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PROTEIN KINASE C MODULATES PHOSPHOLIPASE C AND INCREASES ARACHIDONIC ACID RELEASE IN BRADYKININ STIMULATED MDCK CELLS

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The tumor promoter phorbol ester (PMA) has been shown to stimulate protein kinase C (PKC) in MDCK cells. At the concentrations that produce stimulation of PKC, PMA (100μM) inhibits BK-induced I1,4,5P_3 (IP_3) formation and calcium transients in these cells. 1-5-isoquinolinyl-2-methyl-piperazine (H7) a known inhibitor of PKC in MDCK cells reverses the effect of PMA on BK-stimulated IP_3 formation and Ca^{+2} transients in these cells. PMA also stimulates arachidonate release which can be inhibited by preincubation with H7. A dual mechanism of regulation by PKC at the level of phospholipase C (down regulation) and phospholipase A_2 (stimulation) is suggested in these cells. © 1988 Academic Press, Inc.

The sequential mechanisms by which bradykinin (BK) induces cellular responses such as calcium mobilization, arachidonic acid release, prostaglandin biosynthesis and membrane depolarization in renal epithelial cells have not been clearly elucidated. We have previously shown in rabbit papillary collecting tubule cells (RPCT) the rapid hydrolysis of phosphatidylinositol bisphosphate (PIP_2) upon BK stimulation (1); and recently have demonstrated the activation of a membrane-associated phosphatidylinositol specific phospholipase C (PLC) coupled to the BK receptor through a pertussis sensitive Gi-like protein in Madin Darby canine kidney cells (MDCK) and in RPCT cells (2,3). In MDCK cells BK stimulates arachidonic acid release and PGE_2 biosynthesis concomitant with the

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rapid mobilization of intracellular calcium (4,5). Arachidonic acid release can occur directly by the action of phospholipase A_2 (PLA_2) on membrane phospholipids (6) or from inositol-containing phospholipids by sequential action of phospholipase C and diacylglycerol lipase (7). In some systems, for example platelets, the activation of protein kinase C (PKC) can modulate the calcium signalling system through the phosphorylation of an IP3 phosphomonoesterase (8,9,10). On the other hand, there is evidence which suggests that the activation of phospholipase A2 in renal epithelial cells can be regulated by PKC (11,12).

We evaluated the role of PKC in the modulation of BK stimulated PLC in MDCK cells by utilizing phorbol esters and H7 (1-(5-isoquinolenyl-sulfonyl)-2-methylpiperazine dihydrochloride) which has been shown to inhibit certain intact cellular responses via inhibition of C-kinase (13), and HA1004 a weak C-kinase inhibitor that also inhibits markedly cyclic nucleotide dependent protein kinases (14,15). We designed the present study to examine the effect of phorbol esters and PKC inhibitors on the BK induced I1,4,5P3 formation, calcium mobilization and arachidonic acid release in MDCK cells.

MATERIALS AND METHODS

Materials; Myo-[2-^{3}H]-Inositol-(20 Ci/mol); [^{3}H]Inositol 1 phosphate(1 Ci/mmol), [^{3}H]Inositol 1,4 bisphosphate(1 Ci/mmol) and [^{3}H]Inositol 1,4,5, trisphosphate(1 Ci/mmol) were obtained from Amersham. [^{3}H]Inositol 1,3,4 trisphosphate was obtained by selective removal of the 5-phosphate of [^{3}H] Inositol 1,3,4,5 tetrakisphosphate NEN^R (5 Ci/mmol) with inositol polyphosphate-5-phosphomonoesterase from human platelets kindly provided by P. Majerus (Washington University, St. Louis). T-150 and 24 well flasks for tissue culture were from Falcon-Becton Dickinson. Dulbecco's modified Eagle's medium and F-12 Ham's-inositol-free medium were prepared by the Cancer Research Center Washington University (St. Louis). Bradykinin and all the other reagents were from Sigma.

Cell culture and phosphoinositide hydrolysis: MDCK cells obtained from American Type Culture Collection were used from passage 48 through 60. Cells were plated and grown to confluency for 48 hours in inositol free media in the presence of myo-[^{3}H] inositol, 4 μCi/well. On the day of the experiment the media was removed and the cells were washed three times with HBSS Hepes pH 7.4 and then incubated in the presence of buffer

alone (control conditions), BK 10-7 M for 15 seconds, PMA for 5 minutes and PMA plus BK. The reaction was stopped by the addition of MeOH:CHCl3 (2:1), the cells were scraped from flasks, centrifuged at 2000 xg for 10 minutes and the pellet was saved for protein determination. The aqueous phase was chromatographed by HPLC to separate the water soluble inositol polyphosphates. Chromatography was performed on a Pharmacia Mono Q™ HR 5/5 anion exchange column using two Waters 6000A pumps and U6K injector. The mobile phase consisted of 20 mM ethanolamine pH 9.5 (buffer A), and 5mM calcium sulfate plus 500 mM sodium sulfate in buffer A (buffer B). The gradient was stepwise increased in a convex manner (Waters No. 3) from 25 to 100 mM sodium sulfate over 40 minutes as in Fig. 1. Samples coeluting with authentic standards for [^{3}H]IP, [^{3}H]I1,4P_2, [^{3}H]I1,3,4P_3 and [^{3}H]I1,4,5P_3 were collected and counted by liquid scintillation spectrometry.

Calcium measurements: Cells grown in serum free media were trypsinized for 5 minutes and cell suspensions were washed twice with HBSS Hepes containing: 140mM NaCl, 5mM KCl, 1mM $MgCl_2$, 1.2mM $CaCl_2$, 15mM glucose and 25mM Hepes, pH 7.4. Cells were loaded with Fura-2 by addition of 1 μM Fura2-AM to the cell suspension (1-2 x 10^6 cells/ml) for 40 minutes at 37°C in an atmosphere of 95% O_2, 5% CO_2. The cells were then centrifuged at 500x g to remove extracellular dye. Calcium transients were measured on a Photon Technology Incorporated (PTI) spectrofluorometer with dual excitation at 340nM and 380nM and quantified at emission wavelength of 510 nM. In each experiment calibration for Fmax and Fmin was obtained by addition of 1% Triton and EGTA 5 mM, respectively, to the cell suspension. Cytosolic calcium was calculated using the standard procedure for dual wavelength measurements of Fura-2 fluorescence utilizing a dissociation constant (Kd) of Fura-2 for Ca^{2+} of 224 nM at 37°C (16). Experiments with phorbol esters and kinase inhibitors were carried out at 37°C by preincubating for 2 minutes prior to BK stimulation of the cells in suspension and continually stirred to ensure rapid equilibration of agonists.

Arachidonic acid release was measured in confluent monolayers. Cells were washed with HBSS Hepes on the day of the experiment and incubated in the same buffer containing 1 mg/ml of fatty acid free albumin in the presence of BK for 30 seconds, PMA for 5 minutes and PMA plus BK. Arachidonic acid was extracted by addition of $CHCl_3$:MeOH

(2:1), after addition of 10 ng of octadeurated AA as internal standard. Arachidonic acid released was isolated on octadecylysilyl reversed phase C-18 columns (Baker Co) by the method of Powell (17) and converted to the pentafluorobenzoyl ester by addition of 10 ul of 35% pentafluorobenzyl bromide in acetonitrile and 10 ul of disopropylethylamine and heated at 45°C for 30 minutes. The reactants were then evaporated to dryness under N2 and reconstituted with heptane for mass spectrometry. The mass was determined by mass spectrometry operating in the negative ion chemical ionization mode and monitoring m/z 303 for protium and m/z 311 for deuterium which represents loss of the pentafluorobenzoate ion (M^+ -181). Gas chromatography was carried out on a 25 meter cross linked OV-1 (Hewlett Packard) capillary column, 0.31 mm internal diameter and programmed from 85°C to 260°C at 30°C/min. Injection temperature was kept at 265°C and source temperature at 100°C. Quantitation was performed on a Hewlett Packard 5985B mass spectrometer.

RESULTS AND DISCUSSION

The HPLC anion exchange system for separation of polyphosphoinositides as shown in Fig.1 allowed us to study independently the kinetics of formation of the inositol 1,4,5 trisphosphate and the 1,3,4 isomer. Interestingly, in our labelled studies as well as in our previous study with mass determination (18) in the presence of BK there was a rapid

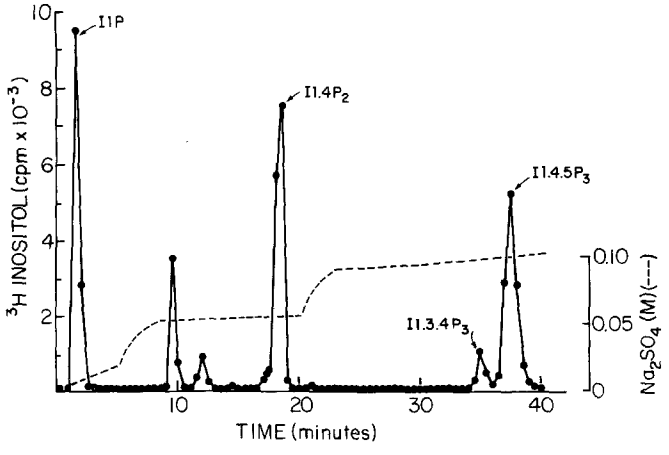
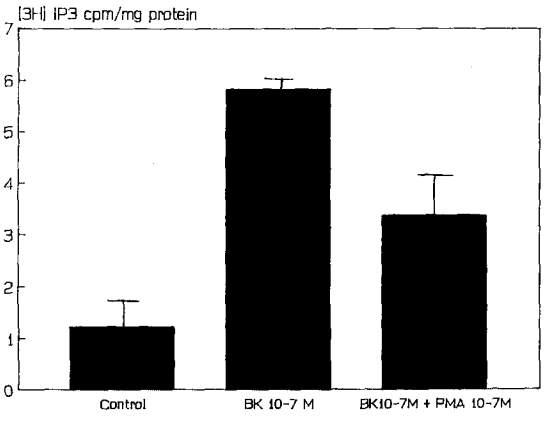
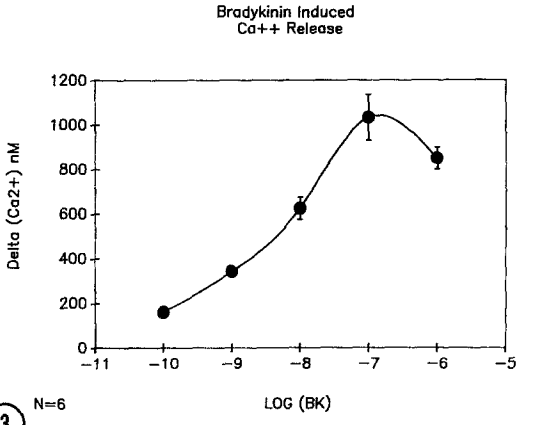


Fig. 1 HPLC separation of inositol polyphosphates on a Mono Q column. The retention times for the inositol phosphates were IP1 2 min; I1.4P_2 18 min, I1.3.4P_3 35 min and I1.4.5 P_3 37 min.



(2)



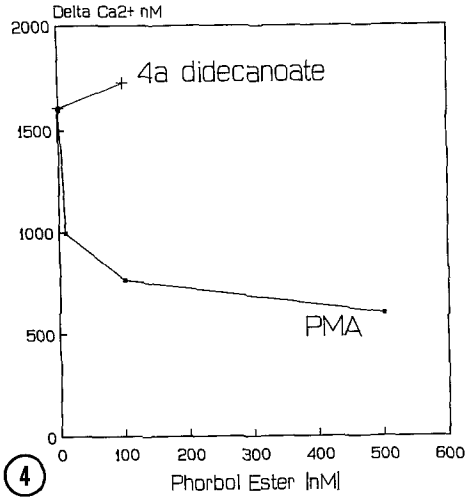
N=6

(3)

Fig. 2 **Effect of PMA on BK induced I1,4,5P_3 formation** cells were labelled with myo-^{3}[H] inositol for 48 hours and then incubated in the presence of buffer alone (control); BK 10^{-7}M (BK) for 15 seconds and PMA for 5 minutes followed by BK (PMA and BK).

Fig. 3 **Dose response relationship of Bradykinin-induced calcium transients.** Values are expressed as delta changes(nM) over basal cytosolic calcium.

formation of inositol 1,4,5 trisphosphate consistent with its role as a second messenger. Preincubation with PMA 10^{-7}M resulted in inhibition of the BK induced IP_3 formation. This effect was rapid and detected as early as 2 minutes after PMA (100 nM) addition (Fig. 2). We also found that PMA did not affect basal levels of IP_3 ruling out that PMA effect was caused by depleting the agonist sensitive pool of membrane polyphosphoinositides. Bradykinin stimulated a dose dependent increase in calcium transients in MDCK cells in suspension as shown in Fig. 3. The maximal Ca^{2+} peaked within 10 seconds followed by a slower decay, toward baseline in about 60 seconds. At lower BK concentrations these responses were slower to peak and there was not complete recovery to baseline. Preincubation with phorbol myristate acetate (PMA) at different concentrations for 2 minutes prior to BK stimulation produced a dose dependent inhibition of the BK induced calcium transients; while 4 alpha-didecanoate an inactive tumor promoting phorbol at similar concentrations had no effect on the BK induced Ca^{2+} transients (Fig. 4). PMA or 4 alpha didecanoate did not have any significant effect on the basal cytosolic calcium as measured by Fura-2.



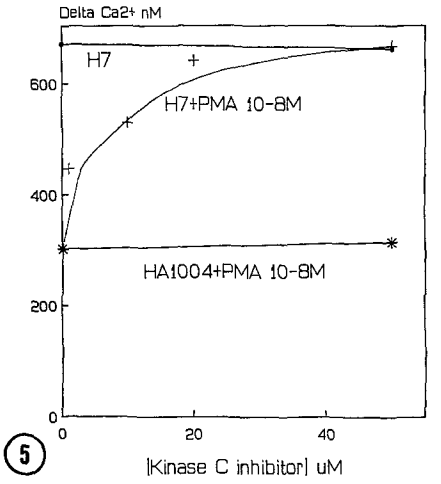


Fig. 4 **Dose response curve for phorbol ester mediated inhibition of bradykinin induced calcium transients.** MDCK cells in suspension were preincubated at 37°C for 2 minutes in the presence of PMA (o) or 4-alfa didecanoate (+) prior to bradykinin stimulation.

Fig. 5 **Effects of protein kinase inhibitors on PMA inhibition of calcium transients.** MDCK cells were preincubated for 2 minutes at 37°C at various concentrations of H7 (+) and HA1004 (-), then followed by PMA preincubation and BK stimulation.

Pretreatment with H7 for 2 minutes before the addition of PMA reversed in a dose dependent manner the PMA inhibition of the BK induced calcium transients with a K_i of 12 μM consistent with the published K_i for this C-kinase inhibitor (13,14); suggesting the effect of PMA was occurring through activation of protein kinase C. To exclude this effect was due to inhibition of cAMP kinase we used HA1004, another kinase inhibitor, which has a published K_i for cAMP dependent kinase of 4×10^{-5}M. In our hands concentrations up to 5×10^{-5}M there was no reversal of the PMA effect on Bk induced calcium mobilization, see Fig. 5. It is of some significance that the Ki for HA1004 on the cAMP dependent protein kinase is 2.3×10^{-6} M (15).

In this study we have shown that the inhibitory effects of PMA on IP_3 formation and calcium mobilization are most likely due to activation of protein kinase C. In several types of cells, activation of PKC leads to attenuation of receptor stimulated PLC activity (19,20) and this effect is presumed to be mediated *in vivo* by the diacylglycerol

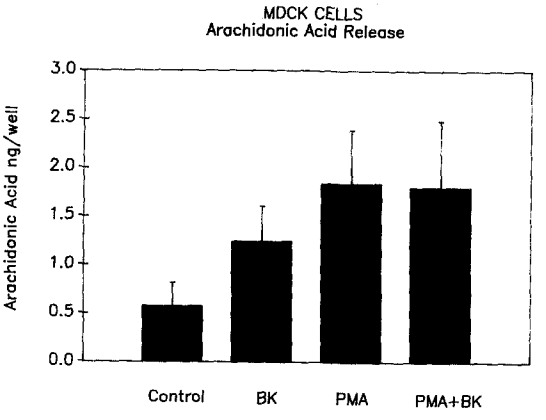


Fig. 6 **Arachidonic acid release in MDCK cells.** Cells were exposed to HBSS buffer alone (control); bradykinin 10^{-7} M (BK) for 30 scs.; phorbol myristate acetate (PMA) 10-7 M for 5 minutes and then pretreated with PMA for 5 min. prior to BK stimulation for 30 scs. (PMA plus BK).

produced after PIP_2 hydrolysis. In our hands, pretreatment of intact MDCK cells with PMA does not cause significant inhibition of PKC activity in plasma membranes prepared after PMA pretreatment. The mechanism of PMA mediated inhibition of BK responses remains to be established but phosphorylation of a Gi like protein coupling PLC to BK receptor by PKC has been reported in other systems (21).

We also tested the effect of PMA on the stimulation of arachidonic acid release. BK produced stimulation of AA release in confluent monolayers of MDCK cells within 30 seconds. PMA at similar concentrations and time course that produced inhibition of Ca^{2+} responses stimulated the basal release of arachidonic acid (Fig. 6). This stimulation was inhibited by pretreatment with H7 (100 μM). Two mechanisms for the activation of PLA_2 have been suggested, one regulated by PKC and the other as a result of an increase in the concentration of intracellular Ca^{2+}. The stimuli for release of AA in our experiments reflects the mechanism controlled by PKC stimulation instead of an increase in the concentration of intracellular Ca^{2+}. These two mechanisms, Ca^{2+} and PKC may act in concert to stimulate arachidonic acid release. The precise role of PKC is not completely understood but its activation could occur through phosphorylation of PLA_2 modulatory protein or through direct activation of the enzyme in the presence of low calcium concentrations.

The lack of complete inhibition of the Ca^{2+} responses may suggest that BK induced calcium transients may not be due entirely due to IP_3 mobilization and PKC could play a role in the opening of a Ca^{2+} channel as suggested in neuronal cells (23).

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