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INTERACTIONS OF α-METHYLFLUORENE-2-ACETIC ACID WITH ADENYLATE CYCLASE*

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Abstract—α-Methylfluorene-2-acetic acid (MFA), a new anti-inflammatory agent, enhanced the stimulation of adenylate cyclase activity from guinea pig heart by isoproterenol, epinephrine and norepinephrine, but did not cause increases in either basal or histamine-stimulated activity. Propranolol blocked the effects of isoproterenol plus MFA on heart cyclase activity. MFA did not enhance PGE_1- or PGE_2-stimulated cyclase activity from guinea pig lung; effects of MFA on isoproterenol-stimulated activity were obscured by a marked inhibition of basal activity. *d*-MFA and *l*-MFA were equipotent enhancers of isoproterenol-responsive heart cyclase activity. Increases in isoproterenol-stimulated cyclase activity from heart in the presence of MFA appeared reversible; decreases in basal activity were irreversible. The action of MFA was not due to inhibition of cyclic nucleotide phosphodiesterase activity, nor did MFA appear either to affect catecholamine degradation or to antagonize an endogenous cytoplasmic inhibitor of catecholamine action. It is possible that MFA perturbed the membrane milieu of the cyclase complex so as to have enhanced catecholamine binding to hormone receptors or coupling of the binding event to cyclic AMP synthesis.

THE ABILITY of hormones to stimulate the activity of adenylate cyclases from mammalian tissues is well known.[1] The enhancement of mammalian cyclase activity by compounds not usually considered hormones has also been reported. Among these compounds are choleratoxin,[2–5] endotoxin,[6] detergents,[7–9] sulfonylurea hypoglycemic agents[10–13] and high concentrations of short-chain alcohols[13–15] or polar aprotic solvents.[16] We present here the results of investigations of the interactions of cyclases with a new nonsteroidal anti-inflammatory agent, α-methylfluorene-2-acetic acid[17,18] (MFA†), that is capable of enhancing the stimulation by catecholamines of adenylate cyclase activity from guinea pig heart without a stimulatory effect on basal activity (activity measured in the absence of exogenous hormone).

MATERIALS AND METHODS

Several batches of the free acid of MFA (synthesized by Dr. E. T. Stiller[17]) and one lot of the sodium salt (contributed by Dr. S. D. Levine) were examined. The effects of these preparations on the isoproterenol-induced stimulation of cyclase activity from guinea pig heart were confirmed with a batch of the free acid of high purity (> 99 per cent). Most of the studies were carried out with the sodium salt (purity ca. 95 per cent), which was soluble in aqueous media. The free acid was dissolved in dimethylsulfoxide (DMSO), and a 5-μl aliquot was added to the assay mixture.

* A preliminary account of some of these studies has been presented previously: I. Weinryb and I. M. Michel, Abstract, 164th American Chemical Society Meeting, New York City, August 31, 1972.

† MFA refers to the racemate unless otherwise specified.

This volume of DMSO (0·85 per cent of total assay vol.) was routinely added to the control assays, although it did not affect cyclase activity.

Standard assay reagents and hormones were obtained as previously described.[19–21] The following compounds were also used: propranolol HCl (Ayerst), phenylbutazone (Ciba-Geigy), indomethacin (Merck), D-naproxen (Syntex), tranylcypromine HCl (Smith, Kline & French), and pyridoxal 5′-phosphate (Nutritional Biochemicals). D-Pheniprazine (K. A. Losee) and SQ 11579 (4-[3-(dimethylamino)propyl]-3,4-dihydro-α-methyl-3-phenyl-2*H*-1,4-benzothiazine-2-methanol) (Dr. J. Krapcho and C. F. Turk) were contributed by Squibb Institute chemists.

Duplicate assays of adenylate cyclase activity were carried out as reported previously,[19,20] with [α-^{32}P]ATP as substrate and an isolation procedure involving the use of [^{3}H]cyclic AMP as a recovery standard, chromatography on Dowex 50 ion-exchange resin, and treatments with nascent $BaSO_4$.

Adenylate cyclase fractions were suspensions of once-washed pellets obtained by centrifugation at 1000 *g* for 15 min of homogenates of (ventricular) heart and lung tissues from male guinea pigs.[19]

Under these conditions of preparation and assay, cyclase fractions from guinea pig heart were consistently responsive to isoproterenol and histamine, but were not significantly stimulated by glucagon (Schwarz/Mann).

RESULTS

Figure 1 shows the effects of α-methylfluorene-2-acetic acid (MFA) on basal and isoproterenol-stimulated cyclase activities from guinea pig heart. Over the concentration range 1 to 6 mM, the cyclase activity in the presence of 10 μM isoproterenol increased steadily in the face of a significant decrease in basal cyclase activity. The total activity in the presence of isoproterenol rose from 45 to 140 per cent above

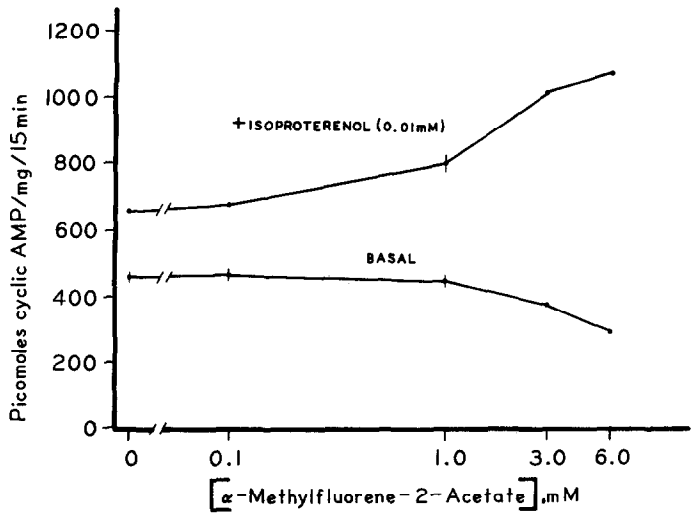

FIG. 1. Basal and isoproterenol-stimulated activities of adenylate cyclase from guinea pig heart in the presence of α-methylfluorene-2-acetic acid (MFA).

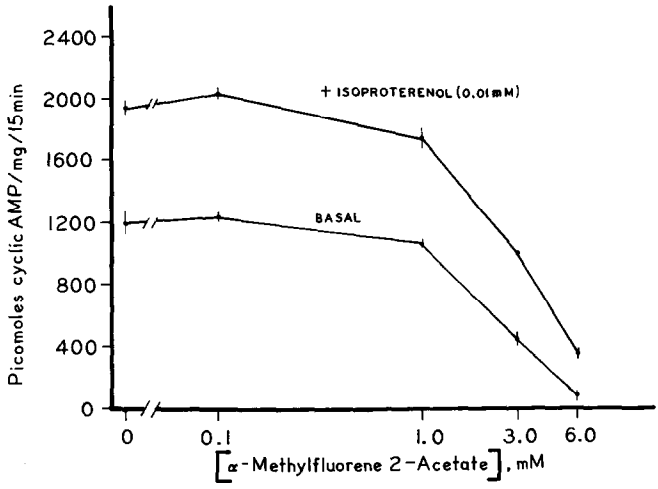

FIG. 2. Basal and isoproterenol-stimulated activities of adenylate cyclase from guinea pig lung in the presence of MFA.

the initial basal activity; alternatively, the hormonally induced activity in the presence of 6 mM MFA was 260 per cent higher than the corresponding basal activity. In other experiments the cyclase preparations appeared to be more sensitive to the effects of MFA, and maximal activity in the presence of isoproterenol was noted at about 1 mM MFA.

The effects of MFA on basal and isoproterenol-stimulated cyclase activities from guinea pig lung are presented in Fig. 2. In contrast to the interaction with the heart enzyme, 1 mM MFA did not enhance the effect of isoproterenol on lung cyclase activity, and higher concentrations markedly inhibited both basal and total activities. MFA, at the highest concentration tested (1 mM), also did not enhance the stimulation of lung cyclase activity by either 0·1 mM PGE_1 or PGE_2.

Additional studies with the heart enzyme revealed that the cyclase activity stimulated by histamine was not enhanced by 1 mM MFA (Table 1). Furthermore, heart cyclase activities stimulated by 100 μM epinephrine or norepinephrine were enhanced by MFA (Fig. 3) in a manner similar to that for stimulation by isoproterenol. These results, then, suggested that the site of action of MFA, with regard to

TABLE 1. EFFECT OF MFA ON STIMULATION OF CYCLASE ACTIVITY FROM GUINEA PIG HEART BY HISTAMINE

Addition to assay	Cyclase activity*
None (basal)	637 ± 13
+0·1 mM MFA	622 ± 66
+1·0 mM MFA	555 ± 21
+0·1 mM Histamine	1527 ± 23
+Histamine + 0·1 mM MFA	1294 ± 4
+Histamine + 1·0 mM MFA	1413 ± 20

* Picomoles cyclic AMP/mg protein/15 min. Values are means of duplicate assays ± range.

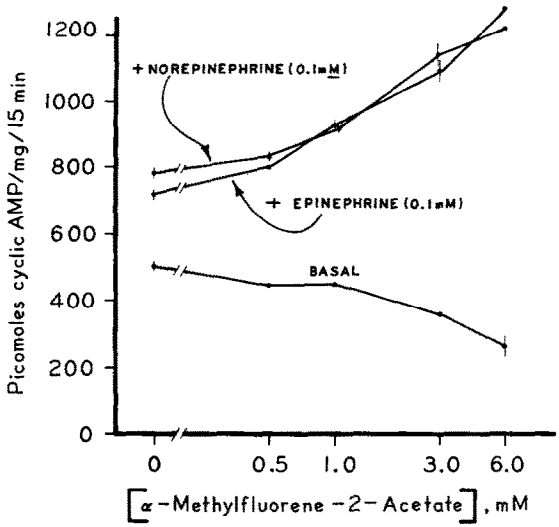

FIG. 3. Basal, epinephrine-stimulated and norepinephrine-stimulated activities of adenylate cyclase from guinea pig heart in the presence of MFA.

its stimulatory effects, was at the catecholamine receptor site. This conclusion was supported by the demonstration that the β-adrenergic antagonist, propranolol, which did not affect basal activity, completely blocked both the β-adrenergic stimulation of heart cyclase activity by isoproterenol and the enhancement of this stimulation by MFA (Table 2).

The effectiveness of MFA in enhancing only catecholamine-responsive cyclase activity from heart suggested the possibility that it hindered degradation of the hormones by inhibiting catechol-*O*-methyltransferase or monoamine oxidase activities in the cyclase preparations. Although this seemed unlikely, since neither enzyme activity is primarily associated with a 1000 *g* particulate membrane fraction,[22,23] the possibility was tested by observing whether the catechol-*O*-methyltransferase inhibitor, pyridoxal-5′-phosphate,[24] or the monoamine oxidase inhibitors, tranylcypromine[25,26] and pheniprazine,[27] could mimic the effects of MFA on heart cyclase activity. Pyridoxal-5′-phosphate and pheniprazine were found not to affect the relative stimulation by isoproterenol, although both compounds inhibited basal activity at 1 mM, whereas 1 mM tranylcypromine inhibited the response to isoproterenol

TABLE 2. INTERACTION OF ISOPROTERENOL, PROPRANOLOL AND MFA—EFFECTS ON CYCLASE ACTIVITY FROM GUINEA PIG HEART

Addition to assay	Cyclase activity*
None (basal)	516 ± 6
+0·01 mM Isoproterenol (IP)	735 ± 9
+IP + 0·01 mM propranolol (PR)	466 ± 41
+IP + 6 mM MFA	1216 ± 3
+IP + PR + MFA	420 ± 9

* Picomoles cyclic AMP/mg protein/15 min. Values represent means of duplicate assays ± range.

TABLE 3. EFFECTS OF INHIBITORS OF CATECHOLAMINE DEGRADATION ON ADENYLATE CYCLASE ACTIVITY FROM GUINEA PIG HEART

Addition to assay	Cyclase activity*	% Stimulation by isoproterenol
None (basal)	226 ± 2	
10 μM Isoproterenol (IP)	327 ± 6	45
0·1 mM Pyridoxal-5′-phosphate (PP)	221 ± 2	
1·0 mM PP	163 ± 12	
10 μM IP + 0·1 mM PP	322 ± 4	46
10 μM IP + 1·0 mM PP	244 ± 4	49
1·0 mM Tranylcypromine (TC)	223 ± 5	
10 μM IP + 1·0 mM TC	265 ± 8	18
1·0 mM Pheniprazine (PZ)	160 ± 5	
10 μM IP + 1·0 mM PZ	235 ± 5	49

* Picomoles cyclic AMP/mg protein/15 min.

(Table 3). None of the compounds enhanced isoproterenol-stimulated heart cyclase activity as did MFA.

The stereospecificity of the action of MFA was investigated by comparing the effects of the *d*- and *l*-isomers of the free acid (Table 4). The isomers were equally effective in enhancing the catecholamine-induced stimulation of total cyclase activity from guinea pig heart.

Because MFA possessed anti-inflammatory activity, several other nonsteroidal anti-inflammatory agents were examined for effects on heart cyclase activity (Table 5). Phenylbutazone and SQ 11579 (4-[3-(dimethylamino)propyl]-3,4-dihydro-α-methyl-3-phenyl-2*H*-1,4-benzothiazine-2-methanol, hydrochloride)[28,29] inhibited isoproterenol-induced cyclase activity, whereas indomethacin and naproxen showed some

TABLE 4. EFFECTS OF *d*- AND *l*-MFA ON ADENYLATE CYCLASE ACTIVITY FROM GUINEA PIG HEART

[MFA] (mM)	Basal cyclase activity* (B)	Total cyclase activity*† (T)	T/B
d-Isomer			
0	465 ± 5	669 ± 4	1·44
0·33	414 ± 10	655 ± 20	1·58
0·67	406 ± 7	670 ± 15	1·65
1·0	400 ± 15	721 ± 13	1·80
3·0	255 ± 13	663 ± 3	2·60
l-Isomer			
0	417 ± 8	610 ± 11	1·46
0·33	394 ± 5	633 ± 20	1·61
0·67	411 ± 5	676 ± 27	1·64
1·0	406 ± 10	745 ± 2	1·83
3·0	286 ± 0	690 ± 37	2·41

* Picomoles cyclic AMP/mg protein/15 min. Data represent the mean ± range of duplicate determinations.

† Isoproterenol (0·01 mM) was present in all assays.

TABLE 5. COMPARISON OF EFFECTS ON HEART CYCLASE ACTIVITY OF MFA AND OTHER SELECTED ANTI-INFLAMMATORY AGENTS

Compound concn (mM)	%Change	
	Basal activity	Isoproterenol-stimulated activity*
MFA 0·1	−2	+22
MFA 1·0	−12	+133
Indomethacin		
0·1	−27	+12
1·0	−51	+42
Phenylbutazone		
0·1	+1	+6
1·0	+21	−100
Naproxen		
0·1	−7	+29
1·0	−7	+28
SQ 11579†		
0·1	+17	−92
1·0	−15	−76

* Calculated from the equation: % Change =

$$\{[(T_c/B_c) - 1]/[(T_o/B_o) - 1] - 1\} \times 100,$$

where T and B are total and basal cyclase activities, respectively, and the subscripts refer to activities in the presence (c) and absence (o) of compound.

† 4-[3-(Dimethylamino)propyl]-3,4-dihydro-α-methyl-3-phenyl-2*H*-1,4-benzothiazine-2-methanol, hydrochloride.

stimulatory effects at 1·0 mM which did not, however, approach that of MFA in magnitude.

The reversibility of the action of MFA was studied in an experiment involving incubation of a cyclase preparation with 6 mM MFA for 22 hr at 4°. Another aliquot of the same enzyme preparation was incubated with buffer as a control. Both treated and untreated cyclase were then diluted into appropriate reaction mixtures (residual MFA concn approx. 0·5 mM) and assayed for activity (Table 6). Since 0·5 mM MFA

TABLE 6. REVERSIBILITY OF THE ACTION OF MFA ON HEART CYCLASE ACTIVITY*

Addition to assay	Cyclase activity†	
	Treated	Untreated
None (basal)	228 ± 1	447 ± 15
+6 mM MFA	93 ± 1	192 ± 9
+0·01 mM Isoproterenol (IP)	505 ± 6	772 ± 23
+IP + MFA	747 ± 3	1092 ± 21

* One ml of heart cyclase preparation (treated) was diluted with 1 ml of 12 mM MFA; another cyclase aliquot (untreated) was added to buffer. After 22 hr at 4°, both mixtures were diluted into reaction mixtures for assay such that the residual MFA concn in the assays of treated enzyme was approx. 0·5 mM.

† Picomoles cyclic AMP/mg protein/15 min. Values represent the mean of duplicate determinations ± range.

TABLE 7. EFFECT OF MFA ON RECOMBINED CYCLASE FRACTIONS FROM GUINEA PIG HEART*

Addition to assay	Cyclase activity†		
	Strained 20% homogenate	Pellet (washed five times)	Washed pellet plus supernatant from homogenate‡
None (basal)	238 ± 6	345 ± 2	204 ± 6
+0·01 mM Isoproterenol (IP)	346 ± 8	511 ± 16	285 ± 3
+1 mM MFA	261 ± 10	331 ± 1	204 ± 5
+IP + MFA	444 ± 6	601 ± 18	348 ± 5

* Fractions were stored at 4° for 22 hr before assay.

† Picomoles cyclic AMP/mg protein/15 min. Values are means of duplicate assays ± range. Fractions were diluted to the following protein concentrations prior to assay: homogenate, 5·2 mg/ml; washed pellet, 5·3 mg/ml; washed pellet plus supernatant, 6·4 mg/ml.

‡ Supernatant fraction was that obtained after centrifugation of whole homogenate at 1000 × *g* for 15 min.

did not inhibit basal cyclase activity significantly, the substantial decrease in basal activity of the preparation treated with MFA relative to the untreated control suggested that high concentrations of MFA inhibit basal activity irreversibly. Further addition of 6 mM MFA inhibited the basal activity of both treated and untreated enzyme. The treated cyclase was 66 per cent more responsive to isoproterenol than the untreated fraction, a result consistent with the presence of residual (0·5 mM) MFA in the treated enzyme aliquot. Thus, the effect of MFA on stimulation of heart cyclase activity by isoproterenol appeared reversible. Additional MFA enhanced the isoproterenol-stimulated activities of both fractions in similar proportion.

The possibility that MFA antagonized the effect of an endogenous, cytoplasmic inhibitor of catecholamine action on heart cyclase was explored by examining the effect of the supernatant fraction from centrifugation at 1000*g* of a whole homogenate on the enhancement by MFA of the stimulation by isoproterenol of cyclase activity from a preparation washed five times (Table 7). Protein concn of the fractions were adjusted to comparable values to avoid effects on hormonal stimulation due to concentration. The relative effect of MFA on the washed cyclase preparation was not significantly altered by the addition of the supernatant fraction.

DISCUSSION

The investigations recorded here favor the conclusion that MFA enhanced the stimulation of adenylate cyclase activity from guinea pig heart by catecholamines via an interaction at the catecholamine receptor of the plasma membrane-associated enzyme complex. The data were also consistent with a second (inhibitory) effect of MFA, at the locus of the catalytic unit, leading to decreased basal activity at sufficiently high MFA concn. The action of MFA on heart cyclase activity resembled that of endotoxin on liver or spleen cyclase activity,[6] insofar as catecholamine responsiveness was increased, whereas basal activity tended to decrease. It is not clear, however, that the stimulations by MFA and endotoxin have any mechanistic features in common.

The enzyme preparations from heart possessed significant cyclic nucleotide phosphodiesterase activity.[20] The stimulatory effects of MFA were unlikely to be due to inhibition of phosphodiesterase activity because: (1) assays were carried out in the

presence of 10 mM theophylline or 1 mM unlabeled cyclic AMP; (2) basal cyclase activity was not enhanced; and (3) histamine-responsive cyclase activity was not enhanced.

Data presented above also appear to rule out the possibility that MFA acted by inhibition of catecholamine degradation, or by competition with a cytoplasmic inhibitor of catecholamine stimulation to which the cyclase complex was exposed by the act of homogenization. We cannot, however, exclude the possibility that MFA, by virtue of the hydrophobic nature of the fluorene moiety, perturbed the membrane milieu of the catecholamine receptor so as to enhance either binding of catecholamine to the cyclase complex or coupling of the binding events to the turnover of ATP at the catalytic unit(s). This latter proposal is similar in some respects to that suggested by other workers to explain the stimulation of basal cyclase activity by sodium fluoride and detergents.[7,8] It is also consistent with the ability of MFA to interact with and stabilize erythrocyte membranes.[18]

The ability of MFA to enhance the catecholamine responsiveness of cyclase preparations from tissues other than guinea pig heart has not been examined extensively. It may be argued that concentrations of MFA greater than 1 mM indeed enhance the responsiveness of cyclase from guinea pig lung to isoproterenol (Fig. 2), but basal and isoproterenol-stimulated activities are so markedly decreased that caution is necessary in reaching a firm conclusion. Of particular interest would be the examination of the interaction of MFA with cyclases from leukocytes, in view of the demonstration of anti-inflammatory activity in animals,[18] and because catecholamines that act as β-adrenergic agonists to stimulate adenylate cyclases have been reported to antagonize reactions characteristic of immediate[30] and delayed hypersensitivity.[31] Whether or not the enhancement of the effect of β-adrenergic agonists on cyclase activity in subcellular fractions is the basis of the anti-inflammatory activity observed *in vivo* for MFA would remain to be proven, particularly in view of the apparently suprapharmacologic concentrations of MFA necessary (≥ 1 mM) to affect heart cyclase activity, at least. In any event, the apparent specificity of action of MFA with regard to the catecholamine receptor of adenylate cyclase, as deduced from studies on the enzyme from heart, may prove useful in delineating hormone receptor–membrane coupling–catalytic subunit relationships of cyclases from a variety of tissues.

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