MOLECULAR PHARMACOLOGY, 18, 199–209

β-Adrenergic-Sensitive Adenylate Cyclase in Choroid Plexus: Properties and Cellular Localization

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Received February 25, 1980; Accepted April 9, 1980

SUMMARY

NATHANSON, J. A.: β-Adrenergic-sensitive adenylate cyclase in choroid plexus: Properties and cellular localization. *Mol. Pharmacol.* **18:** 199–209 (1980).

Because of physiological evidence suggesting the possibility of adrenergic regulation of choroid plexus secretion, a detailed study was undertaken to identify, characterize, and localize β-adrenergic-stimulated adenylate cyclase in broken cell preparations of cat choroid plexus. The enzyme was GTP ($EC_{50} = 2 \times 10^{-6}$ M)- but not calcium-dependent and was activated by low concentrations of isoproterenol ($K_a = 1.4 \times 10^{-7}$ M). Isoproterenol, in the presence of Mg^{2+}, increased the maximal reaction velocity without altering the K_m for ATP. The optimal ratio of added Mg^{2+}/ATP was 4–8/1. Mn^{2+} and, to a lesser extent, Co^{2+} (but not Cu^{2+}) could also support enzyme activity. Among receptor agonists, isoproterenol was most potent, followed in order by epinephrine ($K_a = 1.6 \times 10^{-6}$ M), norepinephrine ($K_a = 2.5 \times 10^{-5}$ M), phenylephrine ($K_a > 2.5 \times 10^{-5}$ M), and dopamine ($K_a = 2 \times 10^{-4}$ M). Isoproterenol activation was blocked by low concentrations of (±)-propranolol ($K_i = 2.7 \times 10^{-9}$ M) but only by higher concentrations of (+)-propranolol ($K_i = 3 \times 10^{-6}$ M), fluphenazine ($K_i = 1.2 \times 10^{-7}$ M), or phentolamine ($K_i > 10^{-3}$ M). Among the more selective β-adrenergic agents, the relatively β_2-selective agonists, zinterol ($K_a = 2.3 \times 10^{-8}$ M) and OPC 2009 ($K_a = 1.3 \times 10^{-7}$ M), were much more potent and effective than the β_1-selective agonist, prenalterol. Salbutamol, terbutaline, and orciprenaline were of intermediate potency and, along with zinterol, acted as partial agonists. Among several antagonists (IPS 339, H35/25, butoxamine, metoprolol, *p*-oxyprenolol, atenolol, and practolol), the calculated inhibitory constants correlated well with those obtained for the same agents in blocking isoproterenol-stimulated adenylate cyclase and IHYP binding in lung. Both agonist and antagonist data indicated that the majority of the adenylate cyclase-associated β receptors in choroid plexus were β_2. Anatomical studies indicated that β-adrenergic-sensitive adenylate cyclase activity was greater in fourth ventricle than lateral ventricle choroid plexus. Cell separation experiments, utilizing several different procedures, consistently demonstrated substantial enrichment of hormone sensitivity in fractions enriched in epithelial as compared with vascular elements. The results suggest a possible β_2-adrenergic regulation of choroid plexus secretory epithelium.

INTRODUCTION

Recent evidence suggests that the sympathetic nervous system may have a regulatory role in the secretion of cerebrospinal fluid (CSF) from the choroid plexus. Histochemical studies of the choroid plexus have demonstrated a dense adrenergic innervation (which disappears following sympathectomy), and electron microscopic studies have shown that nerve terminals containing dense core vesicles lie in close approximation to the choroid secretory epithelium and its underlying capillary network (1, 2). In physiological experiments, electrical stimulation of the sympathetic chain has been shown to alter CSF pressure and production (2, 3), effects which are mimicked by the direct application of norepinephrine to the ventricular fluid bathing the choroid plexus (2). Taken together, these data provide indirect evidence for the existence of adrenergic receptors in the choroid plexus. Furthermore, because of the frequent association of adrenergic (particularly, β-adrenergic) receptors with membrane-bound adenylate cyclase, these data also suggest that sympathetic regulation of the choroid plexus

This research was supported by a grant to the author from the McKnight Foundation and by a Pharmaceutical Manufacturers Association Foundation Research Starter Grant.

0026-895X/80/050199-11$02.00/0

might be associated with activation of a β-adrenergic-sensitive adenylate cyclase. This latter possibility is supported by preliminary biochemical studies (4) as well as by the observation that cholera toxin, which activates choroid plexus adenylate cyclase *in vitro*, increases CSF secretion when given intraventricularly (5).

Further evidence for the existence of a choroid plexus β-adrenergic-sensitive adenylate cyclase, as well as knowledge of this enzyme's pharmacological and biochemical characteristics, would be of value in studies of choroid plexus physiology and in the development of drugs with the potential for altering CSF secretion. The following study presents the first detailed description of β-adrenergic-sensitive adenylate cyclase in mammalian choroid plexus. Furthermore, it provides evidence that this enzyme is enriched in cell fractions containing choroid plexus epithelium, a localization which raises the possibility of an adrenergic regulation of choroid epithelial cell physiology.

MATERIALS AND METHODS

Drugs and reagents. All common reagents were of analytical grade and were purchased from Sigma Chemical Co. Other drugs and chemicals were obtained from the following sources: (−)-isoproterenol, (−)-epinephrine, (−)-norepinephrine, dopamine, (−)-phenylephrine, (±)-propranolol, ATP (crystalline) from equine muscle, ATP (crystalline, Grade 1) by phosphorylation of adenosine, bovine serum albumin (BSA) (fraction V), hyaluronidase (type V, 1090 u/mg), and guanosine 5′-triphosphate (GTP) (type I from equine muscle) from Sigma; trypsin (1:250) from GIBCO; collagenase (125 u/mg) from Worthington; phentolamine, metoprolol, and *p*-oxprenolol from CIBA-Geigy; zinterol from Mead Johnson; atenolol from Stuart Pharmaceuticals; prenalterol (H133/22 = H80/62) and H 35/25 ((±)-erythro-4′-methyl-α-(1-isopropylaminoethyl)-benzylalcohol) from Hässle; butoxamine from Burroughs–Wellcome; salbutamol from Schering; orciprenaline (metaproterenol) from Boehringer Ingelheim; terbutaline from Astra; IPS 339 from Hässle and Prof. G. LeClerc; practolol from Dr. J. Douglas; D-propranolol from Ayerst; and fluphenazine from Squibb.

Tissue. Male mongrel cats were anesthetized with ether and perfused transcardially with 1000 ml of cold phosphate-buffered saline (PBS) after cross-clamping of the descending thoracic aorta. In some cases, nembutol anesthesia was used, with similar biochemical results. Choroid plexus was dissected from lateral, third, and fourth ventricles, then rinsed twice in cold PBS, and larger choroidal vessels were removed. Unless specified, all the choroidal tissue (approx 80–100 mg) from a single cat was pooled and homogenized, 15 mg/ml, in cold 6 mM Tris–maleate, pH 7.4. (In preliminary experiments (4), we found that rabbit, dog, and calf choroid plexus also contained β-adrenergic-stimulated adenylate cyclase activity, but the degree of stimulation in these species was somewhat less than in the cat. Rat choroid plexus showed considerably less activity.)In those experiments comparing β-adrenergic-sensitive adenylate cyclase activity in choroid plexus with that in cerebral vasculature, two preparations of cerebral blood vessels were used. The first (pial vessels) consisted of the intracranial portions of the internal carotid and vertebral arteries and the proximal portions of anterior, middle, and posterior cerebral arteries. The second (intraparenchymal cerebral microvessels) was prepared from the whole brain as previously described by this and other laboratories (6, 7). Basically, the procedure consisted of a sequence of mechanical fragmentation, discontinuous sucrose density gradient centrifugation, and sieve filtration. The purity of the microvessel fraction was monitored by light microscopy and determination of the activity of the vascularly enriched enzymes, alkaline phosphatase and γ-glutamyl transpeptidase (6, 8). Vessel fractions were homogenized (20 mg/ml) in 6 mM Tris–maleate, pH 7.4. Ependymal tissue, used in some experiments, was obtained from the lateral ventricular walls by blunt microscopic dissection and homogenized, 20 mg/ml, as before.

Adenylate cyclase. Unless specified otherwise, cyclic AMP formation was measured in tubes containing (in 0.3 ml) 80 mM Tris–maleate, pH 7.4, 10 mM theophylline, 8 mM $MgCl_2$, 0.03 mM GTP, 2 mM ATP, and tissue homogenate (1 mg wet weight), plus test substances as indicated. ATP derived from equine muscle (with trace vanadium) was used throughout, except in the GTP dose–response studies. In these latter experiments, ATP prepared by phosphorylation of adenosine was utilized. This ATP had no detectable GTP as assayed by high-performance liquid chromatography (J. W. Kebabian, personal communication). In some experiments, 0.1% ascorbic acid was added to reduce the possibility of differential oxidation of the various added amines. Results in the presence of ascorbic acid were similar to those seen in the absence of any antioxidant.

The enzyme reaction (4 min at 30°C) was initiated by the addition of ATP, stopped by heating to 90°C for 2 min, and then centrifuged at 1000*g* for 15 min to remove insoluble matter. Cyclic AMP content in the supernatant was determined by binding assay (9) using a protein fraction from calf adrenal cortex. Appropriate buffer blanks were utilized in all cases. In certain experiments, such as those testing the effects of metal cofactors on adenylate cyclase activity, the cyclic AMP-containing supernatant was partially purified on Bio-Rad AG50W-X8 resin, as described previously (10), prior to binding assay. (We have previously shown that micromolar concentrations of certain divalent cations can exert differential inhibitory effects on binding of cyclic AMP to the adrenal binding protein and thereby introduce error in the determination of adenylate cyclase activity (10).) Protein concentration was determined by the method of Lowry *et al.* (11), using bovine serum albumin as a standard.

Under the preceding conditions, enzyme activity was linear with time at least up to 6 min and linear with respect to protein concentration from one-half to two times that used in the standard incubation. Under these same conditions, cyclic nucleotide phosphodiesterase activity, determined by the method of Filburn and Karn (12), was nearly completely inhibited.

Calculations. Inhibitory constants (K_i) for the various adrenergic blockers were calculated from two types of experiments. Both yielded similar values. In the first, the

concentration of agonist (isoproterenol) was held constant and the concentration of antagonist was varied. Assuming competitive inhibition, the K_i was calculated from the equation (13)

$$K_i = IC_{50}/[1 + (S/K_a)], \quad [1]$$

where IC_{50} is the concentration of antagonist necessary to give 50% inhibition of isoproterenol-stimulated activity, S is the concentration of agonist, and K_a is the concentration of isoproterenol (1.4×10^{-7} M) necessary for half-maximal activation of cat choroid plexus adenylate cyclase activity.

In the second method (used with some but not all antagonists) the concentration of antagonist was held constant and the concentration of isoproterenol was varied. The K_i was calculated (13) from the equation

$$K_i = I/[(K_a'/K_a) - 1], \quad [2]$$

where I is the concentration of antagonist and K_a' and K_a are the concentrations of isoproterenol necessary to cause half-maximal stimulation of choroid adenylate cyclase activity in the presence and absence of antagonist, respectively.

Separation of choroidal vascular and epithelial cells. Combined lateral, third and fourth ventricular choroid (approx 90 mg) was washed once in PBS, cut into several pieces (approx 5 mg each), and placed in a small (12-ml) conical glass centrifuge tube in 4 ml of dissociation medium containing 10 mM Hepes, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.7 mM Na_2HPO_4, 5.6 mM glucose, and 0.1% trypsin or other enzyme (see the following). The tube was incubated for 15 min at 30°C with intermittent tituration, following which the tissue was allowed to settle, the supernatant, enriched in suspended epithelial cells, was transferred to a cooled (4°C) test tube, and horse serum was added to a final concentration of 10%. Fresh dissociation medium was added to the original tube containing sedimented tissue and the dissociation procedure repeated twice more. The combined cell suspensions from the three dissociations were filtered through a 100-μm-mesh-size nylon screen, sedimented at 100*g* for 5 min, and washed twice with the previous dissociation medium lacking enzymes or BSA. Excessive incubation time, temperature, or agitation led to decreased hormone stimulation. In other experiments it was found that incubation with 0.05% hyaluronidase and 0.012% collagenase, in lieu of trypsin (and minus BSA), also yielded successful cell separations.

One aliquot of the cell suspension was fixed in 10% buffered formalin; another was used for determination of total cell count and proportion of cells excluding trypan blue, employing standard hemocytometer techniques. The remainder of the cells were pelleted and homogenized (10 mg/ml) in 6 mM Tris–maleate, pH 7.4, for determination of adenylate cyclase activity. The remaining, partially de-epithelialized choroid vasculature was washed twice, an aliquot taken for histology, and then homogenized, 10 mg/ml, as before.

Some cell separations were carried out in the absence of digestive enzymes, utilizing a calcium-free buffer containing 10 mM Hepes, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.7 mM Na_2HPO_4, 5.6 mM glucose, 1% BSA, and 2.25 mM EDTA. Choroid was incubated at 4°C for 20 min with intermittent tituration. Suspended cells were removed, the procedure was repeated twice more, and dissociated cells were collected and washed as previously. In still other experiments, cat choroid plexus was separated into an "epithelial cell" and a "capillary" fraction using another nonenzymatic procedure, similar to that employed for isolating cerebral microvessels from cat brain (5): freshly dissected choroid was gently fragmented by hand in a Teflon glass homogenizer (0.2-mm clearance) in 2 ml of cold 25 mM Tris–maleate buffer, pH 7.4, containing 140 mM NaCl and 0.5% BSA, and then centrifuged (25,000 rpm, SW27 rotor) on a discontinuous 1.0/1.35/1.5/1.7/2.25 M sucrose gradient. The vessel fraction (1.7/2.25 M interface) was washed and collected against a 100-μm nylon screen, while the remaining, less dense, nonvessel fractions were combined, diluted with medium, and collected by centrifugation at 50,000*g*. Both fractions were homogenized as previously.

For light microscopy, smears of the fixed cell suspensions were air dried, washed in methanol, and stained with hematoxylin and eosin. Intact and de-epithelialized choroids were fixed in 10% buffered formalin, then embedded in paraffin, and 10-μm sections were stained as previously. Photographs were taken at 400× magnification.

RESULTS

As shown in Fig. 1 (left-hand curve), isoproterenol caused a dose-dependent increase of adenylate cyclase activity in broken cell preparations made from fresh choroid plexus. Under the standard incubation conditions, the K_a for isoproterenol stimulation (average ± SEM for all experiments) was $1.4 \pm 0.1 \times 10^{-7}$ M (range,

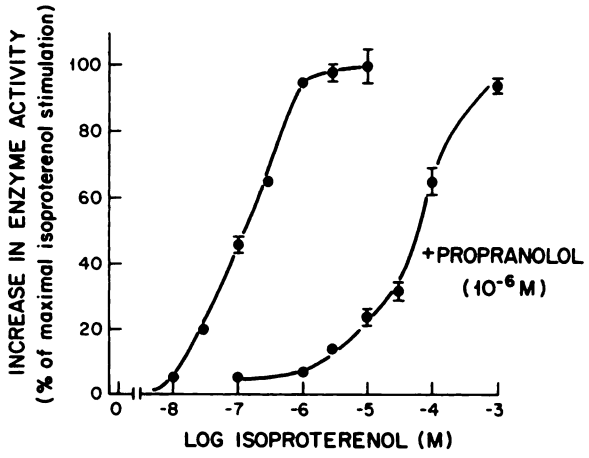

FIG. 1. *Activation of cat choroid plexus adenylate cyclase activity by (−)-isoproterenol in the absence and presence of 10^{-6} M (±)-propranolol*

Activity is expressed as a percentage of the stimulation seen in the presence of a saturating concentration (10^{-4} M) of isoproterenol alone. In this, as well as in all subsequent figures, the values shown at each point are the means and ranges for replicate samples, each assayed for cyclic AMP content in duplicate. At those points which lack error bars, the range was within the size of the symbol. In the experiment shown, basal activity was 23.4 ± 4.3 pmol/mg protein/min, and the stimulation due to 10^{-4} M isoproterenol was 43.3 ± 2.3 pmol/mg protein/min.

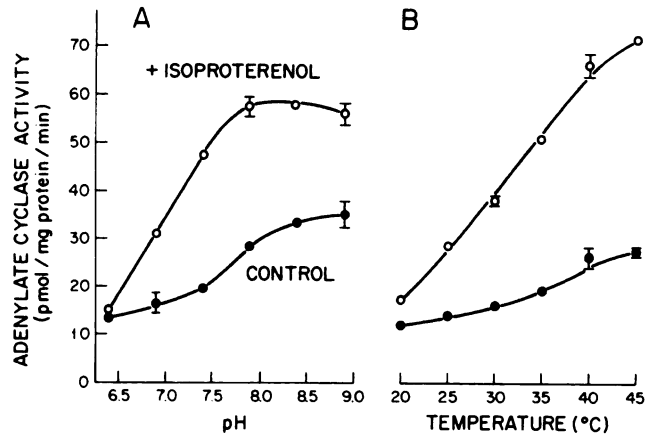

FIG. 2. *Effect of pH (A) and incubation temperature (B) on basal* (●) *and isoproterenol-stimulated* (○) *adenylate cyclase activity*

Incubation conditions were, otherwise, as described in Materials and Methods. Isoproterenol concentration was 10^{-4} M.

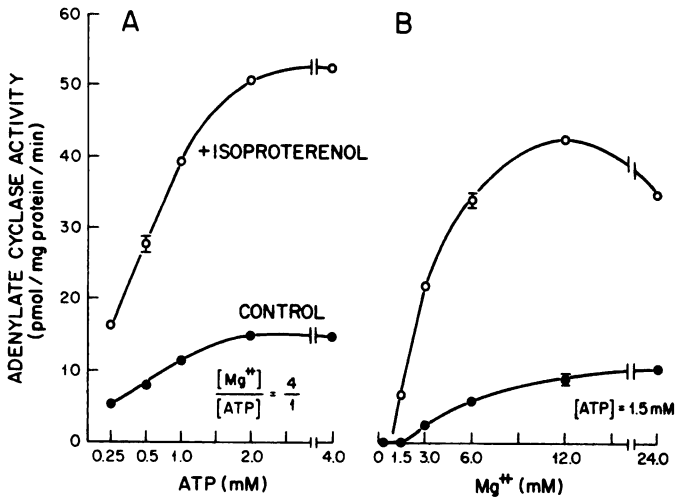

FIG. 3. *Effect of ATP and Mg^{2+} concentration on choroid plexus adenylate cyclase activity in the absence* (●) *and presence* (○) *of* 10^{-4} M isoproterenol

In the experiment shown in A, increasing amounts of Mg^{2+} and ATP were added at a fixed 4/1 molar ratio. In the experiment shown in B, the ATP concentration was fixed at 1.5 mM and the Mg^{2+} concentration was varied from 0.375 to 24 mM, in effect increasing the molar ratio of Mg^{2+} to ATP from 1/4 to 16/1.

1.2–1.5 × 10^{-7} M), and the maximal stimulation was 399 ± 37% of basal activity (range, 208–580%).

Biochemical Properties of Enzyme Stimulation

Effect of pH. The effect of varying the pH of the reaction mixture is shown in Fig. 2A. Stimulation of enzyme activity by 10^{-4} M isoproterenol was observed at all pH values tested above 6.9. Optimal stimulation, in terms of percentage activation as well as absolute increase over basal activity, occurred at pH 7.4.

Effect of temperature. Figure 2B shows the effect of varying the incubation temperature (20–45°C) on basal and isoproterenol-stimulated enzyme activity. The absolute increment of hormone-sensitive activity increased with increasing temperature up to 45°C. An Arrhenius plot (not shown) of the basal enzyme data yielded a straight line throughout the experimental temperature range, with a calculated energy of activation for the adenylate cyclase reaction of 6.4 kcal/mol. In the presence of 10^{-4} M isoproterenol, the Arrhenius plot was linear from 25 to 40°C and yielded a calculated energy of activation of 10.9 kcal/mol. These values are comparable to those seen for certain other hormone-sensitive adenylate cyclases (e.g., Ref. 14).

In two experiments, fresh choroid was frozen at −90°C for 2 weeks, then thawed, homogenized, and assayed as previously for isoproterenol-sensitive enzyme activity. Hormone stimulation was present but was somewhat reduced (average V_{max} = 264% of control) compared to the average stimulation (399% of control) for fresh tissue. All subsequent experiments described in this paper used fresh tissue.

Effect of ATP and Mg^{2+}. In some experiments (Fig. 3A), increasing amounts of Mg^{2+} and ATP were added to the reaction mixture at a fixed 4/1 molar ratio. Under these conditions, absolute activity (both basal and isoproterenol stimulated) increased up to a concentration of 2 mM ATP (8 mM Mg^{2+}), above which activity plateaued. In both the absence and the presence of isoproterenol, the K_m of the enzyme for ATP was similar (approximately 0.75 mM). In other experiments (Fig. 3B), the ATP concentration was fixed at 1.5 mM, and the Mg^{2+} concentration was varied from 0.375 to 24 mM, in effect giving an increasing molar ratio of Mg^{2+} to ATP. Neither basal nor hormone-stimulated activity was detectable at or below a molar ratio (Mg^{2+}/ATP) of 0.5. Increasing concentrations of Mg^{2+} preferentially augmented isoproterenol stimulation relative to basal activity, with an optimal Mg/ATP ratio of between 4 and 8.

Effect of calcium and EGTA.[1] Calcium ion is known to be a regulator of hormone-sensitive adenylate cyclase (15). In some tissues, such as brain (16, 17), micromolar concentrations of calcium (similar to those present in whole tissue homogenates) stimulate basal enzyme activity. Chelation of this calcium with EGTA (which can also chelate other divalent cations) selectively reduces basal but not hormone-stimulated adenylate cyclase activity. The net result is an apparent potentiation of hormone stimulation (expressed as a percentage of basal activity).

In the present experiments, increasing concentrations of calcium were added to whole choroid homogenates in the presence and absence of 10^{-4} M isoproterenol. Also, in the absence of exogenous calcium, EGTA (10^{-3} M final concentration) was added in the presence and absence of 10^{-4} M isoproterenol. As shown in Fig. 4, and unlike the results described previously for brain, EGTA increased both basal and hormone-stimulated enzyme activity. In the absence of EGTA, but in the presence of increasing concentrations of calcium up to 10^{-4} M, basal and isoproterenol-stimulated activity gradually decreased. At 10^{-3} M calcium, both basal and hormone-stimulated activities were markedly inhibited. This inhibition of hormone activation with increasing concentrations of calcium suggests that the observed EGTA stimulation of enzyme activity may be due to chelation of endogenous calcium

[1] Abbreviation used: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid.

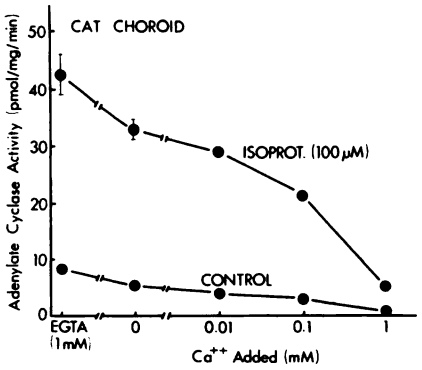

FIG. 4. *Effect of EGTA or added calcium on choroid plexus adenylate cyclase activity in the absence and presence of 10^{-4} M isoproterenol*

EGTA was present only where noted and was omitted from the tubes containing added calcium.

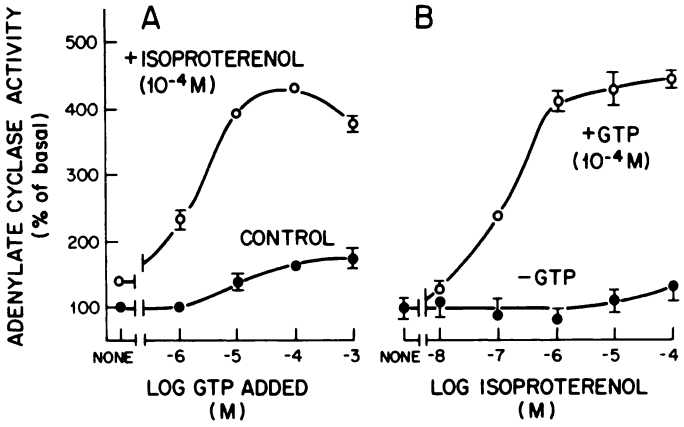

FIG. 5. *Effect of GTP on choroid plexus adenylate cyclase activity*

In the experiment shown in A, the GTP concentration was varied in the absence (●) and presence (○) of 10^{-4} M isoproterenol. Basal enzyme activity in the absence of GTP and isoproterenol was 19.3 ± 1.1 pmol/mg protein/min. In the experiment shown in B, the isoproterenol concentration was varied in the absence (●) and presence (○) of 10^{-4} M GTP. Basal enzyme activity in the absence of isoproterenol and GTP was 8.24 ± 0.5 pmol/mg protein/min.

(or possibly some other divalent cation) normally present in the homogenate.

Other divalent cations. Table 1 compares the effects of substituting Mn^{2+}, Co^{2+}, or Cu^{2+} for Mg^{2+} in the standard reaction mixture. Compared to Mg^{2+}, Mn^{2+} caused an approximate doubling of basal adenylate cyclase activity. Isoproterenol-stimulated activity, however, was the same; thus, in terms of fold-stimulation, Mg^{2+} was twice as effective as Mn^{2+}. In the presence of Co^{2+}, basal activity was about the same as with Mg^{2+}, but isoproterenol stimulation was reduced to about 60% that of Mg^{2+}-dependent activity. Cu^{2+} completely inhibited both basal and isoproterenol-sensitive enzyme activity.

GTP dependence. Figure 5A shows the effect of increasing concentrations of GTP on basal and isoproterenol-stimulated enzyme activity. In the absence of exogenous GTP, hormone stimulation was only 15% of that seen in the presence of 10^{-4} M GTP. In other words, isoproterenol stimulation was almost totally GTP dependent. The EC_{50} of GTP for hormone stimulation (in the presence of an optimal concentration of isoproterenol) was 2×10^{-6} M. In the presence of concentrations of isoproterenol less than 10^{-5} M (Fig. 5B), hormone stimulation was completely GTP dependent. Because of this total lack of stimulation in the absence of GTP, it was not possible to determine whether or not GTP was affecting the K_a of the enzyme for the hormone, as has been reported by some investigators working with other tissues (18).

TABLE 1

Effect of various divalent cations on choroid plexus adenylate cyclase activity

Values shown are the means ± ranges for replicate samples, each assayed for cyclic AMP content in duplicate. In the presence of manganese, cobalt, or copper, magnesium was omitted from the standard incubation mixture.

Metal salt (8 mM)	Enzyme activity (pmol/mg protein/min)	
	Basal	+Isoproterenol (10^{-4} M)
$MgCl_2$	7.6 ± 0.1	44.1 ± 0.2
$MnCl_2$	14.3 ± 1.2	44.8 ± 3.3
$CoCl_2$	8.0 ± 0.1	28.0 ± 2.9
$CuCl_2$	0.0 ± 0.5	0.0 ± 0.1

Pharmacological Properties of Enzyme Stimulation

Agonists. Among the various catecholamines tested (Fig. 6A), isoproterenol (ISO) was the most potent, with a K_a of 1.4×10^{-7} M. (However, see also the effects of zinterol and OPC 2009, under selective agents.) A Hofstee plot of isoproterenol stimulation (not shown) was linear, suggesting activation of a single class of receptors (see also the following). Epinephrine (EPI) and norepinephrine (NE) also both fully activated enzyme activity, but epinephrine ($K_a = 1.6 \times 10^{-6}$ M) was about 15 times more potent than norepinephrine. Norepinephrine ($K_a = 2.5 \times 10^{-5}$ M), in turn, was about 10 times more potent than dopamine (DA) ($K_a > 2 \times 10^{-4}$ M). The α-adrenergic agonist, phenylephrine (PE), caused a small but reproducible stimulation of enzyme activity (30% that of isoproterenol) with a K_a of greater than 2×10^{-5} M. This order of amine potency (isoproterenol > epinephrine > norepinephrine > phenylephrine) is consistent with the stimulation of a β-adrenergic receptor with sensitivity more like that predominating in vascular and bronchial smooth muscle (β_2) than in heart, fat, and small intestine (β_1) (19).

To aid in determining whether the preceding agonists were activating the same or distinct receptors, the additive effects of these amines on adenylate cyclase activity were investigated. Table 2A shows that there was no additivity between combinations of isoproterenol with epinephrine, norepinephrine, dopamine, or phenylephrine. These data, combined with those from antagonist studies (following), suggest (though do not prove) that the observed stimulation by these five amines is the result of the activation of the same class of receptors.

Antagonists. The stimulation of choroid plexus adenylate cyclase activity by isoproterenol was inhibited by low concentrations of the β-adrenergic antagonist, propranolol (PROP) (calculated $K_i = 2.7 \times 10^{-9}$ M). This was

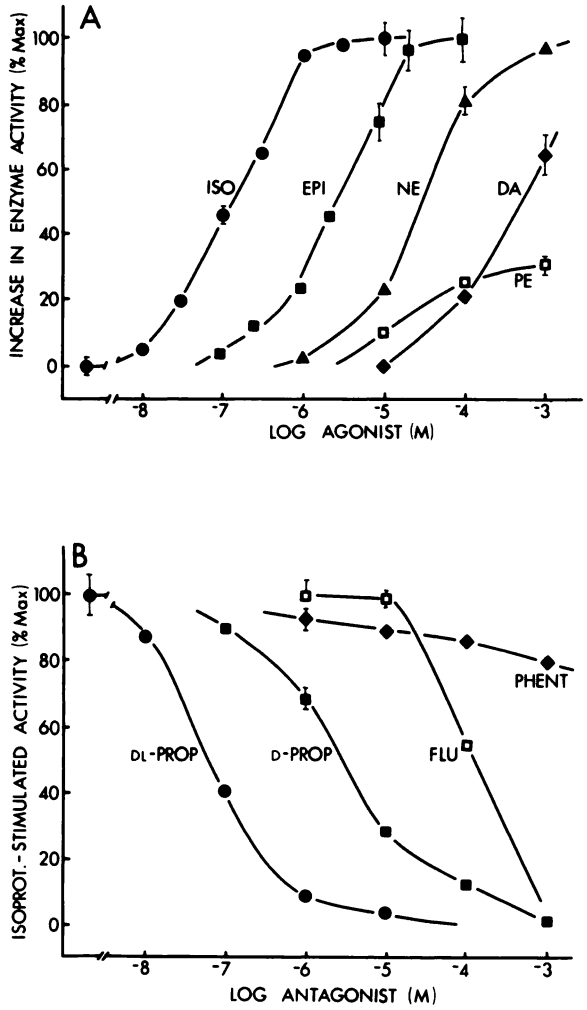

FIG. 6. *Effects of various agonists (A) and antagonists (B) on choroid plexus adenylate cyclase activity*

This figure presents data obtained from a number of different experiments. In A, activity for each agonist is expressed as a percentage of the maximal isoproterenol stimulation observed in the particular experiment in which that agonist was tested. In B, stimulation (above that seen in the presence of antagonist alone) is expressed as a percentage of the stimulation seen in the presence of 3×10^{-6} M isoproterenol alone. In both A and B, the values obtained were consistent from experiment to experiment. See text for abbreviations.

true in experiments in which the agonist was held constant and the antagonist concentration was varied (Fig. 6B) as well as in experiments in which the antagonist was held constant and the isoproterenol concentration varied (Fig. 1). Experiments of the latter type were consistent with competitive inhibition. Antagonism by propranolol was stereoselective, the (+) isomer being approximately 50 times less potent than the racemic mixture (Fig. 6B, Table 3).

The dopamine antagonist, fluphenazine (FLU) (Fig. 6B), was a weak inhibitor of isoproterenol activation ($K_i = 5.4 \times 10^{-6}$ M) compared to its known potency at dopamine receptors (typically, $K_i = 8 \times 10^{-9}$ M) (20). As might have been expected from the weak agonist activity of phenylephrine, the α-adrenergic antagonist, phentolamine (PHENT), caused only a small inhibition of isoproterenol stimulation, even at high concentrations.

TABLE 2

Effects, on choroid plexus adenylate cyclase activity, of various amines present alone or in combination

Shown is the stimulated increase over the activity present in the absence of added amine. In part A this basal activity was 7.59 ± 0.1 pmol/mg protein/min, and in part B (a separate experiment), 11.7 ± 0.3 pmol/mg protein/min. Values represent the means ± ranges for replicate samples, each assayed for cyclic AMP content in duplicate. With the exception of dopamine and phenylephrine, the concentration of amine used was that which gave maximal or near-maximal stimulation when tested alone.

Agent	Cyclic AMP increase
	pmol/mg protein/min
A	
Isoproterenol (10 μM)	36.5 ± 0.2
Epinephrine (100 μM)	35.7 ± 0.5
Norepinephrine (100 μM)	27.2 ± 1.3
Phenylephrine (100 μM)	9.1 ± 2.2
Dopamine (100 μM)	3.6 ± 0.1
Isoproterenol + epinephrine	34.6 ± 2.0
Isoproterenol + norepinephrine	30.9 ± 1.5
Isoproterenol + phenylephrine	30.4 ± 2.5
Isoproterenol + dopamine	35.9 ± 0.2
B	
Isoproterenol (10 μM)	23.1 ± 1.8
Zinterol (10 μM)	12.6 ± 0.8
Salbutamol (100 μM)	7.3 ± 0.3
Orciprenaline (100 μM)	6.0 ± 0.3
Isoproterenol + zinterol	12.3 ± 2.0
Isoproterenol + salbutamol	12.3 ± 2.0
Isoproterenol + orciprenaline	14.5 ± 2.8
Zinterol + salbutamol	7.3 ± 1.8
Zinterol + orciprenaline	11.6 ± 0.3
Salbutamol + orciprenaline	7.8 ± 1.8

Selective β_1- and β_2-adrenergic agents. To investigate the characteristics of the choroid β-adrenergic receptor in more detail, a number of β-agonists and antagonists, with relative selectivity for either β_1- or β_2-adrenergic receptors in other tissues, were tested for their ability to stimulate choroid enzyme activity (Fig. 7A) or to block activation by isoproterenol (Fig. 7B).

Among the agonists, zinterol (ZIN) ($K_a = 2.3 \times 10^{-8}$ M) and OPC 2009 ($K_a = 1.3 \times 10^{-7}$ M), known to have relatively high β_2 selectivity (21, 22), were the most potent compounds tested. On the other hand, the β_1-

TABLE 3

Calculated inhibitory constants for antagonists of isoproterenol-stimulated adenylate cyclase activity in cat choroid plexus

Antagonist	Calculated K_i
	M
IPS 339	1.9×10^{-9}
(±)-Propranolol	2.7×10^{-9}
H35/25	1.4×10^{-7}
(+)-Propranolol	1.4×10^{-7}
Metoprolol	4.9×10^{-7}
Butoxamine	1.5×10^{-6}
p-Oxyprenolol	2.3×10^{-6}
Fluphenazine	5.4×10^{-6}
Atenolol	5.8×10^{-6}
Practolol	8.9×10^{-6}
Phentolamine	$>5 \times 10^{-5}$

selective agent, prenalterol (PRE) (23), was almost totally ineffective in activating the enzyme. Salbutamol (SAL) ($K_a = 9.5 \times 10^{-7}$ M), terbutaline (TER) ($K_a = 3.2 \times 10^{-6}$ M), and orciprenaline (ORCI) ($K_a = 1.2 \times 10^{-5}$ M), somewhat less selective β_2 agonists (24–26), were also less effective (and less potent) than zinterol and OPC 2009 in stimulating enzyme activity.

Although some of the selective agents were as potent as isoproterenol, none resulted in as great a maximal stimulation. One explanation for this might be that full activation as seen with isoproterenol results from the stimulation of a mixed population of β_1 and β_2 receptors (on either the same or different cell types). Alternatively, there might be one population of β receptors which the selective agents were not fully effective in activating. The latter possibility seems more likely (however, see Discussion) in view of antagonist studies (following) and the fact that additivity experiments (Table 2B) with various combinations of the selective agents, zinterol, salbutamol, and orciprenaline, resulted in no greater stimulation than any agent alone. In fact, all three of these drugs decreased isoproterenol-stimulated enzyme activity and, thus, acted as partial agonists of the β-adrenergic receptor. Similar effects of zinterol and certain other β_2-selective agents have been reported for isoproterenol-stimulated adenylate cyclase and isoproterenol-stimulated physiological responses in certain other tissues (17, 24, 25).

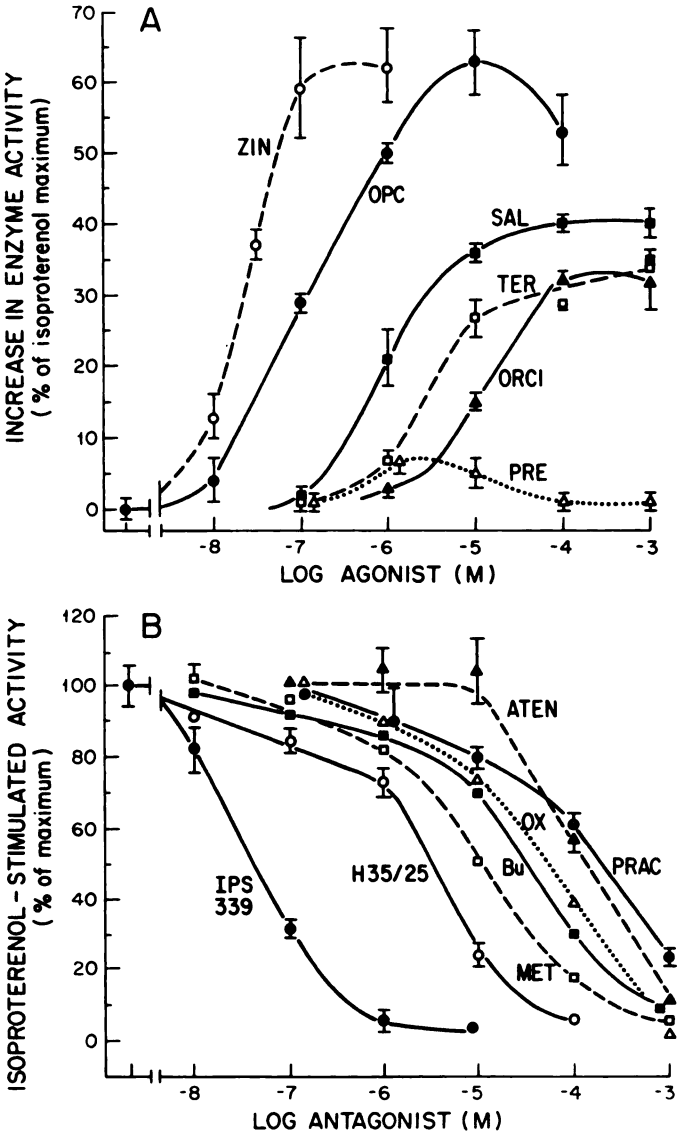

FIG. 7. *Effects of various selective β_1- and β_2-adrenergic agonists (A) and antagonists (B) on choroid plexus adenylate cyclase activity*

Data were obtained from a number of different experiments. In A, activity for each agonist is expressed as a percentage of the maximal isoproterenol stimulation observed in the particular experiment in which that agonist was tested. In B, stimulation (above that seen in the presence of antagonist alone) is expressed as a percentage of the stimulation seen in the presence of 3×10^{-6} M isoproterenol alone. In both A and B, the values obtained were consistent between experiments. See text for abbreviations.

Among the β-adrenergic antagonists (Fig. 7B), the relatively selective β_2 blockers, IPS 339 and H35/25 (27, 28), were the most potent, while the relatively selective β_1 blockers, atenolol (ATEN), practolol (PRACT), and *p*-oxprenolol (OX) (24, 29), were the least potent. Metoprolol (MET) (relatively β_1 selective) and butoxamine (BU) (relatively β_2 selective) (24, 29) were of intermediate potency. Both β_1- and β_2-selective blockers were able to inhibit isoproterenol stimulation at least 95%. Table 3 summarizes the K_i's of all antagonists tested.

Localization of Choroid Plexus β-Adrenergic-Sensitive Adenylate Cyclase

Anatomical distribution. In the cat, the majority of choroid plexus tissue is found attached to the walls of the lateral ventricles and, separately, to the posterior roof of the fourth ventricle. (There is also a small amount in the third ventricle.) Although both lateral and fourth ventricular choroid plexuses receive noradrenergic innervation (1), the gross histology in these two areas differs: The fourth contains closely packed villous folds with a high proportion of secretory epithelium, while the lateral ventricular choroid is more membranous with a smaller proportion of epithelium. In homogenates of these two areas, fourth ventricular choroid plexus consistently showed greater isoproterenol stimulation of adenylate cyclase activity than the lateral choroid (Table 4). This observation provided some indirect evidence that β-adrenergic-sensitive adenylate cyclase activity might be preferentially concentrated in the secretory epithelium (see the following).

Because the choroid plexus is derived, embryologically, as an outpouching from the surface of the cerebral ventricles, it was of interest to see if nonchoroidal ventricular

TABLE 4

Enrichment of isoproterenol-stimulated adenylate cyclase activity in cat fourth ventricular choroid plexus

Choroid plexus tissue	Adenylate cyclase activity (pmol/mg protein/min)	
	Basal	+ Isoproterenol (10^{-4} M)
Lateral ventricle	8.4 ± 1.0[a]	36 ± 1.2
Fourth ventricle	9.0 ± 1.0	77 ± 3

[a] Values shown represent the means ± ranges from replicate samples, each assayed for cyclic AMP content in duplicate.

lining cells (i.e., ependyma) also contain β-adrenergic-sensitive adenylate cyclase activity. In contrast to the stimulation seen in lateral ventricle choroid tissue, we found that a homogenate made from lateral ventricle ependyma showed no isoproterenol stimulation. (Basal activity = 15 ± 1 pmol/mg protein/min; 100 μM isoproterenol-stimulated activity = 16 ± 1 pmol/mg protein/min.)

Cellular localization within the choroid plexus. As noted, the choroid plexus is a highly vascularized tissue consisting of a capillary and arteriolar network (composed of endothelial cells, smooth muscle cells, and—in unperfused tissue—blood elements) surrounded by a single layer of cuboidal epithelial tissue which appears (histologically) like that found in renal proximal tubule. Noradrenergic projections to the choroid plexus lie between the epithelium and the vasculature and, from their position, could possibly innervate either (or both) tissue(s) (2).

Localization of β-adrenergic-sensitive adenylate cyclase to cell type within the choroid might give some idea which tissue(s) receives functional sympathetic innervation. For example, a preferential localization in vascular tissue would suggest noradrenergic regulation of blood flow or vascular permeability, whereas a localization in the epithelium might suggest a regulation of CSF secretion. To localize enzyme activity, a dispersed cell fraction containing epithelial cells was separated from the underlying capillary network using controlled enzymatic digestion with low-dose trypsin (see Materials and Methods). Combined with differential centrifugation and sieve filtration, this procedure resulted in a relatively uniform preparation of cells (90–95% trypan blue excluding) which had a light microscopic appearance identical to that of intact secretory epithelium. The enzymatic activity of this epithelial cell fraction was then compared with that of the remaining de-epithelialized vascular network. (Because animals which were perfused to remove intravascular blood showed no diminution of choroid β-adrenergic-sensitive enzyme activity, it appeared unlikely that the observed hormone stimulation was localized in blood elements. In addition, feline blood, itself, showed very little isoproterenol-stimulated enzyme activity.)

Figure 8A shows the appearance of the choroid plexus prior to treatment, and Fig. 8B shows the appearance after enzymatic digestion with trypsin. Most, but not all, of the darker-staining secretory epithelium has been removed. Figure 8C shows the appearance of the epithelial cell fraction. Isoproterenol-stimulated enzyme activity in homogenates made from tissue shown in Figs. 8B and C are compared in Fig. 8D. Both basal and isoproterenol-stimulated activities were greater in the epithelial

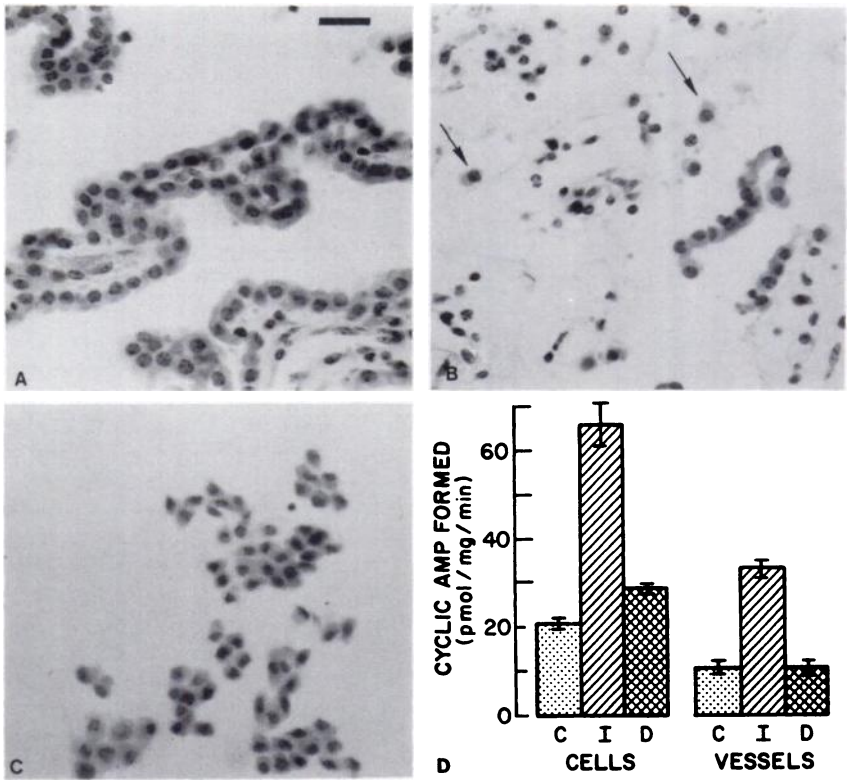

FIG. 8. *Isoproterenol-stimulated adenylate cyclase activity in epithelial cell and vascular fractions derived from cat choroid plexus*

A shows the appearance of the intact choroid tissue with darker-staining cuboidal epithelium surrounding capillaries and arterioles. B demonstrates the appearance of the choroid plexus after incubation with trypsin, as described in Materials and Methods. The tissue is predominantly vascular, consisting of smooth muscle and endothelial cells with dark nuclei and lightly stained cytoplasm. A few darker-staining epithelial cells are still attached (arrows), and a detached string of epithelial cells is seen at right center. C shows the purified epithelial cell fraction consisting almost entirely of secretory cells. D compares the basal and hormone-stimulated adenylate cyclase activity present in homogenates made from the fraction shown in C ("cells") and that shown in B ("vessels"). C = control; I = 10^{-4} M isoproterenol; D = 10^{-4} M dopamine. The bar at the top of A = 50 μm.

cell fraction, as was stimulation due to dopamine. (As described previously, dopamine's stimulation appears due to activation of the β-adrenergic receptor.)

Because proteolytic enzymes have the potential for altering membrane-bound receptors, several other cell separation procedures were utilized which did not involve trypsin. For these experiments, both feline and bovine choroids were used. Treatment with a combination of hyaluronidase and collagenase, which resulted in cell separations histologically comparable to those using trypsin, similarly showed an enrichment of isoproterenol-stimulated enzyme activity in the epithelial cell fraction (Table 5). In another experiment (Table 5), nonenzymatic cell separation of epithelium from underlying vasculature, using a calcium-free EDTA Ringers solution (see Materials and Methods), also showed substantial enrichment in the secretory cell fraction. Finally, in a fourth procedure (Table 5), choroid was separated into a broken cell epithelial fraction and an intact capillary fraction by mechanical fragmentation and sucrose gradient centrifugation, using a method similar to that employed for isolating cerebral microvessels from brain (see Materials and Methods). Here, too, there was selective enrichment of hormone sensitivity in the epithelial cell fraction. Taken together, these various enzymatic and nonenzymatic separation techniques strongly suggest a preferential enrichment of isoproterenol-sensitive adenylate cyclase activity in the choroid secretory epithelium.

It should be noted that three of the four separation procedures showed some hormone sensitivity in the choroid "vascular fraction." Whether this activity was due to undissociated secretory cells, vascular elements, or some other cell type (such as presynaptic nerve terminals) is not clear. In other experiments, we (6, 30) and others (31, 32) have found that superficial pial arteries as well as capillaries isolated from brain parenchyma do contain isoproterenol-stimulated adenylate cyclase activity. Although specific for β-adrenergic agonists, the absolute activity of this capillary enzyme is substantially less than that present in the choroid plexus (6, 30–32). Figure 9 compares the stimulation of adenylate cyclase activity by 10^{-4} M isoproterenol in cat choroid plexus versus homogenates of whole brain, pial arteries, or intraparenchymal microvessels.

TABLE 5

Enrichment, in choroid plexus epithelial cell fractions, of isoproterenol-stimulated adenylate cyclase activity, as shown by various separation procedures

Shown is the increase over basal activity seen in the presence of 10^{-4} M isoproterenol. Basal activities for cell and vessel fractions (respectively) were: for hyaluronidase/collagenase, 7.8 ± 0.2 and 5.9 ± 0.1 (data from calf choroid); for Ca^{2+}-free EDTA, 8.0 ± 0.9 and 2.8 ± 0.2 (data from calf; similar results also obtained with rabbit choroid); and for mechanical dissociation, 7.9 ± 0.4 and 1.4 ± 0.4 (data from cat). All values represent the mean ± range for replicate samples, each assayed for cyclic AMP content in duplicate.

Treatment	Isoproterenol-stimulated adenylate cyclase activity	
	Cell fraction	Vessel fraction
Hyaluronidase/collagenase	11.9 ± 0.7	3.6 ± 0.5
Ca^{2+}-free EDTA	15.1 ± 1.2	2.8 ± 0.3
Mechanical dissociation	12.3 ± 0.8	0.1 ± 0.2

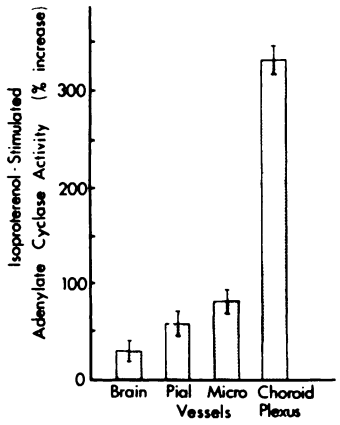

FIG. 9. *Comparative stimulation by isoproterenol of adenylate cyclase activity in broken cell preparations of cat brain, cerebral blood vessels, and choroid plexus*

Pial vessels consisted of larger extraparenchymal superficial arteries; microvessels were small intraparenchymal vessels isolated as described in Materials and Methods. Cat brain consisted of both cortical and subcortical areas, as well as brain stem. Shown is the percentage increase over basal activity due to 10^{-4} M isoproterenol. Basal activities were 37 ± 1 pmol/mg protein/min for brain, 12.3 ± 0.9 pmol/mg protein/min for pial vessels, and 13.8 ± 0.6 pmol/mg protein/min for choroid plexus.

DISCUSSION

In summary, these studies demonstrate the presence of a specific β-adrenergic-sensitive adenylate cyclase in the mammalian choroid plexus. This enzyme is GTP-, but not calcium-, dependent and exhibits pH, temperature, substrate, and metal cofactor requirements as described. Pharmacological characterization reveals a pattern of agonist and antagonist sensitivity similar to that seen in the presence of a β_2-adrenergic receptor. To quantitate this similarity further, Table 6 compares the activation constants of five agonists in stimulating choroid adenylate cyclase activity with the published displacement constants (K_d) for the same agonists in inhibiting the binding of iodohydroxybenzylpindolol (IHYP) to β_2 and β_1 receptors in membrane fractions from lung and heart (33). (These and other recent studies have shown that the majority of β receptors in lung are of a β_2 type, while the majority in mammalian heart are β_1.) The calculated r coefficient (0.99) indicates an excellent correlation ($P < 0.01$) between the agonist characteristics of the choroid enzyme and lung β_2 receptor, as compared with the poor correlation ($r = -0.02$) between choroid and the heart β_1 receptor. Table 6 also compares the calculated inhibitory constants (K_i) for six antagonists in blocking isoproterenol-sensitive adenylate cyclase in choroid plexus with the K_d for the same antagonists in inhibiting IHYP binding in lung or heart. As with the agonists, there is a good correlation ($r = 0.91$, $P < 0.02$) between choroid and lung, as compared with the correlation ($r = -0.07$) between choroid and heart. Table 6 shows additional reported data (33) for β-adrenergic activation and inhibition of lung and heart adenylate cyclase activity. As with the binding data, there is much

TABLE 6

Comparison of β-adrenergic-sensitive adenylate cyclase in choroid plexus with β-adrenergic binding and adenylate cyclase in lung and heart

Agent	Adenylate cyclase K_a or K_i (μM), choroid plexus	K_d for IHYP binding (μM)[a]		Adenylate cyclase K_a or K_i (μM)[a]	
		Lung	Heart	Lung	Heart
	A	B	C	D	E
		(a) Agonists			
Norepinephrine	25.0	9.18	0.82	7.75	1.1
Epinephrine	1.55	1.46	0.90	0.60	1.5
Isoproterenol	0.14	0.10	0.05	0.18	0.12
Salbutamol	0.95	1.55	1.51	1.75	NE[b]
Zinterol	0.023	0.02	1.03	0.065	NE
		(b) Antagonists			
Practolol	8.9	26.0	1.10	9.5	0.75
Atenolol	5.8	6.76	1.59	1.75	0.75
Butoxamine	1.52	3.55	7.03	1.35	6.6
Metoprolol	0.49	3.63	0.22	1.1	0.012
H35/25	0.14	0.84	1.97	0.85	1.35
Propranolol	0.0027	0.0006	0.0017	0.0014	0.0056

[a] Heart and lung data from Minneman *et al.* (24, 33). Comparisons, between columns, of drug potencies yielded the following Pearson correlation coefficients. For agonists: A vs B ($r = 0.99$, $P < 0.01$); A vs C ($r = -0.02$, NS); A vs D ($r = 0.98$, $P < 0.01$); A vs E ($r = 0.28$, NS). For antagonists: A vs B ($r = 0.91$, $P < 0.02$); A vs C ($r = -0.07$, NS); A vs D ($r = 0.87$, $P < 0.05$); A vs E ($r = -0.12$, NS).

[b] No effect as agonist.

better correlation between the choroid and lung enzymes ($r = 0.98$, $P < 0.01$ for agonists; $r = 0.87$, $P < 0.05$ for antagonists) than between the choroid and heart enzymes ($r = 0.28$ for agonists; $r = -0.12$ for antagonists). It should be noted that certain of the drugs used (e.g., salbutamol and butoxamine) demonstrate less β_1 and β_2 selectivity in the binding assays than they do in the activation or inhibition of adenylate cyclase activity. At least in some cases (e.g., with salbutamol) this may be due to dual agonist and antagonist actions of these agents, as we observed in the present study (Table 2) and has been recently reported by others (33).

The above comparisons suggest that the majority of adenylate cyclase-associated adrenergic receptors in feline choroid plexus are of a β_2 type. Because a number of tissues appear to have a mixture of β_1 and β_2 subtypes (24, 33), a small percentage of choroid β_1-adrenergic receptors cannot be ruled out. Furthermore, it should be borne in mind that the results obtained here were with broken cell preparations and that *in vivo* drug selectivity may be influenced by other factors, such as neuronal and extraneuronal uptake mechanisms, susceptibility to degradative enzymes, and membrane solubility. (This last factor may not be quite so important for *in vivo* studies of choroid plexus as it is for brain, since the choroid capillaries are fenestrated and allow free access of drugs from the systemic circulation.)

The present data show an enrichment of β-adrenergic-sensitive adenylate cyclase activity in the epithelial fraction prepared from choroid plexus. Although this fraction appears by light microscopy to be entirely free from vascular and other cell types, we cannot entirely rule out the presence of endothelial cells or of small attached cell fragments (such as nerve terminals). The choroid "vascular fraction," on the other hand, clearly contains a mixture of cell types—endothelium, smooth muscle cells, residual undissociated epithelium, some blood elements, and most likely, adrenergic and other types of nerves. Thus, it is difficult to say with certainty which cell(s) contains the small amount of β-adrenergic-sensitive adenylate cyclase activity observed in this fraction. Furthermore, whether this activity is physiologically significant, compared to the considerable enrichment in choroid secretory cells, is not known.

ACKNOWLEDGMENT

I thank E. J. Hunnicutt, Jr., for expert technical assistance.

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