LEAD CITRATE METHOD OF GUANYLATE CYCLASE AND ITS APPLICATION TO TISSUE RECEIVING RAPID FREEZE SUBSTITUTION FIXATION

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The precise establishment for the subcellular localization of guanylate cyclase would very much aid in clarifying a number of important involvements of cGMP metabolism on biological phenomena. Therefore, as reported, attempts were made to develop a cytochemical method for guanylate cyclase (GCase), using lead citrate as capture agent. Lead citrate acts as an inhibitor of GCase but 21.3% of its activity remains reserved in the tissue. The optimum pH examined on cytochemical sections was between 7.5 to 8.5, the manganese ion working as activator. In the rat retina given immersion fixation, the GCase activity was defined in the space of the disc membranes on the rod outer segment. However, after application of rapid freeze substitution fixation on the retina, the enzyme activity appeared at the cytoplasmic side of the disc membranes, showing good correlation with the biochemical estimations.

Guanylate cyclase (ED 4. 6. 1. 2) (GCase), which catalyzes guanosine triphosphate (GTP) to guanosine 3', 5'-monophosphate (cGMP) and liberates pyrophosphate, is found in a variety of tissues throughout the animal kingdom (4, 23, 27, 59, 67). In earlier reported attempts to separate guanylate cyclase, activity was detected in supernatant fractions (67), while in others it was found that the larger part of the activity was in particulate fraction (22, 23, 27), indicating that the activity of guanylate cyclase is made to move to the supernatant by Triton X-100 (24, 27). In the retina, the function of converting light stimuli to electric activities is said to depend deeply on the concentration of cGMP, the second messenger, to close the ion channel on the plasma membrane of the rod cells. Therefore, an understanding of the mechanism of cGMP function depends on the localization of GCase as well as PDEase in tissue cells. The cytochemical data are especially important, because this knowledge can prevent a disruption of the tissues. For the demonstration of GCase, the lead nitrate method has already

been reported (19). However, another capture agent, lead citrate, has received much attention since 1967 when Professor K. Ogawa and his coworkers introduced it in the field of enzyme histochemistry (34). Attempts have been made therefore, to demonstrate guanylate cyclase using lead citrate with 5'-guanylyl imidodiphosphate as substrate (13), wherein the reliability was checked, testing the inhibition rate by capture agent, susceptibility for fixation, optimal pH, and the effect of activators on immersion fixed tissues. The demonstration of ACase with lead citrate was also done for comparison with the pattern presented by GCase activity. Finally, the sites of the enzyme activity were compared between those found in the immersion fixed and the rapid freeze substituted rat retina (59, 61).

MATERIALS AND METHODS

The tissues of male Sprague-Dawley rats weighing about 200 g and sea urchin sperm (*Temnopleurus torcumcticus*) were used as materials. The rat tissues were fixed in 2% glutaraldehyde in a 0.1 M cacodylate buffer, pH 7.2 which contained 6-8% sucrose for 30 min at 4°C or fixed in 4% formaldehyde

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prepared from paraformaldehyde in the same buffer overnight at 4°C. After washing overnight in 0.1 M cacodylate buffer, pH 7.2, containing 8% sucrose at 4°C, 40 μ frozen or non frozen sections (Vibratome, Oxford; Microslicer, Dosaka EM) sections were made on each of the tissues. Rapid freeze substitution was done using Slammer (Polaron) as indicated before (59).

The sea urchin sperm were fixed free in 0.5, 1.0, and 2.0% glutaraldehyde in 0.1 M in cacodylate buffer, pH 7.2, with 8% sucrose for 5 min at 4°C, using centrifuge.

Cytochemistry

The incubation medium for the cytochemical demonstration of GCase consisted of a 40 mM Tris buffer, pH 7.8, 0.5 mM 5'-guanylyl-imidodiphosphate (GMP-PNP; tetralithium salt, Boehringer Mannheim Yamanouchi, Tokyo), 3.0 mM manganese chloride, 2.0 mM 3-isobutyl-1-methylxanthine (IBMX) (Aldrich Chemical Co. Inc. Milwaukee) or theophylline, 4.0 mM lead citrate (K & K Laboratories), 2.0 mM levo-*p*-bromotetramisole oxalate, and 6–8% sucrose. The incubation medium for adenylate cyclase (ACase) consisted of a 40 mM Tris Maleate buffer, pH 8.5, 0.5 mM 5'-adenylyl-imidodiphosphate (AMP-PNP; tetralithium salt, Boehringer Mannheim Yamanouchi, Tokyo), 7.0 mM magnesium sulfate, 0.5 mM (IBMX), 3.6 mM lead citrate, 1.0 mM levo-*p*-bromotetramisole oxalate, and 6–8% sucrose.

The tissues were incubated for 15–60 min at 37°C in a shaker water bath. At the end of the incubation, the tissues were post fixed in 1% osmium tetroxide (7) for one hr at 4°C, and then dehydrated in a series of ethanol and embedded in epoxy resin, Quetol 812 (32; Nisshin EM). Ultrathin sections, made by an LKB Ultratome, were stained with uranyl acetate and lead citrate (44), and were examined under a JEOL 100B, HU-12A, HU-700 or H-7000 electron microscope.

The control experiments were as follows; in the first, the tissues were incubated in the incubation medium without substrate. In the second, the concentration of manganese chloride, the activator of GCase, was changed from 1 to 3 mM (10). In the third, the pH of the incubation medium was changed from pH 6.0 to 9.5. In the fourth, complete incubation medium contained such inhibitors of alkaline phosphatase as 1–5 mM of ethylenediaminetetraacetic acid (EDTA), 1–3 mM of cysteine (6) or 0.01–0.2 mM of levo-*p*-bromotetramisole oxalate (BrTO; Aldrich Chem. Co. Inc., Milwaukee) (5) was added. In the fifth control, the substrate GMP-PNP was replaced by β -glycerophosphate (β -GP) to examine the contamination of alkaline phosphatase.

Enzymology

For biochemical estimations of GCase activity, sea urchin dry sperm was used. Seminal emission was induced by KCl to sea urchin, and collected on watch glasses. The cGMP content was measured by the radioassay method (39). Samples were incubated in 60 nM of GTP-³H (0.02 μ Ci per nM), 0.3 μ M of cGMP, 0.3 μ M of manganese chloride, 30 μ M Tris buffer, pH 7.7, and 10 to 30 μ g of enzyme protein in a total volume of 0.15 ml, for 15 min at 30°C, with constant shaking, and the reaction was stopped by boiling for 2 min. The whole reaction mixture was applied on a neutral aluminum oxide column. Then this column was washed with 0.05 M Tris buffer, pH 7.4, and drained onto a Dowex 1-X2 column and washed with 10 mM of 0.05 N formic acid. The cGMP was eluted with 6 ml of 0.2 M ammonium formate in 4.0 N formic acid and then radio activity of the samples was determined with a Beckman liquid scintillation spectrometer, LS-233.

RESULTS

Light and electron microscopical localization of GCase

Most of the control experiments of the lead citrate method were done on rat kidney. In the kidney, GCase activity was positive on the brush border of the proximal convoluted tubule and moderate activity was found on glomeruli (Fig. 1). In electron microscopy, the GCase activity was localized on the plasma membrane of the brush border on the proximal convoluted tubules (Fig. 2).

Substrate free control and the control experiments wherein the GMP-PNP was replaced by β -glycerophosphate

No staining was observed on sections incubated in the medium without substrate (Figs. 3, 4). The sections incubated in the medium which contained β -GP replacing GMP-PNP, gave a staining pattern typical of that of ALPase.

As the distribution pattern of GCase resembles that of ALPase, an inhibitor for ALPase BrTO was added to the medium in which GMP-PNP had been replaced by β -glycerophosphate as substrate. The enzyme activity was abolished from the medium with β -GP as substrate. However, as we already saw, the reaction product appeared positive in the medium in which GMP-PNP as substrate was not altered at all.

In rat retina, the GCase activity was positive on the rod outer segment. The site of enzyme activity was in the spaces of the disc membranes (Fig. 5). In the case of GCase, the distribution pattern is quite different from that of ACCase in which the activity was strong on the plasma membrane of the apical process

FIG. 1. GCase activity on the rat kidney cortex. Activities are seen positive on the proximal convoluted tubules. Moderate activities are also observed on the glomerulus. $\times 100$

FIG. 2. Electron micrograph of the rat kidney. GCase activity is seen on the plasma membrane of the brush border of the convoluted tubules. $\times 20,000$

FIG. 3. Substrate free control shows no activity on the proximal convoluted tubules of the rat kidney. $\times 280$

FIG. 4. Electron micrograph of the rat kidney proximal convoluted tubule, incubated in the medium without substrate. Reaction product is not seen on the plasma membrane of the brush border of the proximal convoluted epithelium. $\times 20,000$

FIG. 5. GCase activity in the rod outer segment of the rat retina, immersion fixed with 2% glutaraldehyde for 30 min at 4°C. The reaction product is positive in the cisternal space of the disc membranes. $\times 60,000$

FIG. 6. ACCase activity demonstrated in the rat retina. Strong enzyme activity is seen positive on the plasma membrane of the apical process of pigment epithelium. $\times 8,400$

of the pigment epithelium (Fig. 6).

Inhibition effect of lead ion on GCase

It had been reported that the use of lead ion for capture agent in ACCase histochemistry, based on the claim of complete inhibition by lead ion and also the non-enzymatic hydrolysis of AMP-PNP, has been rejected from biochemical and histochemical studies. However, in the present experiment, with regard to the use of lead ion as lead citrate, the matter of inhibition was reinvestigated by measuring the GCase activity on sea urchin sperm in the presence of various concentrations of lead. Sea urchin sperm was chosen for test material, because it is known to have a high GCase activity and it is in a free cell form on which uniform glutaraldehyde fixation can be certain. As Fig. 7 indicates, with the increase of lead citrate concentration in the medium, a suppression of GCase activity occurred in the tissues fixed in 2% glutaraldehyde for 5 min at 4°C. At 1×10^{-4} M of lead citrate, inhibition was as low as 14.4%; at 1×10^{-3} M, inhibition increased to 42.1%, in 4×10^{-3} M,

which is the concentration of lead citrate in the histochemical reaction medium, inhibition became 78.7%, leaving 21.3% enzyme activity; and in 1×10^{-2} M of lead ion, inhibition reached 84.4%, leaving 15.6% of GCase activity (Fig. 7).

Inactivation of GCase activity after glutaraldehyde fixation

After 5 min fixation by glutaraldehyde in a 0.1M cacodylate buffer, pH 7.2 with 8% sucrose at 4°C, a rapid fall in GCase activity was observed as shown in Fig. 8 (Table 1). An amount of activity as great as 82.6% was lost after fixation with 0.5% glutaraldehyde; with 1% glutaraldehyde fixation, inactivation reached to 88.6% and the inhibition rate reached 92.8% with 2% glutaraldehyde, leaving as small an activity as 7.1% of fresh sea urchin sperm.

Effect of pH on histochemical demonstration of GCase

The GCase activity, estimated by the reaction color on histochemical sections, is shown in Table 2 and in the series of pictures (Fig. 9). The maximal activity of GCase was observed between pH 7.5–8.5 as a function of pH, on rat kidney. However, when in the

FIG. 7. Effect of concentrations of lead citrate on the GCase activity of the sea urchin sperm. Ten to thirty μg of protein was incubated in a total volume of 0.15 ml of 60 nM of GT P^{-3}H (0.02 μCi per nM), 0.3 μM of cGMP, 0.3 μM of manganese chloride, 30 μM Tris buffer, pH 7.7 and increasing concentration of lead citrate, for 15 min at 30°C. The reaction was stopped by boiling for 2 min, and cGMP was measured as described in Materials and Methods.

medium, the substrate GMP-PNP was replaced by β -GP, and in the ALPase medium also, the optimal activity appeared at a higher pH range (Fig. 10; Table 2).

Effect of manganese ion

The biochemical data shows that GCase is activated with the manganese ion (Mn^{++}) (1, 3, 5). To clarify the effect of the manganese ion on histochemical sections, endogenous Mn^{++} ions were first removed applying preincubation in 1×10^{-3} M EDTA, for 30 min at 37°C, and then, the sections were incubated in the medium with Mn^{++} added in various concentrations. The enzyme activity was seen to be negative or slight on the sections incubated without the Mn^{++} ion. With the addition of Mn^{++} , however, positive activity appeared in the sections in concentrations higher than 3 mM (Table 3).

Effect of EDTA

The addition of EDTA (1–2 mM) into the standard medium had little effect on the GCase activity. But in the incubation medium in which substrate GMP-PNP was replaced by β -GP, EDTA blocked the reaction product formation at concentrations higher than 3 mM (Table 4).

Effect of cysteine and levo-*p*-bromotetramisole oxalate on GCase

Since some of the histochemical patterns of GCase closely resemble those of ALPase in the kidney,

TABLE 1. Effect of aldehyde fixation on GCase activity of sea urchin sperm

Aldehyde (%)	p mole/min/mg protein	%
Fresh	47,506.4	100.0
0.5	8,257.6	17.4
1.0	5,410.4	11.4
2.0	3,443.2	7.3

and it has also been known that the ALPase of *E. coli* could cleave to the terminal phosphate of adenylyl imidodiphosphate, producing ADP-NH₂ (63), we were anxious about mistaking ALPase for GCase. However, the addition of the two inhibitors, cysteine (1–5 mM), and L-*p*-BrTO (0.01–1.0 mM) to the GCase had no effect on its activity at all (Tables 5 and 6; Fig. 11). On the contrary, these inhibitors in the medium in which GMP-PNP was substituted by β -GP, abolished the enzymatic reactions completely, showing that the result of GCase was free from ALPase contamination (Fig. 12).

The sites of GCase and ACase on the rat retina

In the retina fixed with conventional immersion fixation with 2% glutaraldehyde in 0.1 M cacodylate

FIG. 8. Effect of glutaraldehyde concentrations for 5 min on GCase activity of sea urchin sperm.

FIG. 9. Effect of pH on GCase activity on histochemical reactions of the rat kidney. Maximal activity of GCase is between pH 7.5–8.5. a: 7, b: 8, c: 9

FIG. 10. Effect of pH in the medium in which substrate GMP-PNP was replaced by β -GP. Higher activity is seen in the section incubated at higher pH. a: 7, b: 8, c: 9

TABLE 2. *Effect of pH on GCase activity*

	6.0	6.5	7.0	7.5	8.0	8.5	9.0	9.5
Guanylate cyclase	— ~ $\pm$	$\pm$	+	++	++	++	+	+
β -Glycerophosphate	—	$\pm$	$\pm$ ~ +	+	+	+	+	+
Alkaline phosphatase	—	—	$\pm$	$\pm$ ~ +	+	+	++	++

TABLE 3. *Effect of manganese ion on GCase activity*

	Mn ²⁺ mM						
	0	0.5	1.0	3.0	5.0	7.0	9.0
Guanylate cyclase	—	$\pm$	+	+ ~ ++	+ ~ ++	+ ~ ++	+ ~ ++

buffer, pH 7.2, for 30 min at 4°C, it was found that the GCase and ACase activities gave different staining patterns. The GCase activity in the retina was positive on the rod outer segment of the visual cell, and the ACase activity was strong on the apical process of the pigment epithelium. The ultrastructural site of GCase is in the space of disc membrane, and that of ACase is on the plasma membrane of the apical process (Figs. 5, 6).

After freeze substitution in 0.5–1.0% glutaraldehyde with 6% tannic acid in acetone at –80°C for 1 hr, the reaction product of GCase was shown clearly on the disc membranes, tightly parallel and leaving no space in the center at the cytoplasmic side in the rod outer segment (Fig. 13).

TABLE 4. *Effect of EDTA on GCase and ALPase*

	EDTA mM				
	1	2	3	4	5
Guanylate cyclase	++	++	+	+	+
Alkaline phosphatase	++	±	–	–	–

DISCUSSION

The histochemical method of ACase (26), and its improvements were also reported (19) by modifying the ATPase medium of Wachstein and Meisel (65). ACase has been investigated in a variety of tissues,

FIG. 11. Effect of *L-p*-BrTO, a potent inhibitor of ALPase, on GCase of the rat kidney. No effect of *L-p*-BrTO is observed. a: 0.01 mM, b: 0.1 mM, c: 1.0 mM

FIG. 12. Effect of *L-p*-BrTO on the medium of GCase, in which substrate GMP-PNP is replaced by β -GP. Marked inhibition of activity is in higher than 0.1 mM. a: 0.01 mM, b: 0.1 mM, c: 1.0 mM

TABLE 5. *Effect of cysteine on GCase activity*

	Cysteine mM					
	None	1	2	3	4	5
Guanylate cyclase	++	+ ~ ++	+ ~ ++	+ ~ ++	+ ~ ++	+ ~ ++
β -Glycerophosphate	++	+ ~ ++	$\pm$	$\pm$	—	—

TABLE 6. *Effect of levo-p-BrTO on GCase activity*

	mM			
	None	Preincubation 1.0	0.1	0.2
Guanylate cyclase	++	++	+ ~ ++	+ ~ ++
β -Glycerophosphate	++	++	+	— ~ $\pm$

FIG. 13. GCase activity on rod outer segment of the rat retina, quick freeze substitution fixed. The enzyme activity is in the cytoplasmic side of the disc membranes.

such as the liver, skeletal and cardiac muscles, and the fungus using ATP as substrate (2, 11, 16, 26, 28, 30, 43, 58, 60, 64, 66).

In 1975, the first attempt on GCase demonstration was reported (53, 54). GCase activity was reported in the rat retina and some other tissues (19,

20, 29, 40, 63, 69). However, LeMay and Jarett (1975) claimed that in the use of the lead ion as capture agent in ACCase histochemistry, the lead ion not only brings about a complete inhibition of the enzyme activity, at the concentration in the reaction medium, but it further acts to catalyze the non enzymatic degradation of the substrate (31). From this point of view, they had concluded that the precipitates appearing after the histochemical incubation on the tissue section were merely non-enzymatic artifact. Similar discussions occurred on the demonstration of $\text{Na}^+ \text{--} \text{K}^+ \text{--} \text{ATPase}$ activity (14, 15, 21, 33, 36, 37, 49, 50, 62). However, Cutler (1975) (12), and Cheng and Farquhar (1975) using *D. discoidium*, human platelets, and rat submandibular gland, found that in the ACCase activity still remaining in the tissue after fixation with glutaraldehyde and during incubation in the presence of lead, there was no detectable non-enzymatic breakdown of substrate and they concluded that the histochemical activity demonstrated on the sections could be reasonably estimated to be ACCase activity (8, 9). The histochemical method for GCase was reported in 1981 by Fujimoto, Toibana and Ogawa (1981), and the inhibitory effect of lead ion was examined, and it was shown that the degree of inhibition was not complete, and that enough enzyme activity remained for the demonstration of cytochemistry (19).

For demonstration of enzyme activity, it is important to select a proper capture agent to get a satisfactory result. On this viewpoint attempts were reported, introducing cadmium, strontium, barium, and cerium (52). Of these metals, cerium is shown to be excellent in the precise localization of reaction product at the ultrastructural level (45–48). Its disadvantage is that it is rather difficult for developing color in

light microscopy (41). Considering lead compounds, besides lead nitrate, lead citrate was introduced to demonstrate enzyme activity at higher pH of ALPase by Professor Ogawa in 1967 (34). Since then, the excellence of lead citrate was proved on other enzymes such as fructose 1, 6-diphosphatase (56), $\text{Na}^+ - \text{K}^+ - \text{ATPase}$ (35), $\text{Ca}^{++}\text{ATPase}$ (1), etc. Therefore, in this report, other attempts to demonstrate GCase were made using lead citrate as capture agent, with 5'-GMP-PNP as substrate. The matter of the inhibitory activity on GCase, was measured on the homogenate of fresh and fixed sea urchin sperm, using radio assay. At 4×10^{-3} M of lead citrate, which is the concentration in the GCase medium, 28.4% of the GCase activities remained, which may be enough to demonstrate enzyme activity cytochemically. More drastic inactivation of GCase was encountered from the sea urchin sperm fixed in 2% glutaraldehyde for 5 min, leaving 21.3% activity in fresh material. To fix a free or cultured cell, the fixation inactivation is usually excessive, compared with immersion fixation, or perfusion fixation. Therefore, GCase activity in the tissue fixed in block more certainly remains in the tissue. Despite the inhibitory effects of lead ion on GCase activity, between 20 to 30% of activity remains, which is sufficient for histochemistry.

The optimum pH examined as pH 7.8, in GCase with lead citrate, and 3 mM Mn^{++} ion as activator, are in good agreement with biochemistry (18). In the medium with lead nitrate, precipitate usually appeared in the medium at a higher pH, but with the use of lead citrate, an almost clear medium appearing at pH 7.8 could be obtained. To avoid a non-specific precipitate, it is important to use a clear medium in histochemistry.

The GCase activity in the kidney was located on the brush border, the distribution site being similar to that of ALPase. *E. coli* ALPase is known to hydrolyze ATP (25) and the terminal phosphate group of AMP-PNP (68). For this reason, the contamination by ALPase was feared in the reaction product of GCase. But *L-p*-BrTO, a potent inhibitor of ALPase, was available to add into the GCase medium (5). On the other hand, in the sections incubated in the medium, β -GP as substrate replacing GMP-PNP, with *L-p*-BrTO, the reaction product formation was completely inhibited. It is thus reasonable to conclude that there was no contamination by ALPase in the GCase activity demonstrated by the lead citrate medium. These conclusions are supported further by the inhibition data of EDTA and cysteine on the media with β -GP and with GMP-PNP.

Unlike ACase, which is considered to be totally

particulate, the major portion of GCase appears to reside in the soluble fraction after the homogenization of most tissues, but there was still some detectable activity in the particulate fraction (39). Since the histochemical sites of GCase are on the plasma membrane, one plausible interpretation of these varying patterns is that GCase may be associated with cellular membranes *in situ* but in some tissues may easily undergo dissociation during the homogenization of fractionation. The increase in enzyme activity appearing after Triton X-100 might support this possibility (38). In the present observation, GCase activity, in the space of the disc membrane of the rod outer segment after immersion-fixed rat retina, became positive at the cytoplasmic side of the disc membranes, in the quick freeze substitution fixed tissue (57). It is reasonable to think that the GCase molecule, which may alter its site during aldehyde fixation, may be stopped of all molecular movement owing to its rapid freezing.

Producing cGMP, GCase is important in cGMP metabolism as well as phosphodiesterase, which decomposes cGMP (3, 17, 42). At photoreception, the rapid fall in cGMP concentration by phosphodiesterase is the key role to close the ion channel in the plasma membrane to produce action potential. Therefore, the site of GCase is important in considering the function of the visual cells. The high GCase activity found in the rod outer segment is in good agreement with the biochemical data (17, 42). Considering the site of cGMP production, as obtained from tissue having received immersion fixation, i.e. the inside of disc membrane, it is difficult to explain, but some modification might occur in the membrane during fixation.

From the consideration of fixation, to keep better morphology as well as to keep as much enzyme activity as possible in the cell, a better technique of fixation was required, a process of fixation preventing the moving of molecules to preserve their living metabolic conditions (51, 55). To this end, the new technique, freeze substitution fixation, developed in our laboratory is useful because all molecular movement can be stopped by quenching within msec, with a reduction in enzyme activity at a minimum (57, 61). The correct determination of the sites of molecules in biological membranes would very much aid in clarifying important involvements of cGMP metabolism as an intracellular effector of messenger for extracellular biological signals.

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