*Biochemical Pharmacology*, Vol. 30, No. 11, pp. 1325–1332, 1981.
Printed in Great Britain.

0006-2952/81/111325-08 $02.00/0


# ACCUMULATION OF ADENOSINE 3′,5′-MONOPHOSPHATE INDUCED BY PROSTAGLANDIN $E_1$ BINDING TO MASTOCYTOMA P-815 CELLS

KIMIO YATSUNAMI, ATSUSHI ICHIKAWA and KENKICHI TOMITA*

Department of Health Chemistry, Faculty of Pharmaceutical Sciences, Kyoto University, Yoshida, Sakyo-ku, Kyoto 606, Japan

(*Received* 11 *September* 1980; *accepted* 25 *November* 1980)

**Abstract**—Addition of prostaglandin $E_1$ ($PGE_1$) to intact mouse mastocytoma P-815 cells stimulated adenosine 3′, 5′-monophosphate (cAMP) accumulation and retarded cellular growth. To study the effects of prostaglandin (PG) binding on cAMP accumulation, specific [$^3$H]PG binding to intact mastocytoma cells was examined. Intact mastocytoma cells have two types of binding sites for $PGE_1$ with high ($K_d = 2.14 \times 10^{-9}$ M) and low ($K_d = 1.05 \times 10^{-8}$ M) affinities and one type of binding site for $PGF_{2\alpha}$. Mastocytoma cells, however, did not have a binding site for $PGA_1$ or $PGD_2$. The order of potencies of nonradioactive prostaglandins in competing with [$^3$H]$PGE_1$ for binding sites was as follows: $PGE_1 \geq PGE_2 >> PGF_{2\alpha} \geq PGA_1 \geq PGD_2 \geq PGI_2$. In contrast, the relative potencies of the prostaglandins in enhancing cAMP accumulation were $PGI_2 \geq PGE_1 \geq PGE_2 >> PGA_1 \geq PGF_{2\alpha} = PGD_2$, indicating that the receptors for E type and I type of PGs were different. Refractoriness of mastocytoma cells to $PGE_1$ stimulation of cAMP accumulation developed within 1 min of incubation of the cells at 37°, but disappeared immediately after decreasing the temperature to below 23°. A change in the number of $PGE_1$ receptors was not observed. cAMP concentrations were quickly restored by increasing temperatures from below 23° to 37°. This refractoriness did not develop in the presence of phosphodiesterase inhibitors. Furthermore, the activity of phosphodiesterase in mastocytoma cells was enhanced within 2 min by $PGE_1$ treatment.

Various effects of prostaglandins (PGs) on the regulation of growth, morphology, and functional differentiation of several cell lines in culture appear to be mediated through the cellular concentrations of adenosine 3′,5′-monophosphate (cAMP). To elucidate the biochemical mechanism of these effects, a number of investigators have examined the binding affinity of PGs to the receptor either in disrupted cell preparations of various tissues [1–8] or in intact cells [9]. Recent studies from this laboratory [10,11] and work by Davis and Ralph [12] have shown that mastocytoma P-815 cells respond to $N^6$, $O^{2'}$-dibutyryl cAMP ($Bt_2$ cAMP) and other compounds that elevate endogenous cAMP concentrations with a reduction in cellular growth rate and an increase in the synthesis of histamine and acidic glycosaminoglycans. The present study further examines some properties of the PG receptor and the interactions between PG binding and cAMP concentrations in intact mastocytoma P-815 cells. A preliminary report of this work has appeared [13].

## EXPERIMENTAL PROCEDURES

*Cells and cell culture.* Mastocytoma P-815 cells [14] were supplied by Dr. M. Potter, National Cancer Institute, NIH, Bethesda, MD, U.S.A., and maintained either in ascitic form in $DBF_1$ [(BALB/c × DBA/2)$F_1$] hybrid mice [15] or in suspension culture in Fischer-Sartorelli's medium supplemented with 5% calf serum (standard medium) [11] as described previously. The cell culture technique in 125-ml Erlenmeyer flasks in an atmosphere of 5% $CO_2$ and 95% air at 37° and the viability test were performed as described previously [11,15].

*Binding assays.* Suspensions of mastocytoma P-815 cells (2–4 × $10^6$ cells) in 75 μl of phosphate-buffered saline (PBS) (pH 7.4) as described by Dulbecco and Vogt [16] were preincubated at 37° for 10 min. Incubations were started by the addition of 25 μl of 1 nM [$^3$H]$PGE_1$ and were carried out for 60 min at 37°, except as noted; they were terminated by the addition of 2.5 ml of cold saline, and the samples were filtered by suction using HAWP 0.45 μm Millipore filters. The filters were then rinsed twice with 5 ml of cold saline, dried, and counted in a mixture of 2 ml of ethylene monomethyl ether and 8 ml of 0.5% 2,5-diphenyloxazole (PPO) in toluene. Nonspecific binding was defined as that binding which was not competed for by 2 μM $PGE_1$. The total radioligand binding minus nonspecific binding was interpreted as specific binding. Binding studies with [$^3$H]$PGA_1$, [$^3$H]$PGF_{2\alpha}$ and [$^3$H]$PGD_2$, each at a concentration of 1 nM, were conducted in a similar manner.

*Measurement of cAMP.* Suspensions of cells (2 × $10^6$) in 0.45 ml of Fischer–Sartorelli's medium were preincubated at 37° for 20 min in the presence or absence of 3-isobutyl-1-methylxanthine (MIX). After the addition of PGs or other drugs to be tested, the mixture in a final volume of 0.5 ml was further incubated at 37° for 10 min, unless otherwise stated. The reaction was terminated by the addition of 0.5 ml of cold 10% trichloroacetic acid, freeze-thawed once, and centrifuged at 500 *g* for 10 min.

* Author to whom correspondence should be addressed.

Table 1. Effect of $PGE_1$ on growth and cAMP content in mastocytoma P-815 cells*

| Addition | Cell growth | | | cAMP | | |
|---|---|---|---|---|---|---|
| | 0 hr | 14 hr | 23 hr | 0 hr | 14 hr | 23 hr |
| | | ($\times 10^5$ cells) | | | (pmoles/$10^6$ cells) | |
| None (control) | 1.8 | 3.8 | 4.8 | 1.0 | 1.0 | 1.0 |
| $PGE_1$ | | 3.1 | 4.3 | | 7.6 | 10.8 |
| $PGE_1$ + MIX | | 2.4 | 2.6 | | 840 | 1300 |

* Mastocytoma P-815 cells ($1.8 \times 10^5$ cells/ml, 30 ml) were incubated in the standard medium with or without 0.055 μM $PGE_1$ and 0.2 mM MIX for 14 and 24 hr at 37°.

Aliquots (0.5 ml) of the supernatant fraction were mixed with 0.5 ml of water, acidified with 0.1 ml of 1 N HCl, and then extracted five times with 2 vol. of water-saturated ether. Aqueous layers were lyophilized, and the residues were dissolved in 0.2 ml of 50 mM sodium acetate buffer (pH 6.2). cAMP in 50 μl aliquots was measured by radioimmunoassay methods of Okabayashi *et al.* [17].

*Assay of cAMP phosphodiesterase activity.* Mastocytoma P-815 cells were sonicated for 10 sec (Ultrasonicator, model 20, Ohtake Seisaku-sho, Tokyo, Japan), and the clear supernatant fraction, obtained by centrifugation at 10,000 *g* for 15 min, was used as the enzyme source. Phosphodiesterase activity was assayed by the method of Thompson and Appleman [18]. The assay mixture (0.4 ml), containing 0.4 μM [$^3$H]cAMP (30 μCi/μmole), 5 mM $MgCl_2$, 40 mM Tris–HCl buffer (pH 8.0) and the enzyme, was incubated at 37° for 10 min.

*Chemicals and reagents.* $PGE_1$, $PGE_2$, $PGA_1$, $PGI_2$ and $PGF_{2\alpha}$ were gifts from the Ono Pharmaceutical Co., Osaka, Japan, and $PGD_2$ was supplied by Dr. T. Shimizu, Department of Medical Chemistry, Faculty of Medicine, Kyoto University, Kyoto, Japan. Prostaglandins, except $PGI_2$, were dissolved (5 mg/ml) in dimethylsulfoxide (DMSO) and diluted with PBS prior to use. A final concentration of DMSO was kept to less than 0.04% (v/v). $PGI_2$ was dissolved (1 mg/ml) in ethanol and diluted with 0.01 M glycine buffer (pH 10.0) prior to use. MIX was obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. and [5,6-$^3$H(N)]$PGE_1$ (74.8 Ci/mmole), [5,6-$^3$H(N)]$PGA_1$ (87 Ci/mmole), [5,6,8,9,11,12,14,15-$^3$H(N)]$PGF_{2\alpha}$ (120 Ci/mmole), [5,6,8,9,12,14,15-$^3$H(N)]$PGD_2$ (100 Ci/mmole), and [ring-2-$^{14}$C]L-histidine (50 mCi/mmole) from Amersham-Searle, Amersham, England. Rabbit antiserum for 2′-succinyl cAMP conjugated with human serum albumin was supplied by Dr. T. Okabayashi, Shionogi Research Laboratory, Shionogi Pharmaceutical Co., Osaka, Japan. Other chemicals of reagent grade were obtained commercially.

## RESULTS

*Effects of prostaglandins on growth and cAMP content of mastocytoma P-815 cells.* As shown in Table 1, the relatively weak inhibitory effect of $PGE_1$ on the growth of mastocytoma P-815 cells was significantly potentiated in the presence of MIX. This effect was accompanied by a marked increase in the cellular content of cAMP from concentrations of less than 1 pmole/$10^6$ cells to 840 and 1300 pmole/$10^6$ cells in 14- and 23-hr incubations, corresponding to 60 pmoles of cAMP formed·($10^6$ cells)$^{-1}$·hr$^{-1}$ (Table 1).

*Time course of cAMP accumulation induced by $PGE_1$.* In the presence of 5.5 μM $PGE_1$, the intracellular concentration of cAMP increased rapidly to a maximum at 30 sec (inset in Fig. 1A) and declined sharply thereafter (Fig. 1A). Concentrations of

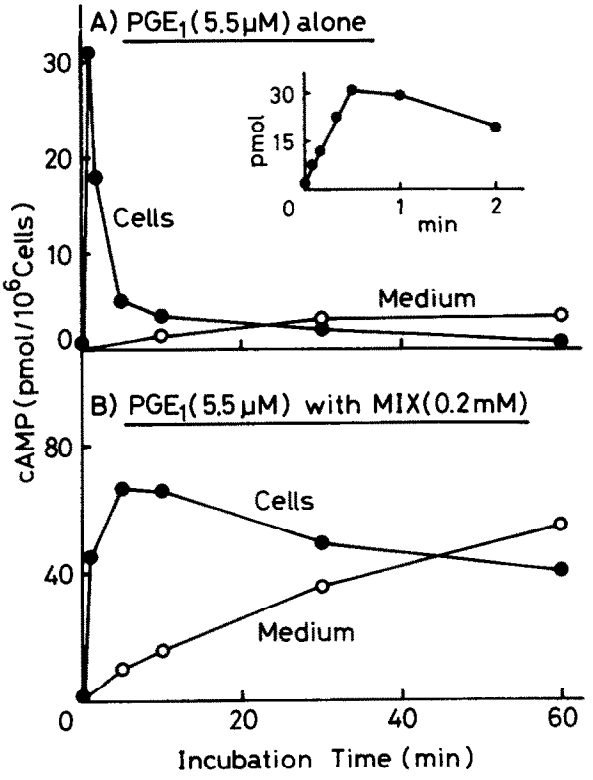


Fig. 1. Time course of intra- and extra cellular cyclic AMP accumulation in mastocytoma cells after the addition of $PGE_1$ in the absence (A) or presence (B) of MIX. Mastocytoma cells ($2 \times 10^6$ cells) were preincubated at 37° for 30 min in Fischer–Sartorelli's medium in the absence or presence of 0.2 mM MIX. Then 5.5 μM $PGE_1$ was added and the cells were incubated for up to 60 min. The intra- (●) and extracellular (○) cAMP contents were estimated as described under Experimental Procedures. Each value is the mean of duplicate determinations.

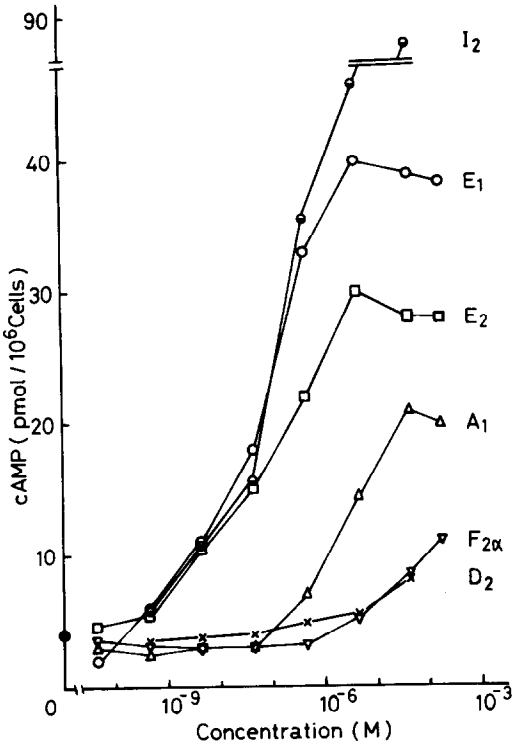


Fig. 2. Dose dependency of the effect of several PGs on cyclic AMP accumulation. Mastocytoma cells ($2 \times 10^6$ cells) were preincubated at 37° for 30 min in Fischer–Sartorelli's medium containing 0.2 mM MIX. Then $PGE_1$ (○), $PGE_2$ (□), $PGA_1$ (△), $PGF_{2\alpha}$ (▽), $PGD_2$ (x) and $PGI_2$ (◒) at various concentrations were added and the cells were incubated for another 10 min. Each value is the mean of duplicate determinations.

cAMP in cells pretreated with $PGE_1$ were not further increased with a second dose of $PGE_1$, even when the cells were washed with fresh medium at 37° to remove the first dose of $PGE_1$ (data not shown). The intracellular cAMP content reached a higher level and declined more slowly in the presence of 0.2 mM MIX than in its absence (Fig. 1B). Concomitantly, release of cAMP into the medium steadily increased and, after 60 min of incubation, the level of extracellular cAMP exceeded that of the the intracellular cAMP.

*Structure–function relationship.* Among several prostaglandin analogues tested, $PGI_2$ and the PGE group were most potent in elevating cAMP concentrations in mastocytoma P-815 cells (Fig. 2). Introduction of a $C_{5-6}$ double bond into the $PGE_1$ molecule ($PGE_2$) slightly reduced the maximum effect. Removal of the hydroxyl group at $C_{11}$ ($PGA_1$) markedly decreased the potency. Furthermore, $C_9$, $C_{11}$-dihydroxy- ($PGF_{2\alpha}$) and 9-hydroxy-$C_{11}$-keto analogues ($PGD_2$) were completely inactive in the concentration range from $10^{-9}$ M to $10^{-5}$ M. The order of potencies of PGs in elevating cAMP concentration was as follows: $PGI_2$, $\geq PGE_1 \geq PGE_2 > PGA_1 \geq PGD_2 = PGF_{2\alpha}$.

*Effect of phosphodiesterase inhibitors on cAMP concentrations.* As shown in Fig. 3, 2 mM MIX maximally potentiated the effect of $PGE_1$ to elevate cAMP concentration by a 39-fold increase over the $PGE_1$ control. The presence of 20 mM theophylline caused a 22-fold increase in cAMP concentration in response to $PGE_1$. In contrast, papaverine was much less effective. The decrease in potentiation of the $PGE_1$ effect on cAMP concentrations caused by pre-exposing the cells to higher doses of MIX (over 2 mM) and theophylline (over 20 mM) was reversed by reducing, respectively, the concentrations of these drugs to below 2 mM and 20 mM by the addition of the medium.

*Time course of* [$^3$H]*$PGE_1$ binding.* At 37° the specific binding of [$^3$H]$PGE_1$ to mastocytoma P-815 cells linearly increased during the first 20 min and reached equilibrium after approximately 45 min, with a half-maximum time of about 12 min, while the nonspecific binding reached equilibrium within 1 min (Fig. 4).

A Scatchard analysis [19] of total $PGE_1$ binding yielded evidence for the existence of at least two independent binding sites (Fig. 5). A high affinity site with $K_d = 2.14 \times 10^{-9}$ M was calculated to have a binding capacity of 12 fmoles/$10^6$ cells, corresponding to 7210 binding sites per cell. A low affinity site with $K_d = 1.05 \times 10^{-8}$ M had a binding capacity of 31 fmoles/$10^6$ cells corresponding to 18,600 sites per cell.

Similar analyses of [$^3$H]$PGF_{2\alpha}$, [$^3$H]$PGD_2$ and [$^3$H]$PGA_1$ binding studies showed that mastocytoma cells had one specific binding site for $PGF_{2\alpha}$ with $K_d = 1.1 \times 10^{-7}$ M, but no site for $PGA_1$ and $PGD_2$.

*Specificity of* [$^3$H]*$PGE_1$ binding.* With the exception of $PGI_2$, the relative potencies of several prostaglandins in displacing [$^3$H]$PGE_1$ from mastocytoma

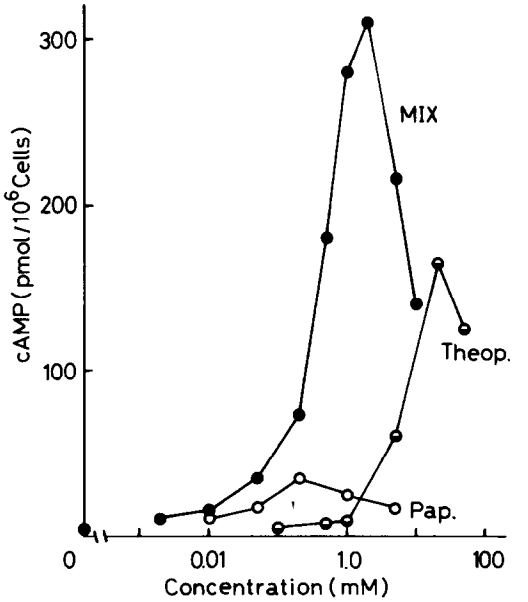


Fig. 3. Effects of several inhibitors of phosphodiesterase on the action of $PGE_1$ on cyclic AMP level. Mastocytoma cells ($2 \times 10^6$ cells) were preincubated at 37° for 30 min in the presence of MIX (●) or theophylline (◒) or papaverine (○) at various concentrations. Then the cells were treated with 5.5 μM $PGE_1$ for 10 min. Each value is the mean of duplicate determinations.

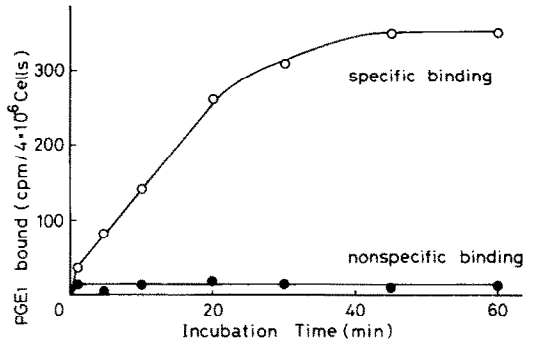


Fig. 4. Time course of [$^3$H]$PGE_1$ binding. Mastocytoma cells ($4 \times 10^6$ cells) were incubated at 37° for various intervals of time with PBS containing 1 nM [$^3$H]$PGE_1$ in the absence (○) or presence (●) of 2 $\mu$M unlabeled $PGE_1$. [$^3$H]$PGE_1$ bound to the cells was estimated as described under Experimental Procedures. The Millipore background values were subtracted. Each value is the mean of triplicate determinations.

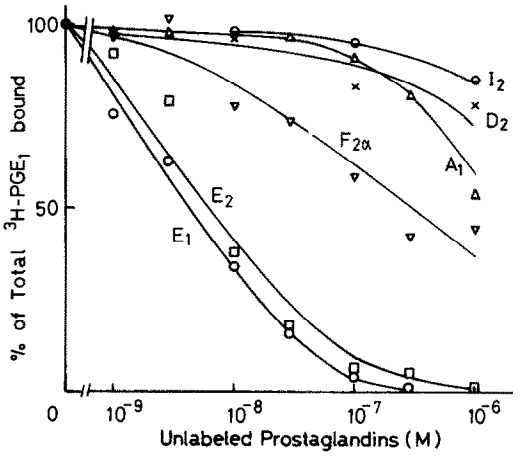


Fig. 6. Specificity of [$^3$H]$PGE_1$ binding. Mastocytoma cells ($4 \times 10^6$ cells) were incubated at 37° for 20 min with PBS containing 1 nM [$^3$H]$PGE_1$ and various amounts of unlabeled prostaglandins: $PGE_1$ (○), $PGE_2$ (□), $PGF_{2\alpha}$ (▽), $PGA_1$ (△), $PGD_2$ (x), and $PGI_2$ (◒). The results are expressed as a percentage of total radioactivity [920 cpm·$10^6$ cells$^{-1}$·hr = 100 per cent] bound in the absence of any competing compound. Each value is the mean of duplicate determinations.

P-815 cells were similar to the order of potencies for elevating cAMP concentrations: $PGE_1 \geq PGE_2 >> PGF_{2\alpha} \geq PGA_1 \geq PGD_2 \geq PGI_2$ (Fig. 6). Both $PGE_1$ and $PGE_2$ completely inhibited [$^3$H]$PGE_1$ binding at $1 \times 10^{-7}$ M. At this concentration, $PGF_{2\alpha}$ inhibited 40 per cent of [$^3$H]$PGE_1$ binding, while $PGA_1$, $PGD_2$ and $PGI_2$ suppressed less than 10 per cent.

*Time course of displacement of bound* [$^3$H]*$PGE_1$ by $PGE_1$.* After the incubation of mastocytoma P-815 cells with [$^3$H]$PGE_1$ (1 nM) for 10, 40 and 120 min at 37°, a large excess of $PGE_1$ was added and the incubation was continued as shown in Fig. 7A. The semilog plots of the remaining counts versus time indicated the presence of at least two kinetic components (rapid and slow) of dissociation. The size of the rapidly dissociating component with a half-time of approximately 3 min seemed to be inversely proportional to the time of preincubation, varying in the range from 70 per cent (10 min) to 40 per cent (120 min) of the original binding. A slowly dissociating component with a half time of about 70 min increased with the time of preincubation, reaching 60 per cent of the initial value after 2 hr of incubation. The dissociation at 0° was insignificant, compared with that at 37° (data not shown). Furthermore, the dissociation rate of a slowly dissociating component, but not of a rapidly dissociating component, increased with the incubation temperature (Fig. 7B).

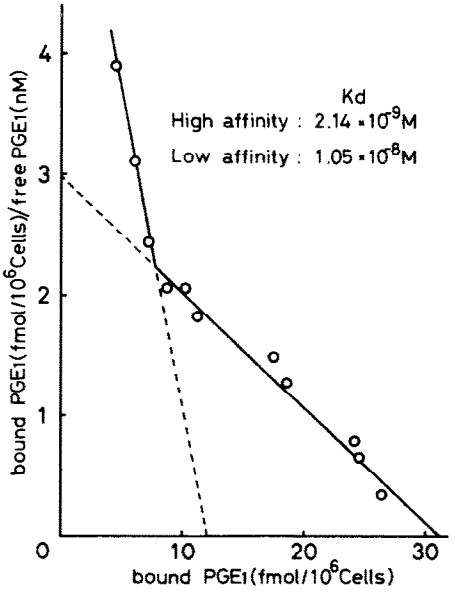


Fig. 5. Scatchard plot of [$^3$H]$PGE_1$ binding. Mastocytoma cells ($4 \times 10^6$ cells) were incubated at 37° for 20 min with PBS containing 1 nM [$^3$H]$PGE_1$ and 0–80 nM unlabeled $PGE_1$. Each value is the mean of triplicate determinations.

*Effect of temperature on* [$^3$H]*$PGE_1$ binding and cAMP concentrations.* The initial rate of [$^3$H]$PGE_1$ binding increased progressively with increasing temperature, 15° to 37°, but the maximum extent of the binding was apparently similar, although the time required to reach the maximum level was different (Fig. 8A).

The cellular accumulation of cAMP was also temperature-dependent. At 15°, cAMP concentrations increased progressively for 60 min. At higher temperatures, the rapid initial accumulation of cellular cAMP was followed by a slow (at 23°), fast (at 30°) and sharp decline (at 37°) (Fig. 8B).

*Effect of changes in temperature on* [$^3$H]*$PGE_1$ binding and cAMP.* As shown in Fig. 9, when the incubation temperature was reduced from 37° to 0° at any given time after the exposure of cells to $PGE_1$ in the absence of MIX, the total (intracellular plus extracellular) cAMP concentrations sharply declined toward the basal level. cAMP concentrations in control cells incubated in the absence of $PGE_1$ also decreased significantly when the temperature was

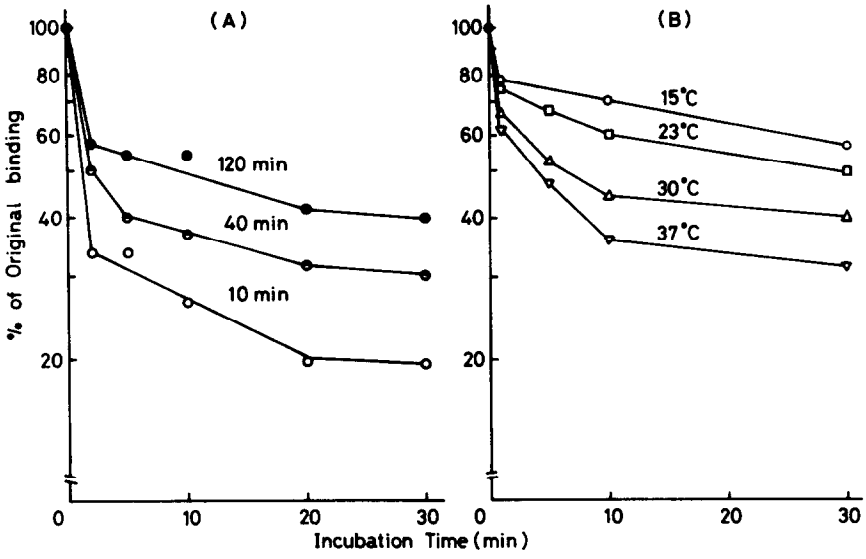


Fig. 7. (A) Time course of dissociation of bound [$^3$H]$PGE_1$. Mastocytoma cells ($4 \times 10^6$ cells) were incubated at 37° for 10 min (○), 40 min (◓) or 120 min (●) with PBS containing 1 nM [$^3$H]$PGE_1$. Then, 2 μM unlabeled $PGE_1$ was added, and the cells were incubated for up to 30 min. (B) Temperature dependency of dissociation of bound [$^3$H]$PGE_1$. Mastocytoma cells ($4 \times 10^6$ cells) were preincubated with PBS containing 1 nM [$^3$H]$PGE_1$ for 60 min at 37°. Then after adding 2 μM unlabeled $PGE_1$ they were incubated for up to 30 min at the indicated temperature: 15° (○), 23° (□), 30° (△), and 37° (▽). Each value is the mean of duplicate determinations. Binding data are expressed as a percentage of the original specific binding prior to addition of 2 μM unlabeled $PGE_1$. Each point is a mean of triplicate determinations.

reduced from 37° to 0° (data not shown). When the temperature was increased from 0° to 37°, cAMP concentrations immediately increased within a minute to the same extent regardless of the length of time after the initial exposure to $PGE_1$ (Fig. 9B). In a parallel experiment, it was found that cAMP accumulation was restored when the temperature was shifted from 37° to below 23° and subsequently re-elevated. The decline of cAMP concentration as the temperature was lowered from 37° to 0° was less sharp in the presence of 0.2 mM MIX than in the absence of MIX. However, the restoration of cAMP concentrations by shifting temperature from 0° to 37° was still observed.

In contrast, $PGE_1$ binding did not decrease but remained constant, after lowering the temperature from 37° to 0°, and it increased again after raising the temperature to 37° (Fig. 9A).

*Effect of MIX on refractoriness.* As shown in Fig. 10, MIX not only potentiated the effect of $PGE_1$ to increase cAMP concentrations, but it also abolished the refractoriness of cells to $PGE_1$ to stimulate cAMP formation. The addition of 0.2 mM MIX immediately re-elevated cAMP concentrations to levels the same as were attained by the initial addition of $PGE_1$ plus MIX regardless of whether MIX was added 10 or 60 min after the exposure of the cells to $PGE_1$.

*Effect of $PGE_1$ on cAMP phosphodiesterase activity.* As shown in Fig. 11, $PGE_1$ rapidly increased the activity of cAMP phosphodiesterase in mastocytoma P-815 cells within 2 min of incubation. The increase in enzyme activity was maintained for about 20 min.

## DISCUSSION

The present data show that mastocytoma P-815 cells have at least two specific binding sites for the E type of prostaglandins, and that the addition of prostaglandins causes an increase in cAMP formation within the cells. Although $PGI_2$ was more effective than the PGE group in stimulating cAMP formation in mastocytoma P-815 cells (Fig. 2), it only slightly replaced the bound [$^3$H]$PGE_1$ (Fig. 6), indicating that the receptor for the PGE group is different from that for $PGI_2$ (Fig. 6). $PGD_2$ is known to be a major cyclooxygenase product and to cause increased cAMP formation in normal rat mast cells [20], but it was ineffective in increasing cAMP concentrations (Fig. 2) and in replacing bound [$^3$H]$PGE_1$ in mastocytoma P-815 cells (Fig. 6). The difference in the responses of mast cells to $PGI_2$ and $PGD_2$ might be due to the altered surface receptor reaction in neoplastic mast cells. The fact that exogenously added $PGI_2$ (or $PGD_2$) stimulated increases in cellular cAMP concentrations suggests the participation of these prostaglandins in the regulation of physiological functions of mastocytoma cells or mast cells. The $K_d$ value for $PGF_{2\alpha}$ ($1.1 \times 10^{-7}$ M) was similar to that for the low affinity site of $PGE_1$, and $PGF_{2\alpha}$ inhibited 40 per cent of [$^3$H]$PGE_1$ binding (Fig. 6). $PGF_{2\alpha}$, however, did not stimulate cAMP accumulation at all (Fig. 2), indicating that the binding of $PGF_{2\alpha}$ to its specific receptor did not induce activation of adenylate cyclase. As shown in cultured mouse fibroblasts [21], $PGF_{2\alpha}$ receptors may couple to guanylate cyclase. Although the existence of two

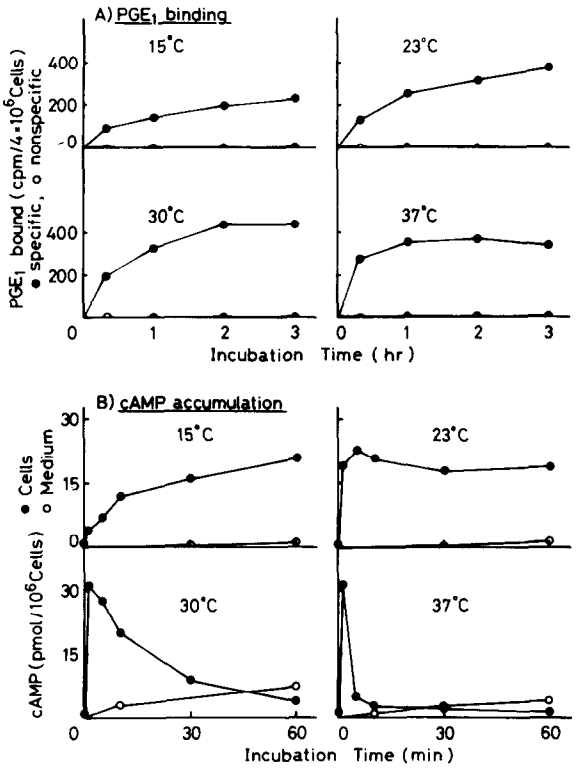


Fig. 8. Temperature dependency of [$^3$H]$PGE_1$ binding and $PGE_1$ response on cAMP accumulation. (A) Mastocytoma cells ($4 \times 10^6$ cells) were incubated with PBS containing 1 nM [$^3$H]$PGE_1$ for up to 3 hr at various temperatures. Specific (●) and nonspecific bindings (○) of [$^3$H]$PGE_1$ were estimated as described under Experimental Procedures. (B) Mastocytoma cells ($2 \times 10^6$ cells) in 0.5 ml of Fischer–Sartorelli's medium were preincubated for 30 min at different temperatures. Then, after adding 5.5 $\mu$M $PGE_1$, they were incubated for up to 60 min. Intra- (●) and extracellular (○) cyclic AMP contents were estimated as described under Experimental Procedures. Each value is the mean of duplicate determinations.

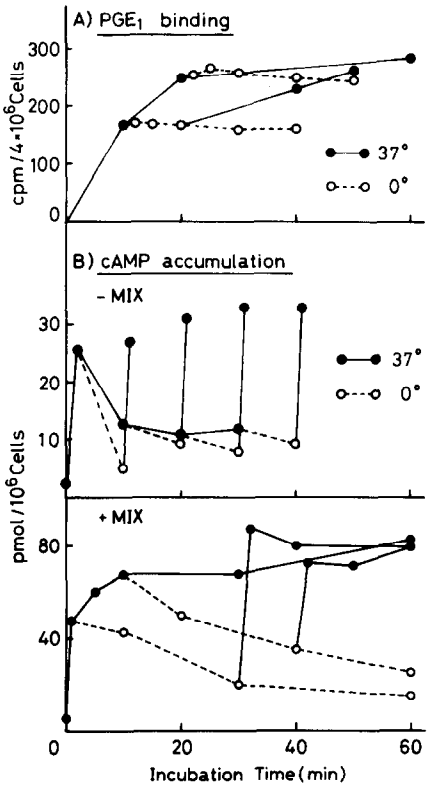


Fig. 9. (A) Effect of temperature shift on [$^3$H]$PGE_1$ binding. Mastocytoma cells ($4 \times 10^6$ cells) were incubated at 37° for various intervals of time in PBS containing 1 nM [$^3$H]$PGE_1$. In parallel experiments, incubation temperature was changed to 0° from 37° (○---○), and then returned to 37° (●——●) at the time points indicated. Each value is the mean of triplicate determinations. (B) Effect of temperature shift on cyclic AMP accumulation by $PGE_1$. Mastocytoma cells ($2 \times 10^6$ cells) were preincubated at 37° for 30 min in 0.5 ml of Fischer–Sartorelli's medium in the absence (−MIX) or presence (+MIX) of 0.2 mM MIX prior to addition of 5.5 $\mu$M $PGE_1$ for up to 60 min. In parallel experiments, incubation temperature was changed to 0° from 37° (○---○), and then returned to 37° (●——●) at the time points indicated.

such heterogenous binding sites for $PGE_1$ (Fig. 5) has been reported in various membrane preparations from other tissues and cells [3–7,22,23], the physiological role of these two binding sites has not been fully clarified. The apparent $K_d$ value of the high affinity binding site ($2.14 \times 10^{-9}$ M in Fig. 5) was similar to the concentration of $PGE_1$ ($5.5 \times 10^{-9}$ M in Fig. 2) required to activate adenylate cyclase in mastocytoma P-815 cells. These data suggest that the high affinity binding site may be associated with the biological effects of $PGE_1$. One possible explanation for two sites with different $K_d$ values could be the localization of the receptors, one being membranous and the other intracellular, as reported for lysosomes of bovine corpora lutea [24]. Therefore, the temperature dependency in the dissociation of the slowly dissociating component (Fig. 7B) may indicate a requirement for energy metabolism in the internalization of the prostaglandin receptor.

There was a striking difference between the time course of $PGE_1$ binding to the cells and that of cAMP accumulation in the absence of MIX. The former

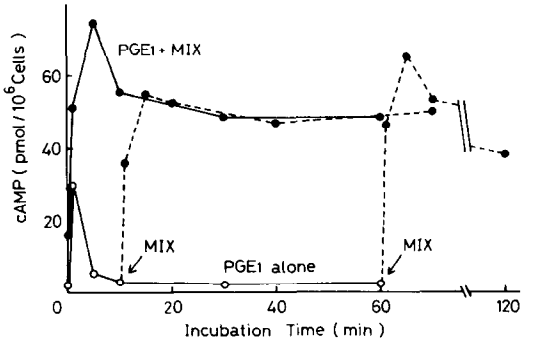


Fig. 10. Effect of MIX on refractoriness induced by $PGE_1$. Mastocytoma cells ($2 \times 10^6$ cells) were preincubated at 37° for 30 min in the absence (○) or presence (●) of 0.2 mM MIX prior to addition of 5.5 $\mu$M $PGE_1$. Incubation proceeded for an additional 60 min. In parallel experiments, after the pretreatment with 5.5 $\mu$M $PGE_1$ for 10 min or 60 min, the cells were treated with 0.2 mM MIX and incubated another 60 min. Each value is the mean of duplicate determinations.

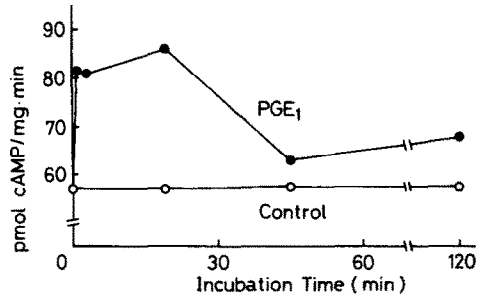


Fig. 11. Effect of $PGE_1$ on phosphodiesterase activity from mastocytoma P-815 cells. Mastocytoma cells ($2.5 \times 10^7$ cells) were incubated with 0.55 $\mu$M $PGE_1$ at 37° for up to 120 min. At the indicated time, cells were quickly washed with cold PBS, sonicated, and assayed for phosphodiesterase activity by the method described under Experimental Procedures. Each value is the mean of duplicate determinations.

increased gradually with a half-time of approximately 12 min at 37° (Fig. 4), whereas the latter rapidly reached the maximum level within 30 sec and decreased sharply thereafter. A similar refractoriness or desensitization of cAMP synthesis to various stimulators is also known in fat cells [25], WI-38 fibroblasts [26,27], macrophages [28], glioma cells [29], astrocytoma cells [30], Walker carcinoma cells [31], and skin [32].

A refractoriness may be mediated by the action of phosphodiesterase in mastocytoma P-815 cells, since the refractoriness was abolished by the addition of MIX (Fig. 10) and since $PGE_1$ stimulated phosphodiesterase within 2 min (Fig. 11) [25,31,33]. The content of total cAMP (cells plus medium) in mastocytoma P-815 cells after 14 hr and 23 hr of incubation with $PGE_1$ plus MIX (Table 1) was 840 and 1300 pmoles/$10^6$ cells, respectively, corresponding to a rate of synthesis of cAMP of 60 pmoles·$(10^6$ cells$)^{-1}\cdot hr^{-1}$. This rate was similar to the maximum rate caused by a single treatment of the cells with $PGE_1$ plus MIX (Fig. 1B). In addition to the inhibition of phosphodiesterase activity, the data shown in Fig. 9 indicate that the temperature shift from 37° to below 20° was effective in abolishing the refractoriness to $PGE_1$ of cell cAMP synthesis, even though [$^3$H]$PGE_1$ binding remained unaltered (Fig. 9A). Numerous studies have indicated an important role of phospholipid metabolism, especially phosphatidylethanolamine, in membrane functions such as $\beta$-adrenergic receptor availability [34,35] and adenylate cyclase activities in various types of mammalian cells [36–38]. The regulatory effect of changes in membranous fluidity, as a result of altered lipid metabolism, on the activity of glucagon-stimulated adenylate cyclase from rat liver has also been discussed [39]. In rat mast cells, the synthesis of phosphatidylcholine, measured by $^{32}$P-labeling during mediator release, was virtually abolished by decreasing the temperature to 20° or lower [40]. Therefore, lowering the temperature might impair the activation of phosphodiesterase by $PGE_1$, or the coupling of $PGE_1$ receptor with adenylate cyclase.

The possibility of impairment of adenylate cyclase itself by low temperatures might be ruled out by the gradual increase of cAMP observed at 15° with time (Fig. 8), but the mechanism seems to be very complex since the temperature can affect many factors such as the lipid environment [41–44].

Since the refractoriness is often hormone- or receptor-specific [45] and is accompanied by a decline in the number of functionally active ligand binding sites [22,46], the present results cannot exclude the possibility that the refractoriness is induced by the desensitization of hormone receptors, even though the binding of [$^3$H]$PGE_1$ was unaffected (Fig. 9A). Further studies on the precise manner in which the $PGE_1$ receptor interacts with adenylate cyclase will require the isolation and characterization of $PGE_1$ receptors.

*Acknowledgements*—We are grateful to N. M, Kneer for critical review of this manuscript and to Y. Ueha for secretarial assistance in the preparation of this manuscript. This work was supported, in part, by grants from the Ministry of Education, Science and Culture of Japan.

## REFERENCES

1. F. A. Kuehl, Jr. and J. L. Humes, *Proc. natn. Acad. Sci. U.S.A.* **69**, 480 (1972).
2. W. V. Moore and J. Wolff, *J. biol. Chem.* **248**, 5705 (1973).
3. R. R. Gorman and O. V. Miller, *Biochim. biophys. Acta* **323**, 560 (1973).
4. M. Smigel and S. Fleischer, *Biochim. biophys. Acta* **332**, 358 (1974).
5. C. V. Rao, *J. biol. Chem.* **249**, 7203 (1974).
6. L. L. Brunton, R. A. Wiklund, P. M. V. Arsdale and A. G. Gilman, *J. biol. Chem.* **251**, 3037 (1976).
7. N. Okamura and H. Terayama, *Biochim. biophys. Acta* **465**, 54 (1977).
8. M. T. Lin and V. Rao, *Biochem. biophys. Res. Commun.* **78**, 510 (1977).
9. B. P. Schauburg, *Biochim. biophys. Acta* **326**, 127 (1973).
10. K. Tomita, A. Ichikawa and K. Esumi, *Fedn. Proc.* **33**, 1392 (1974).
11. A. Ichikawa, K. Esumi, M. Takagi, K. Yatsunami, M. Negishi, K. Yokoyama and K. Tomita, *J. Pharmacobio. Dyn.* **3**, 123 (1980).
12. J. Davis and R. K. Ralph, *Cancer Res.* **35**, 1495 (1975).
13. K. Tomita, K. Yatsunami and A. Ichikawa, *Fedn. Proc.* **38**, 407 (1979).
14. T. B. Dunn and M. Potter, *J. natn. Cancer Inst.* **18**, 587 (1957).
15. A. Ichikawa, H. Kaneko, Y. Mori and K. Tomita, *Biochem. Pharmac.* **26**, 187 (1977).
16. R. Dulbecco and M. Vogt, *J. exp. Med.* **99**, 167 (1954).
17. T. Okabayashi, S. Mihara, M. Nakamura, A. Tanaka and F. Sagawa, *Hakko to Taisha [Amino Acid and Nucleic acid (in Japanese)]*, **24**, 28 (1971).
18. W. J. Thompson and M. M. Appleman, *Biochemistry* **10**, 311 (1971).
19. G. Scatchard, *Ann. N.Y. Acad. Sci.* **51**, 660 (1949).
20. L. J. Roberts, R. A. Levis, R. Hansbrough, K. F. Austen and J. A. Oates, *Biochim. biophys. Acta* **575**, 185 (1979).
21. L. J. Asua, D. Clingan and P. S. Rudland, *Proc. natn. Acad. Sci. U.S.A.* **72**, 2724 (1975).
22. R. J. Lefkowitz, D. Mullikin, C. L. Wood, T. B. Gore and C. Mukherjee, *J. biol. Chem.* **252**, 5295 (1977).
23. L. L. Brunton, M. E. Maguire, H. J. Anderson and A. G. Gilman, *J. biol. Chem.* **252**, 1293 (1977).
24. S. Mirata and C. V. Rao, *Archs. Biochem. Biophys.* **185**, 126 (1978).

25. L. G. Paulson, C. J. Lovell-Smith, V. C. Manganiello and M. Vaughan, *Proc. natn. Acad. Sci. U.S.A.* **71**, 1639 (1974).
26. L. A. Kelly and R. W. Butcher, *J. biol. Chem.* **249**, 3098 (1974).
27. L. A. Kelly, M. S. Hall and R. W. Butcher, *J. biol. Chem.* **249**, 5182 (1974).
28. D. Gemsa, L. Steggemann, J. Menzel and G. Till, *J. Immun.* **114**, 1422 (1975).
29. B. J. Doore, M. M. Bashor, N. Spitzer, R. C. Mawe and M. H. Saier, *J. biol. Chem.* **250**, 4371 (1975).
30. R. Ortmann and J. P. Perkins, *J. biol. Chem.* **252**, 6018 (1977).
31. Y. S. Cho-Chung and S. F. Newcomer, *Cancer Res.* **37**, 4493 (1977).
32. K. Adachi, H. Iizuka, K. M. Halprin and V. Levine, *Biochim. biophys. Acta* **497**, 428 (1977).
33. M. Armiento, G. S. Johnson and I. Pastan, *Proc. natn. Acad. Sci. U.S.A.* **69**, 459 (1972).
34. F. Hirata, W. J. Strittmatter and J. Axelrod, *Proc. natn. Acad. Sci. U.S.A.* **76**, 368 (1979).
35. W. J. Strittmatter, F. Hirata and J. Axelrod, *Science* **204**, 1205 (1979).
36. S. L. Pohl, H. M. J. Krans, V. Kozyreff, L. Birnbaumer and M. Rodbell *J. biol. Chem.* **246**, 4447 (1971).
37. S. Gidwitz, J. E. Pessin, M. J. Weber, M. Glaser and D. R. Storm, *Biochim. biophys. Acta* **628**, 263 (1980).
38. V. H. Engelhard, M. Glaser and D. R. Storm, *Biochemistry* **17**, 3191 (1978).
39. I. Dipple and M. D. Houslay, *Biochem. J.* **174**, 179 (1978).
40. D. A. Kennerly, T. J. Sullivan and C. W. Parker, *J. Immun.* **122**, 152 (1979).
41. M. D. Houslay, T. R. Hesketh, G. A. Smith, G. B. Warren and J. C. Metcalfe, *Biochim. biophys. Acta* **436**, 495 (1976).
42. S. R. Ayad and S. L. Morgan, *Expl. Cell Res.* **109**, 9 (1977).
43. S. R. Ayad and S. J. Foster, *Expl. Cell Res.* **109**, 87 (1977).
44. J. A. Pliego and B. Rubalcava, *Biochem. biophys. Res. Commun.* **80**, 609 (1978).
45. J. P. Perkins, M. M. Moore, A. Kalisker and Y-F. Su, in *Advances in Cyclic Nucleotide Research* (Eds. G. I. Drummond, P. Greengard and G. A. Robison), Vol. 5, pp. 641–660. Raven Press, New York (1975).
46. C. Mukherjee and R. J. Lefkowitz, *Proc. natn. Acad. Sci. U.S.A.* **73**, 1494 (1976).