Coupling of a Single Adenylate Cyclase to Two Receptors: Adenosine and Catecholamine†

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ABSTRACT: A detailed kinetic analysis on the rate of activation of adenylate cyclase by l-epinephrine and by adenosine, separately and combined, was performed. Both ligands were found to induce the activation of adenylate cyclase to its permanently active state in the presence of guanylyl imidodiphosphate (GppNHp). The activation followed strictly first-order kinetics. On the basis of these experiments, it was found that all of the enzyme pool can be activated by the β-adrenergic receptor, but only 60 to 70% of the enzyme can also be activated by an adenosine receptor. The remaining 30 to 40% cannot be activated by adenosine. While previous experiments have led us to conclude that the epinephrine receptor is uncoupled from the adenylate cyclase, it seems that the adenosine receptor is either precoupled to the enzyme or forms a long-lived intermediate of adenosine–receptor–enzyme complex. From the pattern of enzyme activation by the two ligands and GppNHp, it may be concluded that the two ligands, adenosine and the β-agonist, activate the adenylate cyclase through a common guanyl nucleotide regulatory site. This assertion is supported by the finding that both adenosine and l-epinephrine, in the presence of GTP, induce the reversal of the permanently active state, irrespective by which pathway the enzyme was activated.

Adenosine is a potent activator of adenylate cyclase in brain (Sattin and Rall, 1970), cultured human cell lines (Clark et al., 1974; Clark and Seney, 1976; Sturgill et al., 1975), and in neuroblastoma (Blume and Foster, 1975), as well as in rat brain (Gilman and Schrier, 1972), platelets (Mills and Smith, 1971), isolated bone cells (Peck et al., 1974), and in lymphocytes (Wolberg et al., 1975). Turkey erythrocyte adenylate cyclase was found by us also to be activated by adenosine (Sevilla et al., 1977). It is well established that turkey erythrocyte adenylate cyclase is activated by catecholamines through their interaction with β-adrenergic receptors. Thus, it became possible to study the question of activation of adenylate cyclase by two different receptors. This particular problem can be very well studied using the turkey erythrocyte adenylate cyclase system, as the latter is already well explored. Using the nonhydrolyzable GTP analogue, GppNhp,[1] one can study the process of enzyme activation as induced by either a β-agonist or by adenosine, separately, or by the two ligands combined. In this fashion, it becomes possible to analyze a number of basic questions: (a) Is the total pool of adenylate cyclase accesible to the two receptors? (b) Is the mode of enzyme activation by the two ligands additive in nature or do the receptors compete for the same pool of enzyme? (c) Is the pool of the regulatory guanyl nucleotide sites common to the two processes of activation?

Since a number of cell types possess adenylate cyclase and also respond to various hormones, these questions are of general significance.

Materials and Methods

Materials. Pyruvate kinase was from Sigma. PEP was the generous gift of Dr. Zvi Selinger, from our Department, and was neutralized by KOH to a pH of 7.4, and kept frozen at −20 °C.

Adenylate Cyclase Assay and Turkey Erythrocyte Membranes. Adenylate cyclase activity was measured at 37 °C according to Salomon et al. (1974). Assay was routinely performed for 20 min. The assay mixture contained 2 mM [α-^{32}P]ATP, 4 mM $MgCl_2$, 3 units of pyruvate kinase in saturating $(NH_4)_2SO_4$, 5 mM PEP, 10 mM KCl, and 40 mM Tris-HCl, pH 7.4.

Preparation of Membranes. Turkey erythrocyte membranes were prepared as previously described (Steer and Levitzki, 1975).

Activation of Adenylate Cyclase by l-Epinephrine and/or Adenosine and GppNHp. The time course of activation of the enzyme to its permanently active form was measured by preincubating separately 2.8 mg/mL membranes and a solution of 0.1 mM GppNHp and various concentrations of adenosine in 50 mM Tris-HCl buffer, pH 7.4, containing 2 mM $MgCl_2$ and 1 mM EDTA (TME buffer). After equilibration at 37 °C, the two solutions were mixed and, at various times, 70-μL portions were removed into 40 μL of an ice-cold solution containing 8 mM theophylline and 5×10^{-5} M *dl*-PPL. Theophylline is an adenosine antagonist (Clark and Seney, 1976) which stops the process of enzyme activation by adenosine and GppNHp. Theophylline, however, is unable to revert the enzyme from its permanently active state back to its inactive form. Thus, the relation between theophylline and adenosine is similar to that between *dl*-propranolol and l-epinephrine (Levitzki et al., 1976; Sevilla et al., 1976). After completion of activation experiments, tubes were transferred to 37 °C and a 20-min assay was performed. The activation of the enzyme by l-epinephrine and GppNHp was performed as previously described (Tolkovsky and Levitzki, 1978), under identical solution conditions used for the adenosine experiments.

Results

Activation of Adenylate Cyclase by Adenosine and GppNHp. The time course of activation by various concen-

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[1] Abbreviations used are: GppNHp, guanylyl imidodiphosphate; PEP, phosphoenolpyruvate; TME, 50 mM Tris-HCl buffer containing 2 mM $MgCl_2$ and 1 mM EDTA, pH 7.4; PPL, *dl*-PPL, propranolol; Tris-HCl, 2-amino-2-hydroxymethyl-1,3-propanediol hydrochloride; EDTA, (ethylenedinitrilo)tetraacetic acid.

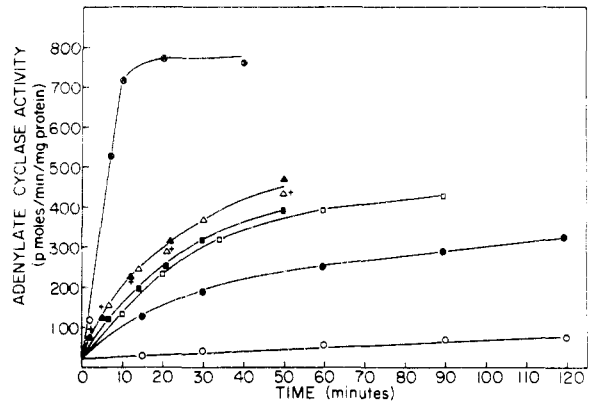

FIGURE 1: Time course of adenylate cyclase activation by adenosine as a function of adenosine concentrations. Membranes were incubated at 37 °C in the presence of 1.0×10^{-4} M GppNHp in TME and the indicated concentrations of adenosine and 1-epinephrine. The final protein concentrations in the preincubation mixture were 1.4 mg/mL. At the times indicated by the points, 70-μL samples were taken out into 40 μL containing 7.5 M theophylline and 5×10^{-5} M *dl*-PPL which were kept at 0 °C. After completion of preincubation, assay of adenylate cyclase activity was performed during 20 min at 37 °C, as described under Materials and Methods: (○) control containing 1 mM theophylline and 1.0×10^{-5} M *dl*-PPL; (●) 1.0×10^{-6} M adenosine; (□) 5.0×10^{-6} M adenosine; (■) 1.0×10^{-5} M adenosine; (△) 5.0×10^{-5} M adenosine; (▲) 1.0×10^{-4} M adenosine; (+) 3.0×10^{-4} M adenosine; (⊗) 1.0×10^{-4} M adenosine.

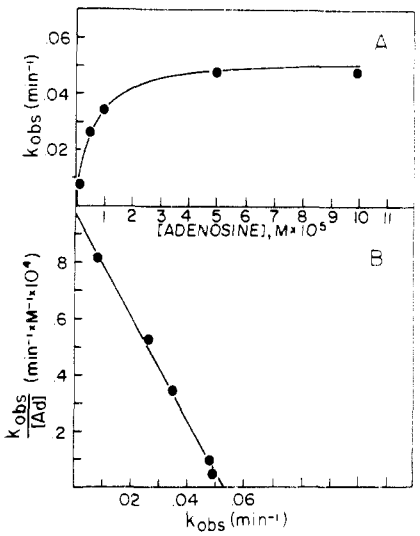

FIGURE 2: The dependence of k_{obsd} characterizing the appearance of the activated species, E′, as a function of adenosine concentration. k_{obsd} values were derived from the slope of the semilogarithmic plot. (A) Direct plot of k_{obsd} as a function of adenosine concentration. (B) Replot in the form of an Eadie plot: $k_{obsd}/[Ado]$ vs. k_{obsd} (eq 2).

trations of adenosine is presented in Figure 1. The rate of activation increases with increasing adenosine concentrations, and saturation of the adenosine effect is found at about 5×10^{-5} M. The maximal level of activation by adenosine is lower than the maximal level of activation produced by 1-epinephrine (Figure 1). The data of Figure 1 are computer fitted to the equation:

$$[E']_t = [E']_{max}[1 - \exp(-k_{obsd}t)] \quad (1)$$

where $[E']_{max}$ was 480 pmol of cAMP mg^{-1} min^{-1} in the presence of adenosine. The maximal activity attainable with 1-epinephrine and GppNHp was found to be 780 pmol of cAMP mg^{-1} min^{-1}. $[E']_t$ is the level of activation at time t.

One observes that at all adenosine concentrations the activation is by a pseudo-first-order process. Since the adenosine effect is saturable (Figure 1), the first-order rate constant is related to k_{obsd} by:

$$k_{obsd} = k\frac{[Ado]}{[Ado] + K_A} \text{ or } \frac{k_{obsd}}{[Ado]} = \frac{k}{K_A} = \frac{k_{obsd}}{K_A} \quad (2)$$

where k is the intrinsic rate constant governing the transition of E to E′ and K_A the dissociation constant for adenosine to the adenosine receptor. The values for k_{obsd} characterizing each adenosine concentration were derived from Figure 1. These, in turn, were plotted as a function of adenosine concentration (Figure 2A).

An Eadie replot of the data presented in Figure 2A is given in Figure 2B. From Figure 2B one can see that k_{obsd} depends on adenosine concentration in a noncooperative fashion, according to eq 2. The dissociation constant for adenosine to its receptor, K_A, calculated from the Eadie plot was found to be 5×10^{-6} M. The intrinsic rate constant, k, characterizing the adenosine activation process (eq 2) was found to be $k \simeq 0.053$ min^{-1}. Thus, adenosine activation is a much slower process than the process of activation by 1-epinephrine, which was found to be 0.4 to 1.0 min^{-1} (Sevilla et al., 1976; Levitzki et al., 1976; Tolkovsky and Levitzki, 1978).

The Effect of Prior Incubation by Adenosine on the Subsequent Ability of 1-Epinephrine to Activate the Remaining Adenylate Cyclase. Due to the inability of adenosine to activate all of the adenylate cyclase pool (Figure 1), it was of interest to examine whether the cyclase pool remaining subsequent to adenosine activation was still accessible to activation by 1-epinephrine. Also, it was of interest to examine the question of whether the two receptors interact with a common pool of cyclase or on separate pools of enzyme. In the latter case, the effect of the two ligands should be additive. We also examined whether the partial activation by adenosine might be due to the presence of *dl*-PPL in the activation medium, thus prohibiting part of the adenylate cyclase from interaction with the adenosine receptor. The results are presented in Figure 3. Inspection of the data reveals that PPL at a concentration of 1.0×10^{-5} M affects neither the rate nor the maximal level of activation induced by adenosine. Neither does 1 mM theophylline affect the rate of cyclase activation by 1-epinephrine nor the maximal level of enzyme activity. Namely, full occupancy by an inhibitor of one receptor species has no effect on the activation of the second receptor species. The addition of epinephrine subsequent to adenosine activation causes complete stimulation of the remaining adenylate cyclase at a rate comparable to the rate of stimulation of 1-epinephrine in the absence of adenosine (Figure 3).

The Effect of Prior Activation by 1-Epinephrine on the Subsequent Activation by Adenosine. From Figure 4 it is clear that adenosine activates a *particular* pool of cyclase within the membrane. It can be seen that as the level of activation by epinephrine increases the subsequent maximal activation level induced by adenosine decreases. The adenosine-induced rate constant of activation, however, is not reduced and remains the one typical for the adenosine-induced reaction. For this experiment (Figure 4), a low epinephrine concentration of 6×10^{-7} M was chosen so that the activation rate would be similar to that produced by saturating adenosine. At different time points along the 1-epinephrine activation curve, the 1-epinephrine-induced process was stopped and the adenosine-induced process initiated by the simultaneous addition of 1.7×10^{-4} M adenosine and 1.6×10^{-5} M *dl*-propranolol, final concentrations. First, the data were plotted on a semilogarithmic plot in order to establish whether the activation pattern

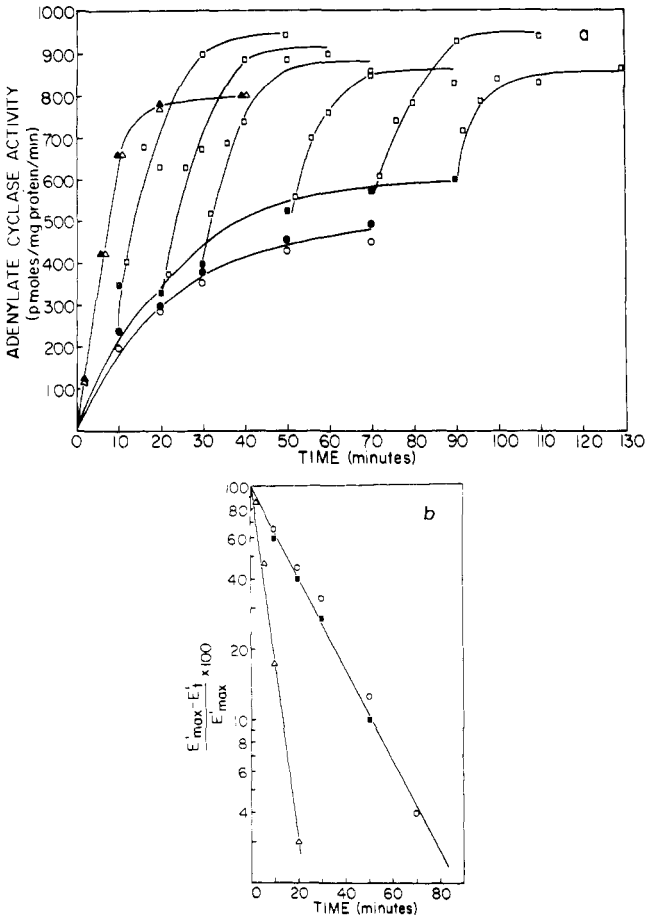

FIGURE 3: The effect of activation by adenosine and the subsequent activation by 1-epinephrine. The experimental procedure is generally that described in Figure 1. At the times indicated, a 70-μL portion of the system activated by 3.0×10^{-4} M adenosine alone (■) was removed into 40 μL of a stopping solution of 5.0×10^{-5} M *dl*-PPL containing 8 mM theophylline kept at 0 °C. To the rest, 70 μL of 1.0×10^{-3} M 1-epinephrine was added to initiate 1-epinephrine activation. At various times, 70-μL portions were removed into the above mixture of PPL plus theophylline. Final 1-epinephrine concentration in the sequential activation experiments was 1.17×10^{-4} M. Final *dl*-PPL concentration in the adenylate cyclase assay was 1.33×10^{-5} M. Final theophylline concentration in the adenylate cyclase assay was 2.2 mM. (a) Direct plot of the data. The activation by 1-epinephrine in the presence of 1 mM theophylline (△) by 1-epinephrine alone (▲). The activation by adenosine in the presence of 1.0×10^{-5} M *dl*-PPL (○) is compared to the activation by adenosine in the absence of propranolol (●): (○) activation by 3×10^{-4} M adenosine in the absence of PPL; (●) activation by 3×10^{-4} M adenosine in the presence of 1.0×10^{-5} M PPL; (△) activation by 1.0×10^{-4} M 1-epinephrine; (▲) activation by 1.0×10^{-4} M 1-epinephrine in the presence of 1 mM theophylline; (□) activation by 1.17×10^{-4} M 1-epinephrine after prior activation by adenosine (■). The lines drawn are theoretical (see text). (b) Pseudo-first-order replot of the results of Figure 3a: (△) 1-epinephrine, 1.0×10^{-4} M (maximal activation level 800 pmol of cAMP mg^{-1} min^{-1}), in the presence or absence of 1 mM theophylline; (○) 3.0×10^{-4} M adenosine in the presence or absence of PPL (maximal activation level was 500 pmol of cAMP mg^{-1} min^{-1}); (■) 3.0×10^{-4} M adenosine in sequential activation experiments (maximal activation level was 600 pmol of cAMP mg^{-1} min^{-1}). The observed rate constant k_{obsd} derived for 1-epinephrine was 0.172 min^{-1} and for adenosine 0.0447 min^{-1} according to eq 1. The theoretical curves of Figure 3a were constructed according to eq 1, inserting k_{obsd} derived from Figure 3b.

was first order with the characteristic rate constants for both processes. Then, a theoretical prediction as to the mode of activation was calculated according to the following equations:

$$[E']_t = [E']_{max_1}[1 - \exp(-k_{obsd}t)] + [E']_{max_2}[1 - \exp(-k_{obsd}t)] \quad (4)$$

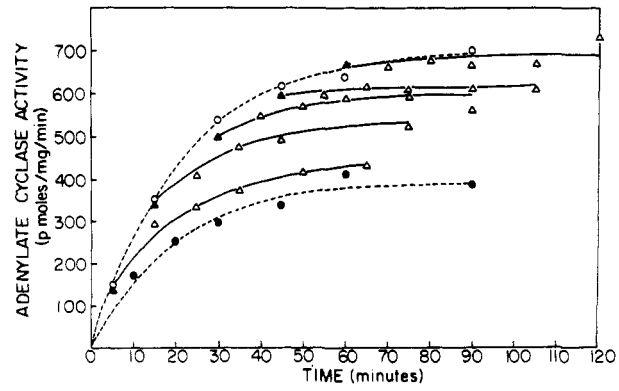

FIGURE 4: The effect of prior activation by epinephrine on the subsequent activation by adenosine. The experimental procedure is exactly as described in Figure 1, except that activation was initiated by a concentration of 6×10^{-7} M 1-epinephrine. At the times indicated (▲), a 70-μL portion was removed into ice-cold 8 mM theophylline and 5×10^{-5} M *dl*-PPL, and 70-μL of 1.62×10^{-3} M adenosine containing 10^{-4} M *dl*-PPL was added. At various times, 70-μL portions were removed into ice-cold 8 mM theophylline containing 5×10^{-5} M *dl*-PPL (△). In the adenosine-activation experiment, where no epinephrine was added, the final concentration of adenosine was 2.7×10^{-4} M. In the sequential activation experiments, final adenosine and propranolol concentrations were 2.14×10^{-4} and 1.32×10^{-5} M, respectively. The different maximal levels of enzyme activity achieved by adenosine vary with the time at which adenosine was added subsequent to the stoppage of epinephrine stimulation. The maximal levels achieved are summarized in Table I: (●) 2.7×10^{-4} M adenosine; (○) 6.0×10^{-7} M 1-epinephrine; (△) 2.7×10^{-4} M adenosine plus 1.32×10^{-5} M propranolol added at times indicated by the filled triangles.

or

$$[E']_t = 400[1 - \exp(-0.475t)] + 300[1 - \exp(-0.0475t)] \quad (5)$$

where 0.0475 min^{-1} is the rate constant governing the activation process by 1-epinephrine in this preparation (at 6×10^{-7} M epinephrine). The maximal specific activity of the permanently activated enzyme of this preparation is 700. This value is obtained with either 1-epinephrine alone or epinephrine and adenosine combined. This value is divided into two: the value 400 pmol mg^{-1} min^{-1} is the maximal specific activity obtained by adenosine alone; 300 pmol mg^{-1} min^{-1} is then the maximal specific activity of the cyclase pool which cannot be activated by adenosine. Thus, at any given time the total activation by 1-epinephrine was calculated as well as the level of activation of each of the cyclase pools. Therefore, at any given time the size of the pool responsive to both adenosine and 1-epinephrine (first half of eq 5) and the pool not responsive to adenosine but responsive to epinephrine (second half of the eq 5) could be calculated exactly. By subtracting the amount of activation already produced by 1-epinephrine on the adenosine-responsive cyclase from the total amount of activation possible (400 pmol mg^{-1} min^{-1}), one is left with the predicted total amount of cyclase which remains accessible to adenosine activation. A comparison of the predicted and observed amount of cyclase which remains accessible to adenosine subsequent to partial activation by epinephrine is given in Table I. The lines in Figure 4 were constructed according to the equation:

$$\Delta[E']_{max} = \Delta[E'][1 - \exp(-k_{obsd}{}^{A}t)] \quad (6)$$

using the maximal level of activation, $\Delta[E']_{max}$, which remains available for adenosine activation (see Table I, column 5) for each degree of activation by 1-epinephrine. The rate constant governing the process of adenosine activation at 2.7×10^{-4} M adenosine, in the absence of preactivation by epinephrine, $k_{obsd}{}^{A}$ was used in eq 4.

TABLE I: Predicted and Observed Levels of Maximal Activation Induced by Adenosine Subsequent to 1-Epinephrine Activation.[a]

time of 1-epinephrine incubat (min)	activat achieved (sp act. in pmol of cAMP min^{-1} mg^{-1})			total extent of Ado activat remain. (sp act. in pmol of cAMP min^{-1} mg^{-1})	
	total	Ado respons. pool	Ado nonrespons. pool	predict. ($t = \infty$)	obsd (t = 60 min)
5	250	185	65	315	295
15	357	204	153	196	190
30	532	304	228	96	100
45	617	353	264	47	30
60	659	377	282	23	15

[a] The equation used to compute the predicted values of activation of the adenosine-responsive enzyme pool by 1-epinephrine was: $[E']_{obsd} = 400[1 - \exp(-0.0475t)2] + 300[1 - \exp(-0.0475)]$. $[E']_{obsd}$ is the observed level of activation, the first term represents the enzyme pool which can be activated by either 1-epinephrine or adenosine, and the second term represents the enzyme pool which can be activated only by 1-epinephrine. The maximal specific activity of this preparation was 700 pmol of cAMP min^{-1} mg^{-1}. The value of $k_{obsd} = 0.0475$ min^{-1} was the pseudo-first rate constant of enzyme activation by 6×10^{-7} M 1-epinephrine, the concentration used in these experiments. The data used to calculate the values in this table are identical to those presented in Figure 6 where more experimental details are given.

Since the adenosine-induced activation is rather slow, long incubation periods were required. Therefore, the rate of adenosine activation was measured on a membrane preparation incubated for various times at 37 °C. The characteristic rate constant governing the activation by adenosine was found to decrease slightly after 45 min of incubation. Therefore, after 45 min a value of 0.045 min^{-1} rather than 0.049 min^{-1} was used as k_{obsd}^{A} and after 60 min a value of 0.040 min^{-1} was used as k_{obsd}^{A}. These rate constants were derived from a pseudo-first-order plot of the control experiments where the rate of andenylate cyclase activation induced by adenosine and GppNHp was measured subsequent to the incubation of membranes for 45 and 60 min, respectively. These experiments were conducted in a similar fashion to those described in Figure 1.

The Combined Action of Adenosine and 1-Epinephrine. When adenosine and epinephrine are present simultaneously in the presence of saturating GppNHp, the results shown in Figure 5 are obtained. Since saturation levels of epinephrine activate at a rate five times the rate found for saturating levels of adenosine, an epinephrine concentration of 6×10^{-7} M was chosen. For comparison, the activation of adenylate cyclase in the presence of each ligand alone is presented. Again, it can be seen that only 70% of the total enzyme pool is accessible to activation by adenosine, whereas all the pool is accessible to activation by 1-epinephrine. From the results it is apparent that the effect of adenosine and epinephrine is not additive (uppermost dashed line). On the contrary, it seems that mainly adenosine is responsible for the activation of the common cyclase pool where the remaining fraction of the enzyme pool is accessible only to the β-adrenergic receptor. The curves of Figure 5 were constructed as described in the legend to that figure. A detailed description of the model, which probably accounts for the mode of activation of a common cyclase pool by the two types of receptors, is given under Discussion.

The Reversal of the Permanently Active State by 1-Epinephrine and Adenosine. The permanently active adenylate cyclase induced by a β-agonist in the presence of GppNHp can be effectively reverted to its inactive state in the simultaneous presence of a β-agonist and GTP (Sevilla and Levitzki, 1977) or in the presence of a β-agonist and ATP (Sevilla et al., 1976; Sevilla and Levitzki, 1977; Levitzki et al., 1976). In Figure 6 it is shown that either 1-epinephrine or adenosine, in the presence of GTP, can induce the reversion of the permanently activated enzyme to its inactive form, irrespective of whether the permenently active form was obtained by 1-epinephrine plus GppNHp or by adenosine plus GppNHp. Similarly, 1-epinephrine plus GTP induce the conversion of the enzyme from its permanently active state to the inactive form, irrespective of whether the permanently active state was attained by adenosine plus GppNHp or by 1-epinephrine plus GppNHp.

Discussion

The Activation of Turkey Erythrocyte Adenylate Cyclase by Adenosine. The ability of adenosine to activate turkey erythrocyte adenylate cyclase is similar to its effects on other adenylate cyclases from different sources (see Introduction). Since turkey erythrocytes possess a β-adrenergic receptor, it becomes apparent that the general question of additivity or nonadditivity in hormone action can be tackled. The turkey erythrocyte system is particularly suitable, since the membrane preparation possesses insignificant basal adenylate cyclase activity. Thus, all the activity is induced by the proper receptor.

Basic Phenomenology of Adenosine Activation. Adenosine, like 1-epinephrine, is capable of activating the adenylate cyclase to a permanently active state in the presence of GppNHp (Figure 1, Sevilla et al., 1977). The process of activation can be stopped by the adenosine antagonist, theophylline, but the latter is incapable of reverting the enzyme back to its inactive state. A similar phenomenon was observed by us with β-agonists, where we have shown (Sevilla et al., 1976; Levitzki et al., 1976) that 1-catecholamines in the presence of GppNHp induce the formation of a permanently active species of adenylate cyclase, the β-blocker, propranolol, which blocks the process of activation but cannot revert the permanently active state back to its inactive form.

The Adenosine Receptor. The kinetics of enzyme activation by adenosine and GppNHp as a function of adenosine concentration reveals a single class of adenosine receptors possessing a dissociation constant of 5.0×10^{-6} M (Figure 2).

Two Receptors and One Cyclase. Only 70% of the available pool of adenylate cyclase can be activated by adenosine, whereas all of it can be activated by β-agonists (Figure 1). It can be shown that when different levels of permanent activation of the cyclase pool are obtained using adenosine plus GppNHp, the remaining portion of the cyclase pool can still be activated by a β-agonist and GppNHp (Figure 3). The level of maximal activation attained by 1-epinephrine and GppNHp is independent of the fraction of adenylate cyclase already in its active state due to the activation by adenosine and GppNHp. Furthermore, the kinetic pattern of enzyme activation of the remaining cyclase, induced by epinephrine plus GppNHp, is independent of the fraction of enzyme already in its permanently active form. When the enzyme pool is activated to different levels by epinephrine and GppNHp, only a *fraction* of the total enzyme remaining in its inactive state can be ac-

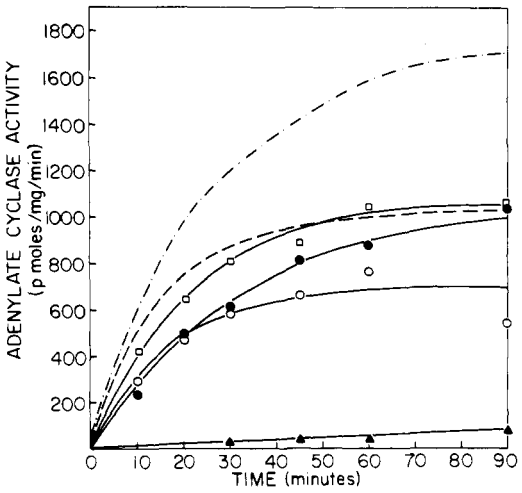

FIGURE 5: The combined activation of adenylate cyclase by l-epinephrine and adenosine. 3.0×10^{-4} M adenosine and 6.0×10^{-7} M l-epinephrine were added simultaneously at zero time to start the joint activation process. GppNHp concentration was 1.1×10^{-4} M. At the times indicated, 70-μL samples were taken out into ice-cold 40 μL of 8 mM theophylline and 5×10^{-5} M *dl*-PPL. Experimental details are given in the legend to Figure 1 and under Materials and Methods. The maximal specific activity attainable by adenosine and GppNHp was 700 pmol of cAMP mg^{-1} min^{-1} and the maximal specific activity attainable by epinephrine was 1070 pmol of cAMP mg^{-1} min^{-1} in this preparation. The curve drawn in each case is theoretical, according to the following formulations: (○) $\sim 3 \times 10^{-4}$ M adenosine alone, $[E'] = 700[1 - \exp(-0.0613t)]$. The value of 0.0613 min^{-1} was derived from a pseudo-first-order semilogarithmic plot of the results. (●) 6×10^{-7} M l-epinephrine alone, $[E'] = 1070[1 - \exp(-0.0313t)]$. The value of 0.0313 min^{-1} was derived from a pseudo-first-order semilogarithmic plot of the results. (▲) 1×10^{-6} M *dl*-PPL and 1 mM theophylline (control); (□) 3×10^{-4} M adenosine + 6×10^{-7} M epinephrine, $[E'] = 700[1 - \exp(-0.0655t)] + 370[1 - \exp(-0.0313t)]$. The component, which depends only on epinephrine, was subtracted from the experimental results according to the kinetics of $370[1 - \exp(-0.0313t)]$. The resulting values were plotted on a semilogarithmic scale, and the observed rate constant of 0.0655 min^{-1} was obtained for the adenosine plus epinephrine component. This value was inserted as the observed rate constant of activation on the common adenylate cyclase pool and the progress curve (continuous curve) was constructed. The line (- - -) was constructed according to: $700[1 - \exp(-(0.0613 + 0.0313(t)] + 370[1 - (-0.0313t)]$, which gives the sum of two terms, a term containing $k_{obsd}{}^{A} + K_{obsd}{}^{H}$ as the rate constant governing the activation of the common adenylate cyclase pool by both receptor species and a second term expressing the activation of the enzyme pool which can be activated exclusively by l-epinephrine. As pointed out in the text, this model is inappropriate, since the rate constant for activation by the hormone in the presence of adenosine must be smaller than the value measured in the absence of adenosine. The line designated - · - was constructed according to the assumption that epinephrine and adenosine activate completely separate adenylate cyclase pools: $1050[1 - \exp(-0.0313t)] + 700[1 - \exp(-0.0613t)]$. This latter model does not fit the data and was rejected on the grounds described in the text.

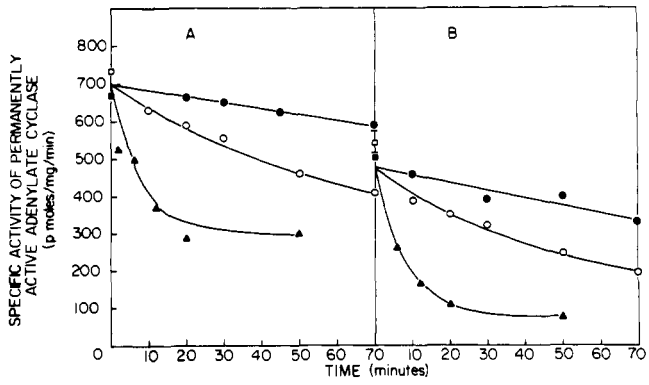

FIGURE 6: The reversal of activation induced by epinephrine and adenosine by the two hormones. Membranes, 1.6 mg/mL, were incubated for 40 min at 37 °C in the presence of either 1.0×10^{-4} M l-epinephrine and 1.0×10^{-4} M GppNHp or 1.1×10^{-3} M adenosine and 1.1×10^{-4} M GppNHp in TME buffer. After activation, a sample was taken out into ice-cold 8 mM theophylline and 5×10^{-5} M *dl*-PPL. Membranes were then washed four times in 30 volumes of TME by centrifugation at 24 000g for 10 min to remove all free GppNHp. After washing, membranes were brought to a concentration of 2.84 mg/mL equilibrated at 37 °C and incubated at 37 °C with either 1.7×10^{-5} M *dl*-PPL plus 1.3 mM theophylline (●), 3×10^{-4} M adenosine (○), or 1.0×10^{-4} M l-epinephrine (▲). Reversal medium in all cases also contained 2.5 mM GTP prepared in TME, 32 units/mL creatine phosphokinase, 20 mM creatine phosphate, and 1 mM $MgCl_2$. At the times indicated, 70-μL portions were removed into ice-cold 40 μL of 10^{-4} M *dl*-PPL and 8 mM theophylline. A zero-time point for each membrane preparation, subsequent to washing, was also taken. The kinetics were analyzed according to a pseudo-first-order process, and the following first-order rate constants were derived: reversal rate constant by adenosine (○), $k_{obsd} = 0.0179$ min^{-1}; reversal rate constant by l-epinephrine (▲), $k_{obsd} = 0.12$ min^{-1}. The curves were constructed according to these values, taking 30 and 75 pmol of cAMP mg^{-1} min^{-1} as the end points of the reversion process of A and B, respectively: (●) reversal in the absence of either adenosine or l-epinephrine but in the presence of 2 mM GTP alone; (□) activity before washes; (■) zero time activity after washes; (A) l-epinephrine preactivated; (B) adenosine preactivated. The slopes of the control reversion were linear with time and were 1.65/min in A and 2.07/min in B.

tivated by adenosine and GppNHp (Figure 4). This fraction can be exactly computed by calculating the fraction of enzyme which can still be activated by both l-epinephrine and adenosine. This is possible since all of the enzyme pool responds to epinephrine as a single species. The fact that 30% of the enzyme pool does not respond to adenosine indicates that part of the adenylate cyclase lacks a recognition domain for the adenosine receptor. All of the enzyme molecules, however, possess a recognition domain for the catecholamine β-receptor.

The Mechanism for Coupling of Two Receptors with One Enzyme. One can derive the action of two receptors on one enzyme pool using the collision coupling model which seems to operate in the turkey erythrocyte membrane β-receptor-dependent adenylate cyclase.

Using the formulation of the collision coupling (Tolkovsky and Levitzki, 1978), one can write the two modes of enzyme activation by the two receptors:

$$AR + E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} (ARE \rightleftharpoons ARE') \xrightarrow{k_3} AR + E' \quad (7)$$

and

$$HR + E \underset{k_5}{\overset{k_4}{\rightleftharpoons}} (HRE \rightleftharpoons HRE') \xrightarrow{k_3} HR + E' \quad (8)$$

where AR is the adenosine–adenosine receptor complex and HR is the catecholamine–hormone receptor complex. Since the intermediates ARE and HRE do not accumulate (Tolkovsky and Levitzki, 1978), one can write that:

$$k_3 \gg k_2, k_5 \quad (9)$$

The rate of formation of the activated form of the enzyme is given by:

$$\frac{d[E']}{dt} = k_3([ARE] + [HRE]) \quad (10)$$

but

$$[ARE] + [HRE] = \frac{k_1[AR][E]}{k_2 + k_3} + \frac{k_4[HR][E]}{k_5 + k_3} \quad (11)$$

From eq 10 and 11 and the inequality of eq 9 one obtains that:

$$\frac{d[E']}{dt} = (k_1[AR][E] + k_4[HR][E]) \quad (12)$$

namely,

$$\frac{d[E']}{dt} = ([E_T] - [E'])(k_1[AR] + k_4[HR]) \quad (13)$$

where $k_1[AR]$ and $k_4[HR]$ are the observed first-order rate constants for the adenosine-activated process and the hormone-activated process respectively. These values depend on the adenosine and the hormone concentration. These values can be written as:

$$k_{obsd}^{A} = k_1[AR] \quad (14)$$

and

$$k_{obsd}^{H} = k_4[HR] \quad (15)$$

where k_{obsd}^{A} and k_{obsd}^{H} are the experimentally observed first-order rate constants. Using these definitions, eq 13 obtains the form:

$$\frac{d[E']}{dt} = (k_{obsd}^{A} + k_{obsd}^{H})([E_T] - [E']) \quad (16)$$

Thus, if both adenosine and epinephrine activate by a collision coupling mechanism where both ARE and HRE are negligible compared to E + E', the rate of activation should be the *combined* rate of activation of the two hormones. From Figure 5 it is clear that the rate of action of the two hormones, when present simultaneously, is much *slower* than the combined activation rate. One is therefore forced to conclude that the collision coupling mechanism, as formulated by eq 7, does not hold for adenosine. We have however shown previously (Tolkovsky and Levitzki, 1978) that a collision coupling mechanism *is* responsible for the activation of adenylate cyclase by catecholamines through the β-receptors. Let us consider another type of collision coupling mechanism which allows both HRE and ARE to accumulate:

$$AR + E \underset{k_2}{\overset{k_1}{\rightleftharpoons}} (ARE \rightleftharpoons ARE') \xrightarrow{k_3} AR + E' \quad (17)$$

and

$$HR + E \underset{k_5}{\overset{k_4}{\rightleftharpoons}} (HRE \rightleftharpoons HRE') \xrightarrow{k_6} HR + E' \quad (18)$$

In this case, we assume that:

$$k_3 \ll k_2 \quad (19)$$

and

$$k_6 \ll k_5 \quad (20)$$

and that:

$$\frac{d[E']}{dt} = k_3[ARE] + k_6[HRE] \quad (21)$$

then

$$\frac{d[E']}{dt} = \left(k_3\frac{[AR]}{K_{AR}} + k_6\frac{[HR]}{K_{HR}}\right)[E] \quad (22)$$

By inserting the conservation equation:

$$[E_T] = [E] + [ARE] + [HRE] + [E'] \quad (23)$$

into eq 21 and 22, one obtains:

$$\frac{d[E']}{dt} = ([E_T] - [E'])\frac{k_3\frac{[AR]}{K_{AR}} + k_6\frac{[HR]}{K_{HR}}}{1 + \frac{[AR]}{K_{AR}} + \frac{[HR]}{K_{HR}}} \quad (24)$$

Equation 24 implies that the combined rate of activation for the two receptors will be indeed smaller than the sum of the separate effects. Equation 24, however, is still inadequate, since we have already shown that HRE *does not* accumulate (Tolkovsky and Levitzki, 1978). We must therefore examine a mechanism which allows ARE to accumulate but does not allow HRE to accumulate.

It follows from the formulations given in eq 17 and 18 that:

$$\frac{d[E']}{dt} = k_3[ARE] + k_6[HRE]$$

$$= k_3\frac{[AR][E]}{K_{AR}} + \frac{k_6k_4[HR][E]}{k_5 + k_6} \quad (25)$$

Since ARE accumulates and HRE does not, the following relations must also hold (see eq 17):

$$k_3 \ll k_2 \quad (26)$$

and

$$k_6 \gg k_4 \quad (27)$$

and since

$$[E_T] = [E] + [E'] + [ARE] \quad (28)$$

one obtains:

$$[E] = \frac{([E_T] - [E'])K_{AR}}{K_{AR} + [AR]} \quad (29)$$

Inserting eq 29 into eq 25, one obtains:

$$\frac{d[E']}{dt} = ([E_T] - [E'])\left(\frac{k_3[AR]}{[AR] + K_{AR}} + k_4\frac{[HR]K_{AR}}{K_{AR} + [AR]}\right) \quad (30)$$

$k_3[AR]([AR] + K_{AR})$ is the observed rate of activation by adenosine alone which can be measured independently. The quantity $k_4[HR]$ is the observed rate of activation by epinephrine when present alone ($k_{obsd}^{H} = 0.0313$ min^{-1}, Figure 5). The observed rate will be *smaller* than the sum of the separate activation rates by a factor of $K_{AR}/(K_{AR} + [AR])$ when epinephrine is present in the activating mixture with adenosine. A satisfactory fit is only obtained when one corrects using the above factor, as described in Figure 5. Indeed, if one attempts to fit the progress curve for the activation of the common pool of adenylate cyclase by both adenosine and l-epinephrine, using the sum of k_{obsd}^{H} and k_{obsd}^{A}, one does not obtain a satisfactory fit to the experimental data (Figure 5). The value of 0.0042 min^{-1} is the difference between the rate constant of simultaneous activation by both ligands (0.0655 min^{-1}) and the rate constant for adenosine activation alone (0.0613 min^{-1}). Since $k_4[HR]$ is (from Figure 5) 0.0313 min^{-1} and $k_4[HR]K_{AR}/(K_{AR} + [AR]) = 0.0042$ min^{-1}, one can calculate K_{AR} from this expression by rearranging it to:

$$k_4[HR]K_{AR} - 0.0042K_{AR} = 0.0042[AR] \quad (31)$$

Since the adenosine concentrations used in the experiment were saturating (Figure 5), $[AR] \approx [R_T]$, one obtains $K_{AR} = 0.155[R_T]$, namely the coupling between the adenosine receptor and the enzyme is quite tight. One can calculate that the receptor concentration is 1.0×10^{-7} to 1.0×10^{-6} M, since turkey erythrocytes possess about 500–700 adenosine receptors per cell (Braun and Levitzki, unpublished).[2]

[2] The concentration of β-adrenergic receptors in turkey erythrocyte membranes is about six receptors/μm^2. Since the thickness of the membrane is about 100 Å or 10^{-6} cm, one can calculate that the concentration of receptors is about 10^{-6} M.

If the adenosine receptor is entirely coupled to the catalytic moiety and at the same time 98% saturated by adenosine (at 3.0×10^{-4} M adenosine) only 2% of the adenylate cyclase remains accessible to activation by l-epinephrine. Thus, adenosine will control the rate of enzyme activation when the two ligands are present simultaneously. The observed rate of activation by adenosine alone is 0.0613 min^{-1} (Figure 5) and in the presence of epinephrine it is 0.0655 min^{-1} (Figure 5). These findings are in accordance with the mechanism whereby the adenosine receptor is tightly coupled to the enzyme, whereas the mode of activation by catecholamines is of the collision coupling type.

The Common Guanyl Nucleotide Site. Both adenosine and l-epinephrine seem to activate the adenylate cyclase through a common guanyl nucleotide subunit. This assertion is supported by the observation that the permanently active form of cyclase induced by either l-epinephrine and GppNHp or by adenosine and GppNHp can be reverted back to its inactive form by either l-epinephrine and GTP or by adenosine plus GTP (Figure 6). The mechanism of reversion involves the displacement of GppNHp from its regulatory site by GTP (Levitzki, 1977; Sevilla and Levitzki, 1977) followed by the hydrolysis of the latter through the GTPase activity (Cassell and Selinger, 1976; Tolkovsky and Levitzki, 1978) of the regulatory site. The findings reported in this study, especially those in Figure 6, imply that adenosine is capable of activating the same GTPase step which can also be activated by the β-agonists. One would expect that adenosine and GTP will cause the reversal of only 60–70% of the permanently active state induced by GppHNp and epinephrine, whereas l-epinephrine and GTP should induce 100% reversal of the permanently active state induced by GppNHp and adenosine. This difference is not apparent from Figure 6. This is probably due to the partial desensitization of the system, caused, most probably, by the long incubations at 37 °C required for these experiments.

The Structure of the Cyclase System. From this study it is apparent that the adenylate cyclase enzyme has a recognition domain for each of the two receptors: the β-adrenergic receptor and the adenosine receptor.

Note Added in Proof

Recently an adenosine receptor directed affinity label was prepared and used to explore directly the question of receptor to enzyme coupling using the same approach used for the study of the mode of coupling of β-receptors to cyclase (Tolkovsky and Levitzki, 1978). These studies (Braun and Levitzki, submitted for publication) demonstrate that the adenosine receptor is permanently coupled to the cyclase in the turkey erythrocyte.

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