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Adenylate Cyclase in Cerebral Microvessels: Action of Guanine Nucleotides, Adenosine, and other Agonists

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SUMMARY

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The isolation of microvessels (capillaries and arterioles) from guinea pig cerebral cortex is described. Adenylate cyclase in microvessel preparations was activated by guanylylimidodiphosphate in a time-dependent manner. The rate of guanyl nucleotide activation was enhanced by adenosine, 2-chloroadenosine, 2-azidoadenosine, norepinephrine, histamine, isoproterenol, prostaglandin E_1, prostaglandin E_2 and prostacyclin. Activation by adenosine analogues was demonstrable only when endogenously-generated adenosine was destroyed with adenosine deaminase. Activation was also in evidence when microvessels were incubated with agonists together with guanine nucleotide, then washed by sedimentation prior to assay, reflecting persistence of the activated state.

INTRODUCTION

Multiple factors are involved in the regulation of blood flow through vascular beds. One important control is likely exerted by neurohormones. Other endogenous substances such as adenosine, adenine nucleotides and prostaglandins may also play a role. Pharmacologically, adenosine is known to be a smooth muscle relaxant and vasodilator. It is highly likely that the nucleoside may function in autoregulation of coronary blood flow (1). Adenosine has been shown to stimulate cAMP[1] formation in a number of tissues (2–5) and its vasoaction is potentiated by agents that inhibit cyclic nucleotide phosphodiesterase (6, 7). These facts, together with the possibility that catecholamine-induced smooth muscle relaxation is mediated by cAMP (see ref. 8), have stimulated interest in the possible involvement of the cyclic nucleotide in adenosine action. While Kukovetz *et al.* (9) have reported a positive correlation between adenosine-induced relaxation of coronary strips and activation of adenylate cyclase, other workers (10–12), using vascular tissues or nonvascular smooth muscle, were unable to demonstrate activation of adenylate cyclase by the nucleoside.

Prostaglandins have important actions on vascular smooth muscle. PGE_1 and PGE_2 are known to activate adenylate cyclase in a variety of cells and tissues. It has been suggested (13) that some of these effects could be mediated by cAMP.

In most instances in which adenylate cyclase has been examined in vascular smooth muscle, large vessels readily accessible by dissection have been used. Tissue from the

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[1] The abbreviations used are: BSA, bovine serum albumin; Gpp(NH)p, guanylylimidodiphosphate; PGE_1, prostaglandin E_1; PGE_2, prostaglandin E_2, PGI_2, prostacyclin, cAMP, adenosine 3′,5′ monophosphate.

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microvasculature has not been extensively examined. Several methods have been developed (14–17) for the isolation of microvascular tissue from brain. In this report we describe the isolation of microvessels (capillaries and arterioles) in highly purified form from guinea pig cerebral cortex. The action of adenosine, several adenosine analogues, histamine, catecholamines and prostaglandins on adenylate cyclase in this preparation is described.

MATERIALS AND METHODS

[^{3}H](G)adenosine 3',5'-cyclic phosphate (40 Ci/mmol) and [α^{32}P]ATP (15–20 Ci/mmol) were purchased from New England Nuclear. ATP, GTP, cAMP, adenosine, 2-chloroadenosine, (−)-isoproterenol, adenosine deaminase (Type I from calf intestinal mucosa), creatine phosphokinase (Type I from rabbit muscle), BSA (FractionV) and dithiothreitol were purchased from Sigma Chemical Co. Gpp(NH)p was purchased from ICN Chemicals. 3-Isobutyl-1-methylxanthine was obtained from Aldrich Chemical Co.; histamine dihydrochloride from Fisher Scientific; (−)-norepinephrine from Serva Chemical Co.; and PGE_1 and PGE_2 from PL Biochemicals. 2-Azido-adenosine was a gift from Dr. R. Haslam, McMaster University; prostacyclin (PGI_2) was obtained through the courtesy of Dr.K. Lederis, Division of Pharmacology, University of Calgary.

Preparation of microvessels. The method used was a modification of that described by Goldstein *et al.* (16). Brains from three female Hartley guinea pigs (300–450 g) were removed following exsanguination and placed on ice. All subsequent procedures were carried out at 4°. The cerebral cortex was thoroughly freed of pial membranes, surface vessels, and white matter and homogenized in a glass homogenizer with a motor-driven teflon pestle (10 strokes) in 5 vol (based on tissue wt) of Krebs-Ringer bicarbonate containing 10 mM glucose and 1% BSA, gassed with 95% O_2–5% CO_2. All subsequent homogenizations were done with a hand-driven pestle. The homogenate was centrifuged at 800 × *g* for 10 min. The loose pellet consisted of two layers, an upper one of finely divided material and a lower granular one of incompletely disrupted neuronal material. After removal of the supernatant fluid, the upper layer was dispersed in a small volume of the above medium and removed with a Pasteur pipet. The lower layer was homogenized in 5 vol of fresh medium (6 strokes by hand) and centrifuged as above. Again the upper layer of finely divided particles was removed and added to the first. The remaining granular bottom layer was dispersed in 3 volumes of medium by homogenization (6 strokes). This was added to the combined particulate suspension from the first two centrifugations, the mixture was further disrupted by homogenization (6 strokes) and centrifuged at 1000 × *g* for 10 min. The resulting pellet was dispersed in 10 volumes (based on initial tissue wt) of the above medium but containing 25% BSA and adjusted to pH 7.5. The homogenate was centrifuged at 3000 × *g* for 15 min. This procedure separated neuronal elements and myelin (which floated as a thick layer on top of a clear supernatant fluid) from a small pellet containing microvessels, nuclei and erythrocytes. The thick floating layer was removed, resuspended in the supernatant fluid and centrifuged again. The resulting small pellet was recovered and combined with the first. This combined residue was suspended in 10 ml of fresh 25% BSA medium and centrifuged at 3000 × *g* for 15 min. The thin film of floating material and the clear supernatant fluid were discarded and the pellet was dispersed by homogenization in 1 volume (based on original tissue wt) of 1% BSA medium. This suspension was passed through a column (1 cm × 1.5 cm diam) of glass beads, 0.45 to 0.50 mm diameter (B. Braun Melsungen). The column was washed with 1% BSA medium until the effluent (as observed microscopically) was free of nuclei and erythrocytes (usually 50–80 ml). Washing of the column was facilitated by carefully stirring the surface of the packed beads with a fine plastic rod. The column was then washed with about 20 ml of 0.25 M sucrose, 20 mM Tris-HCl, 2 mM dithiothreitol, pH 7.5 (hereafter referred to as Buffer A). Removal of nuclei and erythrocytes was monitored by microscopic examination of the effluent. Finally

the glass beads were extruded into a 25 ml beaker and entrapped microvessels were released by stirring the beads repeatedly with small volumes of Buffer A, the suspended microvessels being removed with a Pasteur pipet. Microvessels were then recovered by centrifugation at 3000 × *g* for 10 min, the pellet was suspended by homogenization in 0.35 vol (based on original tissue wt) of Buffer A. The protein content of such preparations was usually about 2 mg/ml (determined by the method of Lowry *et al.* (18)) and represented 500 μg per g of tissue. Preparations were used immediately and a fresh preparation was used for each experiment.

Adenylate cyclase assay. The assay system contained 40 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 5.5 mM KCl, 1 mM 3-isobutyl-1-methylxanthine, 10 mM creatine phosphate, 60 μg creatine phosphokinase, 0.4 mM dithiothreitol, 50 mM sucrose, 50 to 80 μg of microvessel protein and 0.2 mM [^{32}P]ATP (40 dpm/pmol) in a final vol of 150 μl. In some assays 1.2 U of adenosine deaminase was also added. The reaction was initiated by addition of [^{32}P]ATP and the incubation was conducted at 30° for 8 min in 1.5 ml Eppendorf polypropylene tubes. The reaction was terminated by placing the tubes in a boiling water bath for 4 min. Upon cooling ATP (4 μmol) and cAMP (0.14 μmol) were added together with 50 μl of [^{3}H]cAMP solution containing 3 × 10^4 dpm to monitor recovery; final vol 1.1 ml. The tubes were centrifuged at 5000 × *g* for 5 min (Fisher Model 59 centrifuge) and the [^{32}P]cAMP formed was isolated and quantitated by the method of Salomon *et al.* (19). In experiments in which the microvessels were incubated with ligands prior to assay, the procedure was as follows: microvessels (70 to 90 μg protein) were incubated at 30° for various intervals in the adenylate cyclase assay medium (without ATP) with test substances; final vol 150 μl. The reaction was initiated by addition of test compound and terminated by dilution with 1 ml of cold Buffer A followed by immediate centrifugation at 5,000 × *g* for 3 min. The supernatant fluid was removed with a Pasteur pipet, the pellet was dispersed in 1 ml of Buffer A and centrifuged again. Each pellet was suspended (Vortex) in 30 μl of buffer, assay components were added and the assay was conducted as above for 5 min at 30°.

5′-Nucleotidase was determined by the method of Baer *et al.* (20); 2′,3′-cyclic nucleotide phosphohydrolase by the method of Olafson *et al.* (21). Dye uptake into cells was determined using 0.33% trypan blue in Krebs-Ringer bicarbonate (22). Staining was observed microscopically.

RESULTS

Purity and properties of the microvessel preparation. Microscopic examination of the microvessel preparation showed segments of capillaries and arterioles, the former being predominant. Micrographs of two separate preparations are shown in Figure 1. The preparation was free of neuronal elements and membranous debris; occasional nuclei and erythrocytes were entrapped among the vessels, and erythrocytes were also visible within the lumen of the vessel segments. Several preparations were assayed for 2′,3′-cyclic nucleotide phosphohydrolase activity (21). This enzyme is highly active in cerebral cortex, being located in myelin. Microvessel preparations were devoid of this activity providing evidence for the absence of myelinated neuronal fragments. The neuronal material that floated on 25% BSA medium hydrolyzed 30 nmol 2′,3′-cyclic AMP/min/mg at 30°.

The microvessel cells, surprisingly, resisted disruption by homogenization. In fact, suspensions, even after homogenization, consisted of intact segments of vessels with endothelial cells seemingly intact as observed by light microscopy. Even material recovered from the incubation tubes after assay was noticeably intact. However, when treated with trypan blue—a dye which has been used to determine cell viability (22)—at least 95% of the cells were stained as determined by microscopic examination. Uptake of the dye could indicate extensive damage to the surface membrane of the cells. Examination by electron microscopy[2] also revealed damage to the sur-

[2] We are indebted to Dr. W. C. Costerton and Mr. Dale Cooper, Department of Biology, University of Calgary, for the electron microscopic examinations.

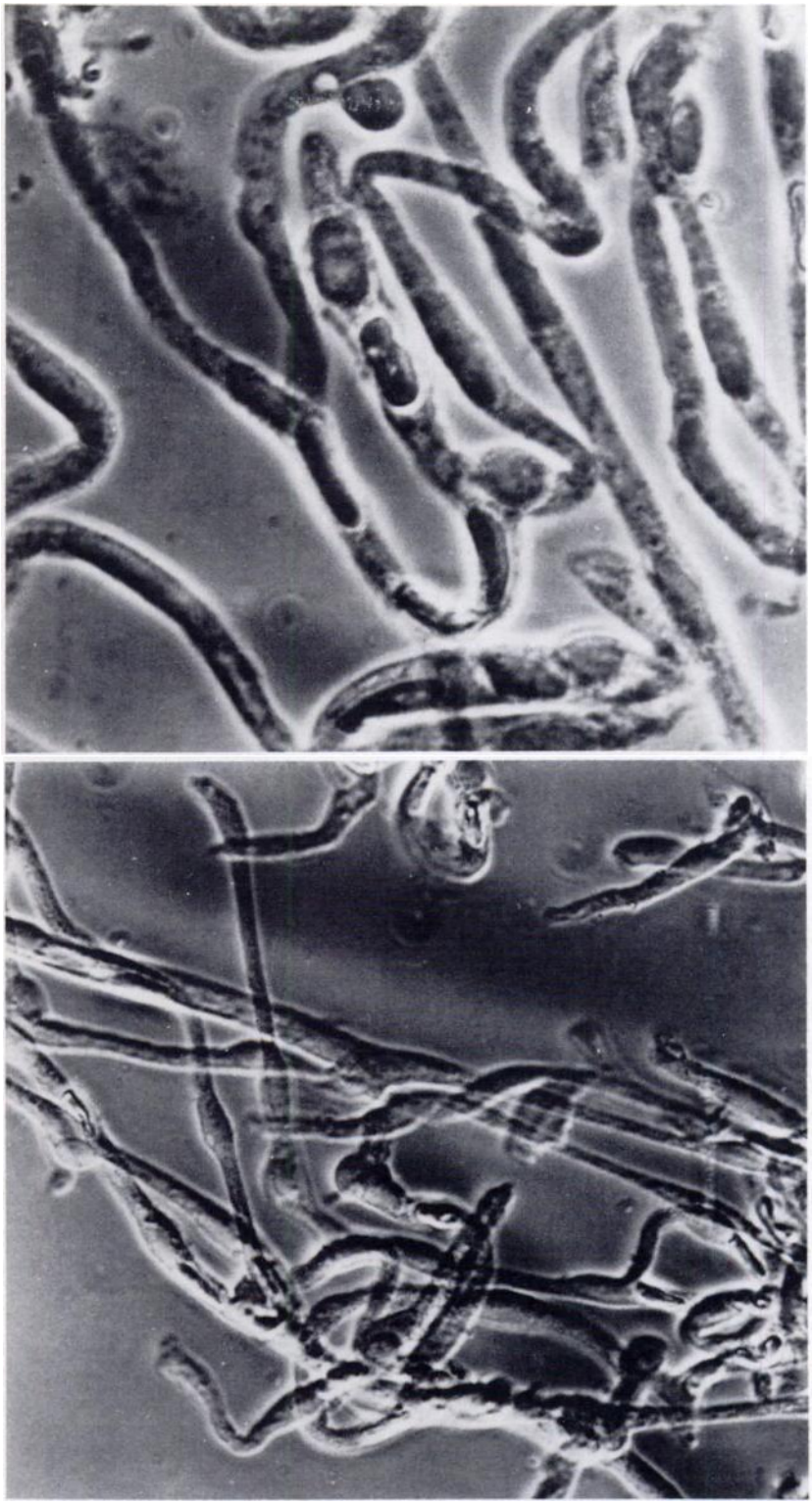

FIG. 1. *Phase contrast micrographs of two separate microvessel preparations showing segments of capillaries* Magnification: × 1140.

face membrane, although both the outer and lumen sides were still well defined. Substrate (MgATP) was obviously accessible to the catalytic site; these preparations, in fact, had slightly better adenylate cyclase activity than other preparations which were extensively homogenized in an all-glass homogenizer until cell disruption was complete.

Effect of adenosine and adenosine analogus on microvessel adenylate cyclase. Basal adenylate cyclase in the preparation was 17.8 ± 1.2 pmol/min/mg at 30°. Gpp(NH)p (30 μM) added directly to the assay increased enzyme activity 4-fold, the apparent K_a was 3 μM. In initial attempts to examine possible effects of adenosine on the enzyme, we failed to observe a significant effect of the nucleoside, or of 2-chloroadenosine or 2-azidoadenosine, either alone or in the presence of GTP. Similarly, these nucleosides had no significant effect on Gpp(NH)p activation (Fig. 2, stippled bars). When adenosine deaminase (1.2 U) was present during the assay, Gpp(NH)p activation was significantly reduced (Fig. 2, compare stippled control bar with hatched bar); basal activity was similarly reduced (data not shown). When 2-chloroadenosine or 2-azidoadenosine was added in the presence of adenosine deaminase, an increase in adenylate cyclase activity was seen (Fig. 2, compare hatched bars with stippled bars). Adenosine, as expected, had no stimulatory action under these conditions. The data indicate that adenosine was generated in sufficient amounts in the assay to activate the enzyme maximally so that exogenous nucleoside (or its analogues) was without significant effect. In the presence of adenosine deaminase, the ability of the 2-chloro- and 2-azido- analogues (which are resistant to deaminase action) to increase Gpp(NH)p-mediated activation was readily apparent. Thus, in the presence of 1 μM Gpp(NH)p and adenosine deaminase, a dose-dependent activation of adenylate cyclase by 2-chloroadenosine was seen (Fig. 3A). Maximal stimulation caused by the nucleoside was about twofold; half-maximal activation occurred at 30 μM. Because the activated state appeared to be stable (see following results) the dose-dependence of 2-chloroadenosine was examined in experiments in which microvessels were incubated with Gpp(NH)p and the nucleoside followed by sedimentation and washing prior to assay in the absence of ligands and in the presence of adenosine deaminase (Fig. 3B). Under these conditions maximal activation produced by the nucleoside was 2.5-fold; half-maximal stimulation occurred at 10 μM. Stimulation of the enzyme by adenosine could also be demonstrated by prior incubation; stimulation was dose-dependent (Fig. 3B), half-maximal activation occurred at approximately 12 μM.

The effect of nucleoside on the rate of Gpp(NH)p activation of the enzyme was also investigated. Microvessel preparations were incubated with Gpp(NH)p in the absence and presence of 2-chloroadenosine or adenosine for various time intervals followed by sedimentation and washing prior to assay in the presence of adenosine deaminase. In Figure 4 it is seen that Gpp(NH)p activation was time-dependent, and under the conditions used, had not reached a maximum up to 30 min. Both 2-chloroadenosine and adenosine increased

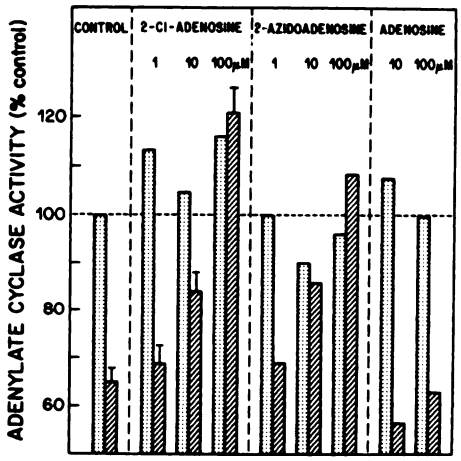

FIG. 2. *Effect of adenosine deaminase on activation of microvessel adenylate cyclase by Gpp(NH)p and adenosine analogues*

Microvessel preparations were assayed in the presence of 1 μM Gpp(NH)p and with the indicated concentrations of adenosine, 2-chloroadenosine and 2-azidoadenosine in the absence (stippled bars) and presence (hatched bars) of 1.2 U of adenosine deaminase. Results are expressed as percent of the Gpp(NH)p activated state in the absence of adenosine deaminase. Values are means from 2 to 3 separate experiments.

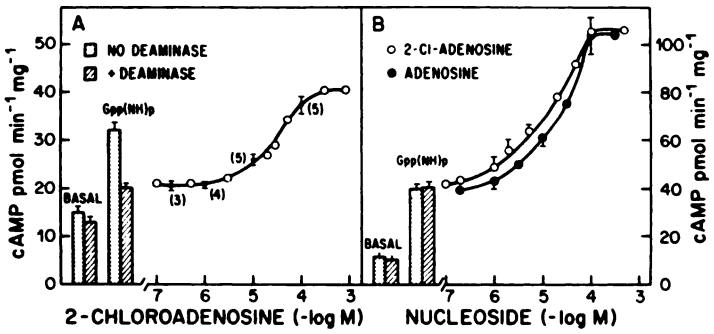

FIG. 3. *Activation of microvessel adenylate cyclase by 2-chloroadenosine*

A. Preparations were assayed in the presence of 1 μM Gpp(NH)p, adenosine deaminase (1.2 U) and varying concentrations of 2-chloroadenosine, which was added directly to the assay. Filled circles are means of the number of separate experiments shown in parentheses; unfilled circles are means of two preparations. B. Preparations were incubated for 10 min with 1 μM Gpp(NH)p and varying concentrations of 2-chloroadenosine (○) or adenosine (●) then sedimented and washed prior to assay as described in MATERIALS AND METHODS. Mean values from three experiments using different membranes are shown with standard errors; other values are the means of two separate experiments. In each panel the bars represent basal and Gpp(NH)p-stimulated activity in the absence (stippled bars) and presence (hatched bars) of adenosine deaminase.

the rate of activation. The maximal level of activation achieved in the presence of the nucleosides was unlikely greater than that achieved by Gpp(NH)p alone; only the rate of activation was affected. Thus theoretically, the concentration-dependent activation by the nucleosides (Fig. 3B) would not be observed after appropriately extended prior incubation. It is significant that when microvessels were incubated with Gpp(NH)p prior to assay, as in Fig. 3B and 4, adenosine was an effective stimulator, even though the final assay contained adenosine deaminase. Obviously, the prior incubation mixture was free of contaminating adenosine, and adenylate cyclase, once activated, remained in the activated state following removal of excess ligands by washing and assay in the presence of adenosine deaminase.

Activation of adenylate cyclase seemed to have an absolute requirement for Gpp(NH)p since 2-chloroadenosine and adenosine alone had no effect (Table 1). It was also observed that when vessels were incubated with Gpp(NH)p and washed prior to assay, addition of adenosine deaminase did not decrease the activity as it did when Gpp(NH)p was added directly to the assay (see Figs. 2 and 3A). This suggested that the Gpp(NH)p-activated enzyme, following washing, might not be susceptible to further activation by adenosine. Microvessel preparations were incubated with 1 μM Gpp(NH)p, then washed and assayed in the presence of 2-chloroadenosine. There was no further activation caused by the nucleoside (Table 1). In contrast, the nucleoside, when added with

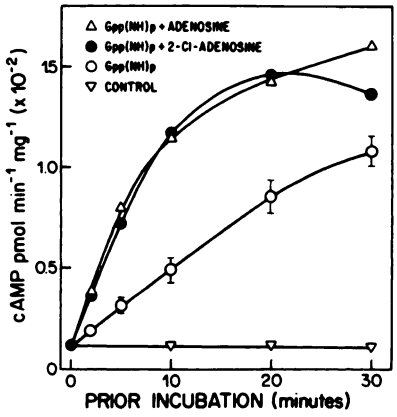

FIG. 4. *Time-dependence of Gpp(NH)p activation in the absence and presence of nucleosides*

Microvessel preparations were incubated for the indicated intervals alone (▽), with 1 μM Gpp(NH)p (○), or nucleotide plus 100 μM adenosine (△) or 2-chloroadenosine (●), then sedimented and washed before assay, as described in MATERIALS AND METHODS. Values with standard errors are means from three separate experiments with different preparations; other values are means of two separate experiments.

TABLE 1

Interaction between Gpp(NH)p and 2-chloroadenosine on adenylate cyclase activation

Microvessel preparations were assayed for adenylate cyclase activity with or without prior incubation with ligands, as described in MATERIALS AND METHODS. The concentrations of ligands present during the prior incubation and assay were: Gpp(NH)p 1 μM, 2-chloroadenosine 100 μM. Values are means from two experiments with different vessel preparations.

Time	Prior Incubation Additions	Assay Additions	cAMP Formed
(min)			*(pmol/min/mg)*
0	—	none	19.8
0	—	adenosine deaminase	17.8
0	—	2-Cl-adenosine	20.0
10	Gpp(NH)p	—	48.0
10	Gpp(NH)p	adenosine deaminase	48.0
10	Gpp(NH)p	2-Cl-adenosine	46.7
10	Gpp(NH)p plus 2-Cl-adenosine	—	124.3
10	Gpp(NH)p	Gpp(NH)p	53.3
10	Gpp(NH)p	Gpp(NH)p plus 2-Cl-adenosine	73.6
10	—	Gpp(NH)p	27.3
10	2-Cl-adenosine	Gpp(NH)p	25.7

Gpp(NH)p to the prior incubation mixture, significantly increased Gpp(NH)p activation. Although Gpp(NH)p-activated washed membranes were unresponsive to 2-chloroadenosine, adenylate cyclase could be further activated in such preparations when Gpp(NH)p was added to the assay, alone or in combination with 2-chloroadenosine. These results suggest that preparations partially activated by Gpp(NH)p were not refractory to further activation after washing, but subsequent activation by 2-chloroadenosine required the presence of the guanine nucleotide. Incubation of microvessel preparations with 2-chloroadenosine alone prior to assay had no effect on the enzyme; when such preparations were assayed in the presence of Gpp(NH)p, activation was equivalent to preparations assayed directly with the guanine nucleotide.

GTP, when added directly to the assay, stimulated adenylate cyclase. Under the usual assay conditions (0.2 mM ATP, 2.0 mM $MgCl_2$), the nucleotide increased activity in a dose-dependent manner causing a maximal stimulation of 1.5-fold at 100 μM (Fig. 5A). In the presence of GTP, 2-chloroadenosine produced a modest increase in activity. When 0.5 mM ATP and 10 mM $MgCl_2$ were present, the stimulatory effect of GTP was increased reaching a maximum of 2-fold (Fig. 5B). Under these conditions

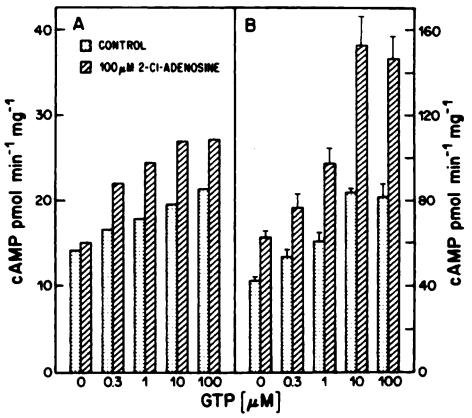

FIG. 5. *Stimulation of adenylate cyclase by GTP*

A. Standard assay conditions were employed (1.2 U adenosine deaminase) and GTP concentration was varied as indicated. Where present, 2-chloroadenosine was 100 μM. Values are means of two separate experiments. B. The assay contained 0.5 mM ATP and 10 mM $MgCl_2$; otherwise the conditions are the same as for A. Values are means ± S.E.M. for 3 to 5 separate experiments. Stippled bars, 2-chloroadenosine absent; hatched bars 100 μM 2-chloroadenosine present.

2-chloroadenosine produced significant stimulation in the absence of GTP, and in its presence (especially at 10 and 100 μM), the nucleoside increased activity approximately 2-fold over that produced by GTP alone.

Effect of histamine, norepinephrine and isoproterenol on microvessel adenylate cy-

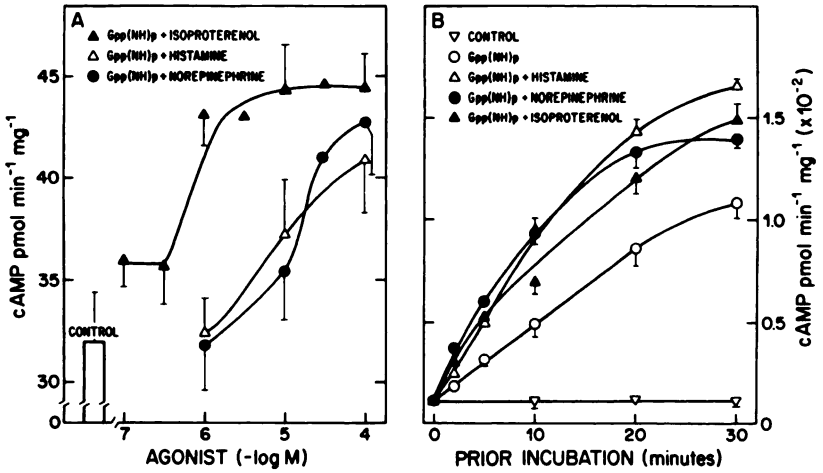

FIG. 6. *Activation of microvessel adenylate cyclase by histamine, norepinephrine and isoproterenol*

A. Microvessel preparations were assayed in the presence of 1 μM Gpp(NH)p, adenosine deaminase (1.2 U) and varying concentrations of histamine (△), norepinephrine (●), and isoproterenol (▲). The unfilled bar represents activity in the presence of Gpp(NH)p alone. B. Preparations were incubated with 1 μM Gpp(NH)p in the absence (○) or presence of 100 μM histamine (△), 100 μM norepinephrine (●) or 10 μM isoproterenol (▲) for the time intervals indicated, then sedimented and washed as described in MATERIALS AND METHODS prior to assay, in the presence of 1.2 U of adenosine deaminase. Values with standard errors are means of three experiments using different vessel preparations; other values are means of two separate experiments.

clase. Histamine, norepinephrine and isoproterenol individually had no effect on microvessel adenylate cyclase. In the presence of 1 μM Gpp(NH)p, a dose-related stimulation of enzyme activity was observed when these agents were added directly to the assay (Fig. 6A). When preparations were incubated with Gpp(NH)p and these agonists, then washed prior to assay, it was found that the neurohormones increased the rate of activation in the presence of the guanine nucleotide (Fig. 6B). That the activation was persistent is apparent from the fact that the activated state was still in evidence after sedimentation and washing of the membranes. Stimulation by 10 μM isoproterenol was completely abolished by 10 μM ±-propranolol and unaffected by the same concentration of phentolamine. Promethazine and cimetidine at 10 μM inhibited the stimulatory action of 100 μM histamine by 84.2 ± 4.4% and 71.9 ± 3.3%, respectively, although neither compound had inhibitory action at 1 μM.

Effect of prostaglandins and prostacyclin. When added directly to the assay in the presence of Gpp(NH)p, PGE_1, PGE_2 and prostacyclin (PGI_2) activated microvessel adenylate cyclase in a dose-dependent manner (Fig. 7). Activation by each was about 1.5-fold greater than that produced

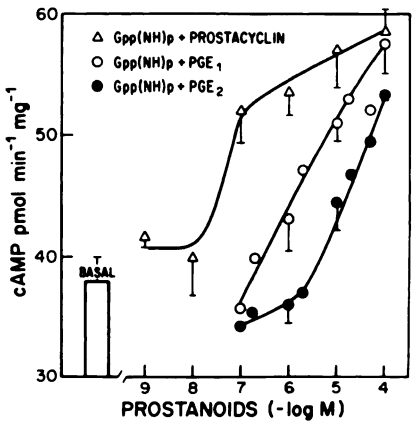

FIG. 7. *Activation of microvessel adenylate cyclase by PGE_1, PGE_2 and prostacyclin*

Preparations were assayed in the presence of 1 μM Gpp(NH)p, adenosine deaminase and varying concentrations of PGE_1 (○), PGE_2 (●) and prostacyclin (△). The bar represents activity in the presence of Gpp(NH)p and adenosine deaminase. Values with standard errors are means from three separate preparations; other values are means of two separate experiments.

by the guanine nucleotide alone. PGI_2 was considerably more potent than either PGE_1 or PGE_2, half-maximal activation occurring at less than 0.1 μM. These agents produced no detectable stimulation when added in the absence of guanine nucleotide (data not shown).

Microvessel adenylate cyclase was stimulated 8- to 10-fold by NaF, maximal stimulation by this anion occurred at 12 mM.

DISCUSSION

The method developed for isolation of microvessels is similar to that used by Goldstein *et al.* (16). We used sequential homogenization to completely disrupt neurons and glia rather than forcing minced tissue through a series of nylon sieves. Homogenization was much more effective in disrupting brain cells and releasing microvessels while preserving their structural identity. The vessels were, in fact, extremely difficult to disrupt by homogenization, especially with a loose-fitting pestle. Nevertheless, the surface membrane of these cells was damaged as evidenced by the rapid uptake of trypan blue and by electronmicroscopic examination. Substrate was readily accessible to the catalytic site; preparations in which vascular segments were preserved had better enzyme activity than those in which all cellular integrity was disrupted. Recovery of microvessels averaged 500 μg of protein per gram of tissue, which was considerably higher than when nylon mesh filtration was used. The use of 0.4 to 0.5 mm glass beads rather than 0.25 mm material (16) greatly speeded up the filtration process. This led to improved purity because nuclei and red cells rapidly passed through the column before aggregation of microvessels occurred.

The criteria described earlier provided evidence of high purity. The possibility of contamination by membranes from other cell types was considered unlikely because the isolation procedure that involved centrifugation at relatively low gravitational forces (3000 × *g*) in a high density medium (25% BSA) was unfavorable for membrane sedimentation. Furthermore, the preparation was purified on a column of glass beads which would not retain small membranous particles. We also examined adenylate cyclase in the neuronal residue which floated on 25% BSA after sedimentation at 3000 × *g*. When assayed under identical conditions, basal adenylate cyclase in this fraction ranged from 200 to 300 pmol/min/mg and was only marginally activated by 1 μM Gpp(NH)p. In the presence of guanine nucleotide, histamine, norepinephrine and isoproterenol elicited less than a 10% increase in activity while activation by adenosine, 2-chloroadenosine, PGE_1 and PGE_2 could not be detected. These findings provide further evidence that adenylate cyclase in the microvessel preparation could not be due to neuronal (or glial) contamination.

The method developed was also effective for isolating microvessels from dog and rabbit cerebral cortex. In these instances, animals were anesthetized with pentobarbital and the brain was perfused through the carotids with oxygenated Krebs-Ringer bicarbonate. Microvessels then isolated were completely free of erythrocytes. Basal adenylate cyclase activities in microvessels prepared from dog and rabbit cerebral cortex were 50 and 20 pmol/min/mg, respectively; the yield of microvessel protein in each was comparable to that from guinea pig.

When added directly to the assay, adenosine alone or in combination with Gpp(NH)p had no effect on the guinea pig microvessel enzyme. It was found that these cells were rich in 5′-nucleotidase (20 to 25 nmol of 5′-AMP hydrolyzed/min/mg at 30°). We considered it likely that adenosine was actively generated from ATP during the assay so that the effects of exogenous adenosine could not be seen. The observation that adenosine deaminase significantly reduced Gpp(NH)p activation provided indirect evidence that the nucleoside was likely to have a stimulatory action on the enzyme. Incidentally, adenosine deaminase could not be detected in microvessel preparations. By using adenosine analogues resistant to adenosine deaminase, dose-related stimulation by 2-chloro- and 2-azidoadenosine was readily demonstrated. Subsequently, it was found that activation by adenosine itself readily occurred when microvessels were incubated with the nu-

cleoside and Gpp(NH)p and then washed prior to assay. Demonstration of such an action of adenosine (as well of the two adenosine analogues) was possible since adenosine would not be generated under the prior incubation conditions and because the activated state was persistent. Subsequent studies with the neurohormones and prostaglandins were also carried out with adenosine deaminase added to the assay. The use of this enzyme to destroy endogenously generated adenosine during adenylate cyclase assays has been previously reported (23).

Previous studies with a variety of tissues have shown that Gpp(NH)p activation of adenylate cyclase is a time-dependent process and hormones, 2-chloroadenosine and PGE_1, act to increase the rate of guanine nucleotide activation (24–27). The effects of histamine, norepinephrine and isoproterenol observed on the microvessel preparation are in accord with this. Significantly the action of adenosine and the two adenosine analogues was analogous to that of the neurohormones, that is, they increased the rate of Gpp(NH)p activation. There seemed to be an absolute requirement for guanine nucleotide for stimulation by all the agonists tested. Moreover, stimulation by agonist required the simultaneous presence of the nucleotide. Thus when the enzyme was partially activated by Gpp(NH)p and the membranes were washed to remove unbound ligand, 2-chloroadenosine had no stimulatory action. However, when both Gpp(NH)p and 2-chloroadenosine were added to partially activated washed membranes, the nucleoside exerted its full effect. As with many other tissues, stimulation of enzyme activity in the presence of Gpp(NH)p was greater than that produced by GTP. Activation of adenylate cyclase in peripheral blood vessels by guanine nucleotides (28) and in capillaries isolated from rat cerebral cortex by catecholamines and dopamine (29) has been reported. The microvessel adenylate cyclase was also stimulated by PGE_1, PGE_2 and prostacyclin when Gpp(NH)p was present. Prostacyclin was much more potent than either PGE_1 or PGE_2. The former compound is synthesized by vascular tissues (30–32) and it has been shown to be a potent vascular smooth muscle relaxant (30, 31, 33). It is known to stimulate cAMP formation in platelets (34, 35) and to activate adenylate cyclase in these cells (35). Whether the adenylate cyclase stimulatory action of any of the agonists examined in the present study relates to their action on vascular tone is unknown at present.

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