CYTOCHEMICAL STUDIES OF NUCLEOTIDYL CYCLASES IN AORTIC ENDOTHELIUM AND SMOOTH MUSCLE CELL

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We demonstrated the localization of adenylate cyclase (ACLase) and guanylate cyclase (GCLase) activities in rat aortic endothelium and smooth muscle cells using a recently improved cytochemical method introduced by Fujimoto et al. (16). Cytochemical effects of some agents (isoproterenol, propranolol, acetylcholine, methylene blue) on cyclase activity were also examined with the direct incubation method in vivo employing the perfusate added with such agents utilizing the unique location of the aorta.

On the endothelial cell, reaction products indicating ACLase and GCLase activities were localized on the cytoplasmic side of: a) the membrane covering the caveolae and vesicles of both luminal and abluminal surfaces, b) the abluminal plasma membrane underneath the mass of stress fiber-like structure, and c) the gap junctional membrane; and localized around the centriole. On the other hand, the basal activity of GCLase in the smooth muscle cell was intense in comparison with that of ACLase, and the reaction products of both cyclases' activities were localized on the cytoplasmic side of the membrane covering the caveolae, the gap junction, the sarcoplasmic reticulum and the rough endoplasmic reticulum, and on the dense bands, the myosin-like filaments and the centriole. ACLase activity in the endothelial cell and smooth muscle cell was activated by isoproterenol and partly inhibited by propranolol. GCLase activity was stimulated by acetylcholine and fairly reduced by methylene blue in both cell types.

Here, we discuss the assessment of our method and the implications of cytochemical localization of cyclase activity in relation to the functional aspects in endothelial and smooth muscle cells.


Abbreviations: cAMP: cyclic 3',5'-adenosine monophosphate; cGMP: cyclic 3',5'-guanosine monophosphate; ACLase: adenylate cyclase; GCLase: guanylate cyclase; AlPase: alkaline phosphatase; AMP-PNP: adenylyl imidodiphosphate; GMP-PNP: guanylyl imidodiphosphate; FA: paraformaldehyde; GLA: glutaraldehyde; PGI2: prostacycline; EDRF: endothelium-derived relaxing factor; ANF: atrial natriuretic factor; Iso: isoproterenol; Prop: propranolol; Ach: acetylcholine; MB: methylene blue.
The role of the normal vascular endothelium has been traditionally assigned to no more than a passive physical barrier between blood and tissues. Currently, it is well recognized that intimal and microvascular endothelium is an active tissue and possesses many biological functions derived from its unique location. Ross and Glomset (53) have suggested that endothelial monolayer plays the critical role in atherogenesis. Endothelial dysfunction may be important in the root of certain systemic disease, apart from thrombosis and vascular disease. With technological advances having allowed investigators to obtain much information, endothelial function has been greatly extented but can be summarized in the following three points: the modulation of vascular smooth muscle tone; the inhibition or activation of platelet adhesion and aggregation; and the control of vascular permeability.

These functions are believed to be closely associated with nucleotidyl cyclases, adenylate cyclase (ACLase) and guanylate cyclase (GCLase) located in the endothelium, for the following reasons. Dibutryl cyclic 3',5'-adenosine monophosphate (cAMP) and dibutryl cyclic 3',5'-guanosine monophosphate (cGMP) are found to be able to increase the permeability of brain microvessels in a dose dependent manner by increasing the number of vesicles (29, 30, 32). Vascular endothelium has the ability to produce prostacycline (PGI₂), a potent inhibitor of platelet aggregation and a vasodilator, which is a powerful activator of ACLase not merely in the platelet but in the vascular endothelium. Its biosynthesis is attenuated by increased cAMP levels in the endothelium, so that ACLase may function as a negative-feedback mechanism producing PGI₂ (4, 27). It is also recognized that dibutyryl cGMP causes the increase of bovine endothelial cell thromboxane production, which is a potent vasoconstrictor and platelet aggregator, without affecting PGI₂ metabolism (64).

Many biochemical studies have demonstrated the existence of β-adrenergic receptor coupled to ACLase activity and GCLase activity in vascular endothelium, whereas cytochemical demonstration of both cyclases' activities has been restricted to the endothelium in microvessels (31, 33, 57–59) and no direct evidence has been published yet on the presence of cyclase in arterial endothelium. With regard to cyclase activity in the endothelium of elastic arteries, its existence is still arguable in biochemical experiments (6, 36, 38, 48).

On the other hand, it is also well accepted that increased cAMP levels or cGMP levels in a vascular smooth muscle cell lead to its relaxation (25). Furchgott and Zawadzki (18) first reported that acetylcholine-induced arterial relaxation depends on the integrity of endothelium. Recently, it has become apparent that several agents (acetylcholine, bradykinine, ATP, ADP, A23187) which relax blood vessels are mediated by endothelium-derived relaxing factor (EDRF), which generates an increase in vascular smooth muscle cGMP (19, 42, 43, 49). PGI₂ is recognized to be another powerful endothelium-producing and -releasing relaxing factor which enhances ACLase activity in vascular smooth muscle (42). It is postulated that β-adrenergic receptors and adenosine receptors (A₂ receptor) linked to ACLase are present in vascular smooth muscle. In hypertension, changes in cyclic nucleotide metabolism have also been observed in rat aorta (2).

These findings illustrate that nucleotidyl cyclase in the vascular smooth muscle cell is also an important participant in the modulation of vascular tone and that
vascular endothelium and smooth muscle cells are functionally coupled. However, enzyme cytochemical studies of adopted vascular smooth muscle cells, showing direct evidence of the relationship of such enzymes with these physiological characteristics have not been published yet.

In these respects, we have demonstrated the localization of ACLase and GCLase in the rat aortic endothelium and smooth muscle cell by using the cytochemical method of Fujimoto et al. (16). In addition, the cytochemical effects of activators and inhibitors of cyclases' activities were evaluated by our original direct incubation method employing the perfusate added with such agents utilizing unique location of aorta.

**MATERIALS AND METHODS**

**Tissue Preparation**

Male adult Sprague-Dawley rats weighing 250–350 g were used. The animals were sacrificed under sodium pentobarbital anesthesia (Somnopentyl, 0.1 ml/100 g b.w., intraperitoneally) by intracardial perfusion with cold 2% paraformaldehyde (FA) in 0.1 M cacodylate buffer containing 0.25 M sucrose, pH 7.4, for 2–3 min. The thoracic and abdominal aortas were then removed, dissected free of connective tissue and adherent fat, cut into rings of less than 2 mm segments, and immersed in the same fixative for 50–60 min at 0–4°C. After fixation tissues were rinsed with the same buffer for 60–120 min.

**Light Microscopy**

Tissues were cut into 15 µm thick sections with an electro-freezing microtome (Yamato Seiki Co. Ltd., Tokyo), and incubated in the following reaction medium at 37°C for 60–80 min within one change of the medium halfway through the incubation time. The incubation media were essentially the same as those described by Fujimoto et al. (16). Medium for ACLase: 80 mM Tris-maleate buffer, pH 7.4; 0.5 mM adenylyl imidodiphosphate (AMP-PNP) (Baehringer Mannhaim GmbH., West Germany); 10 mM NaF; 4 mM MgSO4; 2 mM theophylline; 2 mM lead nitrate; 0.25 M sucrose; 5% V/V dimethyl sulfoxide; 2.5 mM levamisole (Sigma Chem. Co. Ltd., St. Louis) as a potent inhibitor of non-specific alkaline phosphatase (AlPase). Medium for GCLase: 80 mM Tris-maleate buffer, pH 7.4; 0.5 mM guanylyl imidodiphosphate (GMP-PNP) (Baehringer Mannhaim GmbH., West Germany); 3 mM MnCl2; 2 mM theophylline; 2 mM lead nitrate; 0.25 M sucrose; 5% V/V dimethyl sulfoxide; 2.5 mM levamisole.

After washing in distilled water, sections were transferred to 1% colorless ammonium sulfide for 2–3 min, washed again and mounted on the slide glass with glycerine-jelly for light microscopy.

**Electron Microscopy**

Non-frozen and frozen sections of 40 µm in thickness were cut with a Microslicer (Dosaka EM Co. Ltd., Kyoto) and an electro-freezing microtome respectively. Then sections were incubated in the reaction medium in the same way as light microscopic procedure to demonstrate both cyclases. After rinsing three times with the same buffer, sections were postfixed with 1% OsO4 in 0.1 M cacodylate buffer.
buffer for 10 min at 0–4°C. Serial dehydration in graded ethanol and propylene oxide was followed by embedding in Spurr's resin. Ultra-thin sections were cut with an LKB Ultrotome III 8800, stained or unstained with uranyl acetate and viewed under a JEM 100CX electron microscope.

Cytochemical Controls

As cytochemical control, tissue sections were incubated in a substrate-free medium for both cyclases' activities and a medium excluding Mn ions for GCLase activity.

In addition, the cytochemical effects of activators and inhibitors of cyclase activity were assessed by the following procedures. The agent used for ACLase activity was 1 mM d,l-isoproterenol HCl (Iso) (Sigma Chem. Co. Ltd., St. Louis) or 1 mM d,l-propranolol HCl (Prop) (Sigma Chem. Co. Ltd., St. Louis) and used for GCLase activity was 1 mM acetylcholine chloride (Ach) (Wako Pure Chem. Industries Ltd., Osaka) or 0.5 mM methylene blue (MB) (Wako Pure Chem. Industries Ltd., Osaka) as a potent inhibitor of soluble GCLase activity (24).

Prior to the perfusion fixation, 1 mM HEPES buffered Dulbecco's modified Eagle medium containing the above-mentioned agent was perfused intracardially for 5 min at room temperature. Fixative, washing buffer and incubation medium containing the same concentrated agent were used in the following procedure except for incubation medium with isoproterenol by reason of its reaction to the medium. In some experiments, the first perfusion step was omitted or only basal perfusate was used in order to compare the effectiveness of the agents.

RESULTS

Cytochemical observation was carried out by using fixative of 2% FA which had some morphological defects in the ultrastructure because fixation in a mixture
Figs. 1-9. The following symbols are used: L, lumen; N, nucleus; IEL, intra elastic lamina; Mt, mitochondrion; T, T-tubule.

Figs. 1a-c. Light microscopic observations of rat aortic ACLase activity. ×320
a. Basal ACLase activity. Reaction is present on the endothelium and smooth muscle cells.
b. ACLase activity effected by 1 mM Iso. Intense reaction is observed on the endothelium and smooth muscle cells.
c. ACLase activity effected by 1 mM Prop. The reaction of both endothelium and smooth muscle cell is reduced by Prop.

Fig. 2a-c. Light microscopic observations of GCLase activity in rat aorta. ×320
a. Basal GCLase activity. Reaction is observed on the endothelium and smooth muscle cells.
b. GCLase activity effected by 1 mM Ach. Heavy reaction is recognized on the endothelium and smooth muscle cells.
c. GCLase activity incubated without substrate. The reaction on the endothelium and smooth muscle cell is completely lost.
FIGS. 3a-c. Electron microscopic observations of ACLase activity in aortic endothelium.

a. Basal ACLase activity. Slight precipitates are present on the caveolae and vesicles on both luminal and abluminal surfaces. ×61,000

b. Basal ACLase activity. Reaction products are observed on the cytoplasmic side of the membrane covering caveolae and vesicles, and of the abluminal membrane beneath the stress fiber-like structure (*). ×72,000

c. ACLase activity effected by 1 mM Iso. Precipitates localized on the caveolae and vesicles on both luminal and abluminal surfaces are intense. ×53,000
of 0.1% glutaraldehyde (GLA) and 2% FA for 60 min at 0-4°C resulted in faint or no activity of both cyclases. There was no difference in cyclase activity between thoracic and abdominal aorta in endothelium and smooth muscle cells.

Cyclase activity was not affected either in intensity or localization of the reaction even when the agents were added to the incubation medium or to the
fixative. However, all agents, both activators and inhibitors, could have an effect on cyclase activity, to be distinguished from basal activity cytochemically, in the tissue treated with one of these agents from the first step before perfusion fixation.

**Light Microscopy**

Reaction products generated by ACLase activity were observed on both
endothelium and smooth muscle cells (Fig. 1a). Their activity was stimulated by treating them with Iso (Fig. 1b) and partly reduced by treating them with Prop (Fig. 1c). Control experiments in which AMP-PNP was omitted did not show any specific activity.

On the other hand, GCLase activity was localized on both endothelium and smooth muscle cells (Fig. 2a). Their activity was enhanced by adding Ach to the perfusate (Fig. 2b) and there was no reaction product when the medium without substrates (Fig. 2c) or without Mn ions was used.

Electron Microscopy

1. Adenylate cyclase activity on endothelium.

Basal activity of ACLase was localized on the cytoplasmic side of: a) the membrane covering caveolae and vesicles on both luminal and abluminal surfaces (Fig. 3a), b) the abluminal membrane beneath the mass of stress fiber-like structure (Fig. 3b), and c) the gap junctional membrane; and localized around the centriole.

The reaction products of caveolae and vesicles on both luminal and abluminal sides were remarkably increased in the tissue prepared with 1 mM Iso (Fig. 3c) and partially reduced by 1 mM Prop treatment. However, in the activity of the centriole, there was no further effect with such agents. The control experiment, incubated with substrate-free medium, did not show any specific precipitate.

2. Guanylate cyclase activity on endothelium.

In the endothelium, there had been basal activity of GCLase on the membrane covering caveolae and vesicles on both luminal and abluminal surfaces (Fig. 4a), on the abluminal membrane beneath the mass of stress fiber-like structure, on the gap junctional membrane (Fig. 4b) and on the centriole. At high magnification, the reaction products associated with the membrane were found to be on the cytoplasmic side or just on the membrane.

Ach-stimulated intense reaction could be particularly found in the caveolae and vesicles only on the abluminal surface but not on the luminal surface (Fig. 4c). Moreover, it was observed on not only the cytoplasmic side of caveolae and vesicles but also inside of them. Occasionally, scattered precipitate was present in subendothelial space.
On the other hand, MB completely inhibited GCLase activity on the endothelium (Fig. 4d). Control experiments in which GMP-PNP or Mn ions were omitted did not show any specific precipitate on caveolae, vesicles, gap junction nor centriole in the endothelium.

3. Adenylate cyclase activity on smooth muscle cells.

The reaction products of ACLase were recognized on the cytoplasmic side of the membrane covering caveolae (Fig. 5a), of the gap junctional membrane and on the centriole, and a weak or uncertain reaction was observed on the sarcoplasmic and rough endoplasmic reticulum. These reactions had a tendency to be activated by Iso except for the centriole. In addition, Iso-stimulated reaction products were obviously localized on the dense bands and the myosin-like filaments (Fig. 5b), in which basal activity was faint or absent. On the other hand, the basal activity was partly reduced by Prop and there were no reaction products incubated with the medium excluding AMP-PNP as substrate (Fig. 5c).


Basal activity of GCLase was intense in comparison with that of endothelium and appeared on the cytoplasmic side of or right on the membrane covering caveolae, gap junction, sarcoplasmic and rough endoplasmic reticulum, and on the dense bands, myosin-like filaments and the centriole (Figs. 6a-c). The reaction products of centriole were present along the vicinity of microtubules (Fig. 6e). Ach drew more intense reaction but localization of GCLase was essentially similar to that of basal activity. The precipitate indicating GCLase activity induced by Ach was more concentrated on the caveolae than the other localization sites in smooth muscle cells. Moreover, on the caveolae, its reaction was observed on both internal and external sides of the membrane (Fig. 6d).

MB partly inhibited the GCLase activity observed on both the caveolae and centriole and fairly reduced the reaction localized on dense bands and myosin-like filaments. However, on the activity of sarcoplasmic and rough endoplasmic reticulum, MB had no inhibiting effect, or rather a slightly stimulating effect, that is, intense reaction products were sometimes prominent in the luminal space as well as the cytoplasmic side treated with MB (Fig. 6f). Control experiments in which GMP-PNP or Mn ions were omitted did not show any specific precipitate.

These results were summarized in Table 1. From this cytochemical study, there were ultimately no differences in the localization of both endothelium and smooth muscle cells between ACLase activity and GCLase activity. However, some differences in the intensity of reaction between ACLase and GCLase were recognized. In general, the GCLase activity in the smooth muscle cells was the most prominent.

Figs. 5a-c. Electron microscopic observations of ACLase activity in aortic smooth muscle cells.

a. Basal ACLase activity. Slight reaction is recognized on the cytoplasmic side of the membrane covering caveolae. ×51,000
b. ACLase activity effected by 1mM Iso. Tangential section. Reaction localized on the caveolae is activated by Iso. Iso-stimulated activity is also observed on the dense bands (DB), myosin-like filaments and rough endoplasmic reticulum (rER). ×51,000
c. Control experiment incubated in the medium without AMP-PNP. No precipitate is seen in aortic smooth muscle cell. ×34,000
Figs. 6a-g. Electron microscopic observations of GCLase activity in aortic smooth muscle cells.

a. Basal GCLase activity. Tangential section. Reaction products are present on the cytoplasmic side of the membrane covering caveolae and sarcoplasmic reticulum (arrows), on the dense bands (DB) and on the myosin-like filaments (arrowheads). x 62,000

b. Basal GCLase activity. Transverse section. Precipitates are recognized on the cytoplasmic side of gap junctional membrane, dense bands (DB) and myosin-like filaments (arrowheads). x 56,000

c. Basal GCLase activity. GCLase activity is also localized on the rough endoplasmic reticulum. x 42,000

d. GCLase activity effected by 1 mM Ach. Intense reaction stimulated by Ach is observed on the caveolae and along the myosin-like filaments (arrowheads). x 64,000

e. GCLase activity effected by 1 mM Ach. GCLase activity localized on the centriole is unaffected by Ach and present in the vicinity of microtubules. x 72,000
f. GCLase activity effected by 0.5 mM MB. MB partly inhibits the reaction on the caveolae and fairly reduces the precipitate on the dense bands and myosin-like filaments. However, MB tends to stimulate the basal GCLase activity localized on the sarcoplasmic and rough endoplasmic reticulum. ×25,000 Inset: GCLase activity effected by 0.5 mM MB. At higher magnification, intense reaction products are present on the sarcoplasmic (arrowheads) and rough endoplasmic (arrows) reticulum. ×42,000
DISCUSSION

Evaluation of the Method of Cyclase Activity Detection

The fixative consisting of 2% FA preserved both ACLase activity and GCLase activity and permitted their detailed ultrastructural localization in aortic endothelium and smooth muscle cells while 0.1% GLA added to the fixative resulted in no or faint activity in both cell types. Considering that many studies have employed 0.1%-2% GLA as the fixative to detect cyclase activity (17, 41, 55, 56, 58), rat aortic cyclase is supposed to be more affected by fixation with GLA.

The method of perfusion, prior to fixation, used in our experiments in order to evaluate the cytochemical effectiveness of activators (Iso, Ach) and inhibitors (Prop, MB) on cyclases' activities could indicate an available means that causes remarkable change in the amount of precipitate in comparison with basal activity. This method is characterized by the following point: Changes in cytochemical cyclase activity due to intraluminal addition of the agents are determined in intact perfused aorta in vivo. Inasmuch as it utilizes the unique location of the aorta, this method may not be adapted to other sorts of tissue. We employed a high concentration of such agents as seen in other cytochemical studies (17, 55, 57, 58), in the perfusate, and 5 min for perfusion time for these reasons: it is impossible to differentiate the small changes in the amount of reaction products caused by the low concentration of agents, since a cytochemical study is only a qualitative analysis; the high concentration is supposed to allow the agent to pass through the endothelial monolayer to the medial layer as observed in one pharmacological study (35); and many pharmacological studies have shown that the response due to the intraluminal addition of such agents is recognizable on smooth muscle cells within 30 seconds (19, 42). It has been demonstrated that the incubation medium containing 0.1-1 mM Iso or 1 mM Ach stimulated residual cyclase activity cytochemically after fixation (17, 55, 57, 58). However, according to the present results, 1 mM Iso or 1 mM Ach added to the fixative and the washing buffer as well as the incubation medium indeed had some effect on the intensity of the reaction and the localization of the reaction, but these results were not uniformly reproducible. It is well recognized that the receptors are floating in the membrane without restraint and that a signal of hormone or neurotransmitter is translated into the form of the activated cyclase when the hormone-receptor complex is linked with the enzyme (26). Thus, it may be impossible to demonstrate specific hormonal activation of cyclase cytochemically after fixation that results in a stabilization of the membrane structure by building-up networks between proteins (46). These results could illustrate that intact tissue must be required in order to demonstrate the activation of receptor-mediated nucleotidyl cyclase by hormones or neurotransmitters and that the fixation with the activated state of cyclase involves the better preservation of the activity.

The present study was based on the use of AMP-PNP or GMP-PNP as a specific substrate of ACLase or GCLase. AlPase can hydrolyze these substrates and cause false reaction products. However, the incubation was performed with the addition of levamisole, a potent inhibitor of non-specific AlPase, at pH 7.4 in order to eliminate the contamination of this enzyme. The endothelium and smooth muscle cell of larger blood vessels, type usually unspecified, are said to be AlPase-negative
Cyclase Activity in Rat Aorta

Moreover, this study has demonstrated that ACLase activity is stimulated by Iso and partly reduced by Prop and that GCLase activity is enhanced by Ach and inhibited by MB. In these respects, we believe we have precisely grasped the cytochemical activity of both ACLase and GCLase in aortic endothelium and smooth muscle cells.

Ultracytochemical studies have demonstrated the existence of ACLase and GCLase activity in the endothelium and the smooth muscle cells of the microvessel (31, 33, 57-59), whereas most of their results did not go so far as to indicate the detailed localization insomuch as their reaction products were coarse, discontinuous and observed on the external side of the plasma membrane. The results in our study have revealed that the fine and continuous reaction products localized on the plasma membrane are situated on the cytoplasmic side. This finding coincides with the generally accepted model for cyclase in which the catalytic site of the enzyme exists on the interior side of the plasma membrane and the receptor is on the exterior side.

Cytochemical Localization of Endothelial Cyclase Activity

We have demonstrated that the basal activity of ACLase and GCLase in aortic endothelium was localized on the cytoplasmic side of: a) the membrane covering the caveolae and vesicles on both luminal and abluminal surfaces, b) the abluminal membrane beneath the mass of stress fiber-like structures, and c) the gap junctional membrane; and localized around the centriole. In the present study, the intensity of cyclase activity (seen in caveolae and vesicles) was not different between the luminal and abluminal sides. The same results have been obtained in rat brain microvessels (31, 33). However, Wagner et al. (59) reported that ACLase activity was primarily localized on the luminal surface of rat capillary endothelium isolated from epididymal fat. Vorbrodt et al. (58) showed a contrary result, that is, ACLase activity was most prominent on the abluminal side of the endothelium in rat brain arterioles. At the present time, we could not elucidate whether this discrepancy was due to the differences between the functions of each endothelium or between the methods of each experiment.

Our study showed that the ACLase activity localized on the caveolae and vesicles of both sides was stimulated by Iso and partly reduced by Prop. This data which directly indicated the presence of ACLase coupled to β-adrenergic receptors in aortic endothelium is supported by the previous biochemical results (6). Endothelial cell cyclase activity on both the luminal and abluminal surfaces might indicate responses to hormones and neurotransmitters derived from both blood serum and tissue fluid. Furthermore, the inhibition of basal ACLase activity by Prop seems to reflect the participation of small amounts of catecholamine in blood serum or tissue fluid in vivo.

On the other hand, with regard to GCLase activity in aortic endothelium, the activation by Ach is still controversial (6, 36, 48). However, it is well accepted that in aortic endothelium, Ach induces the production and release of EDRF, which is thought to be either a short-lived hydroperoxide of free radical arising as an intermediate product in the oxidation of liberated arachidonic acid or another unsaturated fatty acid by lipoxygenase pathway, and that EDRF, after diffusing from the endothelium to the vascular smooth muscle cell, stimulates the soluble
FIG. 7. Electron micrograph of rat aortic endothelium. Actin-like filaments (5–7 nm in diameter) (arrows) and myosin-like filaments (10–12 nm in diameter) (arrowheads) are recognized in the massed stress fibers above the basal plasma membrane. ×82,000

FIG. 8. GCLase activity enhanced by 1 mM Ach in transverse section of rat cardiac muscle. The reaction products are present on sarcoplasmic reticulum and myosin filaments. ×70,000

FIG. 9. GCLase activity stimulated by 1 mM Iso in tangential section of rat cardiac muscle. Precipitates are seen not only on plasma membrane and sarcoplasmic reticulum but also on I-Z junctions and along myosin filaments (arrowheads). ×43,000
guanylate cyclase in smooth muscle causing an increase in cGMP which then induces its relaxation (19, 42, 49). It is recognized that the GCLase is present mainly in membrane-bound form in the cerebral capillaries (10, 33). Insomuch as atrial natriuretic factor (ANF), a specific activator of particulate GCLase, -induced increases in cGMP are observed in aortic endothelium (54, 61), particulate GCLase also seems to be present in aortic endothelium. In this study, Ach-stimulated intense reaction was found in the abluminal caveolae and vesicles but stimulation of GCLase activity by Ach was not recognized in the luminal caveolae and vesicles. In our method, we could not explain what substance causes GCLase activation, considering that the enhanced reaction products are prominently observed in the abluminal membrane. It is also possible to discuss other kinds of endothelium-derived factors induced by Ach or the existence of different types of GCLase in the endothelium.

MB, a free radical scavenger, has been shown to inhibit the activation of the soluble GCLase induced by nitrovasodilator or EDRF (24, 51). It is also demonstrated that MB inhibits the ANF-induced particulate GCLase activation (40). Results in this study revealed that MB by itself completely inhibited GCLase activity on the endothelium and fairly reduced it on smooth muscle cells. It might indicate that this dye directly acts to inhibit particulate GCLase activity.

Recently, immunohistochemical and morphological studies have revealed that endothelium has massed stress fibers, which extend above the basal plasma membrane, are arranged parallel to the cellular long axis and are composed of thin actin-like filaments (5–8 nm in diameter) and thick myosin-like filaments (10–15 nm in diameter) (14, 28, 60, 62). Ultrastructurally, myosin-like and actin-like filaments were also found in rat aortic endothelium (Fig. 7). It has been suggested that reversible modulation may occur within seconds in response to specific agents (histamine, serotonin, bradykinine, etc.) which indicate receptor-mediated events that act via the endothelial contractile apparatus, leading to subtle changes in junctional microtopography and allowing faster passage of small solutes. This mechanism probably involves activation of the actin-myosin system with the elevation of cytosolic Ca ions in the endothelium (9, 34). These agents also activate cyclase activity and cause the elevation of cyclic nucleotide levels (6) which decreases cytosolic Ca ion levels in the endothelium and mediates the endothelial relaxation like the behavior accepted in vascular smooth muscle relaxation. cAMP and cGMP are also found to be able to increase the permeability in the endothelium in a dose dependent manner accompanied by an increase in the number of vesicles (29, 30, 32). We might suggest that ACLase and GCLase mediate the regulation of both the contractility and permeability of the endothelium in the above-mentioned way. In this respect, cyclase activity observed on the caveolae and vesicles on both luminal and abluminal sides, and on the abluminal membrane beneath the mass of stress fiber is supposed to indicate reasonable localization on aortic endothelium.

Localization of cyclase activity on the cytoplasmic side of the gap junction has been reported by us in various other cell types (16, 17), substantiating an involvement of the cyclase and cyclic nucleotide system in cell to cell communication (12).

Buchwalow et al. have already reported that ACLase activity not activated by adrenalin or NaF was localized on the centrioles in rat thymocyte (5). Our study
agrees with their result and also demonstrates the existence of GCLase activity on the centriole. In view of cAMP accumulation in intact leukocytes enhanced by disruption of microtubules (52), the cyclase localization on microtubules of the centriole might indicate its participation in the modulation of a certain function in the centriole.

**Cytochemical Localization of Smooth Muscle Cyclase Activity**

Results in the investigation of electron microscopy have demonstrated that the basal activity of GCLase was intense in comparison with that of ACLase and that the basal GCLase activity and the Iso-stimulated ACLase activity in aortic smooth muscle cells were localized on the cytoplasmic side of the membrane covering caveolae, gap junction, sarcoplasmic and rough endoplasmic reticulum, and on the dense bands, the myosin-like filaments and the centriole.

Localization of the GCLase activity enhanced by Ach, and the ACLase activity stimulated by Iso on rat cardiac muscle (Figs. 8, 9), which were observed not only on sarcolemma, sarcoplasmic reticulum and gap junctions where cyclase activity has already been recognized (17, 55, 56) but also on myosin filaments and I-Z junctions, confirms our results concerning cyclase activity on the myosin-like filament and the dense bands. Dense bands are regarded as the Z line in smooth muscle cells (20) because thin filaments can be seen to penetrate it, and intermediate filaments are also associated with it.

Recent biochemical evidence may indicate that cyclic nucleotides modulate the vascular smooth muscle relaxation by the following mechanisms. Contraction of smooth muscle may be regulated by Ca-dependent mechanisms: Ca binds to calmodulin and activates myosin kinase leading to phosphorylation of myosin light chains; Ca acts at a second, unknown regulatory site to maintain crossbridge attachment when myosin is dephosphorylated (1, 7, 22). The hypothesis is that activated cyclic nucleotide-dependent protein kinase phosphorylates myosin kinase which puts it results in the inactive state because its binding affinity for the Ca-calmodulin complex decreases (11, 49) and that protein kinase stimulated by cyclic nucleotide activates Ca-ATPase on the plasma membrane and/or sarcoplasmic reticulum membrane and Na-K-ATPase on the plasma membrane which mainly regulate the intracellular Ca concentration (23, 37, 47, 50). Actually, we have demonstrated that Ca-ATPase activity was localized on the plasma membrane and the sarcoplasmic reticulum and Na-K-ATPase was observed on the caveolae in rat aortic smooth muscle cells (39). In consideration of these results, cyclases’ localization on the caveolae, sarcoplasmic reticulum, myosin-like filaments and dense bands seems to support the above hypotheses.

There have been many cytochemical studies reporting the presence of cyclase activity on rough endoplasmic reticulum (8, 13, 45). Our cytochemical findings confirm it but functional relevance is not yet clear. However, as seen in the suggestion mentioned by Poeggel et al. (45), the cyclase localized on rough endoplasmic reticulum is thought to be the active precursor or to modulate the protein production as it is recognized that cAMP regulates the acetylcholine receptor production (3).

In conclusion, the demonstration of endothelial cell cyclase activity on both the luminal and abluminal surfaces may indicate the existence of responses to
hormones and neurotransmitters derived from both blood serum and tissue fluid. Considering that these hormones and neurotransmitters elicit endothelium-dependent vasodilation and directly induce smooth muscle contraction, endothelial cyclase might play an important role acting as a feedback inhibitor of the contraction in vascular smooth muscle cells. However, rat aortic endothelium and smooth muscle cells also have an ectonucleotidase system (39, 44). It is recognized that ATP and ADP are endothelium-dependent vasodilator substances whose mediator, generated in the endothelium, activates GCLase in vascular smooth muscle cells, while 5'-AMP and adenosine are endothelium-independent vasodilators which activate ACLase in vascular smooth muscle cells (19, 42). Biochemically, intracellular cyclic nucleotides of smooth muscle regulate vasodilation. However, the question of whether the cyclase system and nucleotidase system of the endothelium are closely related to the smooth muscle cyclase system and nucleotidase system awaits further biochemical and cytochemical studies.

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