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Biochemical Characterization and Cytochemical Localization of a Catecholamine-Sensitive Adenylate Cyclase in Isolated Capillary Endothelium

(micropinocytosis/AMP-PNP/alloxan/intercellular junctions)

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ABSTRACT **Capillaries were isolated from epididymal fat, and a catecholamine-sensitive adenylate cyclase found in these capillaries was characterized. The effect of various hormones on the accumulation of adenosine 3′:5′-cyclic monophosphate in capillary endothelial cells was determined and the cyclase was found to exhibit mixed alpha and beta characteristics. Cyclase was cytochemically localized in these endothelial cells with 5′-adenylyl-imidodiphosphate as a specific cyclase substrate and alloxan as a specific cyclase inhibitor. Lead imidodiphosphate was precipitated at or near the site of cyclase activity upon hydrolysis of 5′-adenylyl-imidodiphosphate by cyclase. This reaction product was observed primarily on the luminal surface of intact capillaries, in micropinocytic invaginations, in free vesicles within the cytoplasm, and in the intracellular junctions.**

Digestion of epididymal fat with collagenase has been used by several investigators to obtain pure fractions of fat cells (1, 2). Upon centrifugation of the dissociated adipose tissue, adipocytes collect at the top and the vascular and stromal cells are pelleted. This study describes a method for partial purification of the vascular–stromal pellet to obtain a relatively homogeneous preparation of intact capillaries and reports the biochemical characterization and cytochemical localization of a catecholamine-sensitive adenylate cyclase (cyclase) associated with capillary endothelial cells.

A modification of the method of Reik *et al.* (3) was used in the cytochemical localization of capillary cyclase activity. The substrate, 5′-adenylyl-imidodiphosphate, which is specifically hydrolyzed by cyclase but not by other ATPases (4, 5), was used in comparison with ATP; the specificity of localization was enhanced by a reaction in which lead imidodiphosphate is precipitated at or near the site of cyclase activity. Further specificity was achieved by the use of alloxan, which has been shown to selectively inhibit cyclase while sparing other membrane ATPase (6), and thus would be expected to inhibit product formation when 5′-adenylyl-imidodiphosphate is used as substrate.

METHODS AND MATERIALS

Isolation of Capillaries. The distal portion of epididymal fat pads from Sprague–Dawley rats (250–300 g) were removed and placed in Tyrode's basal salt solution (pH 7.2) at 0°, thoroughly minced, and placed in siliconized vials (three fat pads per vial) each containing 40 mg of crude collagenase (Worthington Biochemicals), 6 mg of bovine-serum albumin, and 6 ml of Tyrode's balanced salt solution. The mixture was incubated at 37° for 45 min on a wrist action shaker and centrifuged in siliconized conical tubes (30 × *g* for 2 min). The resulting pellet contained larger blood vessels, stromal cells, and attached fat cells, and was discarded. The supernatant containing the fat cake was decanted and centrifuged (800 × *g* for 2 min). The vascular pellets from the second centrifugation were twice pooled and washed in Tyrode's solution, and collected by centrifugation at 800 × *g* for 2 min. The final pellet was pink; it contained fragments of intact capillary networks with minimal stromal elements, without fat, as monitored by phase-contrast (Fig. 1) and electron microscopy. Pinocytic activity in the capillaries was determined with ferritin as tracer (7).

Enzymology. Capillaries were incubated in Tyrode's balanced salt solution containing 7 mM aminophylline for 5 min at 37°, and the reaction was stopped by boiling for 3 min. Cyclic AMP was extracted by sonication of the suspension of capillaries for 3 min at 150 W and 0°, centrifugation at 6000 × *g* for 30 min, and dilution of the supernatant with Na acetate buffer (pH 6.2) (1:4) for measurement by immunoassay. Cyclic AMP content was measured by a modification of the radioimmunoassay method of Steiner and Kipnis (8). Precipitating antibody was deleted, and antibody to cyclic AMP and bound ^{125}I-labeled succinyl cyclic AMP tyrosine methylester (obtained from Collaborative Research) were isolated by trapping on a 0.45-μm Millipore filter, which was dissolved in dioxane counting solution to which methyl cellosolve had been added. Cyclase and phosphodiesterase activities were measured (9). Protein was determined by the method of Lowry *et al.* (10). Prostaglandins were stored in ethanol at −20°, at a stock concentration of 1 mg/ml. Ethanol was present in some incubation mixtures at a final concentration below 1%, but had no effect on accumulation of cyclic AMP.

Cytochemistry. The incubation medium for the cytochemical localization of cyclase (3) consisted of 80 mM Tris–maleate buffer (pH 7.2), 6% dextran (molecular weight 250,000), 2 mM $Pb(NO_3)_2$, 4 mM $MgSO_4$, 5 mM aminophylline, and either 0.6 mM ATP or 0.5 mM 5′-adenylyl-imidodiphosphate (International Chemical and Nuclear Corporation; Irvine, Calif.) as substrate, with or without alloxan (5 mM). Adenylyl-imidodiphosphate and $PbNO_3$, in the absence of capillary endothelium, did not exhibit spontaneous precipitation. Fresh, unfixed capillaries were incubated for 5

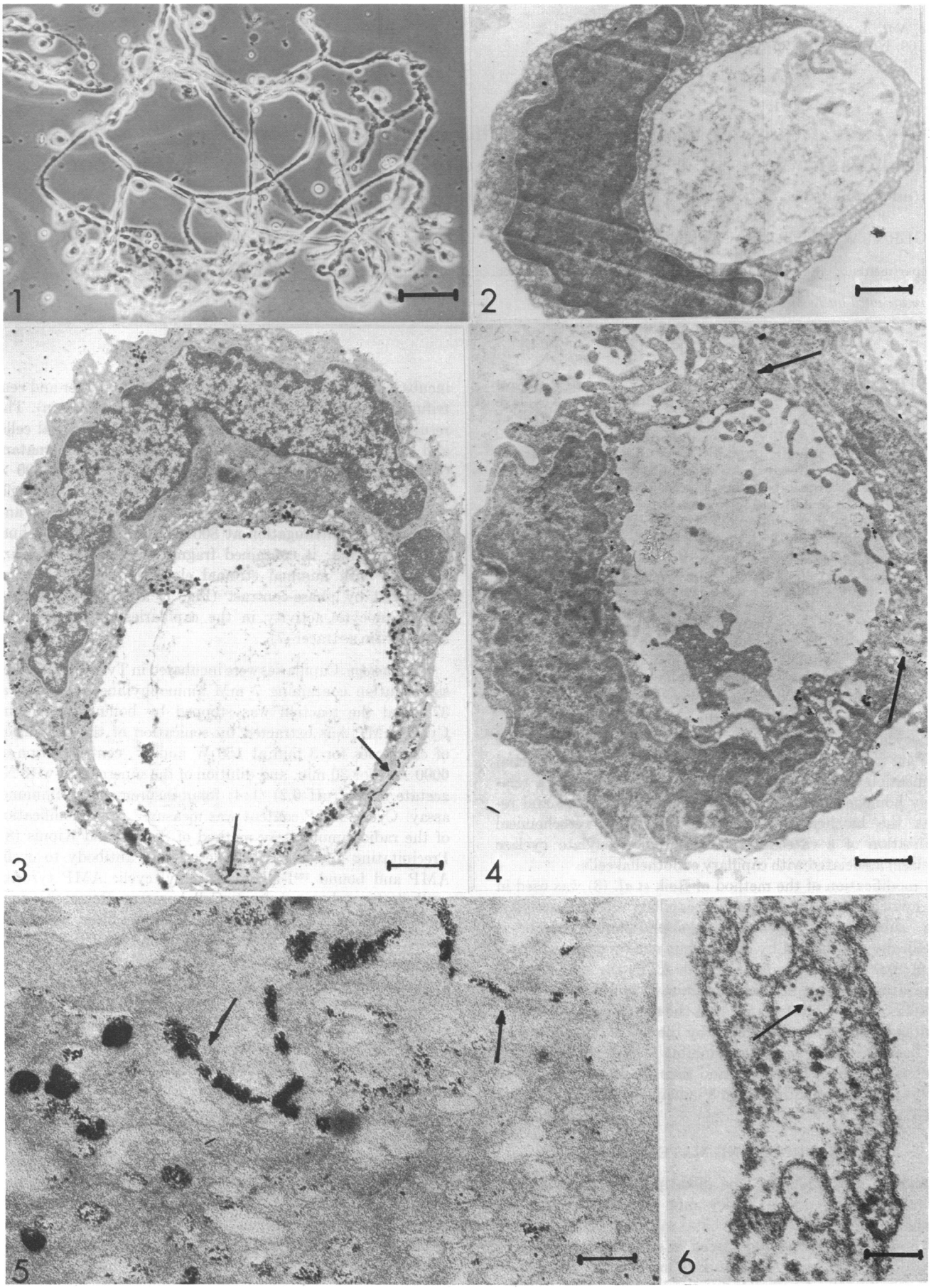

FIG. 1. Phase-contrast micrograph of a capillary preparation from epididymal fat. ×272; scale marker = 40 μm.

min (with ATP as substrate) or for 25 min (with adenylyl-imidodiphosphate as substrate) at room temperature (23°), washed twice in 0.2 M Tris–maleate buffer, and fixed for 30 min in 2% glutaraldehyde in Tyrode's–cacodylate buffer (pH 7.4). After two washes with Tyrode's–cacodylate buffer, the tissue was fixed again in 2% OsO_4 for 1 hr, dehydrated in ethanol, embedded in epon, and sectioned with an LKB 1 ultramicrotome. Counterstained (uranyl acetate) and unstained sections were observed with an Hitachi 11B electron microscope.

RESULTS

Adenylate cyclase measurements

In sonicated or homogenized (Potter–Elvejhem) preparations of fat capillaries, there was modest cyclase activity [9–10 nmol of cAMP per g of protein per 10 min, equal to about 30% of fully activated liver-cyclase activity (11)], and only minimal hormone responsiveness (catecholamines, prostaglandin 1 or 2 produced an activation of about 15% above basal level). However, when tufts of intact capillaries were incubated at 37° for 5 min with 7 mM aminophylline and various hormones or other regulators, marked accumulation of cyclic AMP was observed. The profile of responsiveness of capillary cyclase to hormones is shown in Table 1. Activation was best with isoproterenol and other catecholamines. Prostaglandins E_1 and E_2 produced activation that was slightly less than that seen with isoproterenol. Other prostaglandins (A, B, F_1 alpha, and F_2 alpha) were relatively less effective. The use of alpha and beta agonists indicated a slight predominance of the beta, although good alpha stimulation was also observed (modest inhibition of norepinephrine stimulation was seen with both phentolamine and propranolol, see Table 1). Many hormones that influence cAMP accumulation in fat and other tissues were examined. Melanin-stimulating hormone (10–100 μg/ml), adrenocorticotropic hormone (10–100 μg/ml), insulin (1–20 μU/ml), parathormone (10–100 μg/ml), bradykinin (10–100 μg/ml), and histamine (50 μM) were without significant effect. Phosphodiesterase was present in the capillaries and active, and exhibited characteristic sensitivity to aminophylline and paraverine. In capillary homogenates, phosphodiesterase hydrolyzed 6 mM cAMP per mg of protein per min and was inhibited over 85% by 6.7 mM aminophylline. Profound inhibition (95%) of capillary cyclase activity was observed in the presence of 5 mM alloxan. These inhibitory effects were comparable to those found with alloxan in brain, muscle, liver, and kidney (6).

Cytochemistry

Electron micrographs of capillaries incubated for 25 min at room temperature with adenylyl-imidodiphosphate as a substrate exhibited an electron-dense reaction product (lead-imidodiphosphate) primarily localized at the luminal surface of the endothelial cell membrane (Fig. 4). The reaction product occurred in patches along smooth portions of the luminal membrane, in most pinocytic invaginations, and on the inside surface of micropinocytic vesicles within the cytoplasm (Figs. 4 and 5). A striking and consistent accumulation of product occurred in the intercellular junctions joining the endothelial cells (Figs. 4 and 5). When adenylyl-imidodiphosphate was used as a substrate, 5 mM alloxan prevented appearance of reaction product at any location (Fig. 2). When ATP was used as a substrate, within 5 min of incubation at room temperature, reaction product (lead phosphate and/or lead pyrophosphate) was observed within all pinocytic invaginations, on the luminal and ablumial surfaces of endothelial cells, and within free vesicles within the cytoplasm. Deposits also occurred within the intercellular junctions but were absent from smooth portions of the membrane (Fig. 3). Alloxan did not inhibit formation of reaction product when ATP was used as a substrate, in contrast with its effect on the reaction with adenylyl-imidodiphosphate. In the absence of either nucleotide, no reaction product occurred. In capillaries fixed with formalin (4%) or glutaraldehyde (1%) that showed no cyclase activity, no reaction was obtained with adenylyl-imidodiphosphate, but the pinocytic vesicles showed activity with ATP as substrate, as shown previously. Electron micrographs of capillaries incubated in 1% ferritin revealed ferritin particles sequestered in micropinocytic invaginations and in vesicles within the cytoplasm (Fig. 6).

As might be expected when adenylyl-imidodiphosphate was used as substrate, the rate of appearance of reaction product was noticeably reduced in comparison to the rapidity of product accumulation observed with ATP as substrate. It is emphasized that product accumulation in the presence of adenylyl-imidodiphosphate differed from that seen with ATP in the following ways: (*a*) There was a preponderance of activity on the luminal surface; (*b*) there was a reduction in the number of labeled micropinocytic vesicles; (*c*) there was a noticeable increase in the relative abundance of product accumulation in intercellular junctions; and (*d*) smooth portions of the luminal membrane appeared more heavily labeled with reaction product.

FIG. 2. Electron micrograph of a cross section of a capillary treated with 0.5 mM adenylyl-imidodiphosphate and 5 mM alloxon for 25 min at room temperature. The section is not counterstained. Note the complete absense of reaction product. ×17,000; scale marker = 0.6 μm.

FIG. 3. Cross section of a capillary treated with 0.6 mM ATP for 5 min at room temperature (counterstained section). Reaction product is localized within pinocytic invaginations on the luminal and abluminal surfaces of the endothelial cell, within free vesicles in the cytoplasm, and within intercellular junctions (*arrows*). ×21,000; scale marker = 0.5 μm.

FIG. 4. Cross section of a capillary treated with 0.5 mM adenylyl-imidodiphosphate for 25 min at room temperature (not counterstained). Reaction product is localized in patches along smooth portions of the luminal membrane, in some pinocytic invaginations facing the lumen, in micropinocytic vesicles within the cytoplasm, and in the intercellular junctions (*arrows*). ×21,000; scale marker = 0.5 μm.

FIG. 5. Electron micrograph of high magnification of an endothelial cell treated with adenylyl-imidodiphosphate exhibiting reaction product in an intercellular junction (*arrows*) and within internalized micropinocytic vesicles. ×97,000; scale marker = 0.01 μm.

FIG. 6. Section through the wall of an isolated capillary treated with 1% ferritin for 20 min at 37°. Ferritin (*arrow*) becomes sequestered within internalized micropinocytic vesicles. ×121,000; scale marker = 80 nm.

TABLE 1. *Cyclic AMP accumulation in rat epididymal capillaries*

Hormone	Concentration	pmol of cAMP per mg of protein
None	—	1.2 (±0.14)
Norepinephrine	20 μM	3.9 (±0.54)
Norepinephrine	50 μM	4.2 (±0.43)
Epinephrine	50 μM	4.6 (±0.38)
Isoproterenol	20 μM	5.5 (±0.61)
Phenylephrine	20 μM	2.4 (±0.26)
Phentolamine	20 μM	1.5 (±0.07)
Propranolol	20 μM	1.1 (±0.06)
Norepinephrine and propranolol	20 μM (of each)	2.4 (±0.30)
Norepinephrine and phentolamine	20 μM (of each)	2.7 (±0.21)
*Glucagon	100 μg/ml	2.4 (±0.11)
Vasopressin	50 μM	3.0 (±0.19)
Prostaglandins		
PGE_1	10 μg/ml	3.5 (±0.30)
PGE_2	10 μg/ml	3.4 (±0.22)
*PGF_1 alpha	10 μg/ml	2.1 (±0.11)
*PGF_2 alpha	10 μg/ml	2.2 (±0.09)
†PGA	10 μg/ml	1.8 (±0.26)
†PGB	10 μg/ml	1.9 (±0.14)

Values are averages for five or more determinations (±SD). For conditions of incubation, see *Methods*. Assays were in duplicate and agreed within 6%. Data were analyzed statistically by Student's *t*-test. All stimulations were significantly different from baseline ($P < 0.001$, except for $*P < 0.01$ and $\dagger P < 0.05$). PG, prostaglandin.

DISCUSSION

The procedure used for the localization of cyclase in capillaries provides certain advantages in interpreting the data. The substrate, 5′-adenylyl-imidodiphosphate, is highly specific for cyclase and is not hydrolyzed by other membrane ATPases (4, 5). This eliminates the contamination by specious reaction product (lead phosphate) that predominates when ATP is used as a substrate (3). Specificity of the alloxan inhibition provides an additional degree of confidence in determining the enzymic source of the lead precipitate (6). The availability of both a specific cyclase substrate and inhibitor combine to produce a more reliable cytochemical probe than was previously available for cyclase (3).

Enzyme measurements and cytochemical determinations were performed with fresh, unfixed tissue, and therefore effects of fixation did not intrude upon data interpretation. The cellular homogeneity of the capillary preparation (Fig. 1) reduced the possibility of diffusion of ATP or reaction product from surrounding cells. Since the capillaries are present as intact tubes, a diffusion gradient between luminal and abluminal compartments could account for the accumulation of reaction product primarily in relation to the luminal side of the endothelial cells. However, if such a diffusion gradient exists, it would result in a higher concentration of substrate in the abluminal compartment, which in turn would favor a higher concentration of reaction product at the abluminal surface, an effect opposite to the observed. Also, the presence of reaction product through the entire length of the intercellular junctions suggests free access of substrate to both surfaces of endothelial cells.

A preponderance of cyclase activity at the luminal surface of the capillaries seems appropriate since it is this surface that initially comes in contact with circulating hormones. The ability of receptor-cell membranes to specifically bind and concentrate hormones (13) might provide an enhanced capacity for the transport of hormones across endothelial barriers by micropinocytic vesicles (7, 14–17). Intracellular junctions, which also exhibit cyclase activity, might likewise function as channels for hormone transport (15). These data do not allow a strict classification of capillary adrenergic receptors as pure alpha or beta in the classical sense. There is activation of capillary cyclase by both alpha and beta stimulators, and although there is some beta predominance, the receptor is best described as having mixed properties.

The absence of response of endothelial cyclase to fat cyclase hormones is biochemical evidence of minimal contamination by fat cell membranes. Epididymal fat cells produce about 0.12 nmol cAMP per mg of protein per 10 min (8), an amount significantly lower than that found for capillary cyclase. The absence of cyclase-hormone responsiveness in broken-cell preparations has been observed in various other tissues (18), and probably represents the fact that regulatory components of cyclase are often damaged in homogenization processes. The rather high basal activities of cAMP found in capillaries is intriguing. It is not possible to decide at this time whether this reflects the result of hormone release with sacrifice or whether normal endothelial function requires a high steady state of capillary cyclase activity. Indeed, the capillary-isolation process may itself stimulate cyclase activity.

The high capillary-cyclase activity must alert investigators to the possibility that this enzyme could be the source of a significant fraction of cAMP produced by highly vascularized tissues. A catecholamine-sensitive cyclase present in capillaries in high titer is an intriguing component of the endothelial cell membrane. Prostaglandin, glucogon, and vasopressin responses are probably intrinsic to the capillaries but could possibly represent contamination by other cell types (mast cells, pericytes, etc.). Designation of specific physiological correlates for the function of this cyclase and their possible relation to micropinocytosis or other transport functions of capillary endothelium awaits further study.

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