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Activation of cAMP-dependent Protein Kinase in Epidermis by the Compounds which Increase Epidermal cAMP

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Pig epidermal slices were incubated with various compounds which increased epidermal cAMP (adenosine 3′,5′-monophosphate), and the change in cAMP-dependent protein kinase activity ratio was studied by the method of Cherrington et al (J Biol Chem 251:5209-5218, 1976) with modification.

Epinephrine (5×10^{-5} M), histamine (10^{-4} M) and adenosine (10^{-3}M), potent agonists of epidermal adenyl cyclase, fully activated the protein kinase (PK) during an incubation of 30 to 45 seconds, that was much shorter than that required for maximal cAMP accumulation under the same conditions (5 min). With such a brief stimulus, the epidermal cAMP-PK system did not become refractory and responded to repeated stimuli. Prostaglandin E_2 (PGE_2) and isobuthylmethylxanthine (IBMX) and ethanol only partially activated the enzyme. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and theophylline which were much less effective in increasing epidermal cAMP, activated the enzyme to the same extent as PGE_2 and IBMX respectively.

These results suggest that protein kinase activation takes place in response to a cAMP increase in small locus of the cell. Such an increase in cAMP can be very small or even not measurable when measured as total cAMP in the tissue homogenate. Also, increases above this level may not be physiologic.

It is concluded that measurement of cAMP-dependent protein kinase activity ratio is a more direct and more sensitive way to study the effect of compounds which act through cAMP mediated mechanisms.

Activation of cyclic adenosine 3′,5′ monophosphate (cAMP)-dependent protein kinase (PK) is the only known mechanism for cAMP mediated hormone action in various mammalian tissues. The amount of enzyme activated by endogenous cAMP and the amount which is available for activation by exogenous cAMP can be monitored by measuring the activity ratio of this enzyme in the absence and presence of excess cAMP in the assay system. This approach has been used by several investigators [1-4] studying the effects of cAMP elevating agents in various tissues. It has been validated extensively by Cherrington et al [1] using liver parenchymal cells.

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Abbreviations:
cAMP: cyclic adenosine 3′,5′-monophosphate
DMSO: dimethylsulfoxide
IBMX: isobuthylmethylxanthine
PGE_2: prostaglandin E_2
$PGF_{2\alpha}$: prostaglandin $F_{2\alpha}$
PK: protein kinase

Using pig epidermal slices we found that maximal activation of PK required less time and less stimulator as compared with that for maximal cAMP accumulation via adenyl cyclase. The finding is in accord with the rapid PK response in other organs [1-6]. In addition, PK unlike adenylate cyclase was not refractory to further stimulation.

MATERIAL

Pig epidermal slices were obtained from the backs of medium-sized pigs using a Castroviejo keratome set at 0.2 to 0.3 mm in thickness. The slices (over 80% epidermis) were kept at 4°C, cut into 7 × 7 mm squares and used within a few hours. ATP, cAMP, mixed histone (type H II S) were obtained from Sigma (St. Louis, Missouri) and isobutylmethylxanthine (IBMX) from Aldrich Chemical Company (Milwaukee, Wisconsin). γ-^{32}P-ATP (S.A.: 35 Ci/mmol) was obtained from New England Nuclear (Boston, Mass.). Phosphocellulose paper was a product of Whatman (Clifton, N.J.).

EXPERIMENTAL PROCEDURE

Measurement of the cAMP-dependent protein kinase activity ratio requires strict precautions to avoid artificial activation or inactivation [1]. A relatively high salt concentration in the homogenization buffer is needed to prevent reassociation and resulting inactivation of the enzyme subunits. It also prevents an association of the free catalytic subunit (the active form of the enzyme) with the particulate fraction. Too much homogenization must be avoided in order not to dissociate and concomitantly activate the enzyme. The use of two much tissue or too long incubation should also be avoided for the same reason.

A single epidermal square was carefully homogenized for 8 seconds at 0°C using a conical glass homogenizer with 250 μl of a homogenization buffer containing 10 mM potassium phosphate buffer pH 6.8, 10 mM EDTA, 0.5 mM IBMX and 150 mM KCl (approximately 30 mg of tissue/ml). Although the homogenization was not complete, longer processing was not done because of a slight but steady increase in the activity ratio (15% increase by 20 seconds homogenization). The homogenate was centrifuged at 2,000 rpm for 20 min and the supernatant was used for the assay.

The reaction mixture containing 5 μmol of potassium phosphate buffer pH 6.8, 0.5 μmol of magnesium acetate, 0.5 μmol of NaF, 12.5 nmol of EGTA, 100 μg of histone, 1 nmol of ATP (containing 0.25 to 1 μCi of γ-^{32}P ATP) and 0 or 0.25 nmol of cAMP was preincubated for 5 min at 30°C, then 10 μl of the enzyme preparation was added and incubated for 5 min at 30°C in a total volume of 50 μl. The reaction was stopped by addition of glacial acetic acid to a final concentration of 30%. Thirty μl of the mixture was applied on a 2 × 2 cm piece of phosphocellulose paper, which was washed with tap water for 75 min, dehydrated with acetone and counted in liquid scintillation counter [7, 8]. Protein in the supernatant was measured by the method of Lowry et al [9] using human serum albumin as a standard.

Due to the very low enzyme activity, the ATP concentration in the reaction system was reduced to 0.02 mM from the original 0.2 mM [1] in order to increase the sensitivity of the assay (a 10-fold increase of radioactive ATP instead of the reduction of total ATP was impractical). Since the ATP concentration required for maximal activity of the skin enzyme was about 0.15 mM and we were obliged to work under the optimal ATP concentration, preliminary experiments were done to ensure that the tissue amount and incubation time were within the linear portion of the reaction as well as in the steady part of the enzyme activity ratio.

All the assays were carried out in duplicate, and the results were

expressed as an activity ratio (activity without cAMP/activity with cAMP = total activity).

RESULTS

Since the epidermal cAMP level is known to change quickly after removal of the tissue from the body [10], it was necessary to examine the change in the protein kinase activity ratio following sampling and storage of the skin. Storage of the tissue in Hank's balanced salt solution at 4°C caused a slight gradual decrease in the protein kinase activity ratio in the initial 60 min, but no loss in total enzyme activity was observed over a 3-hr period. When the tissue was transferred into Hank's salt solution at 37°C after 40 min storage, the activity ratio increased slightly for the first 5 min, then gradually decreased to a stable level within 20 to 30 min. This demonstrated the importance of preincubation for 20 to 30 min at 37°C to stabilize the protein kinase activity ratio. Therefore, in the following experiments, a 7 × 7 mm epidermal square was preincubated in Hank's balanced salt solution at 37°C for 30 min, then transferred to the incubation medium consisting of Hank's solution with various test agents at 37°C for the indicated period. After the incubation, the epidermal square was quickly frozen between flat faces of Dry Ice, and its PK activity was assayed as described in the "Methods" Section.

Figure 1 demonstrates the increase in the protein kinase activity ratio induced by incubation with 5×10^{-5} M of epinephrine. The ratio reached 1.0 (full activation) within 30 seconds, and remained elevated during the incubation period for 30 min. Lower concentrations of epinephrine showed a dose dependent effect as shown in Fig 2. Much longer incubation with 5×10^{-5} M epinephrine had been needed to maximally elevate the epidermal cAMP level (5 min), and this longer incubation had caused refractoriness of adenyl cyclase to epinephrine [11]. Incubation of epidermal squares with 10^{-4} M of histamine for 45 seconds fully activated PK (Fig 3). These effects of epinephrine and histamine on the PK ratio were blocked by preincubation of the epidermal squares with a 10 times higher concentration of propranolol and methiamide respectively (Fig 4). But addition of these antagonists at the incubation stage did not block PK activation by agonists. Aden-

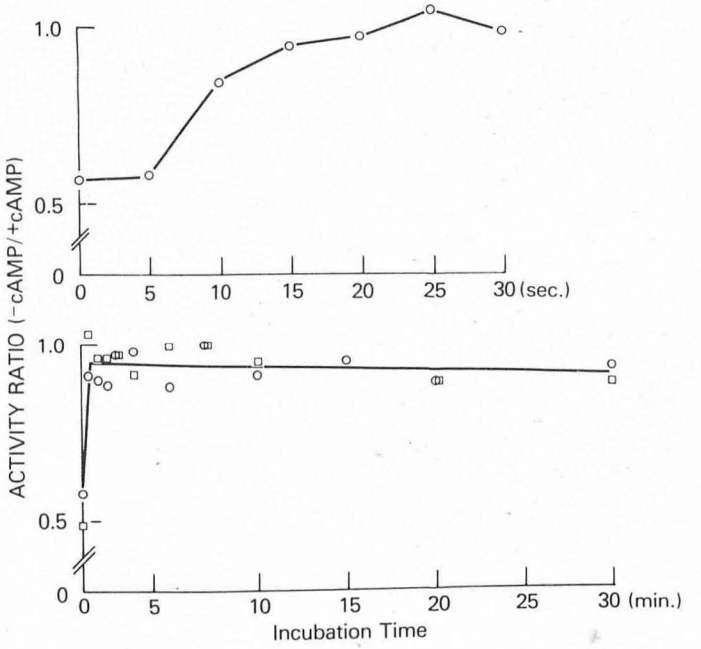

FIG 1. Effect of incubation with epinephrine on protein kinase activity ratio. The pieces of epidermis were preincubated in Hank's salt solution at 37°C for 30 min, then transferred to Hank's solution containing 5×10^{-5} M epinephrine. Two different symbols in the lower figure indicate 2 series of experiment.

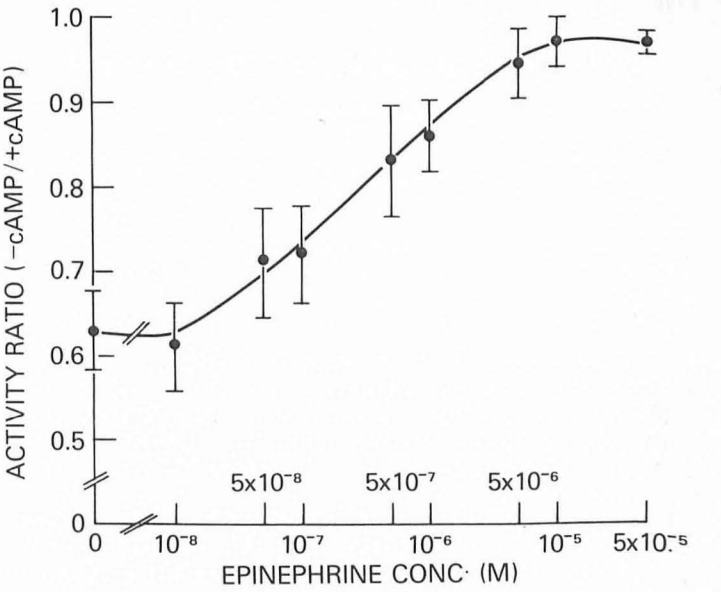

FIG 2. Dose dependent effect of epinephrine on protein kinase activation. The tissue was incubated for 30 seconds with various concentrations of epinephrine. Average ± SEM of 3 experiments.

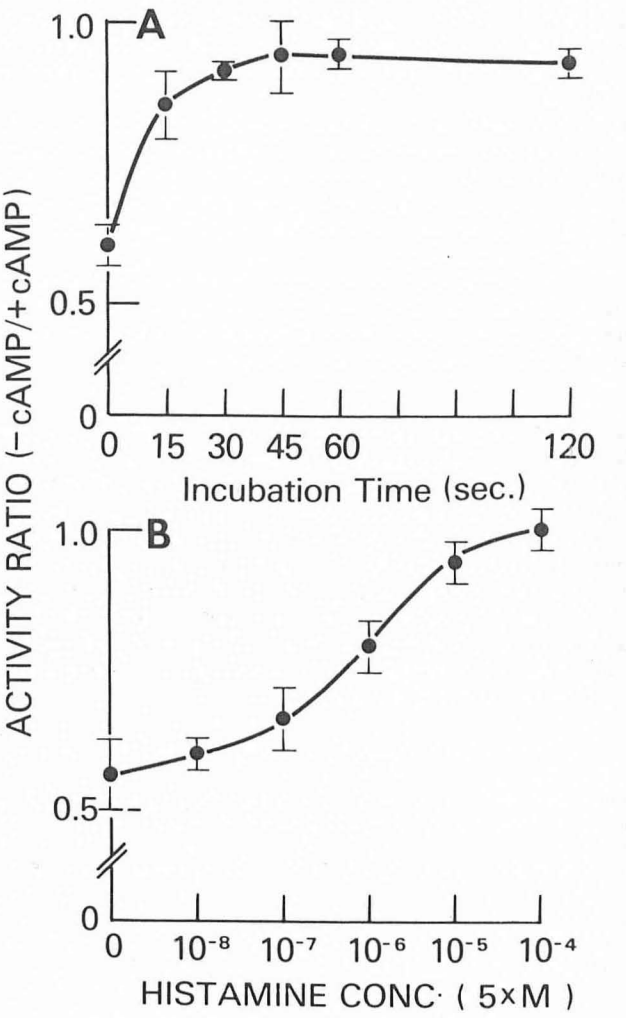

FIG 3. Effect of histamine on protein kinase activation. *A*, Incubation with 10^{-4} M histamine. *B*, Incubation for 45 seconds. Average ± SEM of three experiments.

osine at 10^{-3} M also activated PK almost fully within 45 seconds (Fig 5). The incubation period required for the maximal effect of histamine and adenosine on PK was also much shorter than the incubation time necessary for maximal cAMP accumulation.

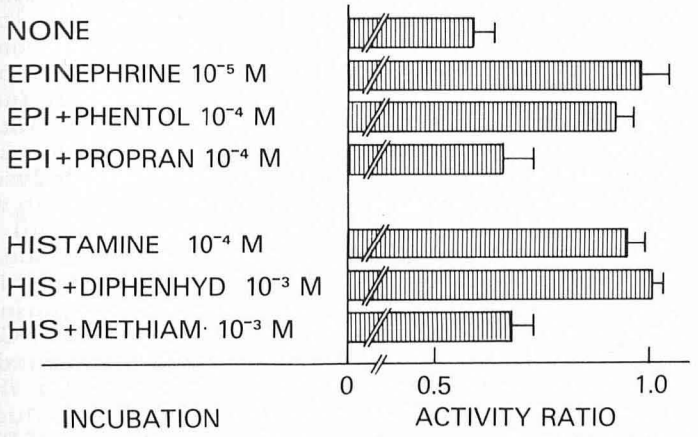

FIG 4. Effect of adrenergic blocking agents and antihistaminic agents on protein kinase activation by epinephrine or histamine. The tissue was incubated with antagonists for 5 min at 37°C, then agonist was added and incubated for another 5 min. Average ± SEM of 4 experiments.

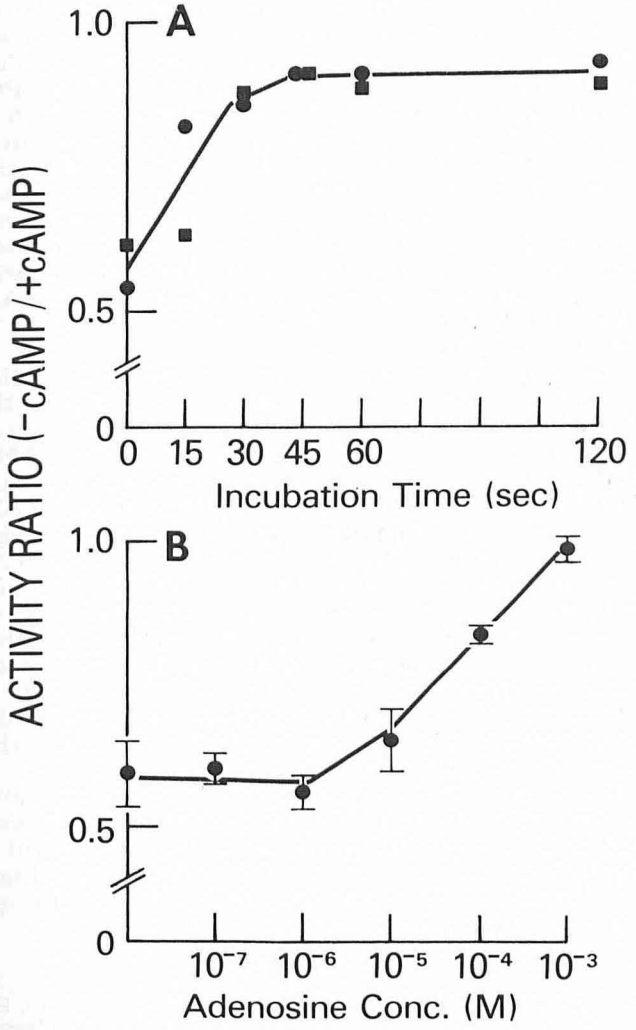

FIG 5. Effect of adenosine on protein kinase activation. *A*, Incubation with 10^{-3} M adenosine. *B*, Incubation for 45 seconds. Average ± SEM of 3 experiments.

When the epidermal square was stimulated with epinephrine for 30 seconds and then transferred to plain Hank's solution, the protein kinase activity ratio gradually decreased and returned to the initial level in 20 min. Addition of IBMX to the Hank's solution prevented this decrease (Fig 6*A*). Figure 6*B* demonstrates that the epidermal cAMP-PK system can respond after such a short stimulation by epinephrine if it is repeated 20 min later. This repeated stimulation of PK was also observed using histamine or adenosine (Fig 6*C*). The effects of IBMX and theophylline are shown in Fig 7. IBMX elevated the PK activity ratio, which reached the maximal value of 0.75 at 1 min and then decreased slightly with prolonged incubation (even though the cAMP level has been reported to continue

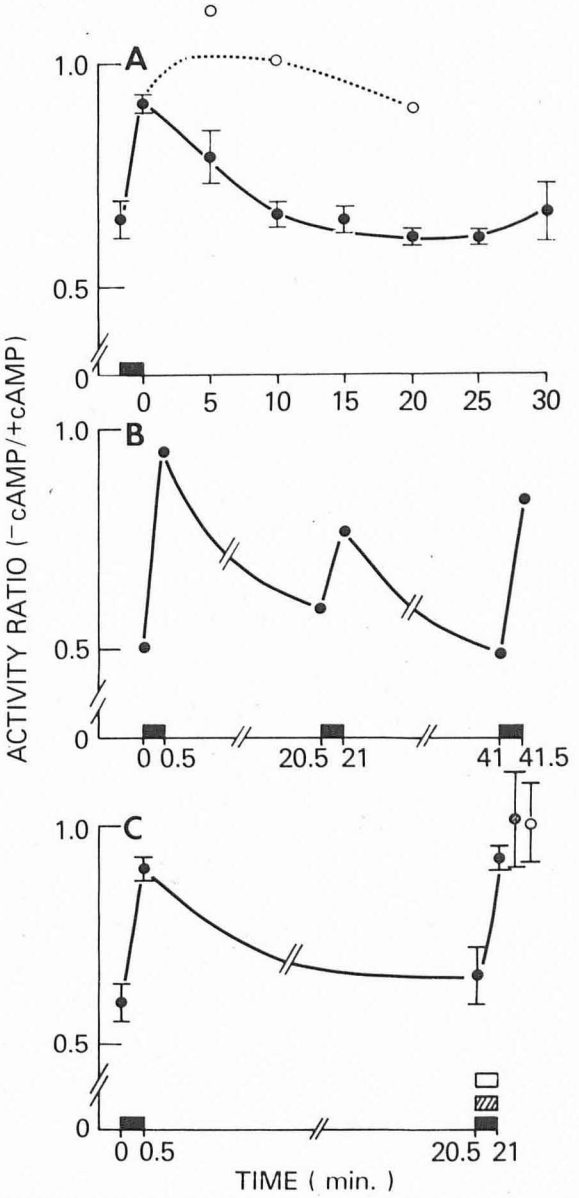

FIG 6. Effect of repeated stimulation by epinephrine on protein kinase activity ratio. *A*, The tissue was incubated with 5×10^{-5} M epinephrine for 30 seconds, transferred to plain Hank's solution and incubated for the indicated period at 37°C. *Open circle* shows the result with the tissue transferred to the Hank's solution containing 2 mM IBMX. Average ± SEM of 3 to 4 experiments. *B*, The tissue was stimulated with the same concentration of epinephrine up to 3 times with 20 min interval in plain Hank's solution. Average of 2 experiments. *C*, The first stimulation was done with epinephrine. Then 20 min later, the second stimulation was done by either epinephrine (*solid*), 10^{-4} M histamine (*shaded*) or 10^{-3} M adenosine (*open*). Average ± SEM of 4 experiments.

rising during this period) [12]. Theophylline, which by itself had shown no effect on the epidermal cAMP level, did activate PK to the same extent as IBMX. PGE_2 at a concentration of 5×10^{-5} M activated PK to a maximal activity ratio of about 0.8 within 1 min. $PGF_{2\alpha}$ which compared with PGE_2 had been much less effective in causing an epidermal cAMP increase [13], was as effective as PGE_2 in elevating the PK activity ratio (Fig 8).

The effects of ethanol and dimethylsulfoxide (DMSO) were also studied at concentration ranges often used in experimental systems. Ethanol in concentrations as low as 0.5% increased the PK activity ratio, while DMSO did not (data not shown). We have previously reported that 5% ethanol raised epidermal cyclic AMP levels *in vitro* [14].

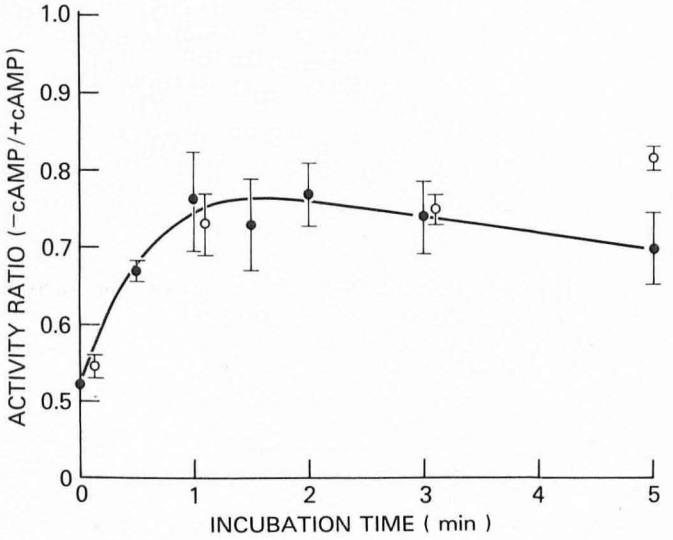

FIG 7. The effect of IBMX and theophylline. The tissue was incubated for the indicated period with 2 mM isobuthylmethylxanthine (IBMX) (*solid symbol*, $n = 6$) or 10 mM theophylline (*open symbol*, $n = 4$).

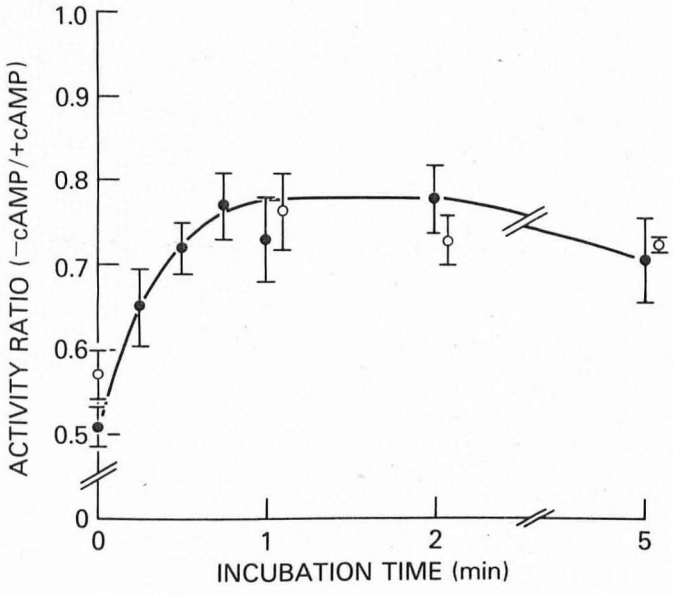

FIG 8. Effect of PGE_2 and $PGF_{2\alpha}$ on protein kinase activity ratio. The tissue incubated for the indicated period with 5×10^{-5} M of either PGE_2 (*solid sumbol*) or $PGF_{2\alpha}$ (*open symbol*). Average ± SEM of 4 experiments except 1 min incubation with $PGF_{2\alpha}$ (2 experiments). PGE_2 and $PGF_{2\alpha}$ were generous gift from Dr. V. A. Ziboh, Department of Dermatology and Biochemistry, University of Miami School of Medicine.

DISCUSSION

A system has been described in this paper which allows one to study the activation of cAMP-dependent PK in epidermis by compounds which increase cAMP. The basal activity ratio observed with epidermis was always higher (about 0.5) compared with that of liver parenchymal cells (less than 0.4). This is partly due to the presence of cAMP-independent PK (unpublished data), which accounts for about 20% of the total kinase activity in the soluble fraction when histone is used as a substrate. Since the activity ratio decreased to 0.2 after dialysis, a part of the cAMP-dependent PK in epidermis may be in an activated form endogenously (Nemoto et al, in preparation).

The data presented on the activation of cAMP-dependent PK concurs with our previous studies on epidermal cAMP increases caused by various compounds including epinephrine: β-adrenergic, [15], histamine: H_2 [16], adenosine [17], PGE [13], IBMX [12] and ethanol [14]. Since the incubation conditions for the cAMP studies were the same as those used in the present study, these results can be directly compared. Much shorter incubation times are sufficient for full activation of PK by epinephrine, histamine or adenosine (30 to 45 seconds) (Fig 1, 3, 5) as compared with incubation periods necessary for maximal cAMP accumulation (5 min). This suggests that only a small increase in total cAMP level is enough for PK activation and that further increases may not be physiological. Refractoriness of epidermal adenylate cyclase, which follows 10 min exposure to epinephrine [11], was not seen with the PK activation after 30 seconds to 1 min stimulation (Fig 7*B*). Theophylline and $PGF_{2\alpha}$ which are less active than IBMX and PGE_2 in increasing epidermal cAMP gave equal responses with PK (Fig 7, 8). These findings indicate that notable changes in the total cAMP level do not necessarily correlate with the active state of PK, and further suggest that small increases in cAMP at small loci in close proximity to the enzyme molecule have physiological importance. These increases may not be detected as a significant rise in cAMP level in the tissue homogenate. A rise in activity ratio without a significant increase in cAMP amount has been reported in *in vivo* ACTH treated liver and adrenal tissue [18].

Recently, Palmer et al raised questions regarding the validity of the original experimental system [19]. According to their data, the extraction buffer cannot prevent the dissociation and concomitant activation of the kinase by endogenous cAMP in the homogenate. Our data with prostaglandins and phosphodiesterase inhibitors would argue against their claim. If cAMP dissociates PK significantly in the homogenate, then higher activity ratios should have been observed with 5 min incubation with PGE_2 or IBMX compared with 1 min incubation (Fig 7, 8), because the cAMP level is known to increase in the 5 min incubation period [12, 13]. $PGF_{2\alpha}$ and theophylline should have given lower activity ratios than PGE_2 and IBMX for the same reason. The discrepancy between the cAMP level and the PK activity ratio observed with prostaglandins and phosphodiesterase inhibitors support the validity of PK activity ratio, which reflects the intracellular activation of PK.

In conclusion, this experimental system can be successfully used to see the outline, if not the exact profile, of the activated state of cAMP-dependent protein kinase in epidermis, and can provide a more sensitive, more direct and more physiological way to study the effect of the compounds which act through a cAMP-PK mediated mechanism.

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