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EFFECT OF ADENYLATE CYCLASE ACTIVATORS AND Mg^{2+} ON THE BINDING AND THE ELECTRON SPIN RESONANCE SPECTRA OF *N*-METHYLMALEIMIDE NITROXIDE IN MEMBRANE PARTICLES FROM THE LIVER FLUKE *FASCIOLA HEPATICA*

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Optimal conditions for activation of adenylate cyclase in membrane particles were studied. Enzyme activation with serotonin (5-hydroxytryptamine), NaF, and guanosine 5′-(3-*O*-thio)-triphosphate (GTPγS) was time- and temperature-dependent. Mg^{2+} was required for enzyme activation. Adenylate cyclase that was activated by NaF or GTPγS was gradually inhibited by *N*-methylmaleimide while enzyme activated with serotonin and GTP responded faster to inhibition by the same sulfhydryl reagent. The enzyme responded in a similar fashion to a spin-labeled *N*-methylmaleimide analog 3-(maleimidomethyl)-2,2,5,5-tetramethyl-1-pyrolidinyloxyl (i.e., *N*-methylmaleimide nitroxide). Binding of the spin label was enhanced following enzyme activation by serotonin, NaF, or GTPγS in the presence of Mg^{2+}. Activation of the enzyme was accompanied by an increase in the strong immobilization peaks in the EPR spectra. Both effects, the increase in binding and in the strong immobilization peaks, can be induced by Mg^{2+} alone. The results indicate that a general conformational change induced by Mg^{2+} may be essential for adenylate cyclase activation.

Introduction

Serotonin (5-hydroxytryptamine) was shown to stimulate motility [1], increase glycolysis and glycogenolysis [2], and activate adenylate cyclase of the liver fluke, *Fasciola hepatica* [3]. The effect of the indolamine on adenylate cyclase has been studied recently in our laboratory [4,5]. Fluke adenylate cyclase, as well as many cyclases from vertebrate cells [6], is a membranous enzyme which appears to have three main components: the receptor that binds serotonin; the nucleotide component that binds GTP and its poorly hydrolyzable analogs; and the catalytic component that converts ATP to cyclic AMP in the presence of Mg^{2+}. Although the intricate details of cyclase activation have not as yet been elucidated, the evidence shows that conformational changes do occur between the different components of the oligomeric complex during enzyme activation. The participation of sulfhydryl groups in enzyme activation has been reported in several vertebrate adenylate cyclases [7–9] as well as in the liver fluke enzyme [10]. It is meaningful to study the nature of these sulfhydryl groups when the enzyme is activated by different activators. The spin-labeling technique earlier described by McConnell and McFarland [11] was used in the present studies. The results indicate that Mg^{2+} is essential for activation of adenylate cyclase by a variety of activators: serotonin, NaF, and the poorly hydrolyzable analog of GTP, guanosine 5′-(3-*O*-thio)triphosphate (GTPγS). We have used the spin probe 3-(maleimidomethyl)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (i.e., *N*-methylmaleimide nitroxide), an analog of *N*-

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methylmaleimide, to study the nature of the sulfhydryl groups' changes during enzyme activation. The results show that membrane particles which have their cyclase activated by any of the activators bind more *N*-methylmaleimide nitroxide and that the reagent was in a highly immobilized environment. These conformational changes can occur as a result of Mg^{2+} alone.

Materials and Methods

The following materials were used in this investigation: *N*-methylmaleimide from Aldrich Chemical Co., Inc.; 3-(maleimidomethyl)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl from Syva, Palo Alto; and the siliconizing material Prosil-28 from PCR Research Chemicals, Inc. Sources of other chemicals and biochemicals were as described previously [4].

Cell free membrane particles were prepared from the liver fluke, *Fasciola hepatica*. The liver flukes were collected from a local slaughterhouse and were incubated in saline media solution overnight [2]. They were frozen on Wollenberger clamps chilled in dry ice and kept in storage at −80°C. For the preparation of membrane particles, frozen flukes were pulverized in a mortar chilled in dry ice and the powder was homogenized in 6 vol. of solution containing 0.33 M sucrose and 1 mM EDTA. The homogenate was centrifuged at 2000 × *g* and the particles were collected after washing twice with the sucrose/EDTA solution as described previously [3].

Activation procedure. Membrane particles were incubated at various temperatures and for different times in an activation mixture containing 0.25 M sucrose, 1 mM EDTA, and indicated activators. In the following paragraphs all Mg^{2+} concentrations are effective concentrations.

Adenylate cyclase activity assay. Adenylate cyclase was assayed by the method of Salomon et al. [12] as described previously [4]. The reaction mixture contained 0.1 M sucrose, 50 mM glycylglycine (pH 7.5), 5 mM phosphocreatine, 0.5 mM 3-isobutyl-1-methyl-xanthine and 0.1 mM Na_2ATP with about 1–2 μCi [α^{32}P]ATP per 0.25 ml assay, 2 mM $MgCl_2$, 5 U creatine phosphokinase, 5 mM dithiothreitol, and 0.02 mM EGTA. Reactions were initiated by the addition of 0.05 ml of membrane suspension (approx. 7 mg protein/ml). The final volume of the reaction mixture was 0.25 ml. Incubations were carried out at the indicated temperature. The reaction was terminated after 10 min and the [^{32}P]cAMP determined as previously described [4]. All values are reported as the average of duplicate incubations.

Spin labeling procedures. Two spin-labeling methods were used. (A) 1 mg *N*-methylmaleimide nitroxide was added to 1 ml sample. After shaking for the indicated time at 10°C, 9 ml of the wash solution was added and the mixture was centrifuged at 10000 × *g* for 10 min at 2°C. The supernate was discarded and the washing repeated three times. The sample was left to stand for 10 min at 0°C between each washing. Lastly, the pellets were suspended in 1 ml of washing solution or distilled water and were homogenized with a motor-driven pestle for 30 strokes on ice. (B) 10 ml tubes were washed with sulfuric acid bichromate solution, rinsed with deionized water, and placed in Prosil-28 solution diluted in water (1/100). They were left in the siliconizing solution for 1 min, rinsed with distilled water, and dried at 70°C for 3 h. 0.2 ml of *N*-methylmaleimide nitroxide in ethanol (5 mg/ml) was placed in each tube. The tubes were continuously rotated in order to coat them evenly while ethanol was evaporated by blowing nitrogen gas into the tube. These tubes were put in a lyophilizer overnight, then filled with nitrogen gas, sealed with paraffin film, and stored at −80°C in a dark box. 1 ml of membrane particles was put in an *N*-methylmaleimide nitroxide coated tube and vortexed for 2 min. The samples were then washed three times to remove excess free spin-label as described under Procedure (A). The first procedure was used in early experiments but was discarded in favor of procedure B because B was more reproducible for short labeling periods. Both methods gave the same ESR spectra.

EPR spectrum measurement. Pyrex disposable micro-sampling pipettes were sealed at one end. 50-μl samples were pipetted into these tubes. The tubes were put into the cavity of the Varian EPR spectrometer E 12 which was connected with a temperature control system and a digital com-

puter. After each scan the data of area, hyperfine splitting, peak height, and line width were calculated by a digital computer (PDP 8/c) according to the program written by Dr. Dicther Recktenwald of the Department of Chemistry.

Results

Conditions for activation of adenylate cyclase

Attempts were made to find optimal conditions for adenylate cyclase activation. This was done by separating the incubation to activate the enzyme from its assay. Fluke membrane particles were first incubated with the activator and aliquots were taken periodically for the adenylate cyclase assay. As was shown previously in the presence of dithiothreitol [4], activation in the presence of NaF was dependent on Mg^{2+} and was time- and temperature-dependent (Fig. 1 and Table I). Maximal activation at 10°C was achieved at 2 h (Fig. 1). The extent of activation was increased at low temperatures. In Fig. 1 it can be seen that maximal activation in the presence of NaF and Mg^{2+} was achieved at 0°C. At higher temperatures (20 to 30°C), the stimulated activity was very much reduced (Fig. 1).

Activation with GTPγS alone or with GTP and serotonin was also dependent on the presence of Mg^{2+} [4]. Fig. 2 shows that maximal activation with GTPγS was almost instantaneous at 30°C. Prolonged incubation at that temperature resulted in a gradual decrease of activity. Unlike activation by fluoride, activation at 0°C with GTPγS was lower than activation at higher temperatures (10 and 30°C). Incubation with GTPγS at 10°C or at 0°C caused a gradual increase in activity with time. Activation of adenylate cyclase by GTP in the presence of Mg^{2+} and serotonin occurred almost instantaneously and the activity gradually decreased during incubation (Fig. 3). Total increase in activity by serotonin and GTP was about one order of magnitude below activation with fluoride or GTPγS. The decay of activity following stimulation by serotonin was faster at higher temperatures than at 0°C.

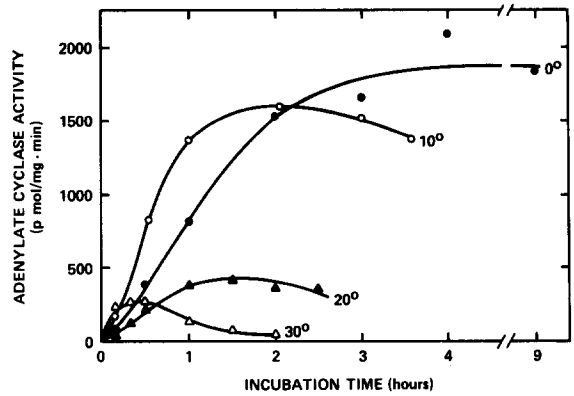

Fig. 1. Activation of adenylate cyclase in membrane particles by NaF and Mg^{2+}. Membrane particles were incubated with 10 mM NaF and 2 mM $MgCl_2$ in the activation mixture at the indicated temperature (°C) and times and assayed as described in Materials and Methods.

TABLE I

ROLE OF Mg^{2+} IN ACTIVATION OF ADENYLATE CYCLASE BY NaF

Membrane particles were incubated with indicated reagents at 0 or 25°C for 2 h. Duplicate aliquots of 50 μl were taken and assayed for 10 min at 30°C as described in Materials and Methods. n.d., not determined.

Additions to activation mixture	Temp. (°C)	Enzyme activity (pmol cAMP/mg per min)		
		Expt. 1	Expt. 2	Expt. 3
Basal activity *		82	82	119
10 mM NaF	0	71	92	93
10 mM NaF+2 mM $MgCl_2$	0	n.d.	9823	20071
10 mM NaF	25	59	114	71
10 mM NaF+2 mM $MgCl_2$	25	n.d.	3675	8228

* Adenylate cyclase activity in the particles without any treatment.

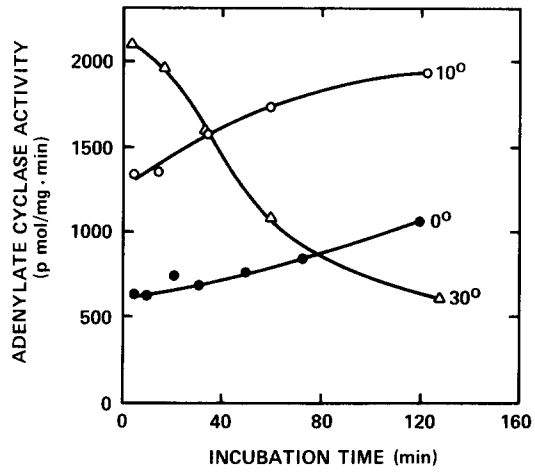

Fig. 2. Activation of adenylate cyclase in membrane particles by GTPγS and $MgCl_2$. Membrane particles were incubated with 0.1 mM GTPγS and 2 mM $MgCl_2$ and assayed as described in Materials and Methods. Temperature in °C.

Inhibition of activated adenylate cyclase by N-methylmaleimide

In order to study the effect of inhibitors on activation of adenylate cyclase, incubations for enzyme activation and for assay were done at the same temperature, 10°C. This was to avoid further

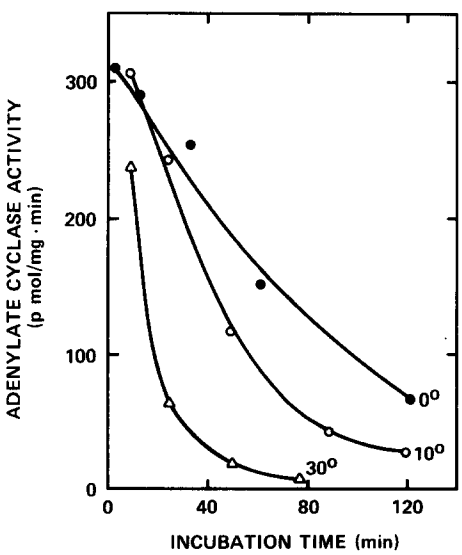

Fig. 3. Activation of adenylate cyclase in membrane particles by serotonin, GTP, and $MgCl_2$. Membrane particles were activated with 0.1 mM serotonin, 0.1 mM GTP and 2 mM $MgCl_2$ and assayed as described in Materials and Methods. Temperature in °C.

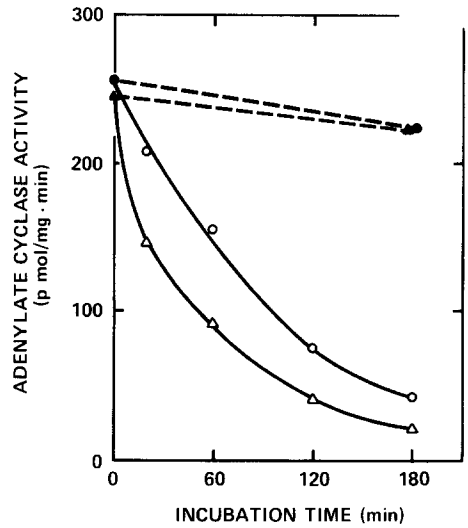

Fig. 4. Inhibition by *N*-methylmaleimide of fluoride- and GTPγS-activated adenylate cyclase in membrane particles. Adenylate cyclase in membrane particles was activated for 2 h at 10°C with 10 mM NaF and 2 mM $MgCl_2$ (●) or 0.1 mM GTPγS and 2 mM $MgCl_2$ (▲) as described in Methods. The membrane particles were then incubated with 5 mM *N*-methylmaleimide at 10°C for the indicated times (○, △). Duplicate aliquots were then taken and assayed as described in Materials and Methods. Broken lines show enzyme activity without *N*-methylmaleimide.

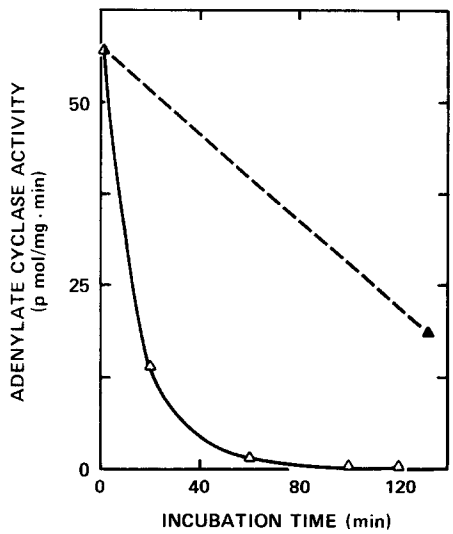

Fig. 5. Inhibitory effect of *N*-methylmaleimide on serotonin-activated adenylate cyclase in membrane particles. Adenylate cyclase in membrane particles was activated with 0.1 mM serotonin, 0.1 mM GTP and 2 mM $MgCl_2$ (▲) for 10 min at 10°C as described in Materials and Methods. The membrane particles were then incubated with 5 mM *N*-methylmaleimide at 10°C for the indicated times (△). Duplicate aliquots were then taken and assayed as described in Materials and Methods. Broken line shows enzyme activity without *N*-methylmaleimide.

significant change in the degree of activation during the assay. Membrane particles which had been activated with GTPγS or with NaF for 2 h were inhibited by *N*-methylmaleimide (Fig. 4). The inhibition was gradual and was almost complete after 3 h. The activity was only slightly reduced during parallel control incubations of the activated enzyme kept at the same temperature.

Adenylate cyclase in membrane particles that were incubated with serotonin, GTP, and $MgCl_2$ for ten minutes were more sensitive to inhibition by *N*-methylmaleimide (Fig. 5). The inhibition was almost complete after one hour of incubation.

Spin-labeling following activation of adenylate cyclase. The particulate enzyme (protein concentration 6.8 mg/ml) was activated with different ligands and was then spin-labeled with 1 mg *N*-methylmaleimide nitroxide powder per ml. Spin-labeling was continued for 2 h at 10°C. Excess *N*-methylmaleimide nitroxide was washed out by centrifugation of the particles four times. After washing, the particles were homogenized in regular homogenizing mixture or distilled water and the EPR spectra were measured (*N*-Methylmaleimide nitroxide inhibited adenylate cyclase activity (Table II) to the same extent as the non-labeled *N*-methylmaleimide (Figs. 4 and 5).). The EPR spectra of the control and the activated particulate enzyme showed weak and strong immobilization peaks (Fig. 6). After incubation with 2 mM $MgCl_2$ and 10 mM NaF (Fig. 6, c), or with 2 mM $MgCl_2$ alone (Fig. 6, b), the strong immobilization peaks (S_c, S_b) became larger than that of the control (S_a) as shown in Fig. 6, a. EPR spectra for GTPγS or serotonin-activated membrane particles in the presence of Mg^{2+} were similar to that of those activated by NaF (not shown). The total binding number of the *N*-methylmaleimide nitroxide was calculated by digital computer from the second integral area of the spectra and was compared with that of binding by control particles. Representative experiments summarized in Table III show that the binding of the spin-label is enhanced

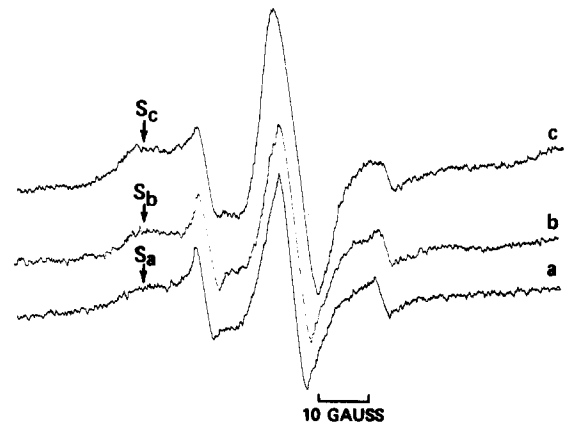

Fig. 6. Effect of adenylate cyclase activation on the EPR spectra of *N*-methylmaleimide nitroxide. Control (a), membrane particles preincubated with 2 mM $MgCl_2$, (b) membrane particles preincubated with 10 mM NaF and 2 mM $MgCl_2$, (c) were prepared as described in Fig. 4. 1 mg of *N*-methylmaleimide nitroxide was added to 1 ml of each of the above preparations. After shaking at 10°C for 3 h, the excess spin-label probe was washed out as described in Materials and Methods. 50-μl aliquots were taken and EPR spectra were measured by using the following settings for the EPR 12 spectrometer: magnetic field, 3257 G; scan range, 100 G; time constant, 1 s; scan time, 16 min; modulation amplitude, 0.5; modulation frequency, 100 kHz; receiver gain, $1 \cdot 10^4$; temperature, 10°C; microwave power, 20 mW; microwave frequency, 9.08 GHz.

TABLE II

INHIBITION OF ADENYLATE CYCLASE BY *N*-METHYLMALEIMIDE NITROXIDE

Adenylate cyclase in membrane particles was pre-activated with indicated activators and then incubated with or without spin-label probe for 3 h at 10°C. Duplicate aliquots of 50 μl were taken and assayed for 10 min at 10°C. 5-HT, 5-hydroxytryptamine.

Activators	Adenylate cyclase activity * (pmol cAMP/mg per min)		Inhibition (%)
	Without spin-label	With spin-label	
10 mM NaF + 2 mM $MgCl_2$	180	21	88
0.1 mM GTPγS + 2 mM $MgCl_2$	204	22	89
0.1 mM 5-HT + 0.1 mM GTP + 2 mM $MgCl_2$	19	0.5	97

* Particulate enzyme was pre-activated with activator and then incubated with or without *N*-methylmaleimide nitroxide for 3 h at 10°C. Assay was also at 10°C.

TABLE III

EFFECTS OF PRE-ACTIVATION OF ADENYLATE CYCLASE ON THE BINDING OF *N*-METHYLMALEIMIDE NITROXIDE TO MEMBRANE PARTICLES

Adenylate cyclase in membrane particles was pre-activated with indicated activator at 10°C. 1 mg of *N*-methylmaleimide nitroxide powder was then added to 1 ml of each sample and shaken for 2 h. The spin-labeled membrane particles were washed four times by centrifuging at 10000× *g* for 10 min and rehomogenized in 1 ml wash solution. 50-μl samples were placed in EPR sampling tubes. EPR spectra were measured as described in Materials and Methods. 5-HT, 5-hydroxytryptamine.

Additions to activation mixture	Total binding number ratio *
Control	100
10 mM NaF+2 mM $MgCl_2$ for 2 h	253
0.1 mM GTPγS+2 mM $MgCl_2$ for 2 h	145
0.1 mM 5-HT, 0.1 mM GTP+ 2 mM $MgCl_2$ for 10 min	207

* Total binding number ratio

$$= \frac{\text{total binding number of activated sample}}{\text{total binding number of control sample}}.$$

Total binding number

$$= \frac{\text{area of EPR spectrum}}{\text{protein concentration (mg/ml)}}.$$

TABLE IV

EFFECT OF PRE-ACTIVATION OF ADENYLATE CYCLASE ON THE BINDING OF SPIN-LABELED PROBE TO PLASMA MEMBRANES

Purified plasma membrane preparations were isolated on sucrose gradient [13]. Adenylate cyclase in the membrane was pre-activated with indicated activator at 10°C. Membranes were then spin-labeled and EPR spectra were measured as described in Table III. Total binding number ratio was computed as in Table III.

Membrane		Total binding number ratio
Pre-treatment	Spin-labeling time (min)	
Control	2	100
10 mM NaF+2 mM $MgCl_2$ at 10°C for 2 h	2	154
0.1 mM 5-HT+0.1 mM GTP+2 mM $MgCl_2$ at 10°C for 10 min	2	155

following enzyme activation by fluoride, by serotonin, by GTP or GTPγS in the presence of Mg^{2+}. The increase in the total binding was also demonstrated in more purified plasma membrane preparations (Table IV) that were isolated on sucrose gradients [13].

TABLE V

BINDING OF *N*-METHYLMALEIMIDE NITROXIDE BY MEMBRANE PARTICLES UNDER DIFFERENT CONDITIONS

Adenylate cyclase in membrane particles was pre-activated with indicated activator at 10°C. 1 ml of each sample was then placed into tubes prepared by procedure B described in Materials and Methods. After shaking for 2 min, samples were washed with 10 vol. of wash solution (0.25 M sucrose and 50 mM glycylglycine) twice and distilled water once by centrifuging at 10000× *g*. Samples were rehomogenized in 1 ml distilled water and EPR spectra were measured as described in Materials and Methods. Total binding number ratio was computed as in Table III. n.d., not determined. 5-HT, 5-hydroxytryptamine.

Sample No.	Additions	Time (min) for activation	Total binding number ratio		
			Expt. 1	Expt. 2	Expt. 3
1	none	10	100	100	100
2	10 mM NaF	120	108	113	103
3	2 mM $MgCl_2$	120	133	152	139
4	10 mM NaF+2 mM $MgCl_2$	120	205	160	148
5	0.1 mM 5-HT	10	104	97	89
6	0.1 mM GTP	10	106	112	106
7	0.1 mM 5-HT, 0.1 mM GTP+2 mM $MgCl_2$	10	143	160	120
8	0.1 mM GTPγS	120	n.d.	87	86
9	0.1 mM GTPγS+2 mM $MgSl_2$	120	n.d.	127	124

TABLE VI

EFFECT OF PRE-ACTIVATION OF ADENYLATE CYCLASE ON THE PEAK HEIGHT OF EPR SPECTRA

Adenylate cyclase in membrane particles was incubated at 10°C with activator for 2 h, and then labeled with *N*-methylmaleimide nitroxide at 10°C. After removing the excess of spin-label by washing four times, EPR spectra were measured as described in Materials and Methods. Strong and weak immobilization peaks were computed from spectra similar to those in Fig. 6.

Activator	Peak height		S/W (%)
	S *	W *	
None	6	18	33
2 mM $MgCl_2$	9	19	47
10 mM NaF+2 mM $MgCl_2$	13	17	76

* S, strong immobilization peak; W, weak immobilization peak.

The question arose as to which component of the activating mixture is responsible for the increase of binding of the *N*-methylmaleimide nitroxide. The results summarized in Table V show that the component which is responsible for major increase in the binding of the spin label is $MgCl_2$. Furthermore, Mg^{2+} appears to be responsible for the increase of the strong immobilized peak of the spin-label. This indicates that an initial conformational change occurred following incubation with Mg^{2+}.

In order to further characterize the effect of activators on the EPR spectra measured, the heights of weak and strong immobilized peaks were determined. Table VI summarizes a representative experiment. The heights of the weak immobilization peaks of EPR spectra in the activated membranes were almost the same as in the control (Table VI). On the other hand, the heights of the strong immobilization peaks as well as S/W (strong immobilized peak height/weak immobilized peak height) were significantly increased in the presence of Mg^{2+} or NaF and Mg^{2+}. The increase of the total binding number shown in Table III is therefore mainly due to more strong immobilized binding, and probably due to more exposure of inner sulfhydryl groups in the membranes.

Discussion

The results reported above indicate that among different activators of adenylate cyclase there are some quantitative and qualitative differences. Activation with NaF and with GTPγS was at least one order of magnitude higher than activation with serotonin and GTP. The response of the enzyme to these activators at different temperatures also revealed some differences. For example, maximum activation by fluoride and by serotonin and GTP occurred at 0°C while maximum activation by GTPγS occurred at 30°C. These differences suggest that the mechanism of activation by these agents may not be the same. It is possible that a rigid microenvironment is favorable to activation of adenylate cyclase with serotonin or NaF, but it may not be the case with GTPγS. Heron et al. [14] reported that microviscosity of the synaptic membranes was increased by in vitro incubation with either cholesteryl hemisuccinate or stearic acid, resulting in an up to 5-fold increase in the specific binding of [^{3}H]serotonin. The possibility also exists that there are differences in the stability of adenylate cyclase with different activators.

The membrane particles appear to undergo a major conformational change during enzyme activation. This is indicated by the marked increase in the binding of *N*-methylmaleimide nitroxide and the consistent increase in the strongly immobilized peak of the EPR spectrum with all the activators. Mg^{2+} was found to be essential for enzyme activation with all the activators tested. Mg^{2+} alone was sufficient to cause the increase in the binding of the spin-label reagent and the increase in the immobilized peak in the EPR. Mg^{2+} therefore may be responsible for the initial conformational change in the membrane. Such a change may result in the spin-labeled probe having access to more exposed inner SH groups in the enzyme.

Since the above described experiments were done on a suspension of membrane particles and not on a purified adenylate cyclase, the changes in the EPR spectra which were demonstrated by the activators may not be a specific phenomenon for the enzyme itself. This is further suggested by the fact that adenylate cyclase constitutes only a minor part of total protein in the membrane. It is possi-

ble, however, that the conformational change in the membrane caused by Mg^{2+} as described above is the overture to activation of the adenylate cyclase by NaF, GTPγS or serotonin with GTP. This is supported by the fact that Mg^{2+} was essential for activation of the enzyme by any of these activators.

Acknowledgements

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