Uptake of Methylchlorpromazine by Brush-Border Membrane Vesicles from Rat Small Intestine[1)]

Hiroshi SAITOH[2)] and Katsumi MIYAZAKI*

Department of Pharmacy, Hokkaido University Hospital, School of Medicine, Hokkaido University, Kita-14-jo, Nishi-5-chome, Kita-ku, Sapporo 060, Japan. Received October 16, 1996; accepted February 20, 1997

The uptake of methylchlorpromazine (MCP), a quaternary derivative of chlorpromazine, was investigated using brush-border membrane vesicles isolated from rat small intestine. MCP was taken up rapidly by the vesicles, a major part of the uptake being due to binding to the membrane. Saturable MCP binding to the brush-border membrane was inhibited strongly by chlorpromazine, moderately by propantheline and imipramine, and slightly but significantly by methylbenactyzine and mepenzolate. However, choline and tetramethylammonium failed to exhibit any such inhibitory effect. The movement of MCP into the intravesicular space was driven by an inside-negative transmembrane electrical potential difference (TEPD) induced by NaSCN or valinomycin. There was no effect of TEPD on MCP binding to the brush-border membrane. The data suggested that both rapid binding to the brush-border membrane and inside-negative TEPD, which is present physiologically across the membrane, play a significant role in the absorptive movements of MCP across intestinal epithelium.

Key words methylchlorpromazine uptake; membrane binding; membrane potential; brush-border membrane; rat small intestine

Using brush-border membrane vesicles from rat small intestine, we previously demonstrated that propantheline, a quaternary ammonium compound (QAC), could permeate the membrane driven by an inside-negative transmembrane electrical potential difference (TEPD).[3)] Since TEPD is present physiologically across the brush-border membrane of intestinal epithelium (cell interior negative), the transport mechanism of propantheline implied its good absorption. However, this QAC was poorly absorbed from rat intestine *in situ*,[4)] indicating that the *in vitro* transport characteristics of propantheline were not directly related to its *in situ* absorption behavior. We have already demonstrated that gastrointestinal mucin is not a major factor in the poor absorption of QACs.[4)]

The presence of an intestinal secretory mechanism which limits the absorption of several QACs has been shown in guinea pig jejunum by Turnheim and Lauterbach.[5)] In a recent paper using excised rat intestine, it was demonstrated that propantheline as well as verapamil is transported much more in the secretory direction than in the absorptive direction.[6)] The secretory-oriented movements of propantheline were found to be mediated by intestinal P-glycoproteins. It has been clarified that intestinal P-glycoproteins are involved in the low oral bioavailability of various compounds.[7-14)] Accordingly, a hypothesis could be offered that propantheline can enter into the cytosol, utilizing the physiological condition of the cell interior being physiologically negative, and then this drug would again be pumped out from the cytosol into intestinal lumen by P-glycoproteins. It is possible that bidirectional movements of propantheline in the absorptive and secretory directions across the brush-border membrane are related to its poor absorption. We observed that the serosal-to-mucosal permeation of propantheline in excised rat intestine was inhibited by various QACs such as methylscopolamine, methylbenactyzine, and mepenzolate (unpublished data). Therefore, various QACs are considered to be common substrates for intestinal P-glycoprotein. However, findings so far are not sufficient to draw a conclusion that QACs can commonly permeate the brush-border membrane driven by TEPD, because our previous studies demonstrated that the uptake of low molecular QACs such as choline and paraquat by brush-border membrane vesicles was not facilitated by TEPD.[15,16)] In this study, we investigated the uptake of methylchlorpromazine (MCP), a quaternary derivative of chlorpromazine, into rat small intestinal brush-border membrane vesicles in order to clarify whether TEPD commonly functions as a driving force for the absorptive movements of relatively larger molecular QACs across the brush-border membrane. There has been a recent report on the absorption of MCP by Kitagawa *et al.*,[17)] indicating that MCP was absorbed to a much lesser extent in rat small intestinal everted sacs.

MATERIALS AND METHODS

Materials [^{14}C]MCP iodide salt was synthesized by New England Nuclear Co. (Boston, MA, U.S.A.). Chlorpromazine hydrochloride, methyl iodide, and valinomycin were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Unlabeled MCP iodide salt was synthesized in our laboratory from chlorpromazine and methyl iodide according to Huang *et al.*[18)] All other chemicals were of the highest grade commercially available and were used without further purification.

Preparation of Brush-Border Membrane Vesicles Brush-border membrane vesicles were isolated from the entire small intestine of male Wistar rats (250—300 g) according to the calcium chloride precipitation method.[19)] The small intestine was excised under ether anesthesia and washed in ice-cold saline. After cutting it longitudinally, the mucosa was gently scraped off with a glass microscope slide. The scrapings (*ca.* 4 g wet weight from 3—4 rats) were homogenized with a Waring blender (Nihon Seiki, Japan) at 16500 rpm for 4 min in 100 ml of 50 mM D-mannitol, 2 mM Tris/HCl, pH 7.1. $CaCl_2$ solution (0.5 M) was added to the homogenate to a final concentration of

* To whom correspondence should be addressed.

10 mM, and the homogenate was kept on ice for 20 min. The homogenate was then centrifuged at $3000 \times g$ for 15 min in a Hitachi 20PR-52 high speed refrigerated centrifuge (rotor; RPR 20-2). The supernatant fluid was centrifuged at $27000 \times g$ for 30 min. The resulting pellet was suspended in 40 ml of an experimental buffer (100 mM D-mannitol, 20 mM 2-(*N*-morpholino) ethanesulfonic acid (Mes)/Tris, pH 6.5) in a glass/Teflon Dounce-type homogenizer with 10 strokes. After the second centrifugation at $27000 \times g$ for 30 min, the fraction of the brush-border membrane was suspended in the experimental buffer at a concentration of 3—4 mg membrane protein/ml. All steps were performed on ice or at 4 °C. The purity of the membrane was routinely evaluated according to the enrichment of alkaline phosphatase (E.C.3.1.3.1.), an enzyme specific to the intestinal brush-border membrane. The specific activity of this enzyme was increased 12-fold in the final membrane suspension compared with the concentration found in the homogenate of the intestinal scrapings. The membrane vesicles were allowed to stand for 1 h at 0 °C before use in order to equilibrate the vesicle suspension.

Uptake Experiments The uptake of [^{14}C]MCP into membrane vesicles was measured by a rapid filtration technique.[20] In a regular assay, the uptake was initiated by mixing 0.1 ml of the experimental buffer containing [^{14}C]MCP with 0.05 ml of the membrane vesicle suspension in a plastic tube. All uptake experiments were carried out at 25 °C. At a specified time, the mixture was diluted with 3 ml of an ice-cold stop solution (150 mM NaCl, 1 mM Tris/HCl, pH 6.5) and filtered under a vacuum through a Toyo GC-50 glass filter (0.5 μm, Toyo Roshi Co., Tokyo, Japan), which was washed previously with 2 ml of an ice-cold stop solution containing unlabelled 1 mM MCP in order to reduce the non-specific adsorption of labeled MCP onto the filter. The filter was washed once with 5 ml of the same ice-cold stop solution and then dissolved with 10 ml of scintillator. The radioactivity was counted using a liquid scintillation spectrometer. In a separate experiment, non-specific adsorption onto the glass filter was determined using the incubation medium instead of the membrane suspension. This value was subtracted from the uptake value determined in the presence of membrane vesicles. The average amount of non-specific adsorption to the pretreated filter was 95.2 ± 12.6 pmol/filter in the uptake experiment of 0.05 mM MCP. Protein concentration was determined by the method of Lowry *et al.*[21] using bovine serum albumin as a standard. Uptake experiments in triplicate were performed using at least three membrane vesicle suspensions prepared freshly.

RESULTS AND DISCUSSION

Uptake of MCP by Brush-Border Membrane Vesicles Figure 1 shows the time course of [^{14}C]MCP uptake at two different concentrations. Although MCP uptake reached steady-state within 5 min at both concentrations, the uptake seemed rapid at 1 mM. In order to distinguish between binding to the brush-border membrane and accumulation into the intravesicular space, the effect of extravesicular medium osmolarity on MCP uptake at

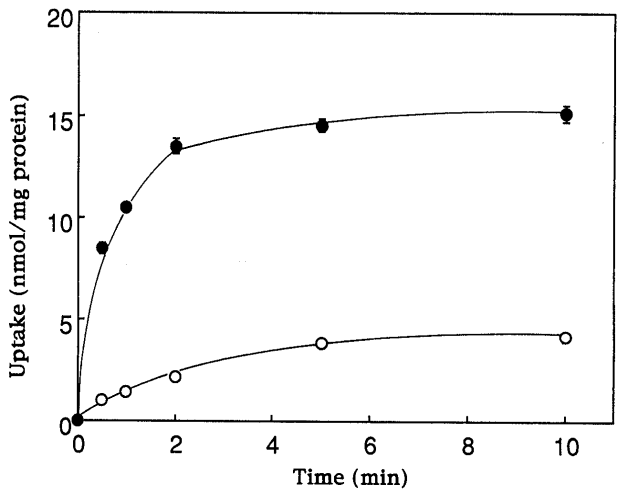

Fig. 1. Time Course of MCP Uptake by Brush-Border Membrane Vesicles

MCP uptake was initiated by mixing 0.1 ml of the experimental buffer containing [^{14}C]MCP with 0.05 ml of vesicle suspension. The final concentration of MCP was 50 μM (○) or 1 mM (●). Each point represents the mean ± S.E. of at least 5 determinations with different preparations of vesicles.

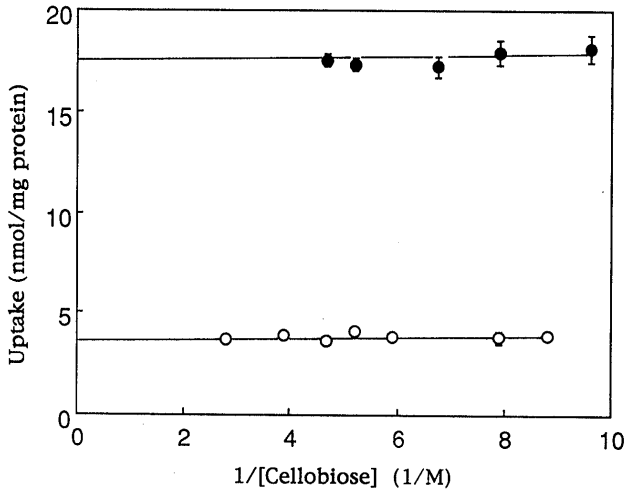

Fig. 2. Effects of Extravesicular Medium Osmolarities on MCP Uptake by Brush-Border Membrane Vesicles

MCP uptake was initiated by mixing 0.1 ml of the experimental buffer containing [^{14}C]MCP and various concentrations of D-cellobiose with 0.05 ml of vesicle suspension. The final concentration of MCP was 50 μM (○) or 1 mM (●). MCP uptake was measured at 30 min after mixing. Each point represents the mean ± S.E. of at least five determinations with different preparations of vesicles.

30 min was investigated using D-cellobiose (75—360 mM) as a membrane-impermeable solute instead of D-mannitol. The equilibrium uptake is normally decreased in inverse proportion to the increase in extravesicular medium osmolarities, because membrane vesicles behave as osmometers. As demonstrated in Fig. 2, however, MCP uptake at 30 min did not change under various medium osmolarities. It was, therefore, suggested that MCP uptake by brush-border membrane vesicles was mainly due to high binding to the membrane. The accumulation of MCP into the intravesicular space was negligible. Apparent vesicular volume caluculated from equilibrium values at 0.05 and 1 mM was about 80 and 20 μl/mg protein, respectively. These values were very large compared with the average vesicular volume of 1 μl/mg protein reported previouly, suggesting a high binding property of MCP to

the brush-border membrane. The manner of MCP uptake was clearly distinct from that of choline[15] and very similar to that of propantheline[22] and chlorpromazine.[23] The difference in MCP uptake at 30 min between two concentrations was at most 4-fold, in spite of a 20-fold difference in the extravesicular concentration, suggesting that MCP binding to the brush-border membrane was saturable. As shown in Fig. 3, although MCP uptake at 10 min was almost linear up to 200 μM, it exhibited a saturable manner beyond 200 μM, being nearly maximum at 1 mM.

Effects of Various Organic Cations on MCP Uptake Table 1 shows the effects of various organic cations on MCP uptake at 10 min by brush-border membrane vesicles. QACs such as propantheline, methylbenactyzine, and mepenzolate, which have hydrophobic tails, exhibited inhibitory effects on MCP uptake. In contrast, low molecular QACs such as choline and tetramethylammonium failed to inhibit MCP uptake, even at 50-fold higher concentrations. Amphiphilic tertiary amines like chlorpromazine and imipramine inhibited MCP uptake to much greater extents than the QACs tested. We already reported that the binding of QACs to the brush-border membrane is not simple adsorption, based on several observations such as 1) the binding of QACs to the membrane was independent of their lipophilicities and rather dependent on the shape of their molecular structures, 2) mutual inhibition between QACs is in a competitive manner, and 3) sialic acid on the membrane plays an important role in the binding of QACs.[24-26] Therefore, it was considered that MCP binding to the brush-border membrane takes place *via* a specialized process like other QACs.

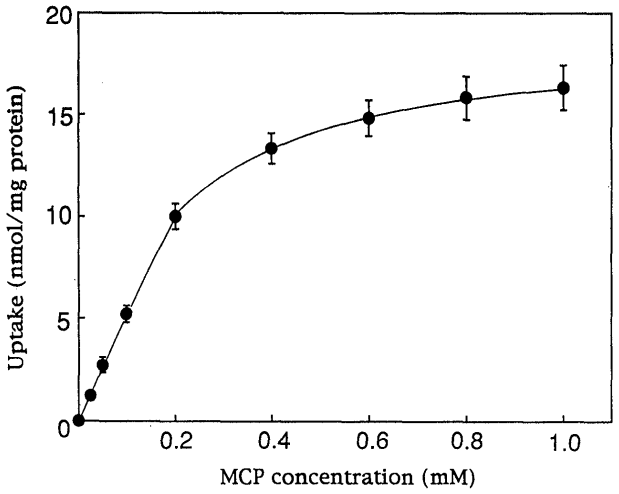

Fig. 3. Effects of Extravesicular MCP Concentrations on MCP Uptake by Brush-Border Membrane Vesicles

MCP uptake over 10 min was measured in a medium containing [^{14}C]MCP at the indicated concentrations. Each point represents the mean±S.E. of six determinations with different preparations of vesicles.

Effects of Inside-Negative TEPD on MCP Uptake by Brush-Border Membrane Vesicles The effect of inside-negative TEPD on MCP uptake was examined by adding NaSCN or Na-gluconate. According to the difference in ion permeability through the intestinal brush-border membrane, the addition of NaSCN rapidly induced an inside-negative TEPD. We demonstrated that the inside-negative TEPD induced by NaSCN greatly stimulated Na^+-dependent D-glucose transport.[16] As shown in Fig. 4A, when NaSCN and MCP were added simultaneously to the vesicle suspension from the start, no facilitated uptake of 0.05 mM MCP was observed. On the other hand, when NaSCN was added to the extravesicular medium after MCP uptake reached a steady state, which was

Table 1. Effects of Various Organic Cations on MCP Uptake by Brush-Border Membrane Vesicles

Inhibitors	% of control
Choline (2.5 mM)	100.0±2.5
Tetramethylammonium (2.5 mM)	100.0±3.6
Propantheline (1 mM)	60.8±4.3[b]
Methylbenactyzine (1 mM)	85.1±2.3[b]
Mepenzolate (1 mM)	90.5±2.1[b]
Chlorpromazine (1 mM)	14.9±4.3[a]
Imipramine (1 mM)	47.3±3.1[a]

MCP concentration was 0.05 mM. MCP uptake was determined after 10 min. *a*) and *b*) $p<0.01$ and 0.05 significantly different from the control, respectively.

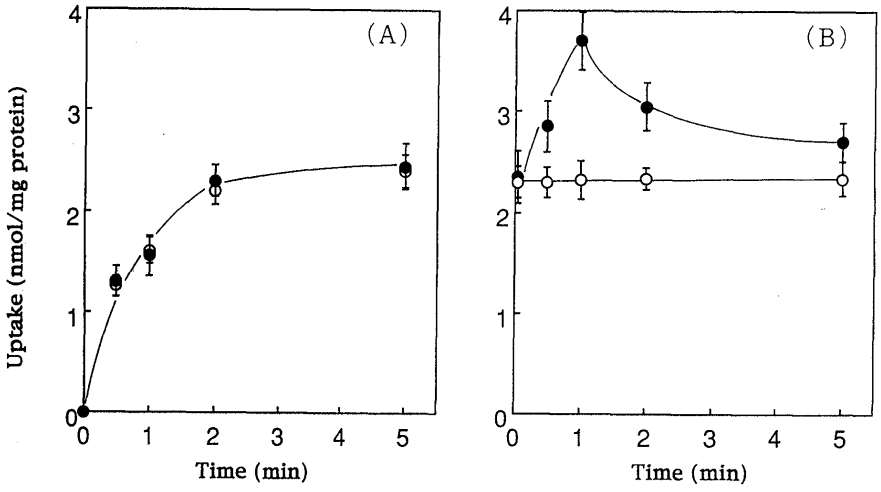

Fig. 4. Effects of NaSCN and Na-Gluconate Gradient on MCP Uptake by Brush-Border Membrane Vesicles

(A) 50 μl of vesicle suspension was incubated with 100 μl of the experimental buffer containing 75 μM [^{14}C]MCP, 150 mM NaSCN (●) or Na-gluconate (○). (B) 50 μl of vesicle suspension was incubated at 25 °C with 50 μl of the experimental buffer containing 100 μM MCP. After 10 min, the mixture (100 μl) was incubated at 25 °C with the experimental buffer containing 50 μM [^{14}C]MCP and 200 mM NaSCN (●) or Na-gluconate (○). Time 0 represents NaSCN or Na-gluconate addition. Final concentration of MCP was 50 μM. Each point represents the mean±S.E. of five determinations with different preparations of vesicles.

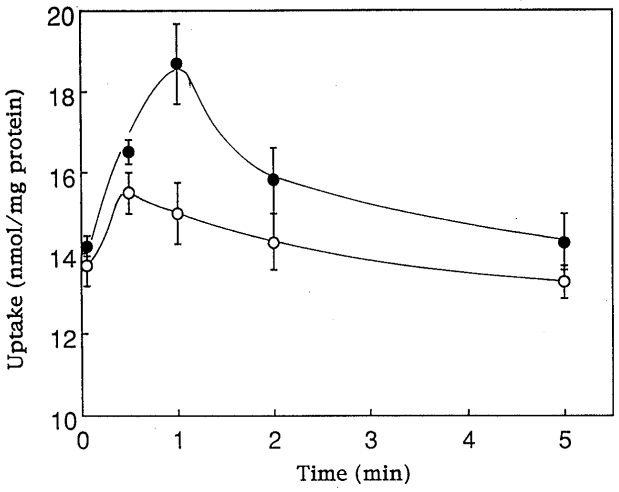

Fig. 5. Effects of Valinomycin-Induced Potassium Diffusion Potential Difference on MCP Uptake by Brush-Border Membrane Vesicles

Membrane vesicles were suspended in the experimental buffer containing 200 mM K-gluconate. 50 μl of vesicle suspension was incubated at 25 °C with 50 μl of the experimental buffer containing 200 mM K-gluconate and 1 mM MCP. After 10 min, the mixture (100 μl) was incubated at 25 °C with 100 μl of the experimental buffer containing 1 mM [^{14}C]MCP with (●) or without (○) valinomycin (50 μg/ml). Time 0 represents valinomycin addition. Final concentration of MCP was 1 mM. Each point represents the mean ± S.E. of five determinations with different preparations of vesicles.

obtained by incubating membrane vesicles with unlabeled MCP for 10 min, an overshoot was observed (Fig. 4B), suggesting that MCP uptake was transiently facilitated. In the case of Na-gluconate addition, no overshoot was observed.

The effect of TEPD on MCP uptake was also examined using valinomycin, a potassium ionophore. When valinomycin is added to brush-border membrane vesicles preloaded with K-gluconate, the potassium diffusion potential (inside negative) is transiently induced. When valinomycin and MCP were simultaneously added to the vesicles preloaded with K-gluconate, there was no facilitated uptake of 0.05 mM MCP. When valinomycin was added to the vesicle suspension after preloading of MCP for 10 min, an overshoot was observed (data not shown). These results were substantially consistent with those obtained from the experiments with NaSCN.

The above results implied that the transiently facilitated uptake of MCP by an inside-negative TEPD occurred *via* the accumulation of MCP into the intravesicular space. In order to confirm it, inside-negative TEPD was induced after MCP binding to the membrane had been saturated by preloading 1 mM MCP for 10 min. As depicted in Fig. 5, a clear overshoot was again observed, indicating that the transiently facilitated uptake of MCP reflected concentrated accumulation into the intravesicular space. It is still possible that the electrical potential gradient temporarily increased the binding capacity of the brush-border membrane to MCP. However, the results in Fig. 4A suggest that inside-negative TEPD had no direct effect on the binding process of MCP to the brush-border membrane. In the absence of valinomycin, weak overshoot was also observed. A possible explanation is as follows: since a buffer without K-gluconate was added to the extravesicular medium after 10 min in the control experiment, the resultant potassium gradient (inside > outside) induced an inside-negative TEPD by the rapid efflux of potassium ion compared with gluconate ion. This TEPD was considered to be much smaller than that induced by valinomycin addition. Accordingly, a large difference in the magnitudes of overshoot in the presence and absence of valinomycin supported the existence of a transport mechanism of MCP driven by inside-negative TEPD.

It can be concluded that QACs like propantheline and MCP, which possess relatively large hydrophobic tails in their structures, can pass through the brush-border membrane driven by a physiological TEPD (cell interior negative). It seems, however, that this mechanism is not applied to QACs lacking hydrophobic tails, such as choline, tetramethylammonium, and paraquat. It should be noted that physiological TEPD across the brush-border membrane is a homeostas in the intestine. Therefore, the intrusion of QACs utilizing physiological TEPD into the cytosol is very inconvenient to the body. It is difficult for the brush-border membrane to exhibit its barrier function against these QACs, because they highly bind to the membrane in the first step of their absorptive movements.

In spite of the specialized absorptive movements of MCP across the brush-border membrane described above, it has been reported that *in vitro* MCP permeation was relatively low compared with other organic cations.[17] Therefore, it is probable that another mechanism such as a P-glycoprotein-mediated efflux system simultaneously functions to restrict MCP absorption, as supposed in propantheline.[6] It should be noted that chlorpromazine is also transported by intestinal P-glycoprotein.[6] Further studies are now underway concerning the involvement of the intestinal secretory system in MCP absorption.

REFERENCES AND NOTES

1) A preliminary part of this study was presented at the 10th Symposium on the Interaction between Biological Membranes and Drugs, Sendai, 1988.
2) Present address: *Department of Pharmaceutics, Faculty of Pharmaceutical Sciences, Health Sciences University of Hokkaido, Kanazawa 1757, Ishikari-Tobetsu, Hokkaido 061-02, Japan.*
3) Saitoh H., Kawai S., Miyazaki K., Arita T., *J. Pharm. Pharmacol.*, **40**, 176—180 (1988).
4) Saitoh H., Hasegawa N., Kawai S., Miyazaki K., Arita T., *J. Pharmacobio-Dyn.*, **9**, 1008—1014 (1986).
5) Turnheim K., Lauterbach F., *J. Pharmacol. Exp. Ther.*, **212**, 418—424 (1980).
6) Saitoh H., Aungst B. J., *Pharm. Res.*, **12**, 1304—1310 (1995).
7) Burton P. S., Conradi R. A., Hilgers A. R., Ho N. F. H., *Biochem. Biophys. Res. Commun.*, **190**, 760—766 (1993).
8) Augustijns P. F., Bradshaw T. P., Gan L.-S. L., Hendren R. W., Thakker D. R., *Biochem. Biophys. Res. Commun.*, **197**, 360—365 (1993).
9) Hunter J., Jepson M. A., Tsuruo T., Simmons N. L., Hirst B. H., *J. Biol. Chem.*, **268**, 14991—14997 (1993).
10) Karlsson J., Kuo S.-M., Ziemniak J., Artursson P., *Br. J. Pharmacol.*, **110**, 1009—1016 (1993).
11) Wils P., Phung-Ba V., Warnery A., Lechardeur D., Raeissi S., Hidalgo I. J., Scherman D., *Biochem. Pharmacol.*, **48**, 1528—1530 (1994).
12) Leu B.-L., Huang J.-D., *Cancer Chemother. Pharmacol.*, **35**, 432—436 (1995).
13) Phung-Ba V., Warnery A., Scherman D., Wils P., *Eur. J. Pharmacol.*, **288**, 187—192 (1995).
14) Aungst B. J., Saitoh H., *Pharm. Res.*, **13**, 114—119 (1996).
15) Saitoh H., Kobayashi M., Sugawara M., Iseki K., Miyazaki K.,

Biochim. Biophys. Acta, **1112**, 153—160 (1992).
16) Nagao M., Saitoh H., Zhang W. D., Iseki K., Yamada Y., Takatori T., Miyazaki K., *Arch. Toxicol.*, **67**, 262—267 (1993).
17) Kitagawa S., Sato K., Sato S., Sasaki M., *Biol. Pharm. Bull.*, **19**, 998—1000 (1996).
18) Huang C. L., Mir G. M., Yeh J. Z., *J. Pharm. Sci.*, **59**, 976—979 (1970).
19) Kessler M., Acuto O., Storelli C., Murer H., Muller M., Semenza G., *Biochim. Biophys. Acta*, **506**, 136—154 (1978).
20) Hopfer U., Nelson K., Perroto J., Isselbacher K. J., *J. Biol. Chem.*, **248**, 25—32 (1973).
21) Lowry O. H., Rosebrough N. J., Farr A. L., Randall R. J., *J. Biol. Chem.*, **193**, 265—275 (1951).
22) Saitoh H., Kobayashi Y., Miyazaki K., Arita T., *J. Pharm. Pharmacol.*, **39**, 9—12 (1987).
23) Saitoh H., Kawai S., Iseki K., Miyazaki K., Arita T., *J. Pharm. Pharmacol.*, **41**, 200—202 (1989).
24) Saitoh H., Kawai S., Iseki K., Miyazaki K., Arita T., *J. Pharm. Pharmacol.*, **40**, 776—780 (1988).
25) Saitoh H., Ebina M., Fukuda S., Iseki K., Miyazaki K., Arita T., *J. Pharm. Pharmacol.*, **41**, 459—463 (1989).
26) Saitoh H., Noujoh A., Chiba Y., Iseki K., Miyazaki K., Arita T., *J. Pharm. Pharmacol.*, **42**, 308—313 (1990).