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MODULATION OF ADENOSINE 3′, 5′-MONOPHOSPHATE CONTENTS OF RAT PERITONEAL MACROPHAGES MEDIATED BY β_2-ADRENERGIC RECEPTORS

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Abstract—The modulation of cAMP contents of rat peritoneal macrophages by β-adrenergic stimulants and blocking agents were studied. The maximum increase of cAMP contents induced by isoproterenol, epinephrine, norepinephrine and hexoprenaline (a selective β_2-adrenergic stimulant) was about 170 ~ 200 per cent and about 100 per cent by salbutamol (a selective β_2-adrenergic stimulant) above the basal level. The activity of phenylepherine, a α-adrenergic stimulant, was very weak. The concentration giving a half maximum stimulation was as follows: isoproterenol; 6.3×10^{-8} M, hexoprenaline; 8.9×10^{-8} M, salbutamol; 3×10^{-7} M, epinephrine; 5.6×10^{-7} M, and norepinephrine; 5.6×10^{-6} M. Taking propranolol as a standard for comparison, antagonists of the isoproterenol induced increase in cAMP in macrophages ranged in their minimum effective concentrations as follows: bufetolol; 1. metoprolol (a selective β_1-adrenergic blocking agent); 1000, practolol (a selective β_1-adrenergic blocking agent); >1000, while in rat hearts bufetolol; 1, metoprolol and practolol; 100. The increase of cAMP by 10^{-5} M epinephrine or 10^{-5} M hexoprenaline in rat peritoneal macrophages was blocked by bufetolol at a concentration of 10^{-7} M or 10^{-6} M, but not by practolol at a concentration of 10^{-4} M. Phentolamine, a α-adrenergic blocking agent, showed no antagonistic activity against isoproterenol in rat peritoneal macrophages.

These observations suggest that the increases in cAMP in rat peritoneal macrophages by catecholamines are mediated by β_2-adrenergic receptors.

Numerous studies suggest that β-adrenergic receptors are linked to adenylate cyclase and that the increase of intracellular adenosine 3′,5′-monophosphate (cAMP) contents is one of the early steps of the sequence initiated by β-adrenergic stimulus [1].

Recently β-adrenergic receptors were classified into two broad subclasses of β_1 and β_2 according to pharmacological data obtained with intact tissues [2]. Receptors found in the heart and adipose tissue are among β_1-type [2], whereas those in the skeletal muscle, liver and trachea are among β_2-type receptor [2, 3]. Biochemical studies demonstrated that the selectivity of β-adrenergic stimulants or blocking agents, observed in pharmacological intact models, is reflected at cAMP system by the similar selectivity [4–7]. Thus it is possible to suppose the type of β-adrenergic receptors of tissue by the effect of β-adrenergic stimulants or blocking agents on cAMP system.

Macrophages play an important role in host defense against infection of certain bacteria and viruses, and in immunity [8–10]. cAMP can modify the function of macrophages such as the release of lysosomal enzymes accompanying with phagocytosis of undigestible particles [11]. Though it was demonstrated that catecholamines could stimulate the adenylate cyclase of macrophages [12], the type of β-adrenergic receptors have not been mentioned.

In the present report, studies were undertaken to identify the types of β-adrenergic receptors which mediates the increase of cAMP by catecholamines in rat peritoneal macrophages.

MATERIALS AND METHODS

Chemicals. 1-Epinephrine bitartrate, DL-isoproterenol hydrochloride, 1-norepinephrine hydrochloride and 1-phenylepherine hydrochloride (Sigma Chemical Co.), phentolamine (Regitine) (Ciba Products Ltd.), liquid paraffin (Iwai Kagaku Co. Ltd.), medium 199 and Hanks' balanced salt solution (Nissui Seiyaku Co. Ltd.), Hepes buffer and calf serum (Flow Laboratories), adenosine 3′,5′-monophosphoric acid (cAMP) (Sigma Chemical Co.), and cAMP assay kit (The Radiochemical Centre, Amersham) (catalogue no.; TRK-432) were used. Other reagents guaranteed were commercially purchased. Bufetolol, propanolol, practolol, metoprolol, hexoprenaline and salbutamol were also used. Test compounds were dissolved in redistilled water immediately before use.

Animals. Donryu strain male rats weighing 150–180 g (Nippon Rat Co.) were used.

Harvest of macrophages. Rats were injected intraperitoneally with 3 ml of liquid paraffin. Four days later the animals were injected intraperitoneally with 15 ml of Hanks' balanced salt solution and the peritoneal cell suspensions were collected. Monolayers of macrophages were prepared from the cell suspensions by the method of Nathan *et al.* [13] with slight modifications. The peritoneal cells were washed twice in cold Hanks' balanced salt solution, and suspended at 2×10^6 cells per ml in medium 199 containing 20% calf serum. Five ml of aliquots of cell suspensions was dispersed into a culture tube (36 cm^2 surface area). After the incubation at 37° for 60 min, the

medium in the tube was withdrawn and the culture was rinsed 5 times with each 5 ml of Hanks' balanced salt solution. Then the adherent cells were detached by a policeman. The cells were suspended at $2 \sim 3 \times 10^6$ cells per ml in medium 199 with 10 mM Hepes buffer (pH 7.4) not containing calf serum and were used as macrophages for the assay of cAMP.

cAMP assay. To 1 ml of cell suspension in a tube, 10 μl of a test solution was added and the cells were incubated at 37° for 2 min under 5% CO_2 in air. After the incubation, the tube was centrifuged at 1200 g for 30 sec and the medium was removed by suction. To the cell pellet 0.1 ml of ice-cold 5% trichloroacetic acid (TCA) was added and the cells were destroyed by agitating with a Vortex mixer and by following with 2 cycles of freezing and thawing. After the centrifugation at 1200 g for 10 min, 75 μl of the supernatant was transfered into a tube and TCA in the supernatant was removed 5 times with each 0.5 ml of water-saturated ether. The aqueous layer was boiled for 3 min to evaporate ether. The concentrations of cAMP of the extract was determined using the cAMP assay kit based on the principle of the competitive binding assay. The standard curve of cAMP was constructed with known amount of cAMP as described above.

Hearts. The concentration of cAMP in slices of rat hearts was determined as reported previously [14]. Rats were sacrificed by blowing on the neck and the hearts were quickly removed. The left ventricular muscle was divided into three segments. The segments were incubated at 37° for 30 min in Krebs–Henseleit buffer [15] containing 10 mM glucose bubbling a gas mixture of 95% O_2 and 5% CO_2. After the incubation, the medium was replaced by the fresh buffer containing 10 mM glucose and 10 mM theophylline, and the segments were incubated for 5 min. Then a test solution was added to the segments and further incubated at 37° for 5 min. At the end of the incubation, the segments were frozen with dry ice–acetone and homogenized in 5% TCA. The homogenates were centrifuged at 1200 g for 15 min and 0.2 ml of aliquots of the supernatant were transfered into tubes. After removing TCA in the supernatant, the concentration of cAMP was determined as described above.

RESULTS

Increase in cAMP in rat peritoneal macrophages by α and β-adrenergic stimulants. Figure 1 shows the time course of the increase in cAMP in rat peritoneal macrophages by 10^{-5} M isoproterenol. As shown in Fig. 1, the increase in cAMP by isoproterenol reached a maximum at 2 min and returned to near a basal level at 5 min. In the following experiments, the drugs were incubated with cells for 2 min.

Figure 2 shows the increase in cAMP in rat peritoneal macrophages by α and β-adrenergic stimulants including hexoprenaline and salbutamol, which are known to stimulate selectively β_2-adrenergic receptors. The maximum stimulation of epinephrine, norepinephrine and hexoprenaline was about 200 per cent above the basal level, which was slightly higher than that of isoproterenol. Salbutamol produced about a half of the maximum stimulation of epinephrine. The concentration giving a half maximum

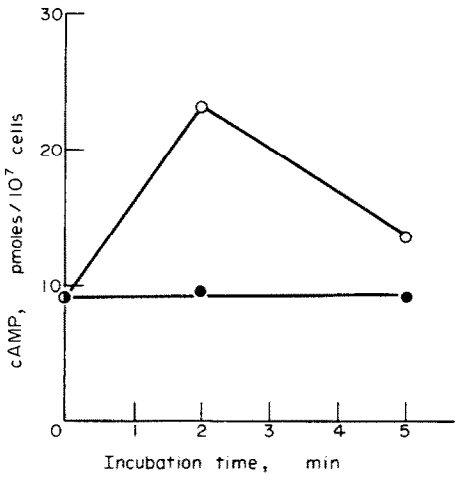

Fig. 1. Time course of 10^{-5} M isoproterenol stimulation of rat peritoneal macrophages cAMP. The drug was incubated with cells for indicated time at 37°. Each point represents the average value of triplicate determinations. (●) Control, (○) Isoproterenol.

stimulation (PC_{50}) obtained graphically was as follows: isoproterenol; 6.3×10^{-8} M, hexoprenaline; 8.9×10^{-8} M, salbutamol; 3×10^{-7} M, epinephrine; 5.6×10^{-7} M and norepinephrine; 5.6×10^{-6} M. The effect of phenylepherine, a α-adrenergic stimulant, was small.

Effects of α and β-adrenergic blocking agents on the increase in cAMP in rat peritoneal macrophages by β-adrenergic stimulants. A blocker and a stimulant were simultaneously added to the cell suspension and were incubated at 37° for 2 min. Propranolol and bufetolol at a concentration of 10^{-7} M or more significantly inhibited the increase of cAMP by 10^{-5} M isoproterenol as shown in Table 1. The effects of practolol and metoprolol, selective β_1-adrenergic blocking agents, were very weak compared with propranolol and bufetolol. Practolol showed no inhibition even at a concentration of 10^{-4} M. Phentolamine, a α-adrenergic blocking agent, at a concentration of

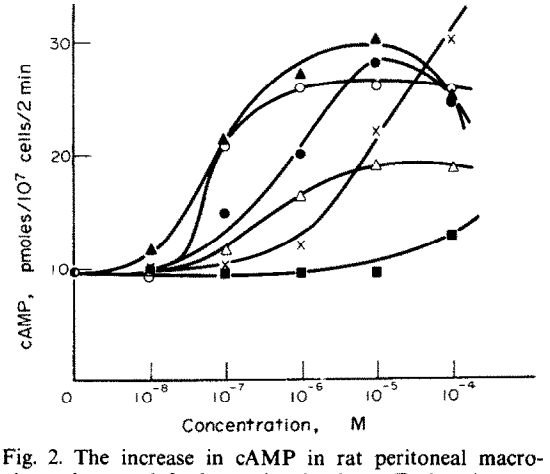

Fig. 2. The increase in cAMP in rat peritoneal macrophages by α and β-adrenergic stimulants. Each point represents the average value of triplicate determinations. (○) Isoproterenol, (●) Epinephrine, (×) Norepinephrine, (▲) Hexoprenaline, (△) Salbutamol, (■) Phenylepherine.

Table 1. Effect of α and β-adrenergic blocking agents on the increase in cAMP in rat peritoneal macrophages by isoproterenol

Compound	Concentration of antagonist (M)	cAMP pmoles/10^7 cells/2 min
Control—no drug		8.9 ± 0.6†
Isoproterenol 10^{-5} M		25.4 ± 0.5
Isoproterenol 10^{-5} M	10^{-8}	24.7 ± 0.9
+ bufetolol	10^{-7}	21.5 ± 0.1*
	10^{-6}	12.0 ± 0.5*
	10^{-5}	10.8 ± 0.5*
Isoproterenol 10^{-5} M	10^{-8}	26.3 ± 0.2
+ propranolol	10^{-7}	20.0 ± 0.2*
	10^{-6}	13.0 ± 0.1*
	10^{-5}	11.6 ± 0.2*
Control—no drug		7.4 ± 0.3
Isoproterenol 10^{-5} M		21.4 ± 0.1
Isoproterenol 10^{-5} M	10^{-7}	22.4 ± 0.6
+ practolol	10^{-6}	24.2 ± 0.4*
	10^{-5}	23.3 ± 1.0
	10^{-4}	25.2 ± 0.7*
Isoproterenol 10^{-5} M	10^{-7}	23.6 ± 0.3*
+ metoprolol	10^{-6}	23.5 ± 0.2*
	10^{-5}	21.1 ± 0.5
	10^{-4}	12.7 ± 0.7*
Control—no drug		6.0 ± 0.2
Isoproterenol 10^{-5} M		17.9 ± 0.1
Isoproterenol 10^{-5} M		
+ phentolamine	10^{-5}	17.8 ± 0.3

Drugs were incubated with macrophages ($2.5 \sim 3 \times 10^6$ per ml) at 37° for 2 min.

* Statistical significance from isoproterenol alone ($P < 0.05$).

† Mean ± S.E. ($n = 3 \sim 4$).

10^{-5} M showed no inhibition. The inhibitory effects of bufetolol and practolol on the increase in cAMP in rat peritoneal macrophages by 10^{-5} M epinephrine or hexoprenaline are shown in Table 2. Bufetolol showed inhibitory effects at a concentration of 10^{-7} M against epinephrine, and at 10^{-6} M against hexoprenaline, whereas practolol did not show any inhibition up to a concentration of 10^{-4} M against both stimulants.

Effects of β-adrenergic blocking agents on the increase in cAMP in rat hearts by isoproterenol. The drugs were incubated at 37° for 5 min with the segments of rat left ventricular muscle in the presence of 10 mM theophylline. Results are shown in Table 3. Propranolol and bufetolol at a concentration of 10^{-7} M significantly inhibited the increase in cAMP by 5×10^{-7} M isoproterenol. Practolol and metoprolol inhibited it at a 100 times higher concentration than that of propranolol or bufetolol.

DISCUSSION

The contents of cAMP of rat peritoneal macrophages obtained 4 days after the injection of liquid paraffin were enhanced by β-adrenergic stimulants. The increases in cAMP by β-adrenergic stimulants were blocked by β-adrenergic blocking agents, propranolol and bufetolol, but not blocked by phentolamine, a α-adrenergic blocking agent. Phenylepherine, a α-adrenergic stimulant, produced only a small increase in cAMP. These observations suggest that the increases in cAMP in rat peritoneal macrophages by β-adrenergic stimulants are mediated by β-adrenergic receptors, but not by α-adrenergic receptors. It is well known that catecholamines stimulate adenylate cyclases of various tissues and increase intracellular cAMP. Catecholamines-stimulated adenylate cyclases of guinea pig peritoneal macrophages were demonstrated by Remold-O'Donnell [12]. It is easy to suppose that the increases in cAMP in rat peritoneal macrophages by β-adrenergic stimulants observed in the present experiments are due to the activation of adenylate cyclase via β-adrenergic receptors.

Beta-adrenergic receptors have been classified into two broad subtypes of β_1 and β_2 based on the order of pharmacological potencies of three classical catecholamines [2]. The order of affinity for β_1-adrenergic receptors is isoproterenol > epinephrine ≥ norepinephrine and for β_2-adrenergic receptors is isoproterenol > epinephrine ≫ norepinephrine. The order of affinity (PC_{50}) of the three catecholamines obtained with the increase in cAMP in rat peritoneal macrophages is isoproterenol > epinephrine ≫ norepinephrine. The affinity of epinephrine is 10 times higher than that of norepinephrine. This result appears to show that β_2-type adrenergic receptors mediate the increase in cAMP in rat peritoneal macrophages by catecholamines.

Recently β_2-adrenergic stimulants, which selectively stimulate β_2-adrenergic receptors, and β_1-adrenergic blocking agents, which selectively block β_1-adrenergic

Table 2. Effect of β-adrenergic blocking agents on the increase in cAMP in rat peritoneal macrophages by epinephrine and hexoprenaline

Compound	Concentration of antagonist (M)	Epinephrine (10^{-5} M)	Hexoprenaline (10^{-5} M)
Control—no drug		7.8 ± 0.5†	
	0	17.2 ± 0.5	18.2 ± 1.1
Bufetolol	10^{-7}	9.8 ± 0.4*	16.7 ± 0.7
	10^{-6}	9.6 ± 0.8*	12.2 ± 0.1*
	10^{-5}	8.6 ± 0.7*	12.1 ± 0.3*
	10^{-6}	17.4 ± 0.4	18.5 ± 1.7
Practolol	10^{-5}	17.0 ± 0.8	20.6 ± 0.4
	10^{-4}	20.5 ± 0.2*	20.4 ± 0.1

Drugs were incubated with macrophages (2.2×10^6 per ml) at 37° for 2 min.

* Statistical significance from the stimulant alone ($P < 0.05$).

† Results are expressed as pmoles cAMP/10^7 cells ± S.E.

Table 3. Effect of β-adrenergic blocking agents on the increase in cAMP in slices of rat hearts by isoproterenol

Compound	Concentration of antagonist (M)	cAMP pmoles/100 mg tissue/5 min
Control—no drug		78.7 ± 1.4†
Isoproterenol 5×10^{-7} M		278.0 ± 0.0
Isoproterenol 5×10^{-7} M	10^{-8}	254.9 ± 10.5
+ bufetolol	10^{-7}	234.3 ± 11.4*
Isoproterenol 5×10^{-7} M	10^{-8}	304.3 ± 10.1
+ propranolol	10^{-7}	216.0 ± 6.1*
Control—no drug		82.0 ± 3.2
Isoproterenol 5×10^{-7} M		309.4 ± 25.6
Isoproterenol 5×10^{-7} M	10^{-6}	319.9 ± 31.3
+ practolol	10^{-5}	184.5 ± 1.0*
	10^{-4}	137.7 ± 8.8*
Isoproterenol 5×10^{-7} M	10^{-6}	271.2 ± 8.4
+ metoprolol	10^{-5}	135.9 ± 14.9*
	10^{-4}	91.5 ± 3.0*

Drugs were incubated with slices of left ventricles of rat at 37° for 5 min in the presence of 10 mM theophylline.

* Statistical significance from isoproterenol alone ($P < 0.05$).

† Mean ± S.E. ($n = 3$).

receptors, have been introduced in experimental and clinical uses. Hexoprenaline [16] and salbutamol [17] are among the former, and practolol [18] and metoprolol [19] are among the latter. Bufetolol [20] and propranolol have no selectivity. These agents allow a distinction between these two subtypes of β-adrenergic receptors.

The degree of maximum stimulation and affinity of hexoprenaline for the increase in cAMP in rat peritoneal macrophages are approximately equal to those of isoproterenol. We reported that the affinity of hexoprenaline for the increase in cAMP in rat hearts (β_1) was 1.3×10^{-6} M, and 7×10^{-8} M in rat lungs (β_2) [14]. The affinity of hexoprenaline obtained with rat peritoneal macrophages is shown to be close to that of the lung. Salbutamol appears to be a partial agonist for the increase in cAMP in rat peritoneal macrophages because the maximum stimulation is about a half of that of epinephrine. This result does not agree to the observation that salbutamol was a full agonist in rat uterus (β_2) [7], while the affinity of salbutamol with rat peritoneal macrophages is equal to that of rat uterus, 3×10^{-7} M [7].

On the other hand, the inhibitory activities of β_1-adrenergic blocking agents, practolol and metoprolol, against the isoproterenol-induced increase in cAMP were observed to be considerably less potent in rat peritoneal macrophages compared with rat hearts.

These results obtained using the selective β-adrenergic stimulants and blocking agents further support the assumption described above that the increases in cAMP in rat peritoneal macrophages by catecholamines are mediated by β_2-adrenergic receptors.

REFERENCES

1. G. A. Robison, R. W. Butcher and E. W. Sutherland, *Cyclic AMP*, pp. 145–231. Academic Press, New York (1971).
2. A. M. Lands, A. Arnold, J. P. McAuliff, F. P. Luduena and T. G. Brown, *Nature, Lond.* **214,** 597 (1967).
3. A. Arnold, J. P. McAuliff, D. F. Colella, W. V. O'Connor and T. G. Brown, *Archs. int. Pharmacodyn. Thér.* **176,** 451 (1968).
4. R. A. Burges and K. J. Blackburn, *Nature New Biol.* **235,** 249 (1971).
5. F. Murad, *Biochim. biophys. Acta* **304,** 181 (1973).
6. R. J. Lefkowitz, *Biochem. Pharmac.* **24,** 583 (1975).
7. Y. Vulliemoz, M. Verosky and L. Triner, *J. Pharmac. exp. Ther.* **195,** 549 (1975).
8. E. Suter and H. Ramseir, *Adv. Immun.* **4,** 117 (1964).
9. G. B. Mackaness and R. V. Blanden, *Progr. Allergy* **11,** 89 (1967).
10. E. R. Unanue, *Adv. Immun.* **16,** 195 (1972).
11. G. Weissman, P. Dukor and R. B. Zurier, *Nature New Biol.* **231,** 131 (1971).
12. E. Remold-O'Donnell, *J. biol. Chem.* **249,** 3615 (1974).
13. C. F. Nathan, M. L. Karnofsky and J. R. David, *J. exp. Med.* **133,** 1356 (1971).
14. K. Ikegami and H. Imamura, *Yakugaku Zasshi* **96,** 1122 (1976).
15. H. A. Krebs and K. Henseleit, *Z. Physiol. Chem.* **210,** 33 (1932).
16. Von, H. Storman and K. Turnheim, *Arzneimittel-Forsch.* **23,** 30 (1973).
17. V. A. Cullum, J. B. Farmer, D. Jack and J. P. Levy, *Br. J. Pharmac.* **35,** 141 (1969).
18. D. Dunlop and R. G. Shanks, *Br. J. Pharmac.* **32,** 201 (1968).
19. B. Ablad, K. O. Borg, E. Carlsson, G. Johnsson, T. Malmfors and C. G. Regardh, *Acta Pharmac. Toxic.* **36,** suppl. V, 5 (1975).
20. M. Nakanishi, H. Imamura, T. Nakamura and T. Kimura, *Yakugaku Zasshi* **91,** 1037 (1971).