Effects of Noradrenaline on the Activation and the Stability of Brain Adenylate Cyclase

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At constant 1 mM-ATP, the Mg^{2+} -saturation curves for adenylate cyclase (EC 4.6.1.1) particulate preparations obtained from corpus striatum and cortex tissues of rat brain show that addition of 0.1 mM-noradrenaline increases the apparent V_{max} for Mg^{2+} by 300% in corpus striatum particles, and by 280% in cortex particles. At 10 mM- $MgCl_2$, the addition of 0.1 mM-noradrenaline increased by 800% the adenylate cyclase activity of corpus striatum particles. At all Mg^{2+} concentrations, the addition of 0.3 mM- $CaCl_2$ suppressed the noradrenaline-induced stimulation of adenylate cyclase of corpus striatum particles, and even resulted in a strong inhibition of the activating effect of Mg^{2+} itself on adenylate cyclase activity of cortex particles. The addition of noradrenaline during a 3 h preincubation of particle preparations of brain cortex at 38°C decreased by more than 4-fold the half-life of the decay of adenylate cyclase activity. The addition of MgATP protected against noradrenaline-induced inactivation.

Previous investigations (Robison *et al.*, 1967; Kakiuchi & Rall, 1968*a,b*) have shown that noradrenaline has a potent stimulatory effect on the accumulation of endogenous cyclic AMP in brain-tissue slices. However, particulate preparations of adenylate cyclase, isolated from brain tissue, have consistently been shown either to lack a response to catecholamines (Rall & Sutherland, 1958) or to show only a slight and varying stimulatory response to these neurotransmitters (Klainer *et al.*, 1962). Consequently, it was decided to study the requirements that could allow for a noradrenaline-induced stimulation of the adenylate cyclase activity of particle preparations from brain tissue. Moreover, since the study of brain adenylate cyclase has been hampered by its lack of stability (Robison *et al.*, 1967; Johnson & Sutherland, 1973), the effects of various preincubation conditions on the stability of adenylate cyclase activity were also studied. As reported herein, it was found that to show a reproducible and high-percentage stimulation of adenylate cyclase by noradrenaline, a high concentration of free Mg^{2+} ions in the incubation medium was necessary. During these studies, a noradrenaline-induced decrease in the stability of the labile adenylate cyclase was also found, and we discuss its possible physiological and pathological significance.

Experimental

Materials

The compounds were from the following sources: noradrenaline, caffeine, oestradiol benzoate and

ATP from Sigma Chemical Co. (St. Louis, MO, U.S.A.); EDTA (disodium salt) and Barbitol (5,5-diethylbarbituric acid) from Fisher Scientific Co. (Springfield, NJ, U.S.A.); Tricine† and Tris from General Biochemicals (Chagrin Falls, OH, U.S.A.); sucrose from Mallinckrodt (Jersey City, NJ, U.S.A.); albumin from Pentex (Kankakee, IL, U.S.A.); cyclic [3H]AMP from Schwartz–Mann (Orangeburg, NY, U.S.A.). Rats were from Camm Research Institute (Wayne, NJ, U.S.A.).

Preparation of Ca^{2+} -treated cyclase particles

Male Sprague–Dawley rats weighing 200 g were stunned and decapitated at 4°C. Cerebral cortex from several brains was sliced and collected in a volume (4 ml/cortex) of ice-cold 10 mM-Tricine/Tris buffer, pH 7.4, containing 2 mM-sucrose. The total fresh tissue weight was determined and the tissue was homogenized in a motor-driven glass homogenizer at 400 rev./min for 2 min. The homogenate was centrifuged at 2400 rev./min for 20 min in an IEC International clinical centrifuge (IEC 809 head) in a 5°C cold-room. The resulting supernatant fraction was discarded and the pellet was washed by resuspension with a vortex mixer in an equal volume of the same homogenizing medium and recentrifuged at 2400 rev./min for 20 min. A second washing step was done by repeating the same procedure with an equal volume of 10 mM-Tricine/Tris buffer, pH 7.4,

† Abbreviations: Tricine, *N*-[2-hydroxy-1,1-bis-(hydroxymethyl)ethyl]glycine; ATPase, adenosine triphosphatase.

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containing 0.5mM-CaCl₂. The pellet was resuspended in 4ml of the same medium. The particulate preparation was used either fresh, when indicated in the legends, or after storage at -70°C and thawing for 30min at room temperature (24°C). Before its experimental use, the Ca²⁺-treated adenylate cyclase particulate preparation (Ca²⁺-treated particles) was washed once more by using the same centrifugation procedure with 10mM-Tricine/Tris, pH7.4, and resuspended in a volume of the same medium needed to dilute the particles to the desired concentration.

Preparation of EDTA-treated adenylate cyclase particles

Ten ovariectomized Sprague-Dawley female rats were subcutaneously injected with 100µg of oestradiol benzoate/kg body wt. for 3 consecutive days. This treatment was not required for the study of adenylate cyclase activation as described in the present paper, but was part of other unpublished experiments. Rats were killed by decapitation as described above, and corpus striatum and cortex tissues were separately collected in 20ml of ice-cold 2mM-Tris/HCl buffer/10mM-NaCl/10mM-KCl/5µM-EDTA, adjusted to a final pH7.4. The total fresh weight was separately determined for cortex and corpus striatum and the tissues were homogenized by the procedure previously described for Ca²⁺-treated particles. Particles were obtained and washed twice in an equal volume of the same solution by using the centrifugation procedure described above for the preparation of Ca²⁺-treated particles. The pellets obtained from both corpus striatum and cortex were separately resuspended in the same buffer and separately stored at -70°C. The protein contents of these membrane preparations were determined by a colorimetric method (Lowry *et al.*, 1951) with bovine serum albumin as the standard. These EDTA-treated adenylate cyclase particulate preparations from cortex and corpus striatum (EDTA-treated particles) were stored at -70°C and used after thawing for 30min at room temperature (24°C).

Assay of adenylate cyclase activity

Unless otherwise indicated, samples of Ca²⁺-treated particles obtained from 20mg fresh wt. of total cortex tissue were incubated for 10min at 15°C in a reaction mixture (1ml) containing: 40µmol of Tricine/Tris buffer, pH7.4, 6.67µmol of caffeine and 0.25µmol of MgATP adjusted to final pH7.4 with Tris. Modifications to the assay medium are indicated in the legends. The incubation was terminated by heating in a boiling-water bath for 5min. Blanks (zero time) were obtained for all assays by incubating a previously boiled particulate prepara-

tion under the same conditions. The reaction mixtures were centrifuged in a Sorvall RC2B centrifuge at 5000g for 10min and the supernatants were collected and assayed for cyclic AMP formed. Whenever immediate measurement was not possible, the supernatants were stored at -70°C and later used for the same assay. Adenylate cyclase activity of EDTA-treated particles was assayed by using the modified assay conditions described under Fig. 1.

Assay of cyclic AMP

Cyclic AMP formed by adenylate cyclase activity was measured by the Gilman (1970) method. This is a saturation assay procedure (Brown *et al.*, 1972) using a 0.4ml reaction mixture containing 50µl of the supernatant from the incubation mixtures (diluted if required to adjust the content of cyclic AMP to less than 5pmol, as well as their corresponding zero time controls), 100µl of 35mM-sodium acetate buffer, pH4.0, containing 11.4µg of albumin, 50µl of cyclic [³H]AMP (NH₄⁺ salt, 0.55pmol, containing 30000c.p.m.), and a mixture of 50µl (8mg/ml) of binding protein prepared from fresh bovine muscle by the method of Miyamoto *et al.* (1969) with 50µl of 5mM-potassium phosphate buffer, pH7.0. The cyclic AMP assay mixture was incubated in an ice bath for 1h, then 1ml of 20mM-potassium phosphate buffer, pH7.0, was added and the mixture was thereafter filtered through a type HA Millipore filter (0.45µm pore size) in a 3025 Millipore Sampling Manifold to separate the precipitated binding-protein-cyclic[³H]AMP complex. The filters containing the precipitate were washed with 3ml of 5mM-potassium phosphate buffer, pH6.0, placed in vials with 10ml of Bray's (1960) scintillator fluid and radioactivity was measured in a Beckman LS-100 scintillation counter. To obtain standard curves for the calculation of the results, standards of known amounts of cyclic AMP were used with every experiment. Results are expressed as nmol of cyclic AMP formed/h per Ca²⁺-treated particles from 1g fresh wt. of total cortex tissue. Cyclic AMP formed by EDTA-treated particles from brain corpus striatum or cortex was assayed by using 100µl samples of supernatants from the incubation mixtures and adjusting the amounts of the other reagents used in the method described above to correspond to a final volume of 0.3ml. Results are expressed in nmol of cyclic AMP formed/h per g of protein in the EDTA-treated particles as determined by a colorimetric method (Lowry *et al.*, 1951), with bovine serum albumin as standard. EDTA-treated particles were used to minimize any contribution by residual metals to the conditions under which the effects of Mg²⁺ and Ca²⁺ on noradrenaline stimulation of adenylate cyclase were assayed. Ca²⁺-treated particles showing

similar properties to Mg^{2+} -treated particles (Brydon-Golz & Bennum, 1975) were used to minimize the contribution that the loss of metals, during preparation of the membranes, could have had in the assay of the specific effects of noradrenaline on the stability of adenylate cyclase.

Assay of ATPase activity

After the incubation step in MgATP described for the assay of the adenylate cyclase activity, 50 μ l samples of the resulting supernatants were taken for the assay of cyclic AMP formed, and 0.6ml samples were taken to measure P_i and also to assay the ATPase activity of the Ca^{2+} -treated particles under identical conditions to the assay of adenylate cyclase activity. P_i was determined by a colorimetric method (Bernhardt & Wreath, 1955) by a modified procedure (Nishizaki & Jagendorf, 1969) by using the modified assay conditions (Bennun & Bennun, 1972) adjusted for the extraction of the 0.6ml sample with 1ml of 2-methylpropan-1-ol/benzene (1:1, v/v). After the extraction of the phosphomolybdate complex, the water phase was removed by suction, and a transparent clear 2-methylpropan-1-ol/benzene phase was obtained after centrifugation at 4000 rev./min in an IEC International centrifuge (IEC 809 head) for 5 min at room temperature. Residual water was completely removed by suction. The A_{320} of the yellow phospho-

molybdate complex was determined with a Zeiss PMQII spectrophotometer in 0.5ml quartz cells, and the values for nmol of P_i released/per particles obtained from 1g of fresh tissue wt. were calculated by using a standard curve.

The standard error of estimates of adenylate cyclase activity are given in the legend to Fig. 1 with the number of independent assays used indicated in parenthesis. In Table 1, the ranges of adenylate cyclase activities for different preparations of Ca^{2+} -treated particles, subsequently to their preincubation under various conditions (spread of results) obtained for independent experiments, as well as the number of experiments, are indicated within parentheses below the values of the S.E.M.

Results

Noradrenaline-dependent stimulation of adenylate cyclase

Mg^{2+} -saturation curves at constant 1 mM-ATP are reported in Fig. 1 for EDTA-treated particles from either corpus striatum (a) or cortex (b), showing that the maximal rate of Mg^{2+} -dependent adenylate cyclase activity (apparent in the presence of Mg^{2+}) is reached for corpus striatum at 15 mM- and for cortex at 25 mM- $MgCl_2$ concentrations respectively. The inclusion of 0.1 mM-noradrenaline in the reaction mixtures increased the apparent v in the presence of

Fig. 1. Mg^{2+} requirement for noradrenaline stimulation of adenylate cyclase

Samples of EDTA-treated-particle preparations from rat brain (a, corpus striatum; b, cortex) containing 76 μ g (a) and 100 μ g (b) of protein determined by the colorimetric procedure of Lowry *et al.* (1951), were incubated at 25°C for 60 min in 40 mM-Tricine/Tris buffer, pH 7.4, 6.67 mM-caffeine, constant 1 mM-ATP and addition of the indicated concentrations of $MgCl_2$ (final volume 0.5 ml). ●, Mg^{2+} alone; ▲, +0.1 mM-noradrenaline; ■, +0.1 mM-noradrenaline and 0.3 mM- $CaCl_2$. The incubation was terminated, blanks were prepared and the cyclic AMP formed was assayed by the procedure described in the Experimental section. The S.E.M. of values for assays in excess of 15 mM- Mg^{2+} are as follows: (a) ●, 83 ± 34 (2); ▲, 304 ± 23 (2); ■, 73 ± 4 (2); (b) ●, 76 ± 7 (4); ▲, 204 ± 14 (4); ■, 27 ± 2 (4).

Mg^{2+} by 300% (corpus striatum) and by 280% (cortex). The kinetic response of the EDTA particles from brain corpus striatum (Fig. 1*a*) also showed a slight decrease in the Mg^{2+} concentration required at 1 mM-ATP for half-maximal activatory effect (apparent K_m in the presence of Mg^{2+}). Consequently, the maximal stimulation of corpus-striatum adenylate cyclase activity reached 800% at the rather physiological concentrations of 1 mM-ATP and 10 mM- Mg^{2+} . Fig. 1*a*) also shows that the joint addition of 0.1 mM-noradrenaline and 0.3 mM- CaCl_2 in the reaction mixture results in suppression of the stimulatory effect of noradrenaline. A more powerful effect by Ca^{2+} ions on the adenylate cyclase activity of EDTA-treated particles obtained from rat brain cortex was observed, because 0.3 mM- CaCl_2 not only suppressed the stimulatory effect of noradrenaline, but also caused a strong inhibitory effect on the stimulation by Mg^{2+} itself (Fig. 1*b*). Consequently, since Ca^{2+} is at a considerably lower concentration than that of Mg^{2+} at most points of the Mg^{2+} -saturation curves, Ca^{2+} shows a much stronger inhibitory effect than the activatory effect of Mg^{2+} on the functional coupling between catecholamine receptor and adenylate cyclase response.

This effect is substantially different from that observed in EDTA-treated particles from rat fat-cells because 0.3 mM- CaCl_2 in the presence of 5–50 mM- Mg^{2+} does not suppress the stimulatory effect of catecholamines (Harris & Bennun, 1976). Fig. 2 shows that when ATP concentration exceeds that of Mg^{2+} , total inhibition of adenylate cyclase activity is observed. Addition of noradrenaline

(Fig. 2*b*) does not result in significant stimulation of adenylate cyclase, even if the Mg^{2+} concentration in the incubation media is increased to 5 mM. Thus the results reported under Figs. 2*a*) and 2*b*) support the finding reported in Figs. 1*a*) and 1*b*) that shows that noradrenaline is only capable of powerful stimulatory effects on adenylate cyclase when Mg^{2+} reaches saturating concentrations.

Fig. 3*a*) shows that in the presence of MgATP the addition of 0.1 mM-noradrenaline to the reaction mixture does not increase the enzyme's $v_{\max}$. The substitution by Mg^{2+} -treated particles of Ca^{2+} -treated particles (Brydon-Golz & Bennun, 1975) in the study of the enzyme's MgATP-saturation curves resulted in the finding of even lower noradrenaline stimulation at a concentration of 0.1 mM. However, the rather non-physiological concentration of 1 mM-noradrenaline was capable of stimulating by 3-fold the adenylate cyclase of Mg^{2+} -treated particles from brain cortex, through a change of K_m , but without modification of $V_{\max}$. (Brydon-Golz & Bennun, 1975). Fig. 3*b*) shows that adenylate cyclase reaches maximal activity at pH 8.5. A rather small noradrenaline-induced stimulation is observed at pH 7.5–7.6. This effect explains, therefore, the slight activatory effect of noradrenaline observed at pH 7.5 (Fig. 3*a*) and its absence at pH 7.4 (Figs. 2*a* and 2*b*). Hence these results consistently support the finding of a requirement for Mg^{2+} at saturating concentrations for a noradrenaline-dependent activation of adenylate cyclase particulate preparations. Moreover, they also explain previous reports that consistently described the response of particulate

Fig. 2. Effect of noradrenaline on adenylate cyclase assay at low Mg^{2+} concentrations

Portions of a Ca^{2+} -treated particulate preparation were incubated under the standard conditions described in the Experimental section, except that the standard reaction mixtures were modified to contain various concentrations of ATP ($\square$, 0.1 mM; $\blacksquare$, 0.4 mM; $\triangle$, 1.0 mM; $\blacktriangle$, 2.5 mM) and the MgSO_4 concentrations indicated at a final pH of 7.4, (a) in the absence or (b) in the presence of 0.1 mM-noradrenaline.

Fig. 3. Effect of noradrenaline on the MgATP saturation and pH curves of adenylate cyclase

Portions of a fresh Ca^{2+} -treated particulate preparation were incubated under the standard conditions described in the Experimental section for the assay of Ca^{2+} -treated particles. In (a) the standard reaction mixtures were modified for increased concentrations of MgATP, adjusted with 10mM-Tris to a final pH7.5 and in (b) 40mM-Tricine was adjusted with Tris to give the final pH values indicated. Assays were done in the absence (●) or in the presence (○) of 0.1 mM-noradrenaline.

preparations of brain adenylate cyclase to catecholamines as being variable and well below the stimulatory effect usually shown by the adenylate cyclases from other tissue sources (Rall & Sutherland, 1958; Klainer *et al.*, 1962; Brydon-Golz & Bennun, 1975).

Noradrenaline-dependent changes in adenylate cyclase stability

As indicated in Table 1, various compounds were included in the preincubation mixture of Ca^{2+} -treated particles and subsequently removed by washing. These washed particles showed significant differences in their adenylate cyclase activity when assayed under identical conditions. Hence particles obtained by a prior preincubation in buffer (control),

when assayed for adenylate cyclase activity in 0.25mM-MgATP, showed a weak and variable stimulation by 0.1 mM-noradrenaline. On the other hand, preincubation in 5mM-MgATP resulted in particles showing adenylate cyclase activity nearly 4 times that of the control particles and lacking a response to the inclusion in the assay conditions of 0.1 mM-noradrenaline. Preincubation with noradrenaline resulted in a pronounced decrease of the adenylate cyclase activity of the particles, but the additional inclusion of 5mM-MgATP resulted in a strong protective effect against noradrenaline-dependent inactivation. Particles obtained after preincubation with 5.0mM-MgSO₄ show during incubation an adenylate cyclase activity which does not significantly differ from that resulting from particles obtained by preincubation in buffer alone (control).

The finding that the stability of adenylate cyclase was affected by noradrenaline and MgATP indicated

Table 1. Effects of preincubation with metabolites and noradrenaline on adenylate cyclase stability

Samples of Ca^{2+} -treated particles from 36mg fresh tissue wt. of total cortex were preincubated at 38°C for 3h in 10mM-Tricine/Tris, pH7.4, with the additions indicated in a final volume of 0.9ml. The preincubation mixtures were centrifuged at 1700rev./min for 15min in an IEC model 809 head at 20°C. The pellets were washed once by the procedure indicated in the Experimental section, with 3ml of the same buffer, and suspended in 0.36ml of the same buffer. Samples of preincubated particles (from 15mg fresh wt. of tissue) were incubated in the absence and presence of 0.1mM-noradrenaline for the assay of adenylate cyclase activity, under the conditions reported in the Experimental section, adjusted to a final volume of 0.5ml. The spread of results and the number of independent experiments done with preparations obtained at different times, are indicated in parentheses below the mean values $\pm$ S.E.M.

Preincubation additions	Cyclic AMP formed (nmol/h per particles obtained per g fresh wt. tissue)	
	Without noradrenaline	With 0.1 mM-noradrenaline
Buffer only	5.8 $\pm$ 1.1 (5.0-7.7) (4)	8.7 $\pm$ 2.8 (5.7-13.4) (4)
5.0mM-MgATP	29.3 $\pm$ 6.7 (19.5-39.0) (4)	30.5 $\pm$ 7.0 (19.5-39.0) (5)
0.5mM-Noradrenaline	0.5 $\pm$ 0.8 (0.0-1.6) (6)	0.6 $\pm$ 0.9 (0.0-1.8) (6)
5.0mM-MgATP + 0.5mM-noradrenaline	8.6 $\pm$ 3.3 (5.0-12.0) (4)	7.0 $\pm$ 4.1 (4.0-13.0) (4)
5.0mM-MgSO ₄	6.1 $\pm$ 1.5 (5.0-7.2) (2)	6.5 $\pm$ 1.0 (5.7-7.2) (2)

the desirability of studying these effects at various times during the course of the preincubations. As described in the legend of Fig. 4, Ca^{2+} -treated particles were preincubated at 38°C with either (a) 0.2 mM-, (b) 0.5 mM- or (c) 1 mM-noradrenaline and then washed before the assay of adenylate cyclase

activity. Fig. 4 shows a rapid decay of adenylate cyclase activity at all the noradrenaline concentrations tested. Inactivation of adenylate cyclase was completed within 2 h. The half-life of the noradrenaline-induced adenylate cyclase decay process was calculated to be 45 min from the moment that the

Fig. 4. Time-dependent effects of noradrenaline on the stability of adenylate cyclase

Samples of a Ca^{2+} -treated particle preparation of brain cortex containing 100 mg of protein determined by the colorimetric procedure of Lowry *et al.* (1951) were preincubated at 38°C in 6.25 ml of 6 mM-Tricine/Tris buffer, pH 7.4, containing the following concentrations of noradrenaline: (a) 0.2 mM; (b) 0.5 mM; (c) 1 mM. Subsequently, 0.5 ml samples from each of these preincubated suspensions were taken at the indicated times and centrifuged at 5000 g for 5 min in a Sorvall RC2B centrifuge. The pellets obtained were resuspended in 10 mM-Tricine/Tris buffer, pH 7.4, with a vortex mixer. By the procedure described in the Experimental section, samples of these washed particles containing 2.4 mg of protein were incubated at 15°C for 10 min (final volume 0.5 ml of the standard reaction mixture), in the absence (●) or in the presence (○) of 0.1 mM-noradrenaline, and adenylate cyclase activity was determined.

Fig. 5. Time-dependent effects of ATP and noradrenaline on the stability of adenylate cyclase

By the procedure described in the legend of Fig. 4, samples of Ca^{2+} -treated particles at the same final protein concentration were preincubated at 38°C in 6 mM-Tricine/Tris, pH 7.4, containing either 0.5 mM-noradrenaline and 5 mM-MgATP (a, ●, ○) or 5 mM-MgATP (a, ▲, △), or 1.0 mM-noradrenaline and 5 mM-MgATP (b, ●, ○). Subsequently, samples were taken at the indicated times and the particles were washed and adenylate cyclase activity was assayed as described, in the absence (----) or in the presence (—) of 0.1 mM-noradrenaline.

first sample of preincubated particles was taken for the assay of its enzyme activity.

Fig. 5 shows the time-course of the effect of 5mM-MgATP on the stability of adenylylase during preincubation at 38°C in the absence and presence of (a) 0.5mM- or (b) 1mM-noradrenaline. The results indicate that the inclusion of MgATP during the first 3h of preincubation was able to protect effectively against the previously shown ability of 0.5M-noradrenaline to induce a rapid decay of adenylylase activity. Moreover, not only did the decay rate of the adenylylase slow down considerably during the first 3h of preincubation, as shown in Fig. 5(a), but activity was also higher than that of the control (Fig. 6). A control for the experiments reported in Figs. 4 and 5 is illustrated in Fig. 6. It shows that only after 3h of preincubation in buffer at 38°C did the Ca^{2+} -treated particles lose 50% of their adenylylase activity. Consequently, noradrenaline appears to play a rather specific role in sensitizing a labile enzyme to heat-dependent effects that affect the enzyme-membrane structure required for adenylylase activity. Consistently, since substrates have been found to protect some enzymes (Schimke *et al.*, 1964), MgATP may decrease the effect of noradrenaline by stabilizing the structure of adenylylase, thus decreasing the sensitivity of the noradrenaline ligand-enzyme membrane structure to heat inactivation.

Fig. 6. Time-dependent effects of preincubation in buffer on the stability of adenylylase cyclase

By the procedure described in the legend of Fig. 4, Ca^{2+} -treated particles at the same final protein concentration were preincubated at 38°C in 6mM-Tricine/Tris, pH7.4. Samples were taken at the indicated times and subsequently the particles were washed and adenylylase activity was assayed as described, in the absence (○) or in the presence (●) of 0.1mM-noradrenaline.

Preincubation-dependent modification of the kinetic properties of adenylylase cyclase

The effects observed during the time-course of the preincubation with noradrenaline and/or MgATP indicated the possibility that a structural modification (Storm & Dolginow, 1973) may have affected the kinetic properties of adenylylase cyclase. Consequently, it was decided to determine if a significant modification of the substrate-saturation curves could be detected for each of the differently preincubated particles, after a preincubation period equal to the half-life of the decay of their adenylylase activities. These studies were particularly important, since, after preincubation, adenylylase cyclase activity had been assayed in 0.25mM-MgATP, which is a concentration below K_m . Thus effects interpreted as inactivating or activating the enzyme could alternatively be attributed to a shift in the substrate affinity constant as a result of desensitizing reactions (Afting *et al.*, 1971; Karadsheh & Ramaiah, 1972). Preincubation in buffer of Ca^{2+} -treated particles at 37°C for 3h 20min, as shown in Fig. 7, resulted in particles with kinetic properties of their adenylylase cyclase similar to those of non-preincubated particles (Fig. 3b). Accordingly, the preincubated particles showed a slight noradrenaline-dependent activation associated with a decrease in K_m of a magnitude similar to that observed in the

Fig. 7. Effect of a preincubation in buffer on the MgATP-saturation curve of adenylylase cyclase

A Ca^{2+} -treated particle preparation from 500mg fresh wt. of cortex tissue was preincubated at 37°C for 3h 20min in 6.2ml of 6mM-Tricine/Tris buffer, pH7.4. By using the procedure described in the legend of Fig. 4, the particles were subsequently washed and resuspended in an equal volume of the same buffer. As described in the Experimental section, portions of the washed particles were incubated with various increasing concentrations of MgATP adjusted to final pH7.5 for the assay of adenylylase activity in the absence (●) or in the presence (○) of 0.1mM-noradrenaline.

substrate-saturation curves of non-preincubated enzyme. In addition, noradrenaline did not change significantly the $V_{\max}$ of the enzyme.

Preincubation of Ca^{2+} -treated particles in 0.1 mM-noradrenaline at 37°C for 45 min (Fig. 8) gave particles that did not show a noradrenaline-induced change of $V_{\max}$, but only some noradrenaline-induced stimulation in the range 1–2 mM-MgATP. On the other hand, as also shown in Fig. 8, preincubation of Ca^{2+} -treated particles in 0.1 mM-noradrenaline for 3 h 20 min gave particles showing a total loss of adenylate cyclase activity over the entire range of MgATP concentration used for the substrate-saturation curve. Fig. 9 shows that preincubation of Ca^{2+} -treated particles in 0.5 mM-noradrenaline and 5 mM-MgATP at 36°C for 150 min produced particles with increased affinity for the substrate MgATP. This decrease in K_m allows the adenylate cyclase of particles preincubated with MgATP and noradrenaline to show 70% of $V_{\max}$ at 0.25 mM-MgATP, whereas the enzyme of non-preincubated or preincubated particles in buffer reached 70% of $V_{\max}$ at 3 times this MgATP concentration. Consequently, desensitizing effects by decreasing K_m for MgATP (Fig. 9) and slower adenylate cyclase decay rate during preincubation

Fig. 8. Time-dependent effect of preincubation with noradrenaline on the MgATP-saturation curve of adenylate cyclase

A Ca^{2+} -treated particle preparation from 440 mg fresh wt. of cortex tissue was preincubated at 37°C in 6.0 ml of 0.1 mM-noradrenaline and 6 mM-Tricine/Tris, pH 7.4. Samples were taken after 45 min (●, ○) and after 3 h 20 min (■, □) of preincubation. By using the procedure described in the legend of Fig. 4, the particles were subsequently washed and resuspended in an equal volume of 6 mM-Tricine/Tris buffer, pH 7.4. As described in the Experimental section, samples of the washed particles were incubated with increasing concentrations of MgATP adjusted to final pH 7.5 for the assay of adenylate cyclase activity in the absence (●, ■) or in the presence (○, □) of 0.1 mM-noradrenaline.

Fig. 9. Effects of preincubation in noradrenaline with and without MgATP on the MgATP-saturation curve of adenylate cyclase

Portions of Ca^{2+} -treated adenylate cyclase particles from 750 mg fresh wt. of cortex tissue were preincubated at 36°C for 2 h 30 min in 9 ml of 6 mM-Tricine/Tris buffer, pH 7.4, containing either 0.2 mM-noradrenaline (▲, △) or 0.5 mM-noradrenaline and 5.0 mM-MgATP (■, □). By using the procedure described in the legend of Fig. 4, the particles were subsequently washed and incubated with increasing concentrations of MgATP adjusted to final pH 7.5 for the assay of adenylate cyclase activity in the absence (▲, ■) or in the presence (△, □) of 0.1 mM-noradrenaline.

in MgATP (Fig. 5) may explain the activatory effect of MgATP during preincubation (Table 1). A similar activating effect by preincubation in MgATP has been previously reported for Mg^{2+} -treated particles (Brydon-Golz & Bennun, 1975). Fig. 9 also shows that preincubation with 0.2 mM-noradrenaline alone gave particles which showed no adenylate cyclase activity over the whole range of substrate concentrations studied. Hence the effect of noradrenaline cannot be explained as an increase in K_m , but as a sensitizer of adenylate cyclase to a thermal decay process, because the noradrenaline-liganded enzyme conformation becomes more labile than that of the enzyme alone.

Comparative thermal stability of adenylate cyclase and ATPase

The effects shown by ATP and noradrenaline during preincubation of adenylate cyclase could be related to a modification of the thermal-stability of the enzyme. Long periods of preincubation at the physiological range of temperature (36 – 38°C) could be compared with the effect on the enzyme's decay over shorter periods of time at a higher temperature (Bennun & Blum, 1966). Hence the heat-stabilities of adenylate cyclase and ATPase (as control) were

Fig. 10. Comparative heat-stability of adenylate cyclase and ATPase activities

A Ca^{2+} -treated particle preparation from 500 mg fresh wt. of cortex tissue was preincubated at 60°C in 6.2 ml of 6 mM-Tricine/Tris buffer, pH 7.4. Samples were taken at the indicated times and subsequently, by the procedure described in the legend of Fig. 4, the particles were washed and resuspended in an equal volume of the same buffer. As described in the Experimental section, samples of the washed particles were incubated at 15°C for 10 min in 0.25 mM-MgATP, final pH 7.5, to determine adenylate cyclase (●) and ATPase (Δ) activities. The cyclic AMP formed and the P_i released were measured as described in the Experimental section. Activity is expressed as a percentage of the initial activity; initial adenylate cyclase activity was 22 nmol of cyclic AMP formed/h per g fresh wt. of total tissue; initial ATPase activity was 20 μmol of P_i released/h per g fresh wt. of total tissue.

Fig. 11. Effect of MgATP on the comparative heat-stability of the adenylate cyclase and ATPase activities

By using the procedure indicated in the legend of Fig. 10, Ca^{2+} -treated particles at the same final concentration were preincubated at 60°C in 0.5 mM-MgATP and 6 mM-Tricine/Tris buffer, pH 7.4. Samples were taken at the indicated times and, as indicated in Fig. 10, the particles were washed and resuspended in 6 mM-Tricine/Tris buffer, pH 7.4, and similarly incubated to determine adenylate cyclase (□) and ATPase (Δ) activities. Initial adenylate cyclase activity was 22 nmol of cyclic AMP formed/h per g of fresh wt. of total tissue; initial ATPase activity was 22.5 μmol of P_i released/h per g fresh wt. of total tissue.

appears to be an effect that can only proceed at temperatures lower than 60°C . On the other hand, Fig. 11 shows that 0.5 mM-MgATP can provide a protective effect against the thermal decay of the ATPase.

Discussion

A hypothesis for adrenergic transmission is based on the role of catecholamines in increasing cyclic AMP concentrations (Greengard, 1972), and the stimulatory action of cyclic AMP on the protein kinases found in neural tissues (Weller & Rodnight, 1970). The first step of this hypothesis (Greengard, 1972) appears to be supported by the finding of a catecholamine-induced increase in endogenous cyclic AMP (Robison *et al.*, 1967; Kakiuchi & Rall, 1968a,b). However, the finding of a direct noradrenaline stimulation of isolated adenylate cyclase, as reported herein, must be distinguished from alterations in endogenous cyclic AMP that may be caused by changes in metabolic conditions. Among the latter are the inhibition of phosphodiesterase-dependent breakdown of cyclic AMP, and regulatory effects on adenylate cyclase through change in $\text{ATP}/\text{Mg}^{2+}$ ratios (Figs. 2a and 2b). The latter

simultaneously studied by preincubating Ca^{2+} -treated particles at 60°C . Fig. 10 shows the thermal-decay curves for ATPase and adenylate cyclase. The similarity of the two curves may indicate that the stability of both enzymes may be dependent on equally temperature-sensitive structural parameters maintained by these enzymes within the membrane. The thermal-decay curve at 60°C shown for adenylate cyclase in Fig. 10 can be compared with those shown in Fig. 6 during preincubation in buffer at 38°C . The progress of the inactivation process affecting adenylate cyclase at both temperatures was also plotted with the ordinate as the logarithmic decay of the initial activity (taken as 100%). The logarithmic plotting (values not included) transform the curves shown for Fig. 6 (38°C) and Fig. 10 (60°C) into linear decays of the initial activity of adenylate cyclase as a function of time. Fig. 11 shows that the inclusion of 0.5 mM-MgATP did not significantly modify the thermal decay curve of the adenylate cyclase activity. Therefore MgATP-dependent activation

experiments appear to emphasize a role for Mg^{2+} as a turn-on control of adenylate cyclase, since, as shown in Fig. 2, the equilibrium-dependent concentrations of free Mg^{2+} ions never reached values that would allow a noradrenaline stimulation of the same magnitude as that observed in the presence of saturating Mg^{2+} (Figs. 1a and 1b).

It appears then that neurotransmitter-dependent stimulation of adenylate cyclase through a change of its apparent v in the presence of Mg^{2+} requires Mg^{2+} at a much higher concentration than ATP. An excess of Mg^{2+} over ATP concentration also shows, without being an absolute requirement, a similar potentiating effect on the stimulation by catecholamines of particulate adenylate cyclase from fat-cells (Harris & Bennun, 1976). As reported in Fig. 1, stimulation by noradrenaline of brain adenylate cyclase is prevented by Ca^{2+} . Nerve cells, in common with most cells (Conway & Hingerty, 1946) contain a high cytoplasmic concentration of Mg^{2+} and a low concentration of Ca^{2+} relative to the concentrations found in mitochondria and in the extracellular fluids (Prince *et al.*, 1972; Rasmussen *et al.*, 1972). Chemical neurotransmission may involve rapid and transient alterations controlling Ca^{2+} gates and/or releasing Ca^{2+} bound to intracellular sites (Prince *et al.*, 1972; Rasmussen *et al.*, 1972). Adenylate cyclase may then function within an integrated turn-on and turn-off control, provided by Mg^{2+} and Ca^{2+} fluxes fulfilling a requirement regarded to be essential for adrenergic synaptic transmission, i.e. that of a limited duration of the effect of neurotransmitters in the postsynaptic membrane. The decrease of K_m of adenylate cyclase by its preincubation with MgATP (Fig. 9) is a desensitizing reaction. It is surmised that this effect may be related to a chelating competition between ATP and R-groups of the proteins within the membrane for the bivalent metals that may participate in the organization of the R-groups of these proteins into regulatory site(s) (Bennun, 1974).

Turnover of the cellular proteins is controlled by factors affecting both protein synthesis and degradation, since the half-life of a protein in mammalian cells is far shorter than the half-life of the cells (Schoenheimer & Rittenberg, 1940). It is known that substrates can retard the rate of degradation of enzymes (Schimke *et al.*, 1964). Integrated control by the environment on phosphatase concentration in *Euglena* has been shown to occur (Bennun & Blum, 1966) through a low-phosphate-concentration-dependent induction of a labile phosphatase and a high-phosphate-concentration-dependent disappearance of the induced enzyme (reversion). The low-temperature requirement which was shown for the thermal decay of the induced phosphatase during the cell's reversion process (Bennun & Blum, 1966) may indicate that the enzyme's decay may become accelerated at higher endogenous concentrations of

phosphate compounds. The biochemical systems involved in decreasing enzyme concentrations are poorly understood at the present, especially those that affect the turnover of membrane components. One of these mechanisms may be a change in the rate of thermal inactivation of adenylate cyclase to adjust its concentration to that required for the physiological and metabolic adaptation of a tissue. This may influence the ability of the cell to translate the actions of hormones and neurotransmitters into changes in cyclic AMP, therefore affecting the cell's metabolism and the induction mechanism of some enzymes (Wicks, 1969; Thoenen, 1972).

Acute stress has been shown to result in a decrease in cyclic AMP content of the hypothalamus and the adenohipophysis. However, systemic stress caused a significant decrease in hypothalamic cyclic AMP and a significant increase in adenohipophysial cyclic AMP (Siegel *et al.*, 1974). It seems that the nature of the stress may have different effects in different areas of the brain. Therefore the effect of noradrenaline on the control of adenylate cyclase activity, and the stability of this labile enzyme (Robison *et al.*, 1967), may have significance in the aetiology of stress-related diseases.

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