Effect of lipophilic cations on thiamine transport system in isolated rat hepatocytes

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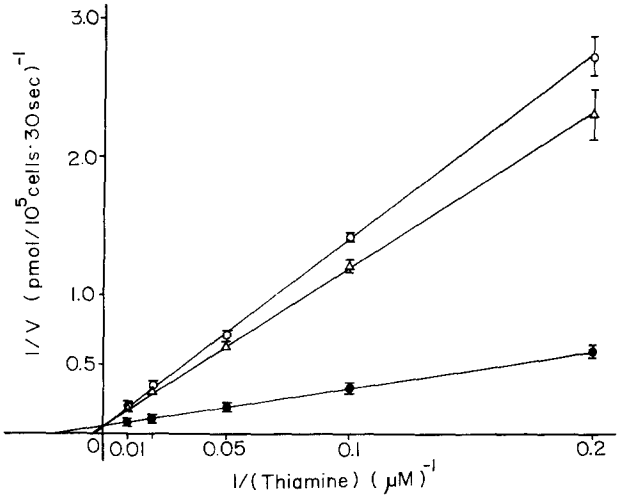
Summary. The effect of lipophilic cations such as triphenylmethylphosphonium, tetraphenylphosphonium and tetraphenylarsonium in addition to dibenzyldimethylammonium on thiamine transport in isolated rat hepatocytes was studied. Lipophilic cations at the concentration 10 μM almost completely inhibited thiamine uptake. Kinetic studies showed that these compounds were competitive inhibitors with a very high affinity. These results suggest that lipophilic cations in addition to quaternary ammonium compounds also share a common binding site for thiamine in isolated rat hepatocytes.
Key words. Thiamine uptake; rat hepatocyte; lipophilic cations.

Thiamine is transported via an active and Na^+-dependent process in isolated rat hepatocytes[2–5]. In our previous studies[4–7], we reported that quaternary ammonium compounds inhibit hepatocyte thiamine uptake, and that the quaternary nitrogen is involved in the binding of these compounds to thiamine carrier[5] and that the presence of a phenyl group in their molecules enhances the affinity of these compounds for the hepatocyte thiamine transport site[7]. Dibenzyldimethylammonium (DDA), a lipophilic cation, is a competitive inhibitor with a K_i of 0.64 μM; the affinity for the thiamine transport system is much higher than with other quaternary ammonium compounds[7]. On the other hand, Barts et al.[8] demonstrated that thiamine and DDA share a common binding site in *Saccharomyces cerevisiae*. Furthermore, they reported that the uptake of other lipophilic cations such as triphenylmethylphosphonium, tetraphenylphosphonium and tetraphenylarsonium also proceed via the thiamine transport system[8]. These results prompted us to investigate the effect of lipophilic cations other than DDA on the thiamine transport system in isolated rat hepatocytes.

Materials and methods. [^{14}C]Thiamine ([thiazole-2-^{14}C]thiamine hydrochloride, 24.3 Ci/mol) was obtained from Amersham International (UK), bovine serum albumin (Fraction V, powder) from Miles, collagenase (CLS IV) from Worthington, dibenzyldimethylammonium, triphenylmethylphosphonium, tetraphenylphosphonium and tetraphenylarsonium from Nakarai Chemicals Ltd (Kyoto). All other chemicals were of reagent grade.

Hepatocytes were prepared from Wistar male rats, each weighing 200–300 g and allowed to eat ad libitum, as previously reported[5] according to a minor modification of the procedure of Seglen[9]. Cells isolated in this manner showed a viability exceeding 90% by the Trypan blue exclusion method. After preincubation for 15 min at 37 °C, the transport of thiamine was initiated in a Corning centrifugation tube (50 ml) by the addition of [^{14}C]thiamine in 3 ml of cells suspended (3.5×10^6 cells/ml) in the Krebs-Henseleit medium containing bovine serum albumin (25 mg/ml), streptomycin (100 μg/ml) and penicillin G (100 units/ml). The incubation and preincubation were carried out under an atmosphere of 95% O_2 and 5% CO_2 at all times. The experiments were terminated by the addition of 15 ml of ice-cold medium. After a separation of the medium from the cell pellets by centrifugation for 5 s at 700 × g, the cell pellets were washed with 10 ml of ice-cold medium, and then recentrifuged for 5 s at 70 × g as described previously[5]. Blank tubes were routinely determined as follows: [^{14}C]thiamine was added to the cell suspensions at 0 °C, and the cells then immediately diluted, centrifuged and washed by the procedure described above.

Cell pellets were extracted by the addition of 1 ml of 6.3% trichloroacetic acid and the radioactivity was measured in a liquid scintillant containing Triton X-100 by means of liquid scintillation spectrometry. The intracellular water space was determined as the difference of ^3H_2O and [^{14}C]inulin distribution ratio in the cell pellets in parallel for each experiment, and calculated to be 2.63 ± 0.222 μl/10^6 cells (n = 45, mean ± SE). Experiments were performed in triplicate and results were corrected for the viability of the cell suspensions. The data obtained



Lineweaver-Burk plots of thiamine uptake in the absence or presence of lipophilic cations. Lineweaver-Burk plots of [^{14}C]thiamine uptake as a function of thiamine concentration over a concentration range from 5 μM to 100 μM were depicted in the absence (–●–) and presence of 0.2 μM tetraphenylphosphonium (–△–) or 0.2 μM tetraphenylarsonium (–○–). Suspensions of liver cells in Krebs-Henseleit medium were preincubated for 15 min at 37 °C. The transport assay was initiated by the addition of [^{14}C]thiamine with and without lipophilic cations, and the mixtures were incubated for 30 s. Subsequent procedure is described in the text. The data presented are corrected for the contribution of nonsaturable uptake by linear regression analysis. Each point represents the mean ± SE of three experiments.

Effect of lipophilic cations on thiamine uptake in isolated rat hepatocytes

Addition	[^{14}C]Thiamine uptake (pmol/10^5 cells per 30 s)	Distribution ratio	% of thiamine uptake
None	4.850 ± 0.110	1.751 ± 0.040	100
Dibenzyldimethylammonium	0.456 ± 0.046[a]	0.165 ± 0.017	9
Triphenylmethylphosphonium	0.113 ± 0.026[a]	0.041 ± 0.009	2
Tetraphenylphosphonium	0.111 ± 0.007[a]	0.040 ± 0.002	2
Tetraphenylarsonium	0.100 ± 0.020[a]	0.036 ± 0.007	2

The uptake of [^{14}C]thiamine was assayed as described in the text. Lipophilic cations (10 μM) were added to cell suspensions simultaneously with 10 μM [^{14}C]thiamine, and the mixtures were incubated for 30 s. The data presented are corrected for the contribution of nonsaturable uptake by linear regression analysis. The distribution ratio is the molar ratio of intracellular thiamine to thiamine in the medium. The results presented are the mean ± SE of 3 experiments. [a] Significantly different ($p < 0.001$) from none.

are presented as mean ± SE. Kinetic paraments (K_t and V_{max}) and SE for these values were calculated as described by Wilkinson[10]. Significant differences were assessed by Student's two-tailed t-test. p-Values less than 0.05 were considered to be significant.

Results and discussion. Thiamine has been shown to be transported by two mechanisms in isolated rat hepatocytes; one is a saturable mechanism with a K_t of 34.1 μM and V_{max} of 20.8 pmol/10^5 cells per 30 s, the second is a nonsaturable mechanism[5]. The table shows the inhibitory effect of several lipophilic cations on thiamine uptake by the saturable mechanism. DDA, triphenylmethylphosphonium, tetraphenylphosphonium and tetraphenylarsonium at the concentration 10 μM inhibited 10 μM thiamine uptake by 91, 98, 98 and 98%, respectively. Barts et al.[8] reported that these lipophilic cations inhibited thiamine uptake in yeast cells. In their experiments, these compounds at the concentration 100 μM inhibited 0.8 μM thiamine uptake by 70-87%. These findings indicate that lipophilic cations are much stronger inhibitors of the thiamine transport system in isolated rat hepatocytes than in yeast cells. Previously DDA was found to be a potent inhibitor with a K_i of 0.64 μM, whose affinity is much higher than that of other quaternary amonium compounds, and to share a common binding site for thiamine in isolated rat hepatocytes[7]. Therefore, we performed Lineweaver-Burk analyses of thiamine uptake in the presence or absence of these lipophilic cations. As shown in the figure, these compounds were also competitive inhibitors.

The values of K_t and V_{max} for thiamine in the absence or presence of 0.2 μM tetraphenylphosphonium were 48.4 ± 3.53 and 202 ± 18.2 μM ($p < 0.001$), 17.4 ± 0.593 and 17.1 ± 1.12 pmol/10^5 cells per 30 s (n.s.), respectively. In the case of tetraphenylarsonium inhibition, the values of K_t and V_{max} in the absence or presence of 0.2 μM tetraphenylarsonium were 48.4 ± 3.53 and 265 ± 38.8 μM ($p < 0.001$), 17.4 ± 0.593 and 19.0 ± 2.18 pmol/10^5 cells per 30 s (n.s.), respectively. K_i values of tetraphenylphosphonium and tetraphenylarsonium were calculated to be 0.06 μM and 0.05 μM, respectively. The apparent affinities of these compounds for the thiamine binding site are about ten times that of DDA. Since these compounds at a concentration of 10 μM almost completely inhibited thiamine uptake, these lipophilic cations were probably also purely competitive inhibitors. These results indicated that lipophilic cations share a common binding site for thiamine in isolated rat hepatocytes.

Although several studies[2-7, 11-17] have been carried out on the thiamine transport system in mammalian cells, no evidence has been provided that lipophilic cations other than DDA share a common binding site for thiamine. The finding in isolated rat hepatocytes is the first such evidence. However, in yeast cells, lipophilic cations have been used to measure the membrane potentials, and the relation between lipophilic cations and thiamine binding sites has been demonstrated[8]. In mammalian cells, lipophilic cations such as tetraphenylphosphonium and triphenylmethylphosphonium have also been used to measure the membrane potentials in thyroid cells[18] and macrophages[19]. Although the thiamine transport system in these cells is still unclear, we should be careful when measuring the membrane potentials with lipophilic cations in mammalian cells as described for yeast cells. Furthermore, our findings indicate that some compounds which have a monovalent cationic group other than the quaternary nitrogen share a common binding site for thiamine in isolated rat hepatocytes.

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2 Chen, C.-P., J. Nutr. Sci. Vitaminol. *24* (1978) 351.
3 Lumeng, L., Edmondson, J.W., Schenker, S., and Li, T.K., J. biol. Chem. *254* (1979) 7265.
4 Yoshioka, K., Nishimura, H., and Iwashima, A., Biochim. biophys. Acta *732* (1983) 308.
5 Yoshioka, K., Biochim. biophys. Acta *778* (1984) 201.
6 Yoshioka, K., Nishimura, H., Himukai, M., and Iwashima, A., Biochim. biophys. Acta *815* (1985) 499.
7 Yoshioka, K., Nishimura, H., and Hasegawa, T., Biochim. biophys. Acta *819* (1985) 263.
8 Barts, P.W.J.A., Hoeberichts, J.A., Klaassen, A., and Borst-Pauwels, G.W.F.H., Biochim. biophys. Acta *597* (1980) 125.
9 Seglen, P.O., Expl Cell Res. *74* (1972) 450.
10 Wilkinson, G.N., Biochem. J. *80* (1961) 324.
11 Hoyumpa, A.M. Jr, Middleton, H.M. III, and Wilson, F.A., Gastroenterology *68* (1975) 1218.
12 Rindi, G., and Ferrari G., Experientia *33* (1977) 211.
13 Spector, R., Am. J. Physiol. *230* (1976) 1101.
14 Nose, Y., Iwashima, A., and Nishino, H., in: Thiamine, p. 157. Eds C.J. Gubler, M. Fujiwara and P.M. Dreyfus. John Wiley & Sons, New York 1976.
15 Komai, T., and Shindo, H., J. Nutr. Sci. Vitaminol. *20* (1974) 189.
16 Menon, I.A., and Quastel, J.H., Biochem. J. *99* (1966) 766.
17 Yamamoto, S., Komaya, S., and Kawasaki, T., J. Biochem. *89* (1981) 809.
18 Grollman, E.F., Lee, G., Ambesi-Impiombato, F.S., Meldolesi, M.F., Aloj, S.M., Coon, H.G., Kaback, H.R., and Kohn, L.D., Proc. natn. Acad. Sci. USA *74* (1977) 2352.
19 Young, J.D.-E., Unkeless, J.C., Kaback, H.R., and Cohn, Z.A., Proc. natn. Acad. Sci. USA *80* (1983) 1357.

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The in vitro characterization of the inhibition of mouse brain protein kinase-C by retinoids and their receptors

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Summary. The mechanism of the in vitro inhibition of Ca^{2+}-, phosphatidylserine-dependent protein kinase C (PK-C)[2] by the purified *holo* (ligand-saturated) forms of cellular retinol-binding protein (cRBP) and cellular retinoic acid-binding protein (cRABP) was studied. We report here that i) the PK-C-inhibitory action of *holo*-cRBP and *holo*-cRABP is due to their respective ligands, all-*trans*-retinol and all-*trans*-retinoic acid; ii) the reduced phosphorylation of the *holo*-retinoid-binding proteins and brain cytosolic proteins is not the result of a retinoid-induced soluble phosphatase or protease activity; iii) retinoids reduce PK-C affinity for calcium and phosphatidylserine in vitro; and iv) the structure-function activity of the retinoids and the specific interaction of these compounds with their binding proteins are important in blocking the activity of PK-C. These observations suggest that the inhibitory effect of retinoids on plasma membrane-associated PK-C activity pays a significant role in defining the early epigenetic aspects of PK-C-dependent tumor promotion and may be a physiological mechanism by which retinoids induce terminal differentiation in cell types that do not express soluble retinoid-binding proteins.
Key words. Retinoids; protein kinase-C; receptors.