Preservation of Sodium-Dependent Iodide Transport Activity By Methimazole and Mercaptoethanol in Phospholipid Vesicles Containing Thyroid Plasma Membranes: with Evidence of Difference in the Action of Perchlorate and Thiocyanate

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Abstract

The effect of methimazole (MMI) and 2-mercaptoethanol (ME) on I^- transport was studied using phospholipid vesicles (P-vesicles) made from porcine thyroid plasma membranes and soybean phospholipids by sonication.

1. When buffer solutions contained either 1 mM MMI or 2 mM ME, I^- uptake by P-vesicles in the presence of external Na^+ was apparently higher than that in the absence of external Na^+ . Na^+ -dependent I^- uptake was inhibited by both ClO_4^- and SCN^- added externally.

2. When PM was treated with 4 mM *N*-ethylmaleimide prior to preparation of P-vesicles, the activity of Na^+ -dependent I^- transport was completely lost even when P-vesicles were incubated in the presence of ME.

3. When neither MMI nor ME was added to buffers, I^- uptake in the presence of external Na^+ was not at all higher than that in the absence of external Na^+ . In these instances, however, I^- uptake was much higher compared than the baseline uptake in the presence of MMI or ME, and was inhibited by external SCN^- and not by ClO_4^- without relation to external Na^+ .

These data indicate that MMI or ME has two distinct effects on our model system of I^- transport. The one is preservation of the Na^+ -dependent I^- transport activity by protecting a sulfhydryl group, and the other is reduction of nonspecific I^- binding to P-vesicles. In addition, ClO_4^- is a more specific inhibitor of thyroid I^- transport than SCN^- , when non-specific I^- oxidation is imperfectly prevented.

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²Abbreviations used are: ME, 2-mercaptoethanol; MMI, methimazole; NEM, *N*-ethylmaleimide; PM, plasma membranes; PTU, propylthiouracil; P-vesicles, phospholipid vesicles.

A theory of Na^+ - I^- cotransport in the thyroid has been proposed on the basis of some experiments using rabbit thyroid slices (Iff and Wilbrandt, 1963), cultured bovine thyroid cells (Bagchi and Fawcett, 1973) and a unique thyroid cell line (Weiss *et al.*, 1984). Recently, a biological model

of the thyroid I^- transport system was developed by the authors (Saito *et al.*, 1982, 1983a, 1983b, 1984, 1985). The model consists of phospholipid vesicles (P-vesicles²⁾) made from the thyroid plasma membranes (PM) and crude soybean phospholipids by sonication. On the basis of the studies using this model system, a specific I^- carrier exists separately from Na^+ - K^+ -ATPase in the thyroid PM. The I^- carrier, a protein in nature, can transport I^- from the external to the internal vesicular space down the concentration gradient of Na^+ . We now propose that I^- transport mediated by this carrier is Na^+ - I^- cotransport. The role of Na^+ - K^+ -ATPase in I^- transport in the thyroid cell is only to pump out Na^+ , generating a concentration gradient of Na^+ across PM.

It is generally believed that thionamide drugs, such as methimazole (MMI) and propylthiouracil (PTU), block peroxidase-catalyzed organic binding of I^- but do not inhibit I^- transport in the thyroid gland. Therefore, they are frequently used in studies of thyroidal I^- transport in order to keep intrathyroidal I^- unbound (Wolff, 1964; Bastomsky, 1974). There are some reports indicating that the drugs may alter I^- kinetics perhaps without decreasing I^- influx. PTU does not decrease I^- uptake but rather increases the thyroid/serum ratio of the unbound I^- concentration *in vivo* (Wollman and Scow, 1953, 1955; Wollman and Reed, 1958). The reason may be that PTU (Wollman and Reed, 1962; Newcomer, 1967) and MMI (Resenberg *et al.*, 1964) decreases thyroid I^- efflux. In this paper we describe the effects of MMI and ME on our model system of I^- transport. In this study, PTU was not used because of low solubility, and ME was used as another reducing agent.

Materials and Methods

Porcine thyroid PM were prepared according to the method described previously (Saito *et al.*, 1982). Phospholipids were prepared from crude soybean phospholipids (L-phosphatidyl choline type IV-S, Sigma) after washing in acetone as described elsewhere (Saito *et al.*, 1982). In the present study, ME was omitted at each step of phospholipid preparation.

P-vesicles were prepared by a sonication technique (Saito *et al.*, 1982). The frozen PM (20 mg protein) were washed once in the choline⁺ buffer (175 mM choline-Cl, 30 mM Tris-HCl and 1.5 mM $MgSO_4$, pH 7.4) and resuspended in 2 ml of the same buffer. The PM suspension was put into a tube containing 100 mg phospholipids and sonicated with a Branson ultrasonic oscillator using 50% duty cycle pulses (20 kHz, 40 watts) for a net 6 min. The resulting clear suspension of P-vesicles was quickly frozen at $-80^\circ C$ and kept for some days (less than a week) until transport assay.

When the effect of *N*-ethylmaleimide (NEM) was studied, washed PM were incubated in 5 ml of the choline⁺ buffer containing 4 mM NEM at $25^\circ C$ for 20 min, while control PM were incubated in the same way with the exception that neither MMI, ME nor NEM was added to the buffer. Then both the control and NEM-treated PM were washed twice with the choline⁺ buffer without MMI, ME or NEM, and then sonicated with phospholipids.

The frozen P-vesicle suspension was thawed at $37^\circ C$ and then kept standing in an ice-water bath. In order to replace the extravesicular buffer solution by a following solution, the suspension (125 μ l) was applied to the first Sephadex G-50 column packed in a disposable plastic syringe. The column was equilibrated with a choline⁺ or Na^+ buffer (175 mM NaCl, 30 mM Tris-HCl and 1.5 mM $MgSO_4$, pH 7.4), which was intended to occupy the extravesicular space during incubation. The bed volume of the column was uniformly adjusted to 2.5 ml in order to obtain a constant factor for P-vesicle dilution. The suspension was allowed to enter the column for 20 sec. Then 100 μ l of the same buffer that equilibrated the column was added. The drops of the buffer solution flowing out of the column were discarded. The syringe containing the column with P-vesicles was then

placed in a plastic tube set in a table type centrifuge. The plastic tube had contained an incubation medium, 0.5 ml of the choline⁺ or the Na⁺ buffer, containing Na¹²⁵I and KI at a final concentration during incubation of 1 μ M. KClO₄ or KSCN was also added to the medium at a final concentration of 1 mM if necessary. Centrifugation was started just 1 min after applying the P-vesicle suspension to the column. Centrifugation was carried out in such a manner that the revolution speed reached 3,500 rpm in 15 sec and remained at that level for another 30 sec. P-vesicles, diluted to 1 ml with the buffer solution contained in the column, were spun down through the column into the plastic tube containing the incubation medium, and incubation was started. The time at which centrifugation was started was designated as "time zero" of incubation. Incubation was carried out at room temperature. At an indicated incubation time, 125 μ l aliquots of the incubation mixture were removed and applied to the second Sephadex G-50 column under the same conditions as the first column. Then the column was centrifuged after adding 100 μ l of the same buffer solution. Radiation in the P-vesicles spun down through the column after removal of extravesicular ¹²⁵I⁻ was measured.

When the effects of MMI and ME were tested, they were added to all the buffer solutions at concentrations of 1 and 2 mM, respectively, throughout the series of experiments.

Assays were carried out in quadruplicate and the results are presented as means $\pm$ SD for 4 assays.

Results

As shown in Fig. 1, Na⁺-dependent I⁻ transport was demonstrated in P-vesicles containing PM in the condition in which 1 mM MMI was present. When P-vesicles were incubated in choline⁺ (Fig. 1B), they took up 0.57 ± 0.03 pmol I⁻/mg protein at 2 min incubation. Externally added ClO₄⁻ and SCN⁻ did not influence I⁻ uptake by P-vesicles. When incubation was carried out in the presence of external Na⁺ (Fig. 1A), P-vesicles took up 1.51 ± 0.06 pmol I⁻/mg protein, apparently more than that in the absence of external Na⁺. External ClO₄⁻ and SCN⁻ depressed this level of I⁻ uptake to that in the case of incubation without external Na⁺.

In the condition in which 2 mM ME is present instead of MMI, Na⁺-dependent I⁻ transport was also observed in P-vesicles (Fig. 2). The level of I⁻ uptake and the effect of ClO₄⁻ or SCN⁻ were observed to

Fig. 1. I⁻ uptake by P-vesicles incubated in the presence of external Na⁺ (A) and external choline⁺ instead of Na⁺ (B). Preparation and incubation of P-vesicles were carried out in the condition in which 1 mM MMI was added to all buffer solutions. P-vesicles were incubated without any inhibitor (○), with external 1 mM ClO₄⁻ (□) and SCN⁻ (Δ).

Fig. 2. I^- uptake by P-vesicles incubated with external Na^+ (A) and without external Na^+ (B). P-vesicles were prepared and incubated in the condition in which 2 mM ME was added to all buffer solutions. P-vesicles were incubated in the absence of any inhibitor (○), in the presence of external 1 mM ClO_4^- (□) and SCN^- (△).

Fig. 3. Effect of NEM treatment of PM on Na^+ -dependent I^- uptake by P-vesicles. Thyroid PM was incubated in 4mM NEM at 25°C for 20 min prior to preparation of P-vesicles. P-vesicles prepared from control (A) and from NEM-treated PM (B) were both incubated in the Na^+ buffer containing 2 mM ME in the absence of any inhibitor (○) and in the presence of external 1 mM ClO_4^- (□). ME was not added to the choline⁺

buffer during preparation of P-vesicles, while it was added to the Na^+ buffer occupying the extravascular space during incubation.

be similar to that in the vesicles incubated in the presence of MMI as shown in Fig. 1.

The effect of NEM treatment was studied and is shown in Fig. 3. Control P-vesicles, prepared in the absence of MMI or ME, were incubated in the Na^+ buffer containing

ME, and they took up 1.75 ± 0.09 pmol I^- /mg protein at 2 min, while they took up 0.69 ± 0.03 in the presence of external 1 mM ClO_4^- (Fig. 3A). P-vesicles made from PM priorly treated with 4 mM NEM for 20 min took up 0.76 ± 0.03 pmol I^- /mg protein at 2 min during incubation in the Na^+ buffer

Fig. 4. I⁻ uptake by P-vesicles incubated in Na⁺ (A) and in choline⁺ instead of Na⁺ (B). Neither MMI nor ME was added to incubation buffers throughout the experiment. Note that the scale in this Figure is 5 times that in Figs. 1 to 3. P-vesicles were incubated in the absence of any inhibitor (○), in the presence external 1 mM ClO₄⁻ (□) and SCN⁻ (Δ).

with ME. The I⁻ uptake was not at all higher than that of 0.80 ± 0.05 pmol/mg protein in the case of inhibition of specific I⁻ transport by externally added ClO₄⁻ (Fig. 3B).

As shown in Fig. 4, P-vesicles took up much more I⁻ in the absence of MMI or ME than in the presence of the reducing agent. When P-vesicles were incubated in the presence of external Na⁺ (Fig. 4A), they took up 3.38 ± 0.20 pmol I⁻/mg protein at 2 min. This I⁻ uptake was inhibited by externally added SCN⁻ (1.44 ± 0.07 pmol/mg protein) but not by ClO₄⁻ (3.36 ± 0.24 pmol/mg protein). When P-vesicles were incubated without external Na⁺ (Fig. 4B), the level of I⁻ uptake by P-vesicles was 3.40 ± 0.21 pmol/mg protein, which was nearly the same as that in the case of the persence of external Na⁺. In this particular case, SCN⁻ also decreased the I⁻ uptake, but ClO₄⁻ did not.

When P-vesicles prepared from only soybean phospholipids were incubated in the absence of reducing agents, I⁻ uptake was also greater than that in the presence of MMI or ME. The I⁻ uptake was also inhibited by SCN⁻ and not by ClO₄⁻. This

phenomenon was similar in both the Na⁺ and the choline⁺ buffers.

Discussion

The authors have been studying the thyroid I⁻ transport system using P-vesicles made from porcine thyroid PM and soybean phospholipids by sonication (Saito *et al.*, 1982, 1983a, 1983b, 1984, 1985). The I⁻ carrier can mediate Na⁺-I⁻ cotransport, as has been postulated on the basis of experiments using thyroid tissue (Iff and Wilbrandt, 1963; Bagchi and Fawcett, 1973; Weiss *et al.*, 1984). The energy source for I⁻ accumulation in the thyroid cells may be a concentration gradient of Na⁺ formed by Na⁺-K⁺-ATPase.

Although it is evident that MMI is not necessary for preservation of the I⁻ transport activity in the thyroid gland, MMI has frequently been used in studies on I⁻ transport in thyroid tissue (Ahn and Rosenberg, 1970; Bagchi and Fawcett, 1973). The use of MMI is recommended for the reason that the movement of only unbound I⁻ can be observed, since it blocks

peroxidase-catalyzed I^- organification without inhibiting specific I^- transport (Wolff, 1964; Bastomsky 1974). Of course, I^- transport in the thyroid tissue has also been observed without using MMI, when both I^- transport and organic binding of I^- are intended to be investigated at the same time (Ahn and Rosenberg, 1970; Saito *et al.*, 1981). But such cases are rather rare.

It has been shown that reducing agents, MMI and ME, do not inhibit Na^+ -dependent I^- transport in our P-vesicles (Saito *et al.*, 1982, 1983a, 1983b, 1984, 1985). In view of the results of the present study, it is obvious that MMI or ME is necessary for the I^- carrier to preserve the action of Na^+ -coupled I^- transport. In P-vesicles prepared without MMI or ME, the activity of Na^+ -dependent I^- transport is recovered, if ME is added later at the beginning of incubation. It is certain that a reducing agent is necessary at the site of interaction between the I^- carrier and I^- including Na^+ and phospholipids. In addition, NEM treatment of PM resulted in complete inactivation of Na^+ -dependent I^- transport. This observation is consistent with the idea that the I^- carrier in the thyroid PM has at least one active sulfhydryl group, as such a theory has been proposed in a certain sugar transport system (Henderson *et al.*, 1983). The role of MMI and ME in maintaining the action is to protect this sulfhydryl group. Although there has been a paper suggesting that there exists a sulfhydryl group in the I^- transport system in the thyroid gland of mice (Anbar and Inbar, 1964), this has not yet been confirmed, since there are many active proteins containing sulfhydryl groups other than the I^- carrier in the whole thyroid cell. Therefore, the observation indicating the presence of a sulfhydryl group in the carrier in this study is a new finding that cannot be obtained without using our P-vesicle system. It is probable that there exists a mechanism supplying a natural reducing

substance to the I^- transport system in the physiological thyroid cells. The I^- -trapping defect may be due to the impairment of such a mechanism, although it is more probable that the disorder is due to structural impairment or loss of the I^- carrier.

Although the authors were not aware of the importance of MMI or ME in preserving Na^+ -dependent I^- transport in earlier studies (Saito *et al.*, 1982, 1983a, 1983b, 1984, 1985), ME was added to the phospholipid preparation. The final concentration of ME in the incubation mixture was at least as high as 0.35 mM. That is enough for the I^- carrier to maintain the Na^+ -dependent I^- transport activity. In the present study, Na^+ -dependent I^- transport was observed at an ME concentration as low as 0.2 mM, although non-specific I^- uptake (see below) was also observed to a small extent (data not shown).

Rapid I^- uptake and a high equilibrium level were observed in the absence of MMI and ME. High I^- uptake in this case may not be due to enhanced I^- transport mediated by the I^- carrier, since specific characteristics such as Na^+ dependency and inhibition by ClO_4^- are lost. In addition, it has been confirmed that P-vesicles made from only soybean phospholipids without thyroid PM components take up more I^- in the absence of any reducing agent than in the presence of ME. The high equilibrium level shown in Fig. 4 indicates that I^- taken up by P-vesicles is bound to something composing the vesicles, since I^- is not concentrated inside the vesicles at the time of equilibrium because of the loss of specific I^- transport. Therefore a portion of the I^- may have been oxidized non-enzymatically and subsequently bound to phospholipids (and protein?) covalently. Such a theory has been proposed by Posner and Ordoñez (1970) on the basis of an experiment using thyroid phospholipids. They

have shown that I⁻ binding to phospholipids is enhanced by adding Fe⁺⁺⁺ and prevented by PTU. Thus, MMI and ME may decrease non-specific I⁻ binding to P-vesicles by inhibiting non-enzymatic oxidation of I⁻.

The authors once reported a trial of solubilization and reconstitution of the I⁻ carrier (Saito *et al.*, 1986). We proposed that the activity of I⁻ counterflow which might contain a disulfide bond was reconstituted into P-vesicles. But the idea should be revised, since specific I⁻ transport was recovered by adding ME in the present study. Non-specific I⁻ binding must have been observed in the experiment on solubilization because of the omission of ME. Solubilization and reconstitution of the I⁻ counterflow activity with a disulfide bond has not yet been established, but it has been confirmed that the I⁻ carrier in sonicated P-vesicles can actually mediate I⁻ counterflow in the presence of ME (Saito *et al.*, 1989).

When P-vesicles prepared without thyroid PM are loaded with a high concentration of stable I⁻ prior to incubation, they take up more labeled I⁻ than P-vesicles not loaded with stable I⁻ in the absence of any reducing agent (unpublished observation). The high I⁻ uptake in this instance cannot be explained by covalent I⁻ binding to phospholipids alone, since I⁻ binding sites of phospholipids should have been occupied with stable I⁻ before incubation. Therefore, I⁻ can readily enter P-vesicles through phospholipid bilayer in the absence of MMI or ME, although it is not clear whether I⁻ permeability increases with or without relation to covalent I⁻ binding to phospholipids. It is possible that covalent binding of I⁻ to phospholipids is reversible, readily resulting in the exchange of bound I⁻ for unbound I⁻. In any case, MMI as well as ME decreases the permeability of I⁻ through the bilayer in addition to inhibition of covalent I⁻ binding to lipids without relation to enzymatic I⁻ organi-

fication in our model system.

There have been some reports stating that PTU (Wollman and Reed, 1962; Newcomer, 1967) and MMI (Rosenberg *et al.*, 1964) decrease I⁻ efflux from the thyroid, resulting in an increase in the thyroid/serum ratio of the I⁻ concentration in *in vivo* experiments (Wollman and Scow, 1953, 1954; Wollman and Reed, 1958). I⁻ efflux has been considered to be reduced as a result of the inhibition of enzymatic I⁻ organification. But another explanation for the increased I⁻ concentration ratio in these *in vivo* experiments can be introduced when the present data are applied. There is usually a considerable amount of I⁻ leakage through the phospholipid bilayer in addition to I⁻ binding to the bilayer of PM, if insufficient reducing agents are present, since the intracellular I⁻ concentration is higher than that outside. The leakage decreases without interfering with the I⁻ carrier, when PTU or MMI is administered, since MMI decreases permeability. We think that an increase in the thyroid/serum ratio of the I⁻ concentration occurs without relation to enzymatic I⁻ organification, since MMI decreases I⁻ permeability in our P-vesicles in which peroxidase cannot act because of the absence of H₂O₂. In any case, the use of MMI in experiments on I⁻ transport using thyroid tissue is recommendable in order to obtain accurate data, by decreasing I⁻ movement not mediated by the carrier in addition to blocking enzymatic I⁻ organification.

ClO₄⁻ and SCN⁻ are both seen to be specific competitive inhibitors of thyroid I⁻ transport on the basis of Lineweaver-Burk plot analysis using sheep thyroid slices (Wolff and Maurey, 1963) and our P-vesicle system (Saito *et al.*, 1983b). But there was a difference in their actions in the present study. ClO₄⁻ inhibited only Na⁺-dependent I⁻ movement, while SCN⁻ inhibited non-specific I⁻ uptake by P-vesicles in the absence of ME in addition to inhibition of

Na⁺-dependent I⁻ transport demonstrated in the presence of ME. Although the precise mechanism of the effect of SCN⁻ remains to be elucidated, diminution of nonspecific I⁻ uptake by SCN⁻ may be related to oxidation or reduction of I⁻, since no such effect was observed in the presence of MMI or ME in the present study. Maloof and Soodak (1966) reported that SCN⁻ can be oxidized and that I⁻ and SCN⁻ oxidation are inhibited by each other. It is probable that the high concentration of SCN⁻ acts as a reducing agent preventing non-enzymatic I⁻ oxidation. Therefore, ClO₄⁻ is better than SCN⁻, when these anions are used as blockers of specific I⁻ transport in the thyroid. SCN⁻ should not be used as a blocker of thyroidal I⁻ transport in the condition in which non-specific I⁻ oxidation is imperfectly prevented.

In conclusion, MMI as well as ME has two distinct effects in our model system of thyroid I⁻ transport. The one is preservation of the Na⁺-dependent I⁻ transport activity, by protecting an active sulfhydryl group in the I⁻ carrier from oxidation. The other is diminution of nonspecific I⁻ binding to phospholipids (and protein?) other than the carrier, by inhibiting non-specific or non-enzymatic I⁻ oxidation. Although both ClO₄⁻ and SCN⁻ act as specific competitive inhibitors of thyroid I⁻ transport, SCN⁻ has an additional effect in the absence of reducing agents. SCN⁻ may inhibit nonspecific I⁻ binding to something composing P-vesicles, presumably by acting as a reducing agent inhibiting non-enzymatic I⁻ oxidation. This idea may be applied to the whole thyroid cells or tissue.

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