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CHARACTERIZATION OF THE PURINE-REACTIVE SITE OF THE RAT TESTIS CYTOSOLIC ADENYLATE CYCLASE*

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Abstract—Naturally soluble rat germ cell adenylate cyclase was inhibited by adenosine and the adenosine analogs, 9-β-D-arabinofuranosyl adenine (AFA) and 2′,5′-dideoxyadenosine (DDA), all of which inhibited hormone-sensitive adenylate cyclases at the "P" site. The IC_{50} values for adenosine and DDA were approximately 0.1 and for AFA, 4.0 mM. The onset of adenosine inhibition was very rapid whether adenosine was added to the enzyme reactant mixture at time zero concomitantly with the addition of substrate or after the enzyme had been activated by the addition of substrate. The adenosine analogs, N^6-methyladenosine (MeA) and N^6-phenylisopropyl adenosine (PIA), which interact with plasma membrane receptors ("R" receptors) for hormone-sensitive adenylate cyclase, had little effect on the activity of the cytosolic adenylate cyclase. Additionally, aminophylline, which has been shown to competitively antagonize adenosine interactions with the plasma membrane "R" receptors but not "P" site interactions, had no effect upon substrate activation of the soluble enzyme and did not prevent adenosine from inhibiting the activity of the enzyme. These data provide evidence for an adenosine regulatory site on the cytosolic enzyme which resembles the "P" site described for membrane bound-adenylate cyclase.

The effects of adenosine on adenylate cyclase activity are mediated at both stimulatory and inhibitory membrane receptors and through a site putatively located on or near the catalytic unit of the membrane-bound hormone-sensitive systems examined to date [1–3]. Because of the diversity of adenosine effects on adenylate cyclase systems from different tissues and to avoid the possible complications introduced by adenosine-related metabolic enzymes (adenosine deaminase [3, 4], adenosine kinase [2], and *S*-adenosylhomocysteine hydrolase [5]), we have utilized a less complicated adenylate cyclase system to examine the effects of adenosine and its analogs on the regulation of adenylate cyclase: the cytosolic rat testicular adenylate cyclase. This form of adenylate cyclase appears to be a "naked" catalytic entity lacking nucleotide regulatory proteins (G/F, [6]). This form of the enzyme is of germ cell origin [7], is not responsive to hormones (luteinizing hormone, follicle stimulating hormone) or guanyl nucleotides, and utilizes manganese-ATP as the substrate, characteristics similar to the solubilized catalytic unit of membrane bound hormone sensitive adenylate cyclase [8–11].

In this study, we report that adenosine, AFA, and DDA inhibited the activity of the cytosolic adenylate cyclase and, in contrast, that adenosine analogs which interact with plasma membrane receptors, N^6-methyladenosine (MeA) and N^6-phenylisopropyl adenosine (PIA), had no effect on the activity of the enzyme. These studies demonstrate that an adenosine regulatory site is located on the enzyme and has the characteristics of the "P" site.

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MATERIALS AND METHODS

Preparation of cytosolic germ cell adenylate cyclase. Sexually mature rats (250–400 g body weight) were decapitated and exsanguinated. The testis and epididymis were removed and placed in isotonic saline on ice. Tissues were either used immediately or quickly frozen and stored at −70°. The testes were decapsulated, dissected free of visible blood vessels, placed in ice-cold 5 mM Tris–HCl buffer, pH 7.2, containing 3 mM $MgCl_2$ and 1 mM EDTA (1 g tissue/4 ml buffer), and disrupted in a Waring blender for 3–4 sec. The resulting homogenate was further homogenized in a tight-fitting ground glass Dounce homogenizer with twenty strokes and then centrifuged at 105,000 *g* for 1 hr. The 105,000 *g* × 60 min supernatant fraction contained the crude cytosolic germ cell adenylate cyclase [7]. Partial purification of the enzyme, to various degree of purity, was accomplished by ion-exchange chromatography, gel filtration, and affinity chromatography using ATP covalently bound to Sepharose. The chromatographic procedures are described in the legends to Figs. 1 and 2.

Assay of adenylate cyclase activity. Adenylate cyclase activity was assayed under conditions of various concentrations of ATP and $MnCl_2$, as noted in the text and legends. Non-radiolabeled cyclic-AMP (1 mM), bovine serum albumin (BSA, 1 mg/ml),

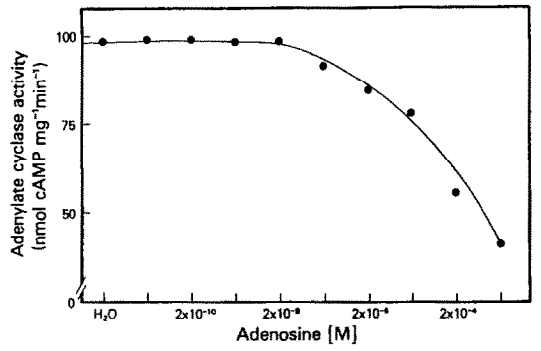

Fig. 1. Effect of adenosine on the activity of the extensively purified naturally soluble germ cell adenylate cyclase. Activity was measured in the presence of 0.2 mM ATP and 5.0 mM $MnCl_2$ for 10 min at 37°, in the absence or presence of increasing concentrations of adenosine as indicated on the horizontal axis. The assay was initiated by the addition of enzyme to the reactant mixture. The enzyme preparation used in this experiment was purified as follows. The cytosol containing the adenylate cyclase prepared in 5 mM Tris–HCl buffer, pH 7.2, containing 3 mM $MgCl_2$, 1 mM EDTA and 0.1 mM phenylmethylsulfonylfluoride was applied to a DEAE-cellulose column (4.2 × 50 cm) equilibrated with the same buffer. The amount of protein applied to the column was 26,000 mg in 26,000 ml. The column was washed with two column vol. of 50 mM Tris–HCl buffer, pH 7.6. (This latter buffer, as well as all Tris–HCl buffers used following this step, contained 25% glycerol, 10 mM 2-mercaptoethanol, 0.1 mM dithiothreitol and 25 μg/ml gentamycin in addition to the components listed above.) Elution was carried out with 2 column vol. of 160 mM Tris–HCl buffer, pH 7.6. Peak activity fractions eluted with the 160 mM Tris–HCl buffer were pooled, dialyzed against 5 mM Tris–HCl buffer, pH 7.6, and then applied to a DEAE-Biogel A column (1.6 × 20 cm) equilibrated with the same buffer. The amount of protein applied to the column was 1400 mg in 500 ml. Washing was carried out with 2 column vol. of 5 mM Tris–HCl buffer, pH 7.6, and elution with 2 column vol. of 60 mM Tris–HCl buffer, pH 7.6. Peak activity fractions eluted with the 60 mM Tris–HCl buffer were pooled, dialyzed against 5 mM Tris–HCl buffer, pH 7.0, containing the components listed above except $MgCl_2$, and then applied to an ATP-Sepharose column (1.5 × 6.8 cm; containing 6 μmol of immobilized ATP/ml of beads) equilibrated with the same buffer. The amount of protein applied to the column was 70 mg in 15 ml. The column was washed with 3 column vol. of the buffer and eluted with 100 mM Tris–HCl, pH 8.2, containing 1 mM ATP in addition to the usual components. Peak activity fractions were pooled and dialyzed against 5 mM Tris–HCl buffer, pH 7.0, and applied to an ATP-Sepharose column (1.5 × 18.6 cm; containing 1.3 μmol of immobilized ATP/ml of beads) equilibrated with the same buffer. The amount of protein applied to the column was 35 mg in 40 ml. Elution was carried out with 5 mM Tris–HCl buffer whose pH was stepwise increased from 7.0 to 7.8. The enzyme activity emerged between pH 7.4 and 7.6. The eluted high specific activity fractions were pooled and dialyzed against 5 mM Tris–HCl buffer, pH 7.6, and concentrated by frontal elution from a DEAE-Biogel A column (0.6 × 3.5 cm) with 100 mM Tris–HCl buffer, pH 7.6. The concentrated enzyme preparation (containing 7.5 mg protein in 4 ml) was applied to a Sephacryl S-200 gel column (5 × 100 cm) equilibrated with 100 mM Tris–HCl buffer, pH 7.6. Elution was carried out with the same buffer.

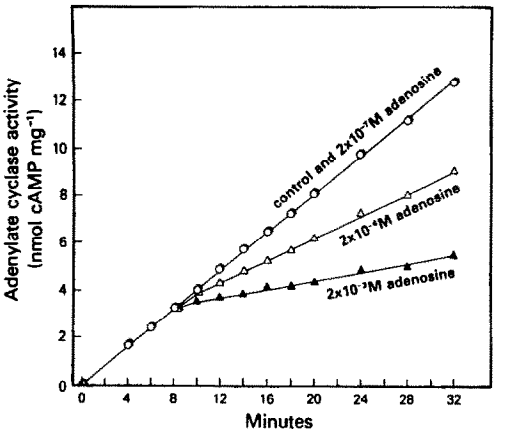

Fig. 2. Effects of adenosine at different concentrations on the kinetics of cytosolic adenylate cyclase. Four tubes were prepared containing ATP (0.2 mM final concentration) and $MnCl_2$ (5.0 mM final concentration), and the reaction was initiated by addition of adenylate cyclase to each tube. Individual time points were obtained by removing aliquots from the reaction mixture and adding them to the stopping solution, and heating at 100° for 2 min. After kinetics had proceeded through 8 min of reaction, buffer was added to one tube (control) and adenosine was added to each of the remaining three tubes to yield the final adenosine concentrations as indicated in the figure, and subsequent kinetics followed as described by removal of aliquots. The enzyme preparation used in this and the preparations employed in the remaining assays were purified by the chromatographic procedures which were, in principle, similar to those described in the legend to Fig. 1. However, slight modifications were introduced, namely, the columns were smaller, the ATP-Sepharose step using columns with low ATP ligand concentrations was omitted, and some of the components added to the Tris–HCl buffer were different. Unless otherwise stated, Tris–HCl buffers contained 3 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol and 15% glycerol. Ion-exchange chromatography was performed using either a DEAE-cellulose or a DEAE-Biogel A column (11.6 × 15 cm). The column was equilibrated with 5 mM Tris–HCl buffer, pH 7.6, and eluted with a stepwise gradient of Tris–HCl buffer, pH 7.6 (1.66 column vol. of each; 25, 50, 75, 100, 125, 175 and 250 mM buffer). Gel exclusion chromatography was performed using Sephacryl S-200 columns (1.6 × 15 cm). The columns were equilibrated with 250 mM Tris–HCl buffer, pH 7.6, and eluted with the same buffer. Chromatography on ATP-Sepharose columns (1 × 5 cm; containing 5.6 μmol of immobilized ATP/ml of beads) was performed using 5 mM Tris-HCl buffer containing the components listed above, except $MgCl_2$. Elution was carried out either with 5 mM Tris–HCl buffer, pH 8.0, containing 2 mM ATP in addition to the usual components, or with a 250 mM Tris–HCl buffer, pH 7.6.

and Tris–HCl (40 mM), pH 7.6, were present in all assays. [^{32}P]Cyclic-AMP was recovered by the method of Salomon *et al.* [12] with slight modifications. All values in tables and figures are the means of duplicate assays and are typically representative results. Protein concentrations were determined by the method of Bradford [13].

Adenosine, methyladenosine, 9-β-D-arabinofuranosyl adenine (AFA), bovine serum albumin, aminophylline, Lubrol PX, Triton X-100, phenylmethylsulfonylfluoride, and inorganic chemicals were purchased from Sigma. Radiochemicals, α-[^{32}P]ATP and [^{3}H]cyclic-AMP, were purchased from

Table 1. Relationship of purification of adenylate cyclase to inhibition by adenosine

Extent of purification (fold)	Addition	24-hr preincubation (% of control)	No preincubation (% of control)
6	Control	100	100*
	8×10^{-4} M Adenosine	106	61
24	Control	98	100*
	8×10^{-4} M Adenosine	105	63
250	Control	99	100*
	8×10^{-4} M Adenosine	71	63
1000	Control	100	100*
	8×10^{-4} M Adenosine	60	62

The adenylate cyclase preparations were either preincubated for 24 hr at 1–4° in the presence of adenosine, or assayed immediately after the addition of adenosine. Activity was measured in the presence of 0.2 mM ATP and 5.0 mM $MnCl_2$ for 10 min at 37°.

* The activity of the non-preincubated control for each enzyme preparation was arbitrarily set at 100%, and the other three values for each enzyme preparation were compared to the "Control" value. The specific activities of control for the 6-, 24-, 250- and 1000-fold purified adenylate cyclase preparations are 0.13, 0.48, 4.89 and 18.95 nmol/mg-min respectively.

Amersham and New England Nuclear respectively. ATP covalently linked to agarose (GATP, Type 4), and 2′,5′-dideoxyadenosine (DDA) were purchased from P-L Biochemicals. Anion exchange gels, DEAE Bio-Gel A (200 mesh) and DEAE cellulose (DE 52), were purchased from Bio Rad, Inc. and Whatman Chemical Separations, Inc., respectively. N^6-Phenylisopropyl adenosine was a gift of Dr. John N. Fain, Brown University.

RESULTS

Inhibition of cytosolic adenylate cyclase by adenosine. Adenosine inhibited the activity of the highly purified cytosolic adenylate cyclase (Fig. 1). Although the IC_{50} was approximately 0.1 mM, inhibitory effects can be seen at concentrations about two orders of magnitude lower (2×10^{-6} M). The inhibitory effect of adenosine was similar to that shown in Fig. 1, provided that the enzyme was purified more than 250-fold over the activity in the crude homogenate (Table 1).

Adenosine deaminase inhibitors such as deoxycorformycin were not included in the assay because they did not alter the effect of adenosine on the activity of the purified enzyme preparations. It was demonstrated that the preparations used were free from contamination by adenosine catabolic enzymes. As shown in Table 1, adenosine was preincubated for 24 hr (at 0–4°) with samples of adenylate cyclase at different stages of purification. When adenosine was preincubated with enzyme preparations purified less than 250-fold, its inhibitory effect was lost. However, when adenosine was preincubated with enzyme preparations purified more than 250-fold, its inhibitory effect remained unchanged.

Kinetics for adenosine inhibition. Time-course assays demonstrated that the onset of inhibition by adenosine was rapid and achieved equilibrium rapidly. Lag times for adenosine inhibition were not experimentally observed for the adenosine concentrations used in the kinetic assay depicted in Fig. 2.

Reversibility of adenosine inhibition. The purified cytosolic adenylate cyclase preincubated with adenosine for 5 min at 37° was dialyzed for 16 hr against 5 mM Tris, pH 7.6, and then assayed. The adenylate cyclase retained full activity after dialysis (Table 2), indicating the reversibility of adenosine interaction

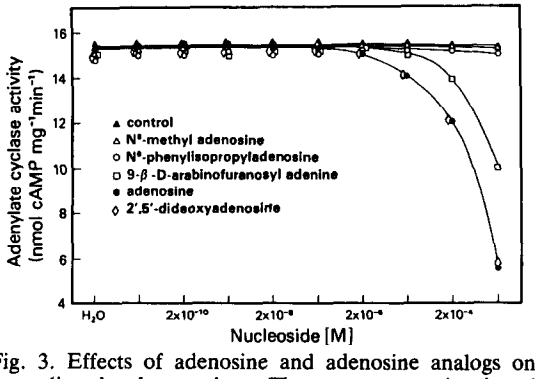

Fig. 3. Effects of adenosine and adenosine analogs on cytosolic adenylate cyclase. The enzyme was incubated for 10 min at 37° with the appropriate concentration of adenosine, or the adenosine analogs N^6-methyladenosine, N^6-phenylisopropyl adenosine, and 9-β-D-arabinofuranosyl adenine, in the presence of 0.2 mM ATP and 5.0 mM $MnCl_2$. Assays were initiated by the addition of enzyme to the reactant mixture.

Table 2. Reversibility of adenosine inhibition of cytosolic adenylate cyclase by dialysis

No.	Preincubation with 1.9 mM adenosine	Dialysis after preincubation†	Addition of 0.8 mM adenosine at assay‡	Percent of control activity
Part A: Preincubation without ATP, $MnCl_2$, and regeneration solution*				
1	+	−	−	66
2	−	−	+	64
3	+	+	−	100
4	−	+	+	67
5	−	+	−	100

No.	Preincubation with 1.9 mM adenosine and substrate	Dialysis after preincubation‖	Addition of 0.8 mM adenosine at assay‡	Percent of control activity
Part B: Preincubation with ATP, $MnCl_2$, and regeneration solution§				
1	+	+	−	100
2	+	+	+	50.5
3	+	−	+	40.2
4	+	−	−	56.3

* Assays were performed using 1.2 mM ATP and 30 mM $MnCl_2$. The specific activity of the control was 25.8 nmol cAMP $\cdot mg^{-1} \cdot min^{-1}$.

† Enzyme and 1.9 mM adenosine were preincubated for 16 hr at 1–4° and then dialyzed against a 2000-fold excess of 20 mM Tris, 1.5% glycerol, 0.5 mM $MgCl_2$ overnight at 4°.

‡ Enzyme was assayed immediately upon addition of adenosine.

§ Assays were assayed with 0.6 mM ATP and 12 mM $MnCl_2$.

‖ The enzyme preparation was pre-equilibrated with 1.9 mM adenosine and 1.3 mM ATP, 1.4 mM $MnCl_2$, and regenerating solution in Tris, $MgCl_2$, EDTA buffer for 10 min at room temperature followed by 2 hr at 4°, and then dialyzed overnight against a 2000-fold excess of 20 mM Tris, 1.5% glycerol, 0.5 mM $MgCl_2$ buffer at 4°.

with the cytosolic adenylate cyclase. Furthermore, the dialyzed cytosolic enzyme was still inhibited to the equivalent extent following dialysis that is seen for the untreated and undialyzed enzyme preparation. Therefore, adenosine inhibitory effects cannot be attributed to a low molecular weight factor or protein which is removed or altered with dialysis. Enzyme preincubated with adenosine for 16 hr without dialysis was inhibited to the same extent as the untreated enzyme assayed with the same concentration of adenosine. Furthermore, addition of Mn^{2+}, ATP, and a regenerating system did not alter the degree of inhibition by adenosine or reversibility of inhibition upon dialysis (Table 2).

Effects of adenosine analogs on the activity of the cytosolic adenylate cyclase. Adenosine analogs, 2′,5′-dideoxyadenosine (DDA) and 9-β-D-arabinofuranosyl adenine (AFA), which inhibit hormone-sensitive adenylate cyclase at the "P" effector site, significantly inhibited the cytosolic germ cell adenylate cyclase (Fig. 3). The potency of AFA was less than that of adenosine, which is the opposite of its potency in hormone-sensitive adenylate cyclases, where AFA exhibits a greater inhibitory effect than adenosine [14]. DDA was as potent as adenosine in its inhibitory effects on cytosolic cyclase activity, whereas in membrane-bound hormone-sensitive adenylate cyclase systems, DDA is usually found to be more potent than adenosine in inhibiting adenylate cyclase activity [14].

In contrast, adenosine analogs which are thought to interact only with plasma membrane receptor sites had minimal affect upon the activity of the cytosolic adenylate cyclase. Both N^6-methyladenosine, which stimulates the L-10 Leydig cell adenylate cyclase [14], and N^6-phenylisopropyl adenosine which inhibits the fat cell adenylate cyclase [15], had little effect upon the activity of cytosolic adenylate cyclase (Fig. 3). Neither preincubation of the enzyme with the N^6-substituted analogs nor the addition of the analogs after the activation of the cyclase by substrate could elicit either stimulatory or inhibitory effects (data not shown). Additionally, aminophylline, which is a competitive antagonist at adenosine "R" receptors [16, 17], did not inhibit the soluble adenylate cyclase, nor did it prevent adenosine from inhibiting the enzyme (Table 3, Fig. 3).

Table 3. Effect of aminophylline on the activity of the naturally soluble adenylate cyclase

Treatment	Activity* (% of control)
Control	100
+ Adenosine†	38
+ Aminophylline‡	98
+ Adenosine† and aminophylline‡	37

* All activities are standardized as percents of control. Control specific activity was 38 nmol $\cdot mg^{-1} \cdot min^{-1}$. Activity was measured with 1.0 mM ATP and 35 mM $MnCl_2$.

† Adenosine concentration, 2×10^{-4} M.

‡ Aminophylline concentration, 2×10^{-4} M.

DISCUSSION

Adenosine and the adenosine "P" site active analogs, 9-β-D-arabinofuranosyl adenine (AFA) and 2′,5′-dideoxyadenosine (DDA), inhibited the naturally soluble enzyme from rat testis. The IC_{50} for adenosine inhibition of approximately 0.1 mM is similar to that reported for adenosine "P" site effects on hormone-sensitive adenylate cyclase [18, 19]. Because the naturally soluble enzyme is free of plasma membrane and the G/F binding protein, the locus for adenosine inhibition of the soluble enzyme must be on the enzyme itself.

The kinetics for adenosine inhibition indicate that the inhibitory effects of adenosine are manifest rapidly and are similar to those seen in hormone-sensitive cyclases [20]. Identical adenosine kinetic effects occurred, within the time scale of our measurements, whether the enzyme had been activated by the addition of substrate prior to, or after, the addition of adenosine. Also, high concentrations of adenosine, preincubated with the enzyme for up to 16 hr, did not inhibit the enzyme to a greater degree than the same concentration of adenosine added to an enzyme preparation at time zero (Table 1). These findings indicate that the interaction of adenosine with the naturally soluble adenylate cyclase occurs under rapid equilibrium conditions and demonstrates no apparent hysteretic effect within the time scale of the assay. Furthermore, adenosine binding was totally reversible by dialysis, even in the presence of substrate, indicating the equilibrium and reversible nature of inhibition of the enzyme by adenosine and its analogs.

The adenosine analogs MeA and PIA had no effect upon the soluble adenylate cyclase. Because these analogs have been shown to exert effects upon hormone-sensitive adenylate cyclase through plasma membrane receptors which are absent here, this finding is not surprising. The inability of aminophylline to affect the cytosolic adenylate cyclase, or to prevent adenosine from inhibiting the cytosolic enzyme, is not surprising since aminophylline exerts its primary effect on the hormone-sensitive adenylate cyclase at the R-site [16, 17]. It can be concluded from these data that the naturally soluble adenylate cyclase from rat testis and the catalytic entity of the membrane-bound adenylate cyclase systems apparently share a similar locus for adenosine action, the "P" site. In both adenylate cyclases, the "P" site is specific for adenosine or adenosine analogs that have an unmodified purine ring. An alteration in the ribose moiety resulted in a negligible effect upon the inhibitory potency of the analog DDA and reduced, but did not eliminate, the inhibitory effects of AFA on the cytosolic enzyme. These are characteristics manifested by the "P" site as described for the membrane-bound hormone-sensitive enzyme.

Whether the naturally soluble enzyme is identical to the catalytic entity of the hormone-sensitive adenylate cyclase is beyond the scope of this report. The published physical and kinetic characteristics of the enzyme isolated from various tissues do not resolve definitively this problem. There are similarities and differences between the catalytic entities derived from different cells types [3, 6, 7, 21–24]. Adenylate cyclases from certain tissues display properties thus far not recognized in other adenylate cyclases. For example, the catalytic component of the bovine brain adenylate cyclase system possesses a site for activation by calmodulin, whereas most adenylate cyclases from other tissues do not [23, 25]. The testis germ cell enzyme displays different behavior toward ATP, present either in solution (when used as substrate in the presence of Mn^{2+}), or immobilized to Sepharose (Braun and Collins, unpublished observation), than displayed by the detergent-solubilized and highly purified cardiac adenylate cyclase [26]. The affinity of the germ cell enzyme for ATP is relatively low, K_m = 0.8 to 0.9 mM [27], whereas the affinity of the cardiac enzyme is far greater, K_m = 0.05 to 0.1 mM [28, 29]. Thus, there may be differences in the structure of the catalytic entities in the various cells.

Previous reports [3, 9] have indicated that the naturally soluble adenylate cyclase is either minimally inhibited or not inhibited at all by adenosine or the adenosine "P" site analog, 2′,5′-dideoxyadenosine. We have attempted to duplicate the assay conditions utilized in those reports. Under conditions identical to those reported, namely, at suboptimal Mn^{2+} concentration (1 mM), adenosine inhibition was not obtained. In addition, we observed that inhibition of the activity of the extensively purified enzyme occurred only in the presence of BSA in the assay mixture (data not shown). Londos *et al.* [9] in their studies indeed used a highly purified cytosolic enzyme preparation; however, the assays were carried out in the absence of BSA.

It has been suggested that the "P" site is either not on the catalytic component of the hormone-responsive adenylate cyclase system, or its expression requires the functional coupling of the catalytic entity to the nucleotide regulatory component [15]. These reports were clarified by the studies of Neer and Salter [23] who have shown that the purine-specific site for adenosine inhibition is on the separated catalytic component of the bovine brain adenylate cyclase. They have found that inhibition by adenosine 9-β-D-arabinofuranoside was greater when the catalytic component was functionally coupled to the nucleotide regulatory entity. This latter finding was considered as suggestive evidence that the activated conformation of the catalytic component may be more susceptible to adenosine inhibition than the basal. Our findings pertaining to the rat testis germ cell enzyme are reminiscent of those pertaining to the catalytic entity of the bovine brain adenylate cyclase as reported by Neer and Salter [23]. The purine specific site for adenosine inhibition is on the enzyme, and it appears that its "activated state" is required to render the enzyme susceptible to inhibition by adenosine. The nature of the BSA requirement for the adenosine inhibition of the highly purified cytosolic enzyme is not clear at the present time. It can be speculated that BSA may stimulate or aid in the induction of an "activated state" of the enzyme *in vitro*, a situation which may be occurring in intact cells and in partially purified enzyme preparations by association of the enzyme with a cytosolic protein(s).

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