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Tissue Levels, Source, and Regulation of 3′-AMP: An Intracellular Inhibitor of Adenylyl Cyclases

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SUMMARY

Tissue levels of 3′-AMP were measured in several rat tissues and the sensitivities of the respective adenylyl cyclases were compared with respect to "P" site-mediated inhibition by 3′-AMP2′-deoxy-3′AMP (2′d3′-AMP), and 2′,5′-dideoxyadenosine. IC_{50} values for these P site inhibitors of adenylyl cyclase varied widely among tissues, e.g., with skeletal muscle being least sensitive to 3′-AMP ($IC_{50} > 170$ μM) and brain being most sensitive ($IC_{50} \sim 10$ μM). These differences were noted when activation was with Mn^{2+} but diminished with Mn^{2+} plus forskolin and conceivably may reflect the distribution of different isozymes of adenylyl cyclase. 3′-AMP levels also varied significantly among rat tissues, with spleen having the highest levels (~280 nmol/g), kidney, liver, heart, and brain having decreasing 3′-AMP content, and skeletal muscle levels being immeasureably low (<0.1 nmol/g). When rats were made diabetic with streptozotocin, the 3′-AMP content of livers increased from ~47 nmol/g in control animals to ~84 nmol/g, a change largely reversed by maintenance of diabetic animals with insulin. The data suggest that tissue 3′-AMP levels may be regulated and in certain tissues may be sufficient to inhibit adenylyl cyclase *in vivo*. Three potential sources of 3′-AMP and 2′d3′-AMP, the most potent naturally occurring P site inhibitors of adenylyl cyclase, were examined. No evidence was found for the formation of either nucleotide from the respective cyclic nucleotide by a unique cyclic nucleotide phosphodiesterase or from the respective nucleoside by a hypothetical adenosine 3′-kinase and ATP. Substantial 3′-AMP and 2′d3′-AMP were formed by spleen and liver homogenates from the respective oligonucleotides (RNA, mRNA, and DNA) in a time- and protein-dependent manner. The data imply the existence of enzymes in these tissues to catalyze the formation of 3′-AMP and 2′d3′-AMP from nucleic acids and suggest that these activities may account for the formation of P site agonists under *in vivo* conditions. The data suggest that these P site inhibitors are a potential link between fluctuations in nucleic acid metabolism and altered sensitivity of membrane-bound adenylyl cyclase to stimulatory signals.

Adenylyl cyclase (ATP-pyrophosphate-lyase, cyclizing; EC 4.6.1.1) comprises a family of hormone-sensitive, membrane-bound enzymes that can be regulated by a number of signals of both extracellular and intracellular origin. The enzyme can be activated or inhibited by cell surface receptors, whose effects are mediated by GTP-binding proteins,[2] G_s or G_i, respectively (1). In addition, adenylyl cyclase may be regulated from within cells. The enzyme from some tissues is activated by Ca^{2+}/calmodulin (2, 3), presumably reflecting changes in cellular free calcium. In addition, mammalian adenylyl cyclases are inhibited via a unique allosteric site by adenosine and analogs of adenosine (4, 5). Because allosteric inhibition occurs with adenosine analogs retaining an intact purine moiety, this site has been designated the P site (6). P site-mediated inhibition of adenylyl cyclase has been demonstrated in intact cells, in membranes, and with detergent-solubilized and purified preparations of the enzyme (4–14). Available evidence suggests that the P site is on the cytoplasmic surface of the enzyme's catalytic moiety (C) (10, 11, 13–15). Although P site-mediated inhibition of adenylyl cyclase does not require the presence of either G_s or G_i, sensitivity to inhibition is dramatically enhanced by activation of the enzyme, for example by stimulatory hormone receptors, activated G_s, Ca^{2+}/calmodulin, or Mn^{2+} (11, 14–16). $Mn^{2+}\cdot C$ and $GTP\gamma S\cdot\alpha_s\cdot C$ complexes are most sensitive to inhibition, whereas forskolin variably affects sensitivity to inhibition, depending on the source of adenylyl cyclase (11, 14–16). Moreover, from kinetic studies (16a) and from studies with a P site-selective affinity ligand (16b), data suggest that the P

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[2] GTP-binding proteins G_s and G_i (equivalent to N_s and N_i) are the guanine nucleotide-dependent regulatory subunits of adenylyl cyclase that mediate, respectively, stimulation and inhibition of the enzyme by stimulatory and inhibitory hormone receptors. These are heterotrimeric proteins consisting of $\alpha_s\beta\gamma$ or $\alpha_i\beta\gamma$ subunits, respectively. C refers to the catalytic subunit of adenylyl cyclase.

ABBREVIATIONS: GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); 2′5′-ddAdo, 2′,5′-dideoxyadenosine; EGTA, ethylene glycol bis(β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HPLC, high performance liquid chromatography; TEA·HCO_3, triethylammonium bicarbonate; 2′d3′-AMP, 2′-deoxy-3′-AMP; 2′d-cAMP, 2′-deoxy-cAMP; 2′d5′-AMP, 2′-deoxy-5′-AMP.

site resides on a cytosolic domain of adenylyl cyclase distinct from the catalytic site.

Uncertain, though, is the physiological role of P site-mediated inhibition of adenylyl cyclase. 2′d3′-AMP and 3′-AMP are the most potent of the naturally occurring P site inhibitors of adenylyl cyclase (14), but it is not known whether or to what extent these 3′-nucleotides may be regulated *in vivo*. Sahyoun *et al.* (17) reported levels of 2′d3′-AMP in several tissues, suggesting that adenine nucleotides other than adenosine might be involved in regulation of adenylyl cyclase. In the present study, we report levels of 3′-AMP in various rat tissues and evaluate several potential cellular sources for its formation. The effects of streptozotocin-induced diabetes and insulin maintenance on 3′-AMP content of rat liver were determined.

Experimental Procedures

Preparation of adenylyl cyclase. Washed particulate and detergent-dispersed adenylyl cyclase from rat brain was prepared essentially as described previously (18). For comparisons of P site sensitivity of adenylyl cyclases from various tissues, washed particulate enzyme was prepared similarly from each. Hearts, spleens, kidneys, livers, brains, and skeletal muscle were excised from seven decapitated rats. Pooled tissues were weighed and then washed with 20 ml of ice-cold buffer containing 3 mM dithiothreitol, 250 mM enzyme-grade sucrose, and 20 mM glycylglycine, pH 7.5. Tissues were then homogenized in 9 volumes (9 ml/g of tissue) of the same buffer by subjecting each to 20 strokes in a glass-glass Dounce homogenizer, except skeletal muscle, which was homogenized with a Polytron homogenizer. Homogenized tissues were centrifuged at 550 × *g* (2,000 rpm; Beckman JA17 rotor) for 10 min at 2°. The supernatant fractions were recentrifuged at 39,000 × *g* (17,000 rpm; Beckman JA17 rotor) for 20 min at 2°. The resulting pellets were resuspended in 1 to 2 volumes of homogenizing buffer and were then stored at −70° until use.

Assay of adenylyl cyclase. Adenylyl cyclase activities were determined essentially as previously described (19, 20). Briefly, reaction mixtures contained 50 mM triethanolamine·HCl, pH 7.5, 1 mM 3-isobutyl-1-methylxanthine, 1 mM dithiothreitol, 1 mg/ml bovine serum albumin, 2 mM purified creatine phosphate, 100 μg/ml creatine kinase, 100 μM ATP, 10 mM $MgCl_2$ or 10 mM $MnCl_2$, and [α-^{32}P]ATP (2–9 × 10^5 cpm), in a volume of 100 μl. Incubations were for 15 min at 30° and reactions were terminated by precipitation with zinc acetate, containing 0.5 mM unlabeled cAMP, and sodium carbonate (20). [^{32}P]cAMP was isolated by sequential chromatography on Dowex-50 and then Al_2O_3 columns, essentially as described by Salomon *et al.* (21). The unlabeled cAMP served for monitoring of the recovery of the labeled cAMP, by measurement of absorbance at 259 nm with an aliquot of the sample. Radioactivity was determined by Cherenkov radiation in a liquid scintillation counter.

Determination of 3′-AMP levels. Rats were killed by decapitation. Tissues were rapidly excised, immediately frozen by clamping at the temperature of liquid nitrogen, and stored at −70° until extraction. Tissues (1 to 2 g, frozen weight) were added to 6 ml of ice-cold extraction buffer, containing 1% (w/v) Lubrol-PX, 5 mM EDTA, 10 mM TEA·HCO_3, pH 7.5, and [^{3}H]3′-AMP (2–5 μCi), and were immediately homogenized with three pulses of 10 sec each with a Polytron homogenizer. Tritiated 3′-AMP was included to allow for correction for sample recovery during extraction and purification procedures. [^{3}H]3′-AMP was found to be unstable to extraction with 10% trichloroacetic acid. Homogenized samples were then boiled for 15 min, diluted further with 3 ml of extraction buffer, and then centrifuged for 20 min at 46,000 × *g* (19,000 rpm; Beckman JA-20.1 rotor). Resulting supernatant fractions were subjected to snake venom 5′-nucleotidase (0.8 mg/ml) for 20 min at 30°, to remove 5′-nucleotides and especially 5′-AMP. The 5′-nucleotidase reaction was stopped by the addition of perchloric acid to a concentration of 0.3 M, which was then neutralized by the addition of K_2CO_3. Samples were clarified by centrifugation at 46,000 × *g* (19,000 rpm; Beckman JA-20.1 rotor) for 15 min. The supernatant fractions were then applied to a DEAE-Sephadex column (1.5 × 5 cm; bicarbonate form). The column was developed with 10 ml of 10 mM formic acid, followed by 5 ml each of TEA·HCO_3 at 0.1, 0.5, and 1 M. 3′-AMP elutes with 1 M TEA·HCO_3. (Columns were regenerated by washing with 20 ml of 1 M TEA·HCO_3, followed by 50 ml of water.) Samples were then concentrated by overnight lyophilization, thereby also removing TEA·HCO_3. Samples were resuspended in 10 mM TEA·HCO_3, pH 7.6, and were purified further by HPLC, first by anion exchange chromatography (Synchropak AX-300, 4.1 × 300 mm) and then on a C18 reverse phase column (Beckman Ultrasphere ODS, 4.6 × 250 mm). Retention times and $A_{259\ nm}$ peak areas were measured with a Waters 990 photodiode-array detector and software. The AX-300 column was washed at 1 ml/min with 10 mM TEA·HCO_3, pH 7.6, for 10 min, at which time the TEA·HCO_3 concentration was increased to ~130 mM over 0.5 min. Adenosine and adenine pass through the AX-300 column and the retention time for 3′-AMP was approximately 18 min. The column was flushed and regenerated between samples with 1 M TEA·HCO_3. Fractions containing [^{3}H]3′-AMP were pooled, concentrated by overnight lyophilization or on a Speed-Vac, and resuspended in ~0.7 ml of formic acid, diluted to pH 2.5, for injection on the C18 column. 3′-AMP was eluted from the C18 column with formic acid, pH 2.5, with a retention time of approximately 16 min. (The column was washed between samples with methanol.) C18 column fractions containing 3′-AMP were pooled, dried, and then resuspended in 100 μl of 50 mM triethanolamine·HCl, pH 7.5, for subsequent assays. Concentrations of 3′-AMP were determined from both the HPLC peak area from the C18 column and the degree of inhibition of Mn^{2+}-activated rat brain adenylyl cyclase, as compared with peak areas and inhibition curves for authentic 3′-AMP.

Assay of cyclic nucleotide 3′-5′-phosphodiesterase. Rat liver and brain were homogenized in 9 volumes of a buffer containing 50 mM glycylglycine, pH 7.5, 10 mM $MgCl_2$, and 2 mM EGTA. Incubations (in 100 μl for 20 min at 30°) contained 10 μg to ~3 mg of homogenate, 1 to 10 μM cAMP, 0.1 μCi of [^{3}H]cAMP, 10 mM $MgCl_2$, 50 mM triethanolamine·acetate, pH 7.4, and 1 mM 3′-AMP, in the presence or absence of snake venom 5′-nucleotidase (0.8 mg/ml). The unlabeled 3′-AMP acted as a trap for labeled product and was used for monitoring of the recovery of product. Experiments were also conducted in the absence of the unlabeled trap to assess any potential product inhibition. Reactions were terminated by the addition of perchloric acid to 0.3 M, and the samples were neutralized with K_2CO_3 before separation of substrate and products by HPLC on AX-300 anion exchange and C18 reverse phase columns, run as described above for the determination of 3′-AMP. Similar experiments were conducted to determine the formation of 2′d3′-AMP from 2′d-cAMP.

Assay for adenosine 3′-kinase. Potential phosphorylation of adenosine at the 3′-position was evaluated. Incubations contained 10 μg to 3 mg of liver or brain homogenate, 1 to 100 μM adenosine, 0.1 μCi of [^{3}H]adenosine, 10 mM $MgCl_2$, 50 mM triethanolamine·acetate, 200 μM ATP, 10 μM *erythro*-9-[2-hydroxy-non-3-yl]-adenine, and 1 mM 3′-AMP, in the presence or absence of snake venom 5′-nucleotidase (0.8 mg/ml). Reactions were terminated by the addition of perchloric acid, and products were analyzed by HPLC. In separate experiments, the possible transfer of ^{32}P from [γ-^{32}P]ATP (~0.1 μCi) to the 3′-position of adenosine was also tested and experiments were also conducted in the absence of unlabeled trap. Similar experiments evaluated the potential formation of 2′d3′-AMP from 2′-deoxy-adenosine.

Formation of 3′-AMP from poly(A). Whether 3′-AMP might be derived from nucleic acids was assessed in homogenates of rat brain, spleen, and liver, by a modification of the method of Razzell (22) for the assay of spleen nucleic acid phosphodiesterase activity. Incubations (in 100 μl for 20 min at 37°) contained 10 μg to 1 mg of homogenate, 0.1 μCi of [^{3}H]poly(A), 0.2 to 5 μM poly(A), 1 mM 3′-AMP, 167 mM ammonium acetate, pH 5.7, and 0.7 M EDTA, in the presence or absence of snake venom 5′-nucleotidase (0.8 mg/ml). In separate

experiments, the formation of 3′-AMP from 5 μM poly(A) was determined in the presence of 1 nCi of tracer [^{3}H]3′-AMP.

Streptozotocin-induced diabetes. Male Sprague-Dawley rats (Charles River Breeding Laboratories), initially weighing 200 g, were used. Diabetes was induced by the intraperitoneal injection of streptozotocin (80 mg/kg of body weight) dissolved in 0.1 M sodium citrate, pH 4.5. Control animals received only the buffer. Blood glucose values were determined 4 days later and animals whose blood glucose exceeded 450 mg/dl were considered diabetic. Insulin-treated rats were given a 10-unit bolus injection of insulin (Eli Lilly porcine insulin), followed by a 6-unit dose every 6 hr thereafter. Rats were killed by decapitation. Tissues were rapidly removed, freeze-clamped at the temperature of liquid nitrogen, and then stored at −70° until they were extracted for nucleotide.

Materials. [α-^{32}P]ATP and [γ-^{32}P]ATP were purchased from DuPont-New England Nuclear or from ICN Pharmaceuticals. [^{3}H]3′-AMP, [^{3}H]2′d3′-AMP, [^{3}H]poly(A), [^{3}H]adenosine, and [^{3}H]2′-deoxyadenosine were from Amersham. [^{3}H]cAMP and [^{3}H]2′d-cAMP were from ICN. Poly(A) and poly(dAdT) were purchased from either Sigma or Boehringer-Mannheim and were purified before use by phenol extraction and ethanol precipitation (23). Purified poly(A) migrated as a single broad band on agarose gel electrophoresis, corresponding to a mean size of ~250 nucleotides (as compared with an RNA ladder from Bethesda Research Laboratories, Inc.). Cyclic nucleotide phosphodiesterase purified from chicken gizzard was a generous gift of Dr. J. N. Wells, Vanderbilt University. DEAE-agarose, Dowex-50, neutral alumina, and all electrophoresis reagents were from Bio-Rad. ATP, GTP, GTPγS, creatine phosphate, and creatine kinase were obtained from Boehringer-Mannheim. Creatine phosphate was purified as previously described (24). Snake venom 5′-nucleotidase, from *Crotalus atrox*, and streptozotocin were from Sigma. Other nucleic acids and nucleotides were obtained either from Sigma or from Boehringer-Mannheim. Other reagents were from commercial sources and were of the highest quality available.

Results

Tissue distribution of 3′-AMP. 2′d3′-AMP and 3′-AMP are the most potent naturally occurring P site inhibitors of adenylyl cyclase, with IC_{50} values of ~1 and ~9 μM, respectively (14). To approach the question of whether the inhibition by these 3′-nucleotides might reflect a physiological role *in vivo*, levels of 3′-AMP were determined for a number of rat tissues (Table 1). Spleen showed the greatest abundance of 3′-AMP, with kidney, liver, heart, and brain having decreasing 3′-AMP contents. 3′-AMP either was absent from skeletal muscle or was present at less than 0.1 nmol/g, the approximate detection limit of the assay. In preliminary studies, we also measured levels of 2′d3′-AMP in extracts of whole rat brains, spleen, and liver, by methods similar to those for 3′-AMP (see Experimental Procedures). Levels of 2′d3′-AMP were not measureable (i.e., <1 nmol/g) in liver, and in spleen and brain they were roughly comparable to levels of 3′-AMP. These values for 2′d3′-AMP compared with levels reported by Sahyoun *et al.* (17), ~2.5 nmol/g of spleen and ~0.8 nmol/ml of packed toad erythrocytes. Those workers did not report levels for other tissues, nor did they report levels for 3′-AMP. If one assumes 50% cell water, 3′-AMP levels in each of these tissues, except for skeletal muscle, would have been sufficiently high to inhibit the respective adenylyl cyclase partially or nearly completely *in vitro* (see below and Table 1). Thus, IC_{50} values and tissue content for 3′-AMP and 2′d3′-AMP were within ranges that would also be consistent with inhibition of adenylyl cyclase via the P site *in vivo*.

Changes in 3′-AMP levels in streptozotocin-induced diabetes. Diabetes significantly distorts regulatory processes in cells and is known to decrease the sensitivity of hepatic adenylyl cyclase to stimulation by glucagon (25–27). The cause of the decreased sensitivity to glucagon is not known but the shift is in the direction that conceivably could result from inhibition of the glucagon/GTP-stimulated form of adenylyl cyclase. Consequently, streptozotocin-induced diabetes was used as a model to determine whether changes in hepatic 3′-AMP content could be induced and whether they could be opposed by maintenance of the animals with insulin. The 3′-AMP content of livers from diabetic rats increased 75%, from 47 ± 8 nmol/g in sham-injected animals to 84 ± 18 nmol/g (Fig. 1). This increase was largely reversed by maintenance of diabetic animals with insulin; 3′-AMP content was 57 ± 15 nmol/g. These findings suggest that levels of 3′-AMP may vary due to changes in circulating hormones, conceivably sufficiently to change the sensitivity of adenylyl cyclase to stimulatory agents.

TABLE 1

3′-AMP content in various tissues and IC_{50} values for inhibition of adenylyl cyclases by 3′-AMP, 2′d3′-AMP, and 2′5′-ddADO

IC_{50} values were determined graphically. For 3′-AMP, values are averages ± standard errors from three experiments. For 2′d3′-AMP and 2′5′-ddAdo, values are averages ± ranges from two experiments. Inclusions were 10 mM $MnCl_2$ and 100 μM forskolin. Initial adenylyl cyclase activities were, in pmol of cAMP (min·mg of protein)$^{-1}$: with Mn^{2+}: spleen, 24.3 ± 0.6; kidney, 5.8 ± 0.3; liver, 4.4 ± 0.4; heart, 17.7 ± 1.1; brain, 801± 119; and skeletal muscle, 8.6 ± 0.9; with Mn^{2+} plus forskolin: spleen, 240 ± 10; kidney, 35 ± 8; liver, 42 ± 12; heart, 160 ± 10; brain, 2430 ± 150; and skeletal muscle, 37 ± 3. 3′-AMP levels are average values ± standard errors (or ± range for $n = 2$) for the number of tissues extracted, shown in parentheses.

Tissue	[3′-AMP]	IC_{50} values			
		3′-AMP		2′d3′-AMP, Mn^{2+} + forskolin	2′5′-ddAdo, Mn^{2+} + forskolin
		Mn^{2+}	Mn^{2+} + forskolin		
	nmol/g	μM			
Spleen	280 ± 47 (8)	60 ± 17	21 ± 2	4.0 ± 0.9	3 ± 0
Kidney	130 ± 37 (4)	29 ± 1	24 ± 7	6 ± 3	1.6 ± 0.5
Liver	47 ± 8 (8)	48 ± 9	12 ± 5	2.3 ± 0.9	0.5 ± 0.1
Heart	3.7 ± 1.0 (3)	15 ± 1	12 ± 4	3.1 ± 0.9	1.1 ± 0.6
Brain	1.8 ± 0.4 (2)	10 ± 2	11 ± 5	3.0 ± 0.7	2.6 ± 0.6
Skeletal Muscle	<0.1 (4)	176 ± 94	37 ± 12	7 ± 3	5.1 ± 0.9

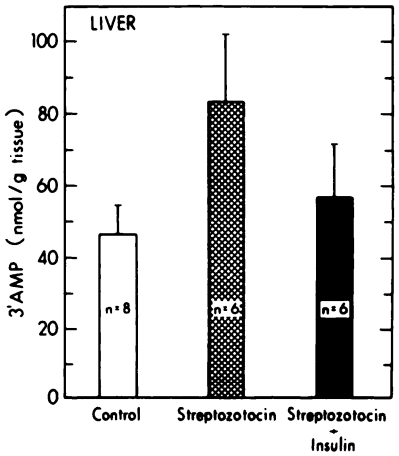

Fig. 1. Effects of diabetes and insulin treatment on levels of 3′-AMP in rat liver. 3′-AMP levels were measured in rat livers from different treatment groups, as described in Experimental Procedures. Values are nmol of 3′AMP/g wet weight ± S.D. for the number of tissues indicated ($p \leq 0.09$ for differences between groups by the *t* test).

Potential sources of 3′-nucleotides. We considered three potential sources of 3′-AMP and 2′d3′-AMP in cells. First, the possibility was assessed that these 3′-nucleotides could be derived, respectively, from cAMP or 2′d-cAMP via a unique phosphodiesterase that would specifically cleave the 5′-phosphoribosyl bond. As shown in Table 2, under conditions allowing substantial formation of 5′-AMP from cAMP and of 2′d5′-AMP from 2′d-cAMP in homogenates of rat brain or liver, consistent with the presence of cyclic nucleotide phosphodiesterase activity, there was no detectable production of either 3′-AMP or 2′d3′-AMP. With neither low (1 μM) nor high (100 μM) concentrations of tritiated cAMP or 2′d-cAMP, either in brain or liver homogenates, could we detect the formation of any labeled 2′d3′-AMP or 3′-AMP (Table 2). In separate experiments (not shown), this lack of formation was found to be independent of the presence of unlabeled 3′-product in the assay (i.e., there was no product inhibition) and was substantiated with a partially purified, Ca^{2+}/calmodulin-dependent phosphodiesterase from chicken gizzard.

Also considered was the possibility that 3′-nucleotides could be formed from ATP and adenosine or 2′-deoxyadenosine by the action of a hypothetical adenosine 3′-kinase (Table 3). Such an adenosine 3′-kinase was sought in two types of experiments. First, whole homogenates of brain or liver were incubated with [γ-^{32}P]ATP and either unlabeled 2′-deoxyadenosine or adenosine and, second, homogenates were incubated with [^{3}H]2′-deoxyadenosine or [^{3}H]adenosine and unlabeled ATP. Neither [^{32}P]- nor [^{3}H]-2′d3′-AMP or 3′-AMP was detected, although labeled 5′-AMP was formed in both brain and liver homogenates and labeled 2′d5′-AMP was formed in brain homogenates (Table 3), consistent with the presence of adenosine 5′-kinase but inconsistent with there being an adenosine 3′-kinase. It is possible, of course, that under conditions other than those we tried such 3′-kinase or unique cyclic nucleotide phosphodiesterase activity might have been observed.

TABLE 2

Formation of adenosine monophosphates from cAMP

Formation of 5′-AMP and 3′-AMP from cAMP and of 2′d5′-AMP and 2′d3′-AMP from 2′d-cAMP were determined as described in Experimental Procedures. The limit of detection of the assay was ~0.1 nmol (min·g of tissue)$^{-1}$. Values are means from three experiments, each performed in duplicate.

Tissue	Substrate concentration	Rate of formation			
		5′-AMP	3′-AMP	2′d5′-AMP	2′d3′-AMP
	μM	nmol of nucleotide (min·g of tissue)$^{-1}$			
Brain	1	0.14 ± 0.06	<0.1	1.3 ± 0.5	<0.1
	10	95 ± 40	<0.1	1.3 ± 0.8	<0.1
Liver	1	1.2 ± 0.5	<0.1	0.4 ± 0.1	<0.1
	10	54 ± 3.3	<0.1	2.8 ± 1.5	<0.1

TABLE 3

Formation of adenosine monophosphates from adenosine or 2′-deoxyadenosine and ATP

Formation of 5′-AMP and 3′-AMP from 1 μM adenosine and of 2′d5′-AMP and 2′d3′-AMP from 1μM 2′-deoxyadenosine were determined as described in Experimental Procedures. The limit of detection of the assay was ~0.1 nmol (min·g of tissue)$^{-1}$. There was also no detectable formation of 3′-adenosine phosphates when 100 μM substrate was used. Values are means from three experiments, each performed in duplicate.

Tissue	Rate of formation			
	5′-AMP	3′-AMP	2′d5′-AMP	2′d3′-AMP
	nmol of nucleotide (min·g of tissue)$^{-1}$			
Brain	140 ± 34	<0.1	141 ± 23	<0.1
Liver	130 ± 2	<0.1	<0.1	<0.1

TABLE 4

Formation of 3′-AMP from poly(A)

Production of [^{3}H]3′-AMP from [^{3}H]poly(A) was determined as described in Experimental Procedures. Comparable values were obtained for the production of unlabeled 3′-AMP from 5 μM poly(A), with 1 nCi of tracer [^{3}H]3′-AMP to monitor recoveries. Data are averages of duplicate values from one of two similar experiments.

Tissue	[poly(A)]	3′-AMP formation
	μM	nmol (min·g of tissue)$^{-1}$
Spleen	0.2	6.0
	1.2	32.0
	5.0	39.0
Liver	0.2	4.7
	1.2	13.3
	5.0	37.0

The third possibility considered was that these adenosine 3′-phosphates could be derived from nucleic acids or nucleic acid degradation products by the action of phosphodiesterases or nucleases that selectively cleave 5′-phosphoribosyl bonds of diester linkages, leaving 3′-phosphorylated nucleosides. In particular, it seemed possible that the ubiquitous tracts of poly(A) found at the 3′ end of most mammalian mRNA molecules could provide a rich cytosolic source of 3′-AMP. With spleen and liver homogenates, 3′-AMP was formed from polyadenylate (Table 4) and 2′d3′-AMP was formed from poly(dAdT) by homogenates of spleen, liver, and brain (not shown). Incubation of these homogenates with 0.2 to 5 μM poly(A) resulted in the production of substantial quantities of 3′-AMP. In each case, product formation was linearly related to incubation time (to 30 min) and to amount of tissue present (from 10 μg to 1 mg of wet weight) (not shown). These data indicate that the enzymic machinery exists in these tissues to catalyze the formation of 3′-AMP and 2′d3′-AMP from nucleic acids and suggest that activities appropriate for the formation of P site agonists may also exist under *in vivo* conditions.

P site-mediated inhibition of adenylyl cyclases from various tissues. Structural requirements for P site-mediated inhibition were defined previously with a detergent-dispersed adenylyl cyclase from rat brain (14). Whether activated by Mn^{2+} or by proteolysis with ninhibin[3] in the presence of GTPγS, the most potent P site-agonists were 2′d3′-AMP, 2′5′-ddAdo, and 3′-AMP, with respective IC_{50} values of ~1, ~3, and ~9 μM (14). Because 2′d3′-AMP and 3′-AMP are naturally occurring and levels of these nucleotides varied substantially among tissues, estimations of IC_{50} values for the most potent P site agonists were made for particulate adenylyl cyclases from a number of tissues, to see whether there might be a relationship between the level of 3′-nucleotide and the sensitivity of the respective adenylyl cyclase to P site-mediated inhibition (Table 1). 2′5′-ddAdo was typically a slightly more potent inhibitor than 2′d3′-AMP, and both compounds were consistently much more potent than 3′-AMP. Of particular note, though, were the observations that sensitivity to inhibition by these compounds varied from one tissue to another and was dependent on the mode of activation of adenylyl cyclase. This is shown for 3′-AMP with enzyme activated with Mn^{2+} alone (Table 1). Sensitivity to inhibition by 3′-AMP varied widely among tissues,

[3] "Ninhibin" is an adenylyl cyclase-activating protease isolated from bovine sperm. It belongs to the acrosin family of trypsin-like serine proteases and was given the name ninhibin due to its apparent effect on N_i (G_i).

with the washed particulate adenylyl cyclase from brain being most sensitive (IC_{50} ~ 10 μM) and the enzyme from skeletal muscle being quite insensitive (IC_{50} ~ 176 μM). In the presence of Mn^{2+} plus forskolin, the tissue differences largely disappeared. Forskolin enhanced the sensitivity to P site-mediated inhibition of adenylyl cyclase from some sources (e.g., spleen, liver, and skeletal muscle) but was without effect on (e.g., heart) or diminished the sensitivity of enzyme from others (e.g., brain). These differences are consistent with previously reported observations with adenylyl cyclases from liver, S49 cyc^- cells, platelets, and brain (11, 15, 16) and might be attributed to differences in catalytic moieties of the enzyme from these different sources.

Discussion

3′-AMP content was determined and found to vary significantly among various rat tissues, with spleen having the highest levels and levels in skeletal muscle being immeasureably low. The adenylyl cyclases from these tissues also exhibited widely varying sensitivities to inhibition by 3′-AMP. This was particularly evident when adenylyl cyclase was activated by Mn^{2+}, whereas the distinctions were diminished in the presence of forskolin, whether 3′-AMP, 2′d3′-AMP, or 2′5′-ddAdo was the inhibitor. The differences in the effect of forskolin on P site sensitivity were consistent with earlier studies and likely reflect the distribution of different isozymes of the adenylyl cyclase family (11, 15, 16). The cellular levels of 3′-AMP and 2′d3′-AMP and the sensitivities of the various adenylyl cyclases to P site-mediated inhibition suggest that this may be a physiologically relevant mode of intracellular regulation of the enzyme (Fig. 2). It is uncertain, though, whether 2′d3′-AMP and 3′-AMP are the only or the most potent naturally occurring P site inhibitors of adenylyl cyclase. Dinucleotides of the general structure dApdN and deoxyadenosine pentanucleotides (d(AGGGG)) are known to inhibit adenylyl cyclase in the 10 to 50 μM range (14). It is possible that physiologically important inhibition of adenylyl cyclase by P site agonists may be due to only one agent or to several agents acting together. Moreover, the physiological agonist cyclase could vary from tissue to tissue, its effectiveness would be dependent on the distribution of the various forms of the enzyme, and its level could be dependent on the metabolic or functional state of the tissue.

This latter suggestion is strengthened by the observations made with diabetic rats. Streptozotocin-induced diabetes in rats is associated with decreased basal adenylyl cyclase activities, decreased responsiveness of adenylyl cyclase to stimulation by hormones and GTP, or a combination of the two effects (25–27). In this report, higher levels of 3′-AMP were noted in livers from animals made diabetic with streptozotocin (Fig. 1). Although this finding is consistent with the observed changes in the regulation of adenylyl cyclase in tissue from diabetic animals, particularly the attenuated response to stimulatory signals, this does not establish a causal relationship between increased 3′-AMP levels and decreased cyclase responsiveness. Diabetes is associated with changes in the levels of a number of membrane proteins (26), as well as altered production and stability of various mRNAs. However, the finding that insulin maintenance tends to lower 3′-AMP levels in liver supports our suggestion that the P site may constitute part of a dynamic cellular control system allowing for intracellular regulation of adenylyl cyclase activity (14). P site agonists could inhibit adenylyl cyclase if the enzyme is activated and, thereby, made susceptible to sufficient on-board concentrations of inhibitor and/or if the level of the inhibitor changes due to altered rates of its formation and/or degradation (Fig. 2).

Sahyoun *et al.* (17) estimated levels of 2′d3′-AMP in rat spleen and toad erythrocytes and speculated that it may be derived from the breakdown of DNA. As reported here, 3′-AMP and 2′d3′-AMP are not produced from cAMP and 2′d-cAMP, respectively, nor are they produced through the phosphorylation of adenosine and 2′-deoxyadenosine. Although we cannot exclude the possibility that these sources may be important in other tissues or under other conditions than those employed here, the fact that these 3′-nucleoside phosphates could be derived directly from nucleic acids *in vitro* indicates the presence of the appropriate enzymes and strongly suggests that this would be their likely source *in vivo*. The intracellular mRNA concentration can be estimated as approximately 2 to 3 μM (28–30). This is the range of concentrations used here to demonstrate the production of 3′-AMP from poly(A) *in vitro* (Table 4) and suggested that the normal cellular concentrations of mRNA and $poly(A)^+$ mRNA would be in a range sufficient for the generation of inhibitory levels of 3′-AMP by nucleases and/or phosphodiesterases (22). Because the steady state levels of 2′d3′-AMP and 3′-AMP reflect a balance between rates of formation and degradation (Fig. 2), the question becomes whether one or both processes are regulated. Changes in the rate of formation could occur if an enzyme were presented varying substrate levels and/or if the catalytic rate changed with a stable substrate level. It is known, for example, that the steady state level of an mRNA is affected by its degradation rate, that the sequential degradation of some mRNAs occurs only after most or all of the poly(A) is removed, and that the control of mRNA turnover seems to be especially important for some cell cycle-regulated genes (28, 31–34). Furthermore, the poly(A) tail length can vary substantially in response to hormonal stimuli [e.g., vasopressin (35)]. Hence, altered mRNA or poly(A) levels could alter 3′-AMP formation. Alternatively, increased 3′-AMP formation could occur through increased 5′-phosphodiesterase activity (forming 3′-AMP) or through a shift in substrate specificity resulting in increased 3′-AMP formation at the expense of 5′-nucleoside monophosphate formation. If any of these processes occur and are regulated, the P site becomes a potential means by which adenylyl cyclase activity could be regulated by other cellular events.

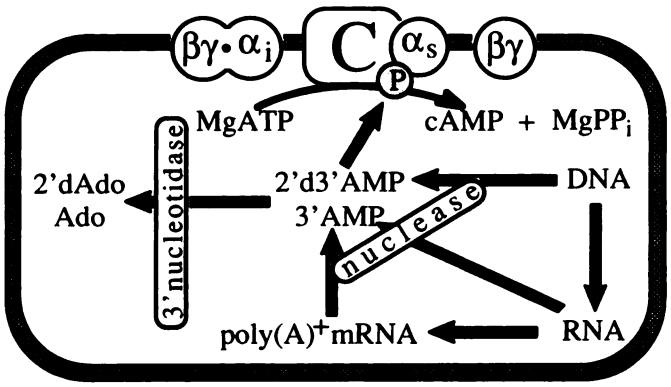

Fig. 2. Regulation of P site agonists and adenylyl cyclase. *Ado*, adenosine; *2′dAdo*, 2′-deoxyadenosine.

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