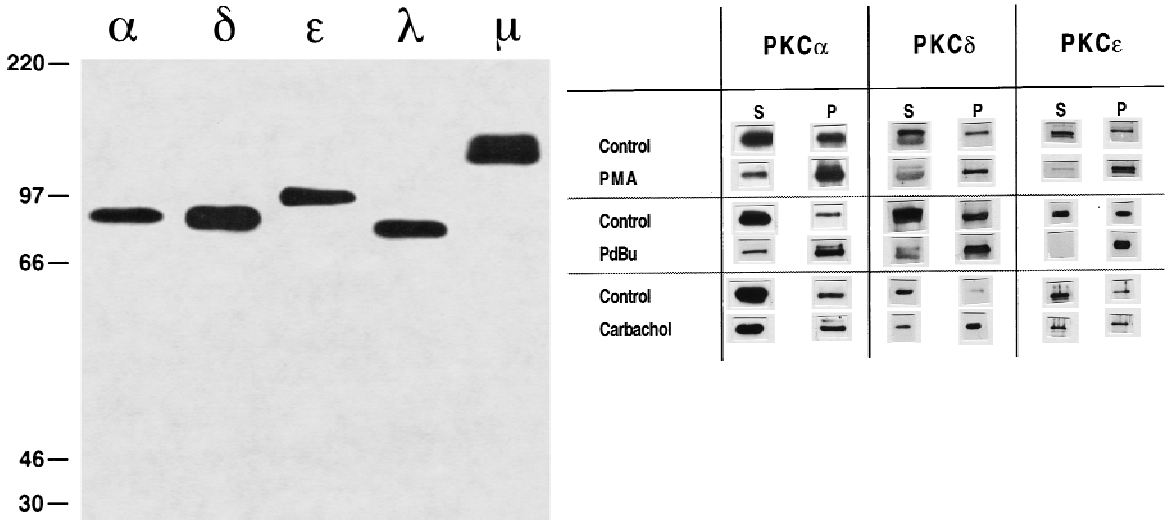

Fig. 2. Effect of phorbol esters and cholinergic agonists on translocation of PKCα, -δ, and -ε.(*A*) Proteins from lacrimal gland acini homogenate were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with the indicated anti-PKC antibodies. The numbers on the left indicate the weight in kiloDaltons and the position of the molecular weight markers. This figure was computer-generated from 5 separate blots. (*B*) Lacrimal gland acini were stimulated with either the phorbol esters, PMA or PdBu (10^{-6} M each for 5 min) or carbachol (10^{-5} M for 1 min). Proteins in the soluble and particulate fractions were separated by SDS-PAGE and transferred to nitrocellulose membranes and probed with the indicated anti-PKC antibodies. Similar results were obtained in at least 2 other experiments.

membranes of the secretory granules. PKCε immunoreactivity was more cytoplasmic than PKCα and binding was seen on the basolateral and apical membranes of acinar cells (Fig. 1*B*). In addition, immunofluorescence was also seen near the apical membrane (arrow, Fig. 1*B*). Double-labeling experiments are needed to identify these structures. PKCε immunoreactivity was also occasionally detected on myoepithelial cells (*data not shown*). PKCδ also had a cytoplasmic distribution (Fig. 1*C*), and binding was also seen on some myoepithelial cells (arrow, Fig 1*D*). The immunofluorescence points on Fig. 1*C* and 1*D* are nonspecific as they were also obtained in the negative controls. The lacrimal gland myoepithelial cells were characterized in our laboratory (Hodges et al., 1997) and by others (Lemullois, Rossignol & Mauduit, 1996) using an antibody against α-smooth muscle actin.

We have identified two additional PKC isoforms present in lacrimal gland: PKCι/λ and PKCμ. PKCι/λ had a cytoplasmic distribution and was present on intracellular membranes, possibly the endoplasmic reticulum or golgi apparatus (Fig. 1*E*). The finding that PKCι/λ is present mostly in cytoplasm was confirmed by Western blotting (Fig. 1*E*). Western blotting showed that 68% of PKCι/λ was in the soluble fraction while 32% of PKCι/λ was detected in the particulate fraction ($n = 5$). PKCμ localization was similar to that of PKCα in that immunofluorescence is clearly membranous, on the basolateral and apical membranes (arrow, Fig. 1*F*), outlining the individual acinar cells in an acinus and an intracellular filamentous network (arrowheads, Fig. 1*F*). The distribution of PKCμ was also confirmed by Western blotting (Fig. 1*F*). Western blotting showed that 25% of PKCμ was in the soluble fraction while 75% of PKCμ was detected in the particulate fraction ($n = 5$). PKCθ and -ζ were not detected by either immunofluorescence or Western blotting techniques (*data not shown*).

The antibodies used for the immunohistochemistry studies were tested by western blotting to show that they recognized only the isoform of PKC they were raised against and did not cross react with the other proteins present in the lacrimal gland (Fig. 2*A*). Other controls for immunohistochemistry included the omission of the primary antibody or the use of preabsorbed antibody using a tenfold excess of the immunizing antigen peptide. In both cases, immunofluorescence was negligible (*data not shown*).

These results show that five isoforms of PKC, three previously identified and two newly identified ones, are present in the lacrimal gland each with a different distribution.

Phorbol Ester- and Cholinergic-Induced Translocation of PKC Isoforms

Classical (-α) and novel (-δ, -ε and -μ), but not atypical (-ι/λ) PKC isoforms, translocate from the soluble to the particulate fraction in response to phorbol esters or other agonists (Kraft et al., 1982; Kraft & Anderson, 1983). Translocation of PKC has been often used as a marker of PKC activation. To determine if lacrimal gland PKC isoforms translocate in response to phorbol esters we used cell fractionation and SDS-PAGE/Western blotting

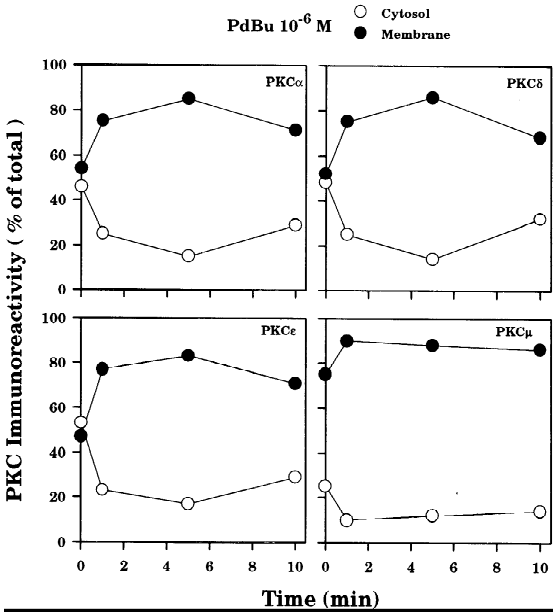

Fig. 3. Effect of time on PdBu-induced translocation of PKCα, -δ, -ϵ and -μ. Lacrimal gland acini were stimulated for the indicated time in the presence of PdBu (10^{-6} M). Proteins in the soluble and particulate fractions were separated by SDS-PAGE and transferred to nitrocellulose membranes and probed with the indicated anti-PKC antibodies. Data are average of 2 experiments.

techniques using antisera specific to PKC isoforms. As shown in Fig. 2*B*, both phorbol 12-myristate 13-acetate (PMA, 10^{-6} M) and phorbol 12, 13 dibutyrate (PdBu, 10^{-6} M) promoted the translocation of all three PKC isoforms from the soluble to the particulate fraction after a 5-min incubation.

Lacrimal gland cholinergic muscarinic receptors are coupled to the hydrolysis of phosphoinositides by PLC and hence produce DAG, the endogenous activator of PKC (Mauduit et al., 1993). Previously, we showed that PKC is involved in cholinergic-induced lacrimal gland protein secretion by using long-term treatment with phorbol esters to downregulate PKC (Zoukhri et al., 1994). Thus, we tested the effect of carbachol, a cholinergic agonist, on the cellular distribution of PKC isoforms. Carbachol (10^{-5} M) translocated PKCδ and -ϵ, but not -α, after a 1-min stimulation (Fig. 2*B*).

Effect of Time on Phorbol Ester-Induced Translocation of PKC Isoforms

The time-dependency of translocation stimulated by PdBu (10^{-6} M) for PKCα, -δ, -ϵ, and -μ is shown in Fig. 3. Translocation was detected at 1 min, maximal at 5 min and still apparent at 10 min for all four isoforms. Prior to addition of agonists, PKCα, -δ, and -ϵ immunoreactivity was almost equally divided between the soluble and the particulate fractions. After a 1-min stimulation, approximately 25% of each isoform was present in the soluble fraction and 75% was in the particulate fraction. At the maximum time (5 min) approximately 15% of each isoform was present in the soluble fraction while 85% was present in the particulate fraction. Translocation persisted for at least 10 min when approximately 30% of the isoforms were present in the soluble fraction and 70% were in the particulate fraction. Translocation of PKCμ was not as dramatic as the other isoforms. Under basal conditions, only a small amount (25%) of PKCμ was detected in the soluble fraction while the majority of PKCμ (75%) was in the particulate fraction. After a 1-min stimulation with PdBu (10^{-6} M), 10% of the isoform was detected in the soluble fraction and 90% in the particulate. Translocation persisted at 10 min when 14% of PKCμ was detected in soluble fraction and 86% in the particulate fraction. The nonreversibility of phorbol-ester-induced translocation is consistent with the slow cellular metabolism of phorbol esters compared to the natural agonist of PKC, DAG. PKCι/λ, an atypical isoform, did not translocate in response to PdBu, as expected (*data not shown*). These results indicate that all PKC isoforms present in the lacrimal gland except PKCι/λ translocated in response to PdBu.

Effect of Concentration on Phorbol Ester-Induced Translocation of PKC Isoforms

The concentration-dependency of translocation with PdBu is shown in Fig. 4. PKCϵ was the most responsive to PdBu (5 min incubation) as translocation was almost maximal (20% in soluble fraction; 80% in the particulate fraction) at a concentration of 10^{-9} M. PKCα was the least responsive with little translocation occurring until the PdBu concentration was increased to 10^{-7} M. PKCδ had an intermediate sensitivity to PdBu translocating a maximum at a PdBu concentration of 10^{-6} M. We did not test the concentration-dependency of translocation of PKCμ with PdBu because translocation with a maximal concentration of PdBu (10^{-6} M) was subtle (*see* Fig. 3).

These results show that PKCϵ is the most responsive to PdBu, PKCδ was less responsive and PKCα was the least responsive.

Effect of Time on Cholinergic-Induced Translocation of PKC Isoforms

Figure 5 shows the time dependency of carbachol (10^{-3} M)-induced translocation of PKC isoforms. As shown, maximal translocation of PKCδ and -ϵ occurred by 30 sec after addition of carbachol (28% of PKCδ in the cytosol down from 54%; 29% of PKCϵ down from 54%, Fig. 5). Translocation was reversible for PKCδ returning almost to control level by 5 min. In contrast, translocation of PKCϵ persisted for as long as measured (10 min) (Fig. 5). PKCμ was also translocated by carbachol

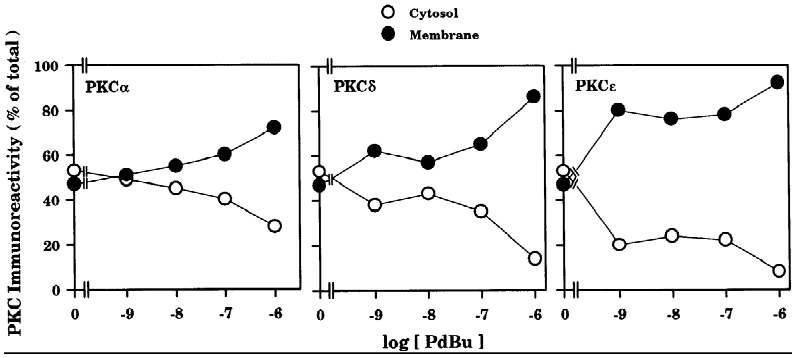

Fig. 4. Effect of PdBu concentration on translocation of PKCα, -δ, and -ε. Lacrimal gland acini were stimulated for 5 min in the presence of increasing concentrations of PdBu. Protein in the soluble and particulate fractions were separated by SDS-PAGE and transferred to nitrocellulose membranes and probed with the indicated anti-PKC antibodies. Data are average of 2 experiments.

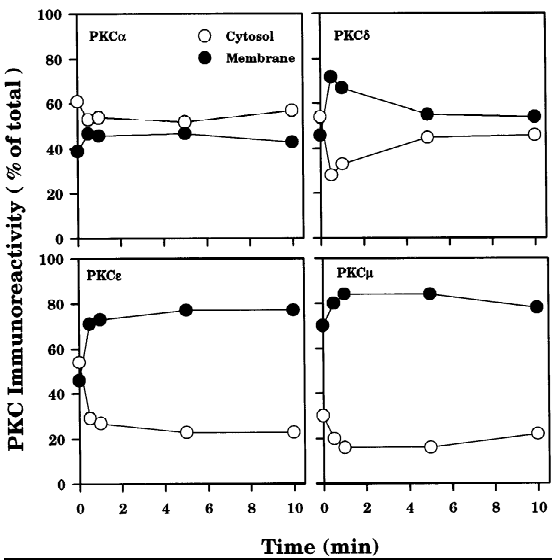

Fig. 5. Effect of time on carbachol-induced translocation of PKCα, -δ, -ϵ and -μ. Lacrimal gland acini were stimulated for the indicated time in the presence of carbachol (10^{-3} M). Proteins in the soluble and particulate fractions were separated by SDS-PAGE and transferred to nitrocellulose membranes and probed with the indicated anti-PKC antibodies. Data are average of 2–3 experiments.

though to a lesser extent than the other isoforms. Maximum translocation occurred by 1 min (16% in soluble fraction, down from 30%; 84% in the particulate fraction, up from 70%). Translocation was reversible, returning by 10 min. Surprisingly, PKCα did not translocate significantly in response to carbachol (Fig. 5). Similarly to PdBu, carbachol did not translocate PKCι/λ (*data not shown*).

These results indicate that cholinergic agonists translocate PKCδ, -ϵ, and -μ in the lacrimal gland.

Discussion

The results of the present study show that the lacrimal gland expresses five isoforms of PKC: a classical isoform, PKCα; three novel isoforms, PKCδ, -ϵ and -μ; and one atypical isoform, PKCι/λ. Using immunofluorescence techniques, we showed that, under basal (unstimulated) conditions, these isoforms were targeted to different loci. PKCα and -μ immunoreactivity was associated with the plasma membrane and with structures at the apical side of the acinar cells that resemble secretory granules. PKCα is a calcium- and phospholipid-dependent isoform, and it is known that calcium, through the C2 domain, facilitates the interaction of classical PKC isoforms with the acidic phospholipids of membranes (Luo et al., 1993). PKCμ possesses a long N-terminal sequence with a potential transmembrane domain (Johannes et al., 1994). Furthermore, a recent study (Dieterich et al., 1996) showed that PKCμ can be activated, in vitro, by phosphatidylinositol 4,5-bisphosphate (PI 4,5-P_2). PI 4,5-P_2 was shown to bind to the pleckstrin homology domains of several signal transducer molecules, and might thus anchor PKCμ, via its pleckstrin homology domain, to a membrane as suggested by Dieterich et al. (Dieterich et al., 1996). The unique location of PKCα and -μ to the apical membrane of the acinar cells suggests a pivotal role for these isoforms in controlling exocytosis in the lacrimal gland.

PKCδ, -ϵ, and -ι/λ immunoreactivity was detected mainly in the cytosol. PKCδ and occasionally -ϵ immunoreactivity was also localized on myoepithelial cells. The role of myoepithelial cells in the lacrimal gland is not yet understood. However, due to their resemblance to smooth muscle cells, it is suggested that they might contract in response to stimuli to help the acinar cells eject their secretory products into the lumen. It is worth noting that lacrimal gland myoepithelial cells express the M_3 muscarinic receptor (Lemullois et al., 1996) and thus stimulation of these receptors may lead to activation of PKCδ and -ϵ. Interestingly, calcium-independent isoforms of PKC were shown to play a major role in contraction of smooth muscle cells (Khalil & Morgan, 1992; Lee & Severson, 1994).

In another series of experiments, using cell fractionation and western blotting techniques we determined the effect of phorbol esters and cholinergic agonists on the cellular distribution of PKC isoforms. All PKC isoforms, except PKCι/λ translocated from the soluble to the particulate fraction in response to phorbol esters. The kinetics of translocation did not show any differences in the ability of PKC isoforms to respond to the phorbol ester PdBu. Translocation occurred at 1 min and was still measurable at 10 min in agreement with the slow metabolism of phorbol esters. However, a difference was noticed when the concentration dependency to phorbol esters was studied. We found that PKCε was the most responsive to PdBu and PKCα the least responsive. In a recent study, using pseudosubstrate-derived inhibitory peptides, we found that PKCα plays the major role in PdBu-induced protein secretion in the lacrimal gland (Zoukhri et al., 1997). In another study, we found that PKCα was the most sensitive to downregulation by PdBu as 31% of the immunoreactivity of PKCα was lost 20 min after addition of PdBu (10^{-6} M) (Zoukhri et al., 1994). PKCδ and -ε immunoreactivity was only decreased by 11% and 7%, respectively (Zoukhri et al., 1994). Thus, as often stated by Nishizuka (Nishizuka, 1995), association of PKC with membranes would not necessarily reflect the state of activation of the enzyme.

Translocation in response to carbachol was subtle and transient for PKCδ and -μ, whereas that of PKCε was more extensive and was still apparent at 10 min. In contrast, carbachol did not stimulate the translocation of PKCα at any of the times measured. This finding is intriguing since we found that this isoform plays a major role in cholinergic-induced protein secretion in the lacrimal gland. Indeed, inhibition of PKCα by its myristoylated pseudosubstrate-derived peptide resulted in 70% inhibition of cholinergic-induced protein secretion (Zoukhri et al., 1997). The first time measured in our translocation studies with carbachol was 30 sec. It is possible that PKCα might have translocated in response to carbachol at an earlier time. Another possibility is that translocation of PKCα is not necessary for it to stimulate protein secretion.

In summary, we showed that lacrimal gland contains two additional isoforms of PKC in addition to PKCα, -δ, and -ε: PKCμ and -ι/λ. We concluded that all these isoforms are differentially localized and that they translocate, except PKCι/λ, differentially in response to phorbol esters and cholinergic agonists. The role of PKCι/λ in the lacrimal gland remains to be studied.

The authors are grateful to Dr. James Zieske for his help with the immunofluorescence technique and for his critical reading of the manuscript. This work was supported by National Institutes of Health grant EY06177.

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