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INHIBITORY EFFECT OF CYCLIC AMP ON PHORBOL ESTER-STIMULATED PRODUCTION OF REACTIVE OXYGEN METABOLITES IN RAT GLOMERULI

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SUMMARY: Studies were conducted to investigate cross-talk between protein kinase C (PKC) and cyclic AMP (cAMP) pathways using rat glomeruli (Glm). Phorbol 12-myristate 13-acetate (PMA), a PKC activator, stimulated production of reactive oxygen metabolites (ROM) in Glm. Forskolin and dibutyryl cAMP (Bt_2cAMP) inhibited production of ROM dose-dependently. In the presence of both Bt_2cAMP and 3-isobutyl-1-methylxanthine (IBMX) an additive effect was observed. Forskolin at 10^{-4} M inhibited translocation of PKC from the cytosol to the membrane. These results demonstrate that cAMP-mediated inhibition can occur at a step distal to PKC activation.

INTRODUCTION: Cross-talk between PKC and cAMP pathways have been demonstrated as several different patterns in various cells (1). In bi-directional control systems two pathways appear to counteract each other (1), and this interaction may be reciprocal in rat parietal cells (2). In intact cells where both pathways function, it has been shown that the link between two pathways is most probably at a step proximal to PKC activation, especially at the inhibitory GTP protein (3,4).

Glm (5,6) and mesangial cells (7-9) have been shown to produce ROM in response to various stimulations including PMA as in other phagocytic cells, allowing a biochemical analysis of this mechanism. Since little is known about cross-talk between the two pathways in the kidney cells, we studied the effects of cAMP agonists (cAMP increasing agents) on production of ROM stimulated by PMA using rat Glm.

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MATERIALS AND METHODS

Preparation of glomeruli: Following decapitation, the kidneys of male Sprague-Dawley rats (200-230g) were perfused with cold phosphate-buffered saline with D-glucose and albumin (PBSGA) (10). After perfusion, kidneys were removed and decapsulated. All subsequent preparations were carried out at 4°C. Kidneys were sliced, and gently squeezed through a stainless steel sieve. Tissues retained on the 74-MIC opening were collected with PBSGA. After centrifugation at 800 rpm for 5 min, supernatant with small fragments were eliminated, and resulting tissues were resuspended with arbitrary volume of PBSGA. The purity of each preparation was evaluated by counting Glm under light microscopy.

Assay of reactive oxygen metabolites: The content of each reaction tube was a mixture of 20 µl luminol (final concentration 1.1×10^{-4} M), 200 µl glomeruli in PBSGA, and 760 µl complete veronal buffer (CVB) (11) with or without reagents to make the final volume 1 ml. ROM were quantitated by chemiluminescence in a LKB 1251 Luminometer at 37°C with constant mixing. The luminometer converts photons with a photomultiplier tube into an electric current. Following the preincubation of a tube for 5 min (with or without reagents), base line chemiluminescence was recorded. Then reaction was started by adding 20 µl PMA (final concentration 1.6×10^{-5} M). After the peak plateau level was reached, the emission of photons was recorded with an integration for 30 sec. Results were given by subtracting the base line chemiluminescence from the peak plateau chemiluminescence. Control values (no reagents) were obtained at each experiment, and data were expressed as % of control.

Measurement of PKC translocation: Glm for one experiment were isolated from 5 rats. PKC activity was determined as described in Tamura et al. (11) with modification. In brief, forskolin or vehicle was added to Glm in suspension (500 µl) during the preincubation period. After 10 min incubation with 10 µl PMA or vehicle, the reaction was stopped by addition of 2.5 ml cold homogenizing buffer containing 20 mM Hepes-NaOH (pH 7.4), 5 mM EGTA, 3 mM $MgCl_2$, 0.1% bovine serum albumin (BSA), 20 µg/ml leupeptin, and 5 mM dithiothreitol (DTT). Glm were disrupted by sonication, and centrifuged at 100,000xg for 60 min. The supernatant was used as a cytosolic fraction. The pellet was resuspended in 3 ml of cold homogenizing buffer containing 0.1% Triton X-100. After sonication, the pellet suspension was left on ice for 60 min and then centrifuged at 100,000xg for 60 min. This supernatant was used as a membrane fraction. Both cytosolic and membrane fractions were diluted 10 times with the cold homogenizing buffer prior to assay. A 20 µl aliquot of sample was added to 80 µl of reaction mixture containing 25 mM Hepes-NaOH (pH 7.4), 10 mM $MgCl_2$, 2 mM DTT, 20 µM ATP, 0.2 mg/ml histone type III-S, 0.5 µCi [γ-^{32}P]-ATP, with or without 1.5 mM $CaCl_2$, 25 µg/ml phosphatidylserine, and 0.5 µg/ml diolein. Incubation was performed at 30°C for 10 min. The reaction was terminated by adding 1 ml of cold stopping solution containing 10% trichloacetic acid (TCA) and 2 mM ATP, followed by addition of 100 µl of 0.63% BSA. After 20 min centrifugation at 800xg, the supernate was discarded and the pellet was resuspended in 0.1 ml of 0.1 N NaOH and immediately reprecipitated with 1 ml of cold stopping solution. The precipitated protein was trapped on a Whatman GF/C filter. Filters were washed with a total volume of 30 ml of cold 5% TCA and dried. The radioactivity remaining on the filter was determined by a liquid scintillation counter. PKC

activity was determined by subtracting the activity measured in the absence of calcium and lipids from that measured in their presence. Each sample assay was performed in triplicate.

Materials and chemicals: Luminol (Wako Chem. Co.) was dissolved in dimethylsulfoxide (DMSO) and directly diluted in CVB. PMA, 4α-phorbol 12,13-didecanoate (PDD) and forskolin (all from Sigma Chem. Co., St. Louis) were dissolved in DMSO and stocked at -20°C. Bt_2cAMP (Yamasa Shoyu Co.) and IBMX (Sigma) were dissolved in CVB. [γ-^{32}P]ATP (specific activity, 3000 Ci/mmol) was purchased from New England Nuclear (Boston, MA).

RESULTS

Typical chemiluminescence responses to control and forskolin-treated Glm both of which were stimulated with PMA are shown in Fig. 1. After a lag period of about 1 min following addition of PMA, chemiluminescence was rapidly increased. Peak plateau level was reached between 10 to 15 min, and declined thereafter. In forskolin-treated Glm, the initial lag was slightly longer and the peak plateau level was lower than the control. PDD, a phorbol ester that does not activate PKC, produces no significant chemiluminescence (data not shown). The diterpene forskolin, which is an effective activator of adenylate cyclase in membrane preparations and intact cells (12), inhibited chemiluminescence in a dose-dependent manner (Fig. 2). Next, we determined the

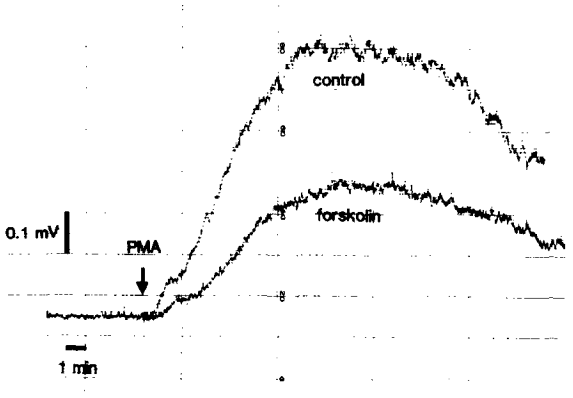

Figure 1. Typical PMA-stimulated chemiluminescence responses of control and forskolin-treated glomeruli. PMA was added at arrow point. Control group contained the same volume of DMSO as in forskolin-treated group.

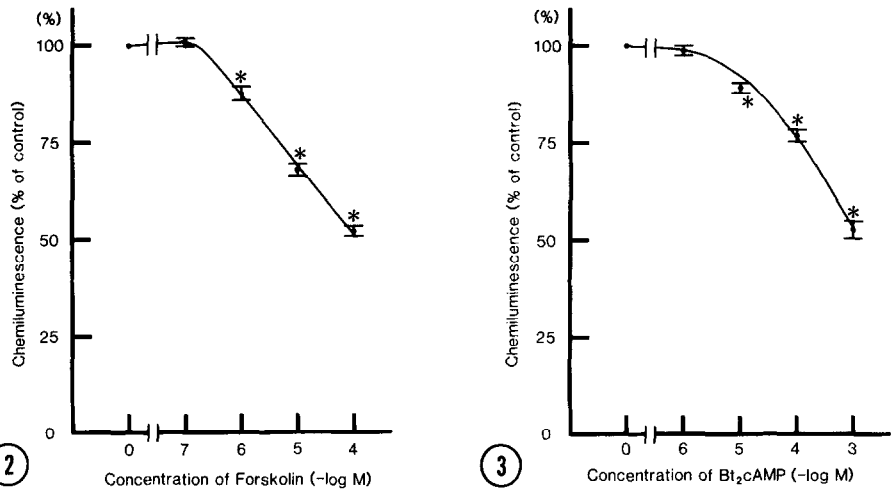

Figure 2. Effect of various concentrations of forskolin on PMA-stimulated chemiluminescence. Data are means ± SE of six experiments from six rats. Control groups contained the same amount of DMSO as in forskolin-treated group. Asterisk denotes significant difference from 10^{-7} M forskolin ($p < 0.001$, t test).

Figure 3. Effect of various concentrations of Bt_2cAMP on PMA-stimulated chemiluminescence. Data are means ± SE of six experiments from six rats. Asterisk denotes significant difference from 10^{-6} M Bt_2cAMP ($p < 0.01$ or higher, t test).

direct effects of Bt_2cAMP (Fig. 3). Bt_2cAMP also inhibited chemiluminescence dose-dependently. Bt_2cAMP at 10^{-4} M or 10^{-4} M IBMX alone had a significant inhibitory effect (75.1 ± 1.5 % or 70.3 ± 1.8 %, respectively) (Fig. 4). Moreover, in the presence of both 10^{-4} M Bt_2cAMP and 10^{-4} M IBMX additive effects were observed (55.7 ± 1.2 %) (Fig. 4). Forskolin at 10^{-4} M significantly inhibited translocation of PKC from the cytosol to the membrane fraction (Table 1).

DISCUSSION

The present study demonstrated that forskolin or cAMP inhibits, in a dose-dependent manner, PMA-stimulated production of ROM in rat Glm, and that forskolin inhibited PMA-stimulated translocation of PKC from the cytosol to the membrane. In other cells including platelets, neutrophils and lymphocytes, cAMP, probably through cAMP-dependent protein kinase (PKA) activation,

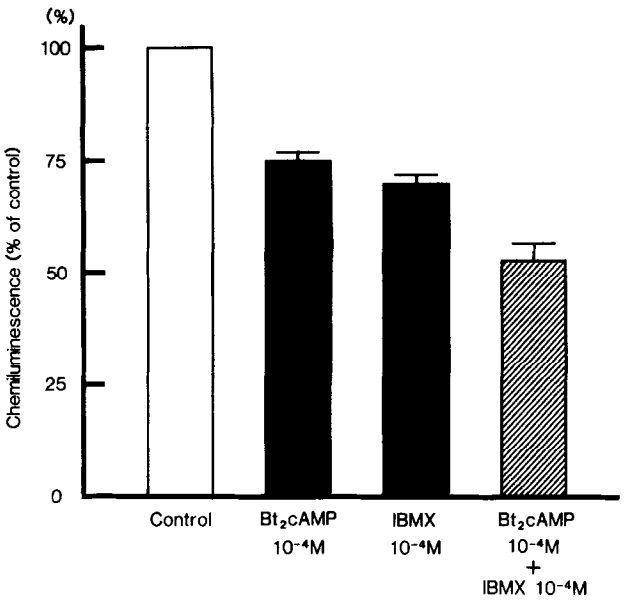

Figure 4. Effect of Bt_2cAMP and IBMX on PMA-stimulated chemiluminescence. Data are means ± SE of five experiments from four rats. In the presence of both agents significant difference were seen from Bt_2cAMP or IBMX alone ($p < 0.001$, t test).

inhibits PKC pathways at a step proximal to PKC activation (1,13). In fact, cAMP inhibited the release of superoxide from neutrophils stimulated by fMet-Leu-Phe, while cAMP had no effects on superoxide production stimulated by PMA or 1-oleyl-2-acylglycerol (14). Histamine inhibits superoxide production stimulated by serum-treated zymosan by increasing cAMP accumulation also in mesangial cells (8). Agents that increase cAMP inhibit phosphatidylinositol (PI) kinase, the enzyme that

Table 1. Effect of forskolin on PKC translocation

	PKC translocation (% PKC in membrane)
Control	34.2 ± 3.0
PMA	74.0 ± 3.2
PMA + forskolin, 10^{-4} M	51.5 ± 4.8*

Values are means ± SE of 6 experiments from 30 rats. Asterisk denotes significant difference from PMA ($p < 0.01$, t test).

catalyzes conversion of membrane PI into PI monophosphate (15), then inhibits PI hydrolysis (16,17). This results in decreased diacylglycerol, a PKC activator (18), and inhibits PKC-mediated cell response.

The mechanism by which cAMP inhibits production of ROM is through inhibition of PKC to the membrane from our study. Since phorbol ester-induced production of superoxide is dependent of translocation of PKC activity to the membrane (19), cAMP may change intracellular distribution of PKC. In fact , cAMP inhibits translocation of PKC activity to the membrane both in basal and PMA-stimulated conditions in rat parietal cells (2), but not in skeletal myoblasts (20).

In conclusion, our present findings demonstrate that cAMP may inhibit production of ROM at a step distal to PKC activation in rat Glm by inhibiting translocation of PKC from the cytosol to the membrane. This cAMP-mediated inhibition differs from the one in leukocytes, in which the inhibition occurs only at a step proximal to PKC activation.

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