PROTEIN KINASE C ISOFORMS IN PRIMARY CELL CULTURES OF RECTAL GLAND TUBULES OF THE DOGFISH SHARK, SQUALUS ACANTHIAS

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Protein kinase C (PKC) plays a crucial role in intracellular signal transduction by linking the hydrolysis of inositol phospholipids to intracellular responses. PKC occurs as multiple, structurally related isoforms of at least 10 subspecies: conventional PKCs (cPKCs), α,β, and γ, are Ca^{2+} and phorbol ester (PE) sensitive, novel PKCs (nPKCs), δ,ε,η, and θ, are Ca^{2+} insensitive but still PE sensitive, and atypical PKCs (aPKCs), α and ζ, are neither Ca^{2+} nor PE sensitive (for review see Nakamura and Nishizuka, J. Biochem. 115, 1029-1034, 1994).

Previous work suggests that PKC mediated pathways are involved in chloride secretion in the shark rectal gland (SRG). Bell and Sargent described high levels of PKC in the SRG compared to rat spleen (Bell and Sargent, Comp. Biochem. Physiol. 87B, 875-880, 1987). Our laboratory and others demonstrated that PE, applied from the apical side to monolayers of primary cell cultures of the SRG mounted in Ussing chambers, induces I_{sc} (Ferro and Valentich, Bull. MBIBL 30, 63-64, 1991 and Lehrich et al., J. Am. Soc. Nephrol. 5, 721, 1994).

In the present study we sought to determine the presence, cellular localization and characteristics of PKC isoforms in SRG. Using specific antibodies and cell fractionation, we identified two distinct isoforms of PKC in the SRG and partially characterized these enzymes. In this report we focus on PKC α, since the characteristics of PKC ζ in the SRG have been described recently by our laboratory (Lehrich and Forrest, J. Biol. Chem., 1994, in press).

Confluent primary cell cultures of SRG tubules were lysed in homogenization buffer containing 62.5 mM Tris HCl pH 6.8, 2% SDS, 10% glycerol, 0.01% bromo phenol blue, β-mercaptoethanol and various protease inhibitors. Lysates were boiled for 7 minutes and then subjected to SDS-PAGE (7.5% acrylamide), and followed by electrotransferrence to Immobilon-P transfer membranes. Membranes were screened with specific monoclonal and polyclonal antibodies to seven different isoforms of PKC (purchased from Gibco and UBI). Particulate and cytosolic fractions were generated as follows: confluent primary cell cultures were homogenized in buffer containing 40 mM tris HCl pH 7.6, 10 mM EGTA, 2 mM EDTA and various protease inhibitors (1 mM benzamidene, 0.5 mM PMSF, 25 μg/ml leupeptin, and 10 μg/ml pepstatin), and were then fractionated by ultracentrifugation at 100,000 g for 1 hr.

We detected two isoforms of PKC in primary cultures of SRG tubules. Using a monoclonal antiserum, PKC α was detected as a single immunoreactive band with a molecular mass of 76 kDa, and PKC ζ, using a polyclonal antiserum, appeared as an immunoreactive doublet band with the molecular mass of 78-82 kDa (Fig. 1, lane 1 and lane 4). The absorption of 2 ng/ml of the monoclonal anti-PKC α antibody with 50 ng purified PKC α enzyme caused a marked significant decrease in immunoreactivity (Fig. 1, lane 2). 100 ng purified PKC α enzyme was sufficient to eliminate immunoreactivity (Fig. 1 lane 3) indicating that the immunoreactive band is due to a specific antibody-antigen interaction. Absorption of the PKC ζ antiserum with a specific PKC ζ peptide that was used to raise the antibody, in a ratio of 1:1, eliminated the immunoreactivity (Fig.1, lane 5), suggesting that the immunoreactivity due to the anti-PKC ζ antiserum is also highly specific.

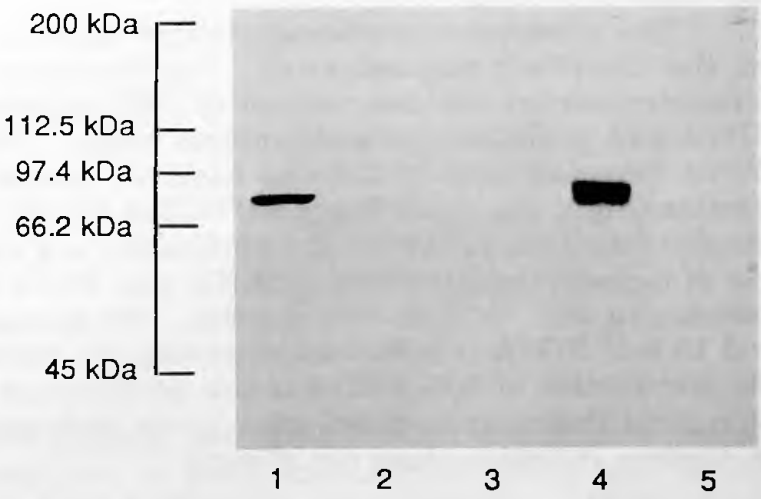

Fig. 1: Five companion lanes in an acrylamide gel were each loaded with identical amounts of cell homogenate, electrotransferred to nitrocellulose and separately probed as follows: 1) PKC α antiserum reveals an immunoreactive band with the MW of 78 kDa. 2) PKC α antiserum plus 50 ng purified rat brain PKC α enzyme leads to the almost complete disappearance of the immunoreactivity. 3) PKC α plus 100 ng purified rat brain PKC enzyme no longer reveals any immunoreactivity. 4) PKC ζ antiserum reveals a doublet of immunoreactivity with the MW of 78-82 kDa. 5) PKC ζ antiserum and PKC ζ peptide in a 1:1 ratio leads to complete absorption of the antibody.

Phorbol ester sensitive PKC isoforms respond to prolonged exposure to PE with downregulation. To address whether the isoforms of PKC detected in the SRG cells are PE sensitive, we performed down regulation experiments. The exposure of primary cultures of SRG cells to PE for 24, 12, and 6 hours revealed a decrease of immunoreactivity for PKC α within 12 hours, and complete disappearance of the detectable enzyme within 24 hours (Fig. 2 A). PKC ζ did not respond to prolonged PE exposure with time dependent down regulation (Fig. 2 B).

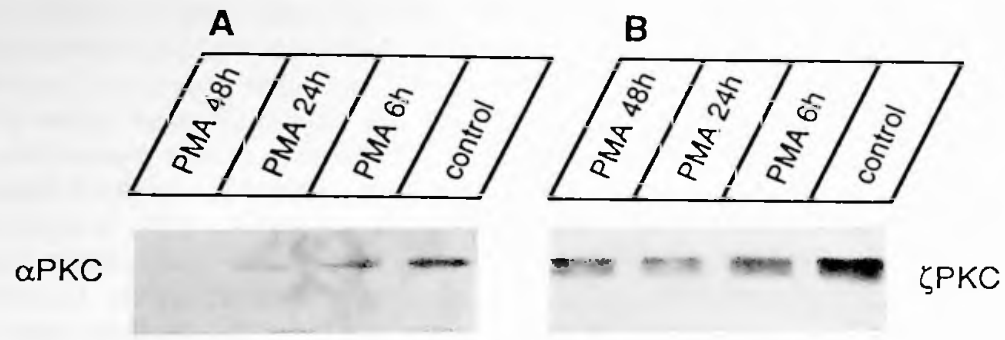

Fig. 2: Cells were incubated with 1μM PE for 6 hr, 12 hr and 24 hr, and then homogenized. Probing of the electrophoresed and electrotransferred cell homogenate with PKC α (A) and PKC ζ (B) antiserum revealed complete downregulation of PKC α and nonsignificant fluctuation of PKC ζ.

The subcellular localization of Ca^{2+}-sensitive isoforms of PKC is dependent on the free Ca^{2+} concentration. To determine the effect of the free Ca^{2+} concentration on the distribution of PKC isoforms in the basal state, we used EDTA and EGTA in our fractionation buffer. Fractionation without EDTA and EGTA revealed that PKC α was localized almost exclusively to the particulate fraction (Fig. 3 A). With 2 mM EDTA and 10 mM EGTA in the buffer, PKC α was equally distributed between the particulate and cytosolic fractions (Fig 3 A). The use of higher concentrations of EDTA and EGTA did not reveal a more complete association with the cytosolic fraction. We assumed therefore that 2 mM EDTA and 10 mM EGTA is sufficient to chelate the free calcium concentration and that the distribution of 50% PKC α to the cytosolic and 50% to the particulate fraction represents the baseline distribution in non-activated cells. PKC ζ was not affected by calcium chelation (data not shown).

To investigate if PKC α undergoes redistribution upon PE treatment, we incubated primary cell cultures with PE prior to fractionation. The presence of 100 nM PE for 15 min induced almost complete translocation of PKC α into the cytosolic fraction (Fig. 3 B). In contrast, PKC ζ was distributed evenly in the particulate and cytosolic fractions, and PE did not change the distribution (data not shown).

The central findings of this study are: 1) Two distinct PKC isoforms are present in primary cultures of SRG tubules, PKC α and PKC ζ, with molecular weights of 76 kDa and 78-82 kDa, respectively. 2) PE downregulates PKC α, but not PKC ζ, suggesting that the shark equivalent of PKC α contains a PE binding domain, while the shark equivalent of PKC ζ does not. 3) PKC α is evenly distributed in the particulate and the cytosolic fractions of the cell in the basal state. Activation with PE leads to a translocation into the cytosolic fraction,

suggesting a cytosolic location for substrates of the shark equivalent of PKC α in the rectal gland.

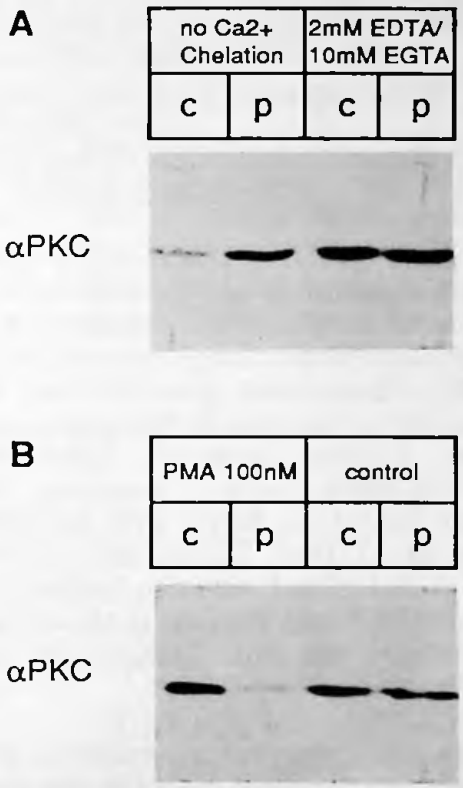

Fig. 3: Particulate and cytosolic fractions were generated and loaded on a 7.5% acrylamide gel and probed with PKC α antiserum. A) Cytosol vs particulate, lysed in fractionation buffer containing no EGTA/EDTA and 10 mM EGTA and 2 mM EDTA. When lysis buffer contains EGTA/EDTA, PKC α is evenly distributed in cytosolic and particulate fractions. B) Cytosol vs particulate fractions after incubation with 100 nM PE for 15 min, lysed in fractionation buffer containing 10 mM EGTA and 2 mM EDTA, and control incubated with DMSO.

Taken together, these data suggest that SRG PKC α and ζ have major characteristics that are representative of their subfamilies (cPKC and aPKC isoforms). However, the observed effect of PE to cause translocation of PKC α from the membrane fraction into the cytosol of the SRG is not conventional. Since PE is capable of inducing Isc when applied to the apical side of SRG monolayers, we speculate that PKC α may translocate from the apical membrane into the cytosol to phosphorylate proteins that are involved in the regulation of chloride secretion.

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