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Fatty acids as modulators of membrane functions: Catecholamine-activated adenylate cyclase of the turkey erythrocyte

(β-adrenergic receptor/membrane fluidity/transition temperature/GTP site/guanylyl imidodiphosphate)

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ABSTRACT **Activation of the adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] from turkey erythrocytes by isoproterenol decreased precipitously below 26°. Certain unsaturated fatty acids enhanced the activation by isoproterenol up to 25-fold at reduced temperatures. The fatty acid also enhanced the formation of a persistent active state of the enzyme which was produced by preincubation with guanosine 5′-(β,γ-imino)triphosphate [Gpp(NH)p]. Once the enzyme had been activated by Gpp(NH)p plus isoproterenol the reaction rate was no longer as temperature sensitive and the fatty acid had little effect. The synthetic Gpp(NH)p apparently substituted for the natural GTP, which is known to play a regulatory role in the adenylate cyclase system. The findings suggest that the function of GTP which is mediated by the hormone is the temperature-sensitive event which is enhanced by the fatty acid. The use of free fatty acid to probe membrane-associated reactions in intact cells and in isolated membrane preparations is proposed.**

The interaction of phospholipids with proteins is of prime importance in many functions of biological membranes. A significant part of the properties of the phospholipids is determined by their fatty acid composition. Attempts have, therefore, been made in recent years to modify the fatty acid composition of the cell membrane in order to learn how it affects various membrane phenomena. Aspects such as transport through the cell membrane (1, 2) and mobility of receptors on the surface of the membrane (3) have been studied. Modification of the lipid composition in these studies required the growth of specific bacterial mutants or tissue cultures and was limited to those fatty acids which the cell could incorporate into the phospholipids (3–5). It is, however, possible to insert, artificially, free fatty acids and their simple derivatives into biological membranes so as to study their effect on membrane properties. Thus, addition of sodium laurate or glyceryl monoolein to secretory granules of rat parotid gland sealed the granule membrane against leakage at low temperatures (6). A similar effect was demonstrated for the membrane of the neurosecretory granules of neurohypophysis (7). Certain fatty acids also enhanced cell fusion (8), acted as crenators of erythrocytes, and protected against hemolysis in hypotonic media (9, 10). Manipulation of membrane functions by insertion of free fatty acids has the following advantage. Experiments are not dependent on the successful esterification of the fatty acid since the free fatty acid can serve as such to modify the physical properties of the membrane. Fatty acids are available at a high state of purity for almost any chain length, position, and number of double bonds and as *cis* and *trans* isomers. Any effect can, therefore, be pinpointed to a specific chemical structure. It should be added that such experiments cannot be done with phospholipids because these are not available in a homogenous form at the wide variety specified above for the fatty acids.

In the present work the above outlined approach has been applied to the catecholamine-activated adenylate cyclase [ATP pyrophosphate-lyase (cyclizing), EC 4.6.1.1] of turkey erythrocytes. It has been amply demonstrated by other investigators that the hormone-activated adenylate cyclase system requires phospholipids for its function (11–14). The present study demonstrates that the insertion of specific fatty acids into the membrane causes a dramatic enhancement of the hormone-activated reaction at incubation temperatures below 26°. The fatty acid apparently acts on the guanyl nucleotide site which functions as a regulator of the adenylate cyclase system (12, 15–17).

MATERIALS AND METHODS

Turkey Erythrocyte Membranes. These were prepared by a modification (17) of the procedure of Rosen and Rosen (18) as described for frog erythrocytes.

Addition of Fatty Acids to the Erythrocyte Membranes. All operations were carried out at 0–4°. A batch of membranes which had been stored under liquid nitrogen was washed and resuspended in 10 mM Tris·HCl buffer (pH 8.1) containing 1 mM dithiothreitol. Fatty acid in absolute ethanol was added to the membrane suspension to give a final concentration of 1 mM (about 1 μmol/3 mg of membrane protein). The final ethanol concentration did not exceed 1% (v/v). After 10 min preincubation at 4° the membranes were assayed for adenylate cyclase activity. Controls without fatty acid received 1% ethanol, which caused a 10% inhibition of activity.

Adenylate Cyclase. Activity was assayed according to Salomon *et al.* (19). The reaction mixture was as previously described (17). GTP, which is required for the hormone-induced adenylate cyclase reaction (12), was not added to the assay system. GTP functions at a very low concentration (12) which is apparently present as a contaminant in the ATP substrate.

Activity remained stable at 4° for several hours. It was linear with amount of protein and time under the conditions described in the experiments, unless otherwise noted. The low basal activity was not subtracted in any of the experiments shown. Each experiment was repeated at least twice. The effects of *cis*-vaccenic acid were repeated more than ten times. Amplification of adenylate cyclase activity by *cis*-vaccenic acid showed up to ±25% variation, depending on the membrane batch. For a single batch the results varied in

Abbreviation: Gpp(NH)p, guanosine 5′-(β,γ-imino)triphosphate

Table 1. Effect of various fatty acids on the activity of turkey erythrocyte adenylate cyclase

Fatty acid	Structural formula	Melting temperature, °C	NaF activation at 20°	NaF activation at 37°	Isoproterenol activation at 20°	Isoproterenol activation at 37°	Isoproterenol activation ratio at 20°, with fatty acid/without fatty acid
			pmol of cAMP mg^{-1} min^{-1}				
None	—	—	30	230	3	60	—
Butyric	C4:0	−8	35	260	3.6	60	1.2
Lauric	C12:0	44	60	320	8.2	50	2.7
Palmitic	C16:0	63	35	290	1.9	70	0.6
Palmitoleic	Δ9-10 C16:1*c*	0.5	74	370	33	90	11
Stearic	C18:0	69	30	260	2	70	0.7
Petroselinic	Δ6-7 C18:1*c*	33	65	340	16	90	5.3
Oleic	Δ9-10 C18:1*c*	16	70	370	38	150	13
Elaidic	Δ9-10 C18:1*t*	44	45	310	5	90	1.6
Cis-vaccenic	Δ11-12 C18:1*c*	13	70	350	49	150	16
Trans-vaccenic	Δ11-12 C18:1*t*	44	37	280	4.8	90	1.6
Linoleic	Δ9,12 C18:2*c*	−5	53	310	12	60	4
Eicosenoic	Δ11-12 C20:1*c*	22	56	300	38	160	13
Erucic	Δ13-14 C20:1*c*	34	36	240	8	90	2.6
Nervonic	Δ15-16 C22:1*c*	43	35	250	5.7	70	1.9

All fatty acids were added at 1 mM concentration. Adenylate cyclase activity was assayed with 145 μg of membrane protein by incubation for 10 min at 37° or 20 min at 20°. The concentration of DL-isoproterenol was 50 μM and fluoride was 10 mM.

the range of ±10%. Protein was determined by the method of Lowry *et al.* (20).

Chemicals and Radiochemicals. *Cis*- and *trans*-vaccenic acid were from the Hormel Institute, Minn.; all other fatty acids were obtained from Sigma; guanosine 5′-(β,γ-imino)-triphosphate [Gpp(NH)p] was from ICN; [^{14}C]oleic acid and [α-^{32}P]ATP were products of Amersham, England.

RESULTS

The hormone-induced adenylate cyclase activity was found to fall steeply when the incubation temperature was reduced from 37° to 20° (Table 1). A much smaller drop in activity was observed for the fluoride-induced activity. Addition of certain fatty acids to the erythrocyte membrane caused a dramatic increase of the activation by the hormone at 20°. The chain length of the fatty acid and the number and position of double bonds proved to be critical for the activation. The highest amplification was achieved with *cis*-vaccenic acid, in the presence of which the reaction induced by isoproterenol was accelerated 16-fold at 20° and only 2-fold at 37°.

Examination of the melting points of the fatty acids indicates that this parameter by itself does not determine the effect, but that the specific molecular structure is also important. Linoleic acid, which had the lowest melting temperature, produced only a modest amplification even when tested in the entire range of concentrations, 0.01–2 mM. In addition, palmitoleic acid was somewhat less effective than vaccenic acid, although the melting temperature of the former was much lower than that of the latter. Fluoride did not show more than a 2.5-fold increase in activity by any fatty acid at 20° or at 37°. The basal rate of the adenylate cyclase obtained in absence of activators was only 3 pmol of cyclic AMP/mg of protein per min at 37°. The activities with isoproterenol and with fluoride were, respectively, 20 and 80 times higher than the basal activity (Table 1). At 20° the basal rate was 1 pmol of cyclic AMP/mg of protein per min. No change in the low basal activity at 37° and at 20° was detected in the presence of *cis*-vaccenic acid. The K_m for isoproterenol at 20° (1.5 μM) was also found not to be affected by the addition of *cis*-vaccenic acid to the erythrocyte membranes.

Labeled [^{14}C]oleic acid was used in order to determine the amount of fatty acid which becomes attached to the membranes. Addition of 0.1–1 mM oleic acid to 3 mg of membrane protein per ml followed by three washes in the cold resulted in the retention of 85% of the added fatty acid. The enhanced enzyme activation at 20° was still demonstrable. The activity increased linearly from 0.1 to 1 mM oleic acid or vaccenic acid with no further increase up to 2 mM. Membranes after addition of [^{14}C]oleic acid were incubated under actual adenylate cyclase assay conditions to find out also whether the fatty acid becomes esterified. At least 95% of the oleic acid attached to the membranes remained in the unesterified form as shown by thin-layer chromatography (21) (data not shown).

It was possible also to affect the adenylate cyclase system in the membrane by adding the fatty acid to the intact erythrocyte. *Cis*-vaccenic acid at 1 mM concentration was added to a 25% (v/v) suspension of cells in 0.14 M NaCl containing 30 mM Tris·HCl (pH 7.4). After hemolysis and several washes the membranes showed an isoproterenol-induced activity at 20°, which was 3-fold that of control ghosts from erythrocytes treated similarly but without the fatty acid (data not shown).

Since the effect of the fatty acids was much more pronounced at 20° than at 37° the temperature dependency was studied in more detail. Arrhenius plots for the isoproterenol- and fluoride-activated adenylate cyclase reactions are given in Fig. 1A and B, respectively. The plot for the catecholamine-activated reaction shows a distinct break at 26° which is not shifted by the fatty acids. However, the fatty acids do decrease the energy of activation considerably below, but also above, the break point. It should be noted that the isoproterenol-induced reaction is amplified 25-fold by *cis*-vaccenic acid at 14°. The fluoride activation also shows a break point at 26°, but in contrast to the hormone

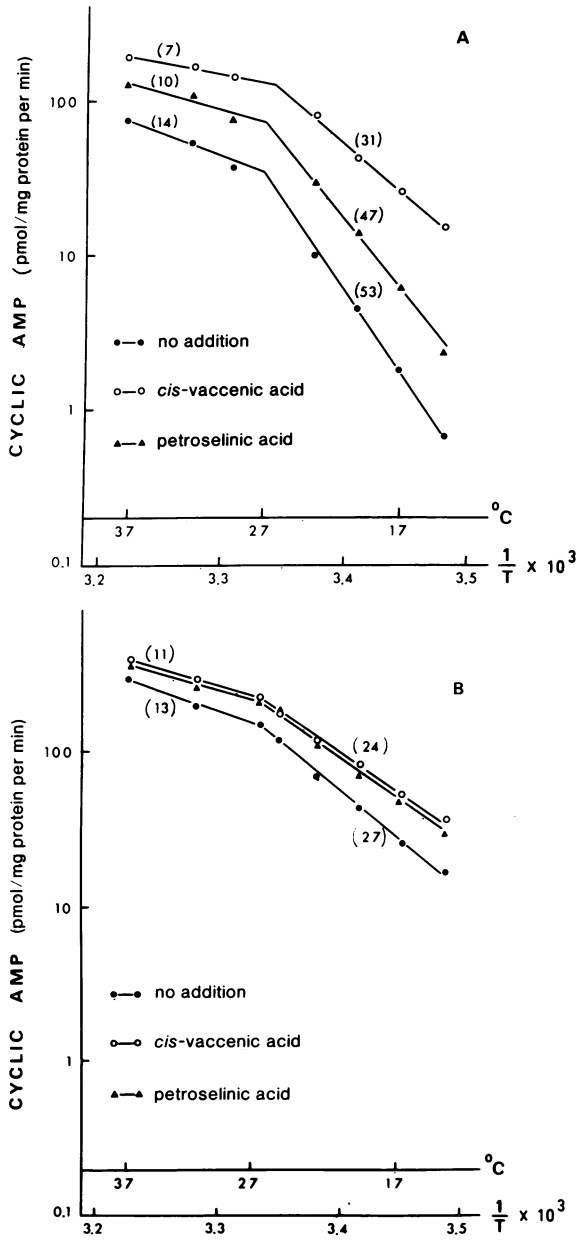

FIG. 1. Temperature dependency of adenylate cyclase activity of turkey erythrocyte membranes. (A) Activation by 50 μM isoproterenol. (B) Activation by 10 mM fluoride. The assay systems contained 170 μg of membrane protein. Incubation time was 20 min for temperature range 14°–20° and 10 min for the temperature range 23°–37°. Numbers in parentheses give the energy of activation ($-E_a$) in kcal/mol.

activation the drop in rate is less steep and the fatty acids cause only an insignificant decrease in the activation energy.

It has recently been shown that the adenylate cyclase system can be activated not only by isoproterenol, or fluoride, but also by Gpp(NH)p, an analog of GTP (16, 17, 22). Preincubation of the membranes with Gpp(NH)p in the presence of hormone produced an active state which persisted after thorough washing, and resisted the subsequent addition of the β-receptor blocking agent propranolol (17). Therefore, the temperature dependency of enzyme preactivated by Gpp(NH)p plus isoproterenol was also studied. Surprisingly, the Arrhenius plot of the enzyme activated by the nucleotide plus hormone shows no break point and the energy of

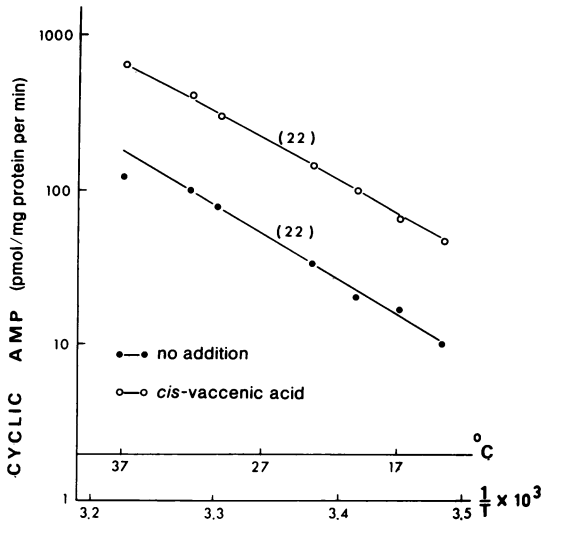

FIG. 2. Temperature dependency of the persistent active state of the adenylate cyclase system produced by preincubation with isoproterenol plus Gpp(NH)p. *Cis*-vaccenic acid was added to the membranes. Subsequently the system received further additions at 0°–4° in the following order: 3 mM $MgCl_2$, 5 μM DL-isoproterenol, and 2 μM Gpp(NH)p. The systems were transferred to 17° for 15 min of preincubation. The period of preincubation was terminated by addition of 20 μM propranolol, followed by transfer to 0°. Adenylate cyclase was subsequently measured at various temperatures as described in Fig. 1 without the further addition of activators. Numbers in parentheses shown in the figure give the energy of activation ($-E_a$).

activation is thus not changed throughout the temperature range studied (Fig. 2). Addition of *cis*-vaccenic acid prior to the activation did not affect the slope of the Arrhenius plot. The reason for the higher absolute activity in the presence of vaccenic acid is due to the fact that formation of the active state in absence of fatty acids is very slow at 17° (Fig. 3) and preincubation was terminated before the maximal activation

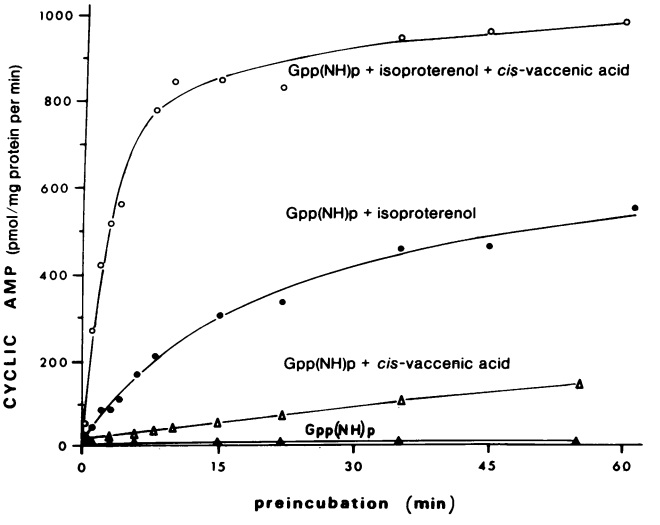

FIG. 3. Effect of *cis*-vaccenic acid on the rate of formation of the persistent active state. *Cis*-vaccenic acid was added to the membranes. Subsequently the membranes, 2.6 mg of protein per ml, were incubated at 17° with 3 mM $MgCl_2$ plus 2 μM Gpp(NH)p, in the presence and in the absence of 5 μM DL-isoproterenol. The systems without isoproterenol contained also 20 μM propranolol. At various times, aliquots of 50 μl were taken from the incubation mixture and transferred to the adenylate cyclase assay system at 37° which contained 20 μM propranolol.

Table 2. Insensitivity to fatty acid of the adenylate cyclase system activated by isoproterenol plus Gpp(NH)p

Exp.	Preincubation	Assay temperature, °C	Adenylate cyclase activity pmol of cAMP/mg of protein per min: Without vaccenic acid (a)	With vaccenic acid (b)	b/a
I	Isoproterenol plus Gpp(NH)p → ± vaccenic acid	37	358	443	1.2
		20	56	71	1.2
II	± Vaccenic acid → isoproterenol plus Gpp(NH)p	37	378	557	1.4
		20	54	102	1.9

Exp. I. Addition of fatty acid after preincubation with isoproterenol plus Gpp(NH)p. Membranes, 2.4 mg of protein per ml, received the following additions in the cold: 3 mM $MgCl_2$, 5 μM DL-isoproterenol, and 2 μM Gpp(NH)p. The mixture was transferred to 25° for 30 min of preincubation followed by addition of 20 μM propranolol and transfer to 0°. The system then received 1 mM vaccenic acid and was further incubated for 10 min in ice. A control without fatty acid was also run. Adenylate cyclase activity without further addition of activators was measured at 20° and 37° (see Table 1). *Exp. II.* The procedure was exactly as under Exp. I but the sequence of incubations was reversed; first vaccenic acid and then isoproterenol plus Gpp(NH)p. A control without fatty acid was run in parallel.

was reached. When preincubation to form the persistent active state was performed at a higher temperature for a longer time vaccenic acid had only a slight effect (Table 2). It should be noticed that the residual effect by the fatty acid is not only small in magnitude but also temperature independent (Fig. 2 and Table 2).

Fatty acid also had a negligible effect when added after the system had been preactivated by Gpp(NH)p plus isoproterenol (Table 2). However, the fatty acid does affect the rate of formation of the persistent active state produced by Gpp(NH)p. As shown in Fig. 3, the fatty acid at 17° enhanced formation of the active state about 12-fold in the system containing Gpp(NH)p without hormone and about 5-fold (initial rate) in the presence of hormone.

DISCUSSION

The temperature dependency of the adenylate cyclase reaction has been examined in previous studies and discontinuities in the Arrhenius plots have been reported (23, 24). However, the causes for these discontinuities remain obscure.

The present experiments on the turkey erythrocyte suggest that the temperature-sensitive reaction of the adenylate cyclase system involves the regulatory GTP site (12, 17, 22). This conclusion is based on the following findings. Fatty acid accelerated the activation of the enzyme by Gpp(NH)p at 17° in the absence of hormone. The more rapid functional introduction of Gpp(NH)p by hormone was also accelerated by the fatty acid. Enzyme preactivated by Gpp(NH)p plus hormone no longer showed any break in the Arrhenius plot. Such enzyme was no longer significantly affected by the addition of fatty acid. It is, therefore, tentatively concluded that the functional introduction of Gpp(NH)p to its site is the temperature-sensitive event. Gpp(NH)p is apparently stable at the regulatory site (17) and, therefore, the extreme temperature sensitivity disappears once the enzyme has been activated by this synthetic nucleotide. In contrast the natural nucleotide, GTP, is probably being degraded at the regulatory site (17) and must be continuously regenerated. Therefore, it is suggested that the break in the Arrhenius plot reflects the repeated functional introduction of GTP, by the hormone, into the regulatory site. The fatty acids apparently facilitate this reaction.

An additional effect of the fatty acids on the hormone receptor cannot be ruled out. However, it is also conceivable that the hormone receptor on the outer surface of the membrane and the GTP site on the inner surface form a complex which spans the membrane and is reversibly coupled to the catalytic unit of the enzyme. Previous evidence already suggested that the hormone activation of the adenylate cyclase is mediated by the guanyl nucleotide site (17). The fact that the fatty acids accelerated both the hormone activation and the Gpp(NH)p activation are in line with such a concept.

It is quite possible that the functional introduction of GTP into the site requires the presence of phospholipids and that the fatty acids thus affect the lipid environment of the regulatory complex. In this respect it should be noted that the enzyme already activated by Gpp(NH)p plus hormone, which is no longer affected by fatty acids, also has no further requirement for phospholipid for activity (22).

The thermal behavior of the lipid bilayer of the membrane (25) was kindly tested by Dr. M. Shinitzky, who determined the microviscosity (26) of the turkey erythrocyte membrane as a function of temperature. The findings indicated a high rigidity of the membrane but with no discontinuity of the Arrhenius plot. Because of the rigidity of the turkey erythrocyte membrane it is perhaps not surprising that unsaturated fatty acids which might increase local fluidity (4, 5, 25) were the most potent in enhancing hormone activation of the adenylate cyclase. While the fatty acids had a rather defined effect within the adenylate cyclase system, it is quite possible that certain other reactions in the turkey erythrocyte membrane might also be affected.

Insertion of free fatty acids and simple lipids into membranes might find use in analyzing other membrane-associated systems. The function of various receptors (acetylcholine, insulin, lectins) and transport systems (ions, amino acids, sugars) comes to mind. In the present work the fatty acid was partially effective even when added to the intact erythrocyte. It may thus be possible to examine certain effects not only in the isolated membranes and vesicles but also at the level of the intact cell.

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