Adenosine Receptor Permanently Coupled to Turkey Erythrocyte Adenylate Cyclase†

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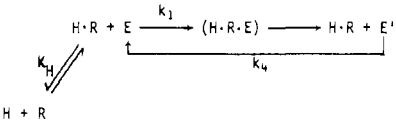
ABSTRACT: The mode of coupling of the adenosine receptor to adenylate cyclase in turkey erythrocyte membranes was probed by two independent approaches. The progressive inactivation of the adenosine receptor by an adenosine receptor affinity label resulted in the proportional reduction in the adenosine plus GppNHp dependent specific activity. In contrast, the intrinsic rate constant (k_3), characterizing the process of adenylate cyclase activation by the adenosine–adenosine receptor complex, is independent of the extent of receptor inactivation. This behavior favors the precoupled mechanism, $A + R{\cdot}E \overset{K_A}{\rightleftharpoons} A{\cdot}R{\cdot}E \overset{k_3}{\underset{k_4}{\rightleftharpoons}} ARE'$, where the receptor R and the enzyme E are permanently coupled to each other and the adenosine A binds to the receptor and induces the first-order process of cyclase activation to its active form ARE′. The finding that adenosine receptor is permanently coupled to the cyclase catalytic unit is corroborated by the observation that the progressive increase in membrane fluidity has no effect on the rate constant (k_3) of adenylate cyclase activation by the adenosine–adenosine receptor complex and that the dose–response curve for adenosine is noncooperative.

We have previously shown that turkey erythrocyte adenylate cyclase can be activated by adenosine (Sevilla et al., 1977). Furthermore, we have shown that a common pool of adenylate cyclase catalytic units can be activated either by β-adrenergic agonists through the β receptor or by adenosine through the adenosine receptor (Tolkovsky & Levitzki, 1978a). We have also found that the β receptor is not physically attached to the cyclase and that the hormone-bound receptor activates the enzyme by a "collision coupling" mechanism which can be described as



(1)

where the process is bimolecular, governed by the rate constant k_1 (Tolkovsky & Levitzki, 1978a,b), HR is the hormone–receptor complex, E is the enzyme, and E′ is the activated state of adenylate cyclase capable of converting ATP to cAMP. k_4 is the monomolecular rate constant characterizing the conversion of the active state of cyclase to its inactive form (Levitzki, 1977; Sevilla & Levitzki, 1977; Tolkovsky & Levitzki, 1978a–c). The "collision" coupling model predicts that the accumulation of the active form of adenylate cyclase obtained in the presence of GppNHp and saturating concentrations of agonist follows the equation (Tolkovsky & Levitzki, 1978a)

$$[E'] = [E_T]\{1 - \exp(-k_1[R_T]t)\} \quad (2)$$

where [E′] is the concentration of the active form of adenylate cyclase, $[E_T]$ is the total adenylate cyclase concentration, and $[R_T]$ is the concentration of receptor. In the presence of GppNHp the enzyme remains in a permanently active form since the conversion of the active state to its inactive form is blocked ($k_4 = 0$; Levitzki, 1977).

Equation 2 predicts (a) first-order kinetics of activation of enzyme in the presence of GppNHp; (b) the apparent first-order rate constant describing the process of activation of the enzyme to its permanently active form which is linearly dependent on total receptor concentration $[R_T]$; this was found to be the case for adenylate cyclase activation by β receptors in turkey erythrocytes (Tolkovsky & Levitzki, 1978a); and (c) the maximal activity attainable, dependent only on total enzyme concentration, namely, $[E'] = [E_T]$, when $t >> k_1[R_T]$.

Obviously, even when the total concentration of receptor is reduced to a very small fraction of its original quantity, all of the enzyme molecules will be activated if one waits long enough. This was indeed shown to be the case for the β-receptor-dependent activation of adenylate cyclase in turkey erythrocytes (Tolkovsky & Levitzki, 1978a).

Knowing the mode of β-receptor coupling to cyclase, we could analyze the mode of coupling of the adenosine receptor to the cyclase by investigating the mode of activation of the two agonists combined. The data could be best fitted (Tolkovsky & Levitzki, 1978c) by a model which assumes that the mode of coupling between the β receptor and adenylate cyclase is of the "collision coupling" type (eq 1) and that the adenosine receptor is permanently coupled to adenylate cyclase. Thus, the mode of coupling between the adenosine receptor and adenylate cyclase can be summarized as

$$A + R{\cdot}E \underset{K_A}{\rightleftharpoons} A{\cdot}R{\cdot}E \overset{k_3}{\underset{k_4}{\rightleftharpoons}} A{\cdot}R{\cdot}E' \quad (3)$$

This scheme predicts that the accumulation of the active form of the adenylate cyclase in the presence of GppNHp[1] is given by

$$[A{\cdot}R{\cdot}E'] = [R{\cdot}E]_T\{1 - \exp(-k_3t)\} \quad (4)$$

We have pointed out (Tolkovsky & Levitzki, 1978a–c, 1979) that such a scheme, in contrast to the "collision" coupling model, predicts that the rate constant of cyclase activation (k_3) by the ligand receptor will remain unaffected by a progressive inactivation of the receptor, whereas the maximal number of catalytic units that can be activated will decrease linearly with the extent of receptor inactivation. In contrast, we have already shown that the opposite situation holds if the mode of coupling between cyclase and receptor is of the "collision" coupling type (eq 1). Both eq 2 and 4 describe a first-order process of accumulation of the activated form of adenylate cyclase. The integrated forms of eq 2 and 4 describe the time

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; GppNHp, guanylyl imidodiphosphate; o-adenosine, α-(adenosine-9)-α′-(hydroxymethyl)diglycolic dialdehyde.

0006-2960/79/0418-2134$01.00/0

course of cAMP accumulation. The latter curve is nonlinear and exhibits a typical lag period which is related to the rate constant of enzyme activation (Tolkovsky & Levitzki, 1978a–c). Thus, by following the time course of cAMP production as a function of agonist and in the presence of GppNHp, one monitors the kinetics of appearance of the activated species of cyclase (see also legend to Figure 5).

The "collision" coupling mechanism predicts that the rate-limiting step of the activation process is the diffusion of the hormone-occupied receptor to the catalytic unit of adenylate cyclase. This process, which takes place in the membrane matrix, should be strongly influenced by its viscosity. Indeed, we found that the rate of adenylate cyclase activation by β agonists increases linearly with membrane fluidity (Hanski et al., 1979; Rimon et al., 1978). The membrane fluidity was increased by the progressive insertion of the fluidizing agent *cis*-vaccenic acid.

In contrast to the "collision" coupling model, in a situation where the association of the receptor is tight, it is expected that the rate of adenylate cyclase activation by the permanently coupled receptor will be weakly dependent or altogether independent on membrane fluidity.

In this communication we provide evidence that the adenosine receptor is permanently coupled to the adenylate cyclase.

Materials and Methods

Materials. Adenosine 5′-triphosphate (GTP-free, chemically synthesized by phosphorylation of adenosine) was obtained from Sigma and purified prior to use on a Dowex 50-X8 column to remove adenosine. ATP (1 mL of 0.1 M) was applied on the Dowex 50-X8 (H^+ form) column containing 2 mL of the resin and eluted with 2 mL of cold water. The eluent was neutralized to pH 7.4 by NaOH at 0 to 4 °C; the concentration of ATP was determined spectrophotometrically at 257 nm (pH 1). [α-^{32}P]ATP was obtained from Radiochemical Centre (Amersham, England). Phosphocreatine, creatine phosphokinase, adenosine deaminase, adenosine, l-epinephrine bitartrate, and *cis*-vaccenic acid were supplied by Sigma. 5′-Guanylyl imidodiphosphate was supplied by ICN Pharmaceuticals. Phosphodiesterase inhibitor 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (RO 20-1724) was a generous gift from Dr. H. Shepard of the Roche Institute.

Preparation of Membranes. Turkey erythrocyte membranes were prepared and stored as previously described (Steer & Levitzki, 1975), and protein was determined according to Lowry et al. (1951).

Adenylate Cyclase Assay. Adenylate cyclase activity was measured at 37 and 25 °C, according to Salomon et al. (1974). A 20-min assay was routinely performed. The assay mixture contained 2 mM [α-^{32}P]ATP, 4 mM $MgCl_2$, 2.2 mg/mL phosphocreatine, 0.2 mg/mL creatine phosphokinase, and 0.3 mM phosphodiesterase inhibitor RO 20-1724 in 40 mM Tris-HCl buffer, pH 7.4.

Synthesis of o-Adenosine. The potential affinity label for adenosine binding sites α-(adenosine-9)-α'-(hydroxymethyl)diglycolic dialdehyde (o-adenosine) was prepared according to Davoll et al. (1946) by periodate cleavage of the ribose ring of adenosine. Figure 1 outlines the synthesis of o-adenosine and its postulated mode of covalent modification.

Covalent Modification of Adenosine Binding Sites. Turkey erythrocyte membranes (5 mL; 0.2 mg of protein/mL) were incubated with o-adenosine in 0.12 M phosphate buffer, pH 7.4, containing 2 mM EDTA and 4 mM $MgCl_2$, at 25 °C. After 5 min, 100 mL of sodium borohydride (20 mg/mL) in 0.1 N NaOH was added (resulting in pH 8.4), and the

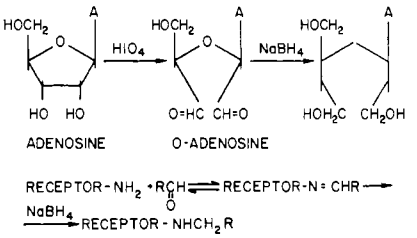

FIGURE 1: Preparation of an adenosine affinity label and its possible mode of interaction with the adenosine site. The bisaldehyde derivative of adenosine, o-adenosine, probably interacts with amino groups on the adenosine receptor. When $NaBH_4$ is added, the hypothetical receptor derivatized Schiff base is reduced to the corresponding secondary amine, whereas the aldehyde side chains in the remaining free o-adenosine are reduced to primary alcohols. Thus, when $NaBH_4$ is added to the mixture of membrane and o-adenosine, both bound and free o-adenosine are reduced instantaneously. Therefore, the amount of o-adenosine irreversibly attached is determined by the dissociation constant of o-adenosine to the adenosine receptor and the concentration of free o-adenosine.

suspension was allowed to stand for 20 min. The modified membranes were washed four times in 40 mM Tris-HCl, pH 7.4, containing 2 mM EDTA and 4 mM $MgCl_2$. Each wash consisted of suspending the membranes in 5 mL of buffer and centrifuging at 10000*g* for 15 min. Finally, the membranes were resuspended in 0.75 mL of the same buffer.

Kinetics of cAMP Accumulation. cAMP production was initiated by mixing the assay ingredients (see above), 1×10^{-3} M adenosine and 1×10^{-5} M GppNHp with the membranes, using the procedure described earlier (Tolkovsky & Levitzki, 1978b). At specific times, subsequent to mixing, samples of 150 μL were withdrawn from the reaction mixture and added to 100 μL of 0.2% $NaDodSO_4$ solution. The amount of cAMP produced was determined according to Salomon et al. (1974). All the components of the reaction mixture were preincubated for 5 min at 25 °C prior to mixing. The cAMP accumulation experiments was also performed at 25 °C when cAMP contents were determined according to Salomon et al. (1974).

The initial estimates for the parameters characterizing the kinetics of cAMP accumulation were obtained as described in Results. The best fit for the experimental equation was performed by using a nonlinear curve fitting procedure (LSQ) (Booth et al., 1959).

Results

Activation of Adenylate Cyclase by Adenosine. Turkey erythrocyte adenylate cyclase was found earlier to be activated by adenosine (Sevilla et al., 1977; Tolkovsky & Levitzki, 1978c). The activation obtained in the presence of 1 mM adenosine was usually three- to fourfold that of the basal activity: 6 to 8 pmol of cAMP (mg of protein)$^{-1}$ min^{-1} at 37 °C. In order to properly estimate K_D for adenosine, it was important to check whether the adenosine added to the reaction mixture is the sole source of adenosine in the assay mixture.

The adenosine present in the adenylate cyclase assay, other than the added adenosine, can originate from two sources: the membranes and the ATP added to the adenylate cyclase assay (Premont et al., 1977). The basal activity measured in the presence of different ATP samples varied from 1.5 to 5 pmol of cAMP (mg of protein)$^{-1}$ min^{-1}. Subsequent to the purification of ATP on a Dowex 50-X8 column, as described in Materials and Methods, the basal activity was consistently found to be 1–2 pmol (mg of protein)$^{-1}$ min^{-1} for all ATP samples checked. In order to remove possible adenosine contamination of the membrane, the membranes were treated with adenosine deaminase. The product of adenosine deamination, inosine, has no effect on the cyclase system. The

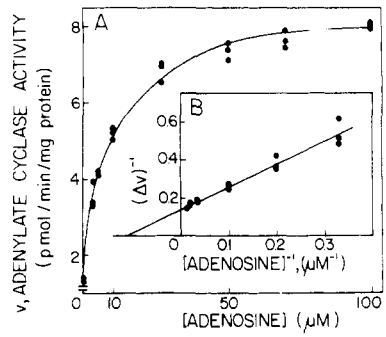

FIGURE 2: Activation of adenylate cyclase by adenosine. Adenylate cyclase assay was performed according to Salomon et al. (1974) at 37 °C in the presence of phosphodiesterase inhibitor RO 20-1724, as described in Materials and Methods. The ATP used in the assay was freed of adenosine prior to the assay by chromatographing it on a Dowex 50 column, as described in Materials and Methods. (A) Saturation curve. (B) Double-reciprocal plot of the same data. Δv is the difference in adenylate cyclase activity in the presence and in the absence of added adenosine (basal activity 1.5 pmol of cAMP min^{-1} mg^{-1}). The maximal l-epinephrine-dependent adenylate cyclase specific activity of the same membranes was found to be 60 pmol min^{-1} mg^{-1} under the same assay conditions.

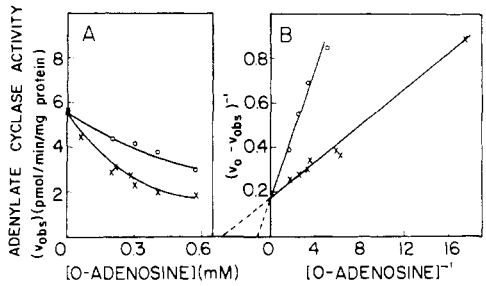

FIGURE 3: Inactivation of the adenosine dependent cyclase by o-adenosine and its protection by adenosine. Turkey erythrocyte membranes were incubated with increasing concentrations of o-adenosine in the absence (X—X) and in the presence (O—O) of 2 × 10^{-5} M adenosine with the subsequent addition of $NaBH_4$ as described under Materials and Methods. The degree of receptor modification is calculated as the difference between the adenosine-dependent specific activity in the absence of added affinity label (v_0) and the observed adenosine-dependent specific activity subsequent to treatment (v_{obsd}) with the specified concentrations of o-adenosine. From the inhibition curve (X—X) by o-adenosine, one can calculate that o-adenosine binds to the receptor with a dissociation constant of 2.4 × 10^{-4} M. From the competitive inhibition of o-adenosine reaction by adenosine (O—O) and the value for the o-adenosine–receptor dissociation constant, one can calculate that the affinity of adenosine to its receptor is 8 × 10^{-6} M, in good agreement with the direct determination of Figure 1. (A) Raw data. (B) Double-reciprocal plot.

membranes were incubated with adenosine deaminase (0.4–1 U/mg) for 10 min at 25 °C. After the removal of the adenosine deaminase, the basal activity was measured and found unchanged. These results show that the turkey erythrocyte membranes are devoid of adenosine contamination.

Figure 2 depicts the dose response of turkey erythrocyte adenylate cyclase to adenosine. The double-reciprocal plot yielded a dissociation constant K_A = 9 μM and a maximal specific activity V_{max} = 7.1 pmol (mg of protein)$^{-1}$ min^{-1}. The K_A values are close to those obtained by Clark et al. (1974) for human astrocytoma cells, for neuroblastoma intact cells (Blume & Foster, 1975), and for human blood platelets (Haslam & Lynham, 1973).

Irreversible Inactivation of Adenosine-Dependent Adenylate Cyclase Activity by o-Adenosine. Treatment of erythrocyte membranes with o-adenosine was conducted as described in Materials and Methods. From Figures 3–5 it can be seen that treatment of the membranes with o-adenosine and $NaBH_4$ results in the irreversible inactivation of the adenosine-dependent adenylate cyclase activity.

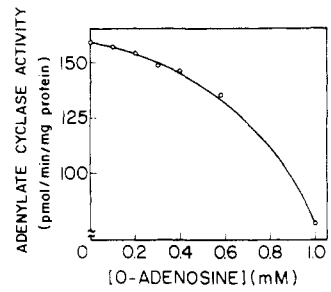

FIGURE 4: Inhibition of NaF-dependent cyclase activity by o-adenosine. From the figure it can be seen that at 0.57 mM o-adenosine, the maximal concentrations of o-adenosine used to inactivate adenosine receptor, the extent of the NaF-dependent activity is less than 15% (135 pmol of cAMP min^{-1} mg^{-1} vs. 160 pmol of cAMP min^{-1} mg^{-1} in the control). Experimental details are given in the text.

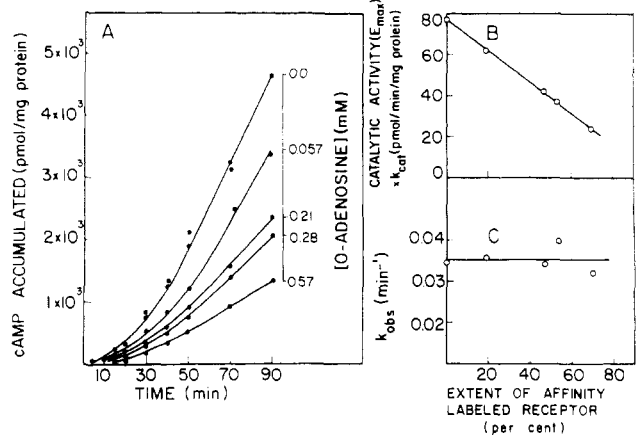

FIGURE 5: Dependence of the rate of adenylate cyclase activation and of its maximal specific activity on the extent of receptor modification. The time course of cAMP accumulation was measured in membranes subsequent to treatment with o-adenosine, in the presence of saturating adenosine and GppNHp, as described under Materials and Methods. The progress curves were all found to be first order. The points represent the average of triplicate determinations, and the curves are the best computer fit according to eq 3. (A) cAMP accumulation subsequent to o-adenosine treatment. (B) The dependence of the maximal adenosine plus GppNHp specific activity as a function of the extent of receptor labeling. The extent of adenosine receptor labeling was measured by the procedure described in Figure 3. The values of $k_{cat}E_{max}$ were obtained from the computer fits of A. (C) The dependence of the rate constant of cyclase activation on the extent of receptor labeling. The values for k_{obsd} were obtained from the computer fits in A.

Although o-adenosine at concentrations higher than 0.6 mM causes a large inhibition (Figure 4) of NaF-induced adenylate cyclase activity, in the concentration range of 0 to 0.6 mM the loss of NaF-dependent cyclase was minor and did not exceed 15%, thus indicating that the catalytic moiety remains intact. Hence, the experiments were limited to this range of concentrations. Within this concentration range the irreversible activation of the adenosine-dependent adenylate cyclase, after the treatment of turkey erythrocyte membranes with o-adenosine and $NaBH_4$, reached 75% (Figures 5A and 5B).

Figure 3B (lower line) shows that the double-reciprocal plot of the amount of enzyme activity inactivated, $v_{intact} = v_0 - v_{obsd}$ vs. affinity label concentration used, yields a straight line. This finding suggests that the degree of inactivation represents the occupancy of the adenosine binding sites by the affinity label (o-adenosine), as though the reduction of the o-adenosine–adenosine receptor complex by sodium borohydride "freezes"

the equilibrium between bound and free o-adenosine existing before the reduction. Indeed, $NaBH_4$ reduces both the bound o-adenosine and the free-o-adenosine (Figure 1).

The apparent dissociation constant calculated for o-adenosine from Figure 3 was found to be 0.24 mM. If o-adenosine acts as an affinity label of adenosine, the latter should specifically protect the adenosine binding sites against the inactivation process. As can be seen in Figure 3, this is indeed the case. Furthermore, Figure 3 also shows that these two substrates compete with each other for the same site. From this competitive relationship between o-adenosine and adenosine, one can calculate a dissociation constant for adenosine of $K_A = 8 \times 10^{-6}$ M, which is in excellent agreement with the direct determination of the affinity of adenosine to the adenosine receptor (Figure 2).

Kinetics of cAMP Accumulation in Membranes Treated with o-Adenosine. When turkey erythrocyte membranes are incubated with adenosine and GppNHp, the adenylate cyclase is activated to its permanently active form. This process can be described

$$[R{\cdot}E'] = [R{\cdot}E]_T\{1 - \exp(k_{obsd}t)\} \quad (5)$$

where R·E′ is the activated enzyme at time t, $[R{\cdot}E]_T$ is the total concentration of enzyme–receptor units, and k_{obsd} is the pseudo-first-order rate constant of activation (Tolkovsky & Levitzki, 1978b).

Since the activated form of the enzyme R·E′ is the species responsible for the production of cAMP, one can follow the time course of accumulation of R·E′ by following the accumulation time course of cAMP. The equation describing the accumulation of cAMP as a function of time is obtained by the integration of eq 3 as follows; namely, the amount of cAMP produced per unit time depends linearly on the amount of active enzyme

$$\frac{\mathrm{dcAMP}}{\mathrm{d}t} = k_{cat}[R{\cdot}E'] \quad (6)$$

where k_{cat} is the turnover number of adenylate cyclase and $k_{cat}[R{\cdot}E']$ is the specific activity of the enzyme. Upon integration of eq 4 using eq 3, one obtains

$$\mathrm{cAMP}_t = k_{cat}[R{\cdot}E]_T t + \frac{k_{cat}[R{\cdot}E]}{k_{obsd}}\{\exp(-k_{obsd}t)t - 1\} \quad (7)$$

where $k_{cat}[R{\cdot}E]_T$ is the maximal specific activity attainable. The quantity $[R{\cdot}E]_T$ is designated E_{max} for convenience, as it represents the total number of catalytic units that can be activated.

Equation 5 predicts a lag time in the accumulation of cAMP where, at longer times when the term $\exp(-k_{obsd}t)$ becomes negligible, the accumulation of cAMP is linear with time. The slope of the linear portion yields the maximal adenylate cyclase activity $k_{cat}E_{max}$, and the intercept on the time axis yields the value of $1/k_{obsd}$.

The cAMP accumulation curves are presented in Figure 5A. Each curve represents membranes pretreated by different concentrations of o-adenosine. The best fit values for k_{obsd} and $k_{cat}E_{max}$ were replotted against the expected extent of affinity labeled receptor (Figures 5B and 5C), calculated from the Michaelis–Menten equation, using a dissociation constant for o-adenosine of 2.4×10^{-4} M (see Figure 3). k_{obsd} at 25 °C for the native membranes was found to be 0.035 ± 0.005 min^{-1}. Furthermore, the rate constant of enzyme activation is *independent* of the adenosine receptor concentration. In contrast, the maximal specific activity attainable ($k_{cat}E_{max}$) is directly proportional to the concentration of the intact adenosine receptor.

Discussion

Affinity of Adenosine to Its Receptor. From the dose–response curve of turkey erythrocyte membranes to adenosine (Figure 2), we can calculate a dissociation constant of $K_A = 9 \times 10^{-6}$ M for the adenosine receptor toward adenosine. This value is in the range of adenosine affinity to the adenosine receptor on other cell types (Clark et al., 1974; Blume & Foster, 1975; Haslam & Rosson, 1975).

Specificity of o-Adenosine as a Specific Adenosine Receptor Affinity Label. From Figures 3 and 4 it is apparent that the bisaldehyde-adenosine (o-adenosine) inactivates specifically the adenosine-dependent adenylate cyclase (Figure 3) but has an insignificant effect on the catalytic moiety (Figure 4) in the range of concentration used. From Figure 3 it is apparent that adenosine protects competitively against the inactivation of the adenosine-dependent cyclase activation by o-adenosine, as predicted if adenosine and o-adenosine compete for the same site. This finding indicates that the action of o-adenosine is not on the coupling between receptor and enzyme. It should be noted at this point that $NaBH_4$ alone has an insignificant effect on the adenylate cyclase system, but destroys the catecholamine-sensitive, adenylate cyclase. This effect is presumably due to a reduction of S–S bonds in the β-adrenergic receptor (unpublished data).

o-Adenosine as a Tool to Delineate the Mode of Coupling between Cyclase and the Adenosine Receptor. From Figure 5 it can be seen that the progressive inactivation of the adenosine receptor by o-adenosine and $NaBH_4$ leads to a proportional decrease of the maximal specific activity without a change in the rate constant of adenylate cyclase activation by the adenosine–adenosine receptor complex. This behavior is predicted by eq 3 which describes a situation where the receptor and the enzyme are permanently coupled to each other.

Effect of Membrane Viscosity on the Mode of Coupling between the Adenosine Receptor and Adenylate Cyclase. We have already shown (Rimon et al., 1978) that the rate constant characterizing the rate of adenylate cyclase activation by adenosine is independent of membrane fluidity (viscosity^{-1}). This result strongly supports the assertion that the adenosine receptor is permanently coupled to the adenylate cyclase. As expected, we have found earlier (Hanski et al., 1979) that the bimolecular process of adenylate cyclase activation by β-agonist-bound β receptors strongly depends on membrane fluidity. Progressive fluidization, however, causes an increase in the specific activity of the adenylate cyclase. This effect is a direct effect of the membrane fluidity on the catalytic unit of the adenylate cyclase system and is separate from the effect of membrane fluidity on the rate of adenylate cyclase activation by its receptor. Indeed, the effect of membrane viscosity on the specific activity of adenylate cyclase is independent of the mode of adenylate cyclase activation. Thus, all three modes of adenylate cyclase activation, i.e., by NaF, l-epinephrine + GppNHp, and adenosine + GppNHp, depend identically on membrane viscosity (Rimon et al., 1978). These dependencies can be superimposed on the same graph (Rimon et al., 1978). This effect of membrane viscosity on the specific activity of membrane embedded enzymes is well documented. Hence, Ca^{2+}–ATPase (Warren et al., 1974), (Na^+,K^+)-ATPase (Kimelberg, 1975), and adenylate cyclase (Klein et al., 1978) reveal a similar dependence of enzyme specific activity on the viscosity of the membrane.

Mode of Coupling of Other Hormone Receptors of Cyclase. The mode of coupling of the adenosine receptor to adenylate cyclase and the mode of coupling of the β-adrenergic receptor

to adenylate cyclase represent two extreme modes of receptor to enzyme coupling. Using the two approaches, (a) affinity labeling and (b) modulation of membrane fluidity, it is probably possible to explore the mode of coupling of other hormone and neutrotransmitter receptors to adenylate cyclase. Furthermore, one can, in principle, employ the diagnostic tests used in the present studies to explore the mode of receptor to signal coupling, also in cases where cyclase is not involved.

Acknowledgments

The authors thank I. Hanski and Dr. G. Rimon for their stimulating discussions. The authors are also indebted to Dr. Meir Shinitzky for help and advice in the measurements of membrane microviscosity using fluorescence polarization.

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