Experiments on the mechanism of the inhibition of mitochondrial Ca^{2+} transport by La^{3+} and ruthenium red[1]

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Summary. The effects of La^{3+} and ruthenium red on the energy-linked uptake of Ca^{2+} mediated by a synthetic neutral Ca^{2+} ionophore have been investigated in rat liver mitochondria. The results indicate that unspecific surface charge effects do not play a major role in the mechanism of inhibition of mitochondrial Ca^{2+} transport by La^{3+} and ruthenium red.

La^{3+} and ruthenium red are powerful inhibitors of the active transport of Ca^{2+} in mitochondria[2,3]. The mechanism of the inhibition has not yet been investigated in detail. It could either be based on a competitive interaction with specific Ca^{2+} transport sites on the mitochondrial membrane, or on non-specific charge effects at the inner membrane surface. Indeed, La^{3+} is known to change the ion permselectivity of artificial lipid membranes from a cation to an anion exchanger[4]. Effects of this type have been described also for di- and trivalent cations (Mg^{2+}, Al^{3+}, etc.), which reduce markedly the rate of valinomycin dependent Rb^+-uptake by yeast cells[5]. A similar effect on mitochondrial Ca^{2+} transport has been reported for the positively charged polyamine spermine[6]. Even if the amounts of polyvalent cations required for a substantial reduction of the rate of uptake is in general rather high, the decrease of the negative surface charge density due to La^{3+} or ruthenium red might still conceivably play a role in the inhibition of Ca^+ transport. This possibility has indeed already been suggested by Scarpa and Azzone[7].

To investigate possible surface charge effects of La^{3+} and ruthenium red, use was made of a neutral synthetic Ca^{2+} ionophore, which selectively transports Ca^{2+} in bulk phases in electrodialysis experiments[8]. Recently, it has been shown that this Ca^{2+} ionophore can transport Ca^{2+} across mitochondrial and other biological membranes[9].

The experiments have provided conclusive evidence that surface charge effects do not play a major role in the mechanism of inhibition of the energy-linked Ca^{2+} transport by La^{3+} and ruthenium red.

Materials and methods: Liver mitochondria were prepared by a conventional method from rats fasted for 12 h[10]. The isolation medium contained 220 mM mannitol, 70 mM sucrose, 10 mM HEPES-Tris, pH 7.4 and 0.5 mg BSA/ml. The Ca^+ accumulation in the presence of increasing amounts of ruthenium red or La^{3+} was measured by using $^{45}Ca^{2+}$ and millipore filtration. The initial rates of Ca^{2+} uptake in the presence of increasing amounts of ruthenium red or La^{3+} were determined using $^{45}Ca^{2+}$ and an inhibitor stop-method. The amount of endogenous Ca^{2+} released by mitochondria in the presence of rotenone was determined by using the Ca^{2+}-indicator Arsenazo III[11]. Ruthenium red was obtained from Fluka and used without further purification. The neutral synthetic ionophore was a gift from Prof. W. Simon, ETH Zurich.

Results and discussion. Table 1 shows the accumulation of Ca^{2+} by energized rat liver mitochondria, in the presence of the synthetic neutral Ca^{2+} ionophore, and of increasing amounts of La^{3+} or ruthenium red. The Ca^{2+} bound to mitochondria in the absence of ionophore was between 2 and 4 nmoles Ca^{2+}/mg protein. This amount was subtracted from the values reported in tables 1 and 2. The accumulation was not influenced by concentrations of La^{3+} in the range of 1–50 µM, or of ruthenium red in the range of 0.8–10 µM. The average amount of Ca^{2+} accumulated via the ionophore was the same with both inhibitors.

In table 2 the initial rates of Ca^{2+} uptake in the presence of the Ca^{2+}-ionophore and of increasing amounts of La^{3+} or ruthenium red are shown. Clearly, the initial rate of Ca^+ uptake remained the same in the presence of concentrations of La^{3+} up to 30 µM, or of ruthenium red up to 10 µM. The rates of uptake were comparable for the 2 inhibitors. It must be stressed that these concentrations of inhibitors are 10–50 times higher than those required to block almost completely the energy-linked uptake. Only when La^{3+} was added in concentrations in excess of 50 µM,

Table 1. Maximum levels of Ca^{2+} accumulated by rat liver mitochondria in the presence of the neutral synthetic ionophore, and of varying amounts of $LaCl_3$ or ruthenium red

Inhibitor	Ca^{2+} uptake after 2 min (nmoles/mg protein)
2 µM La^{3+}	10
5 µM La^{3+}	12
10 µM La^{3+}	12
40 µM La^{3+}	7
50 µM La^{3+}	8
60 µM La^{3+}	6
70 µM La^{3+}	1
0.8 µM RR	7
1 µM RR	10
4 µM RR	8
5 µM RR	7.8
10 µM RR	6.6

1 mg of rat liver mitochondrial protein were incubated for 8 min at 25 °C with 2 µM rotenone (final vol. 1 ml). Then, 20 µM $^{45}Ca^{2+}$ and different amounts of La^{3+} or ruthenium red were added. After these additions, mitochondria were incubated for 2 min with 40 µM of the synthetic neutral Ca^{2+} ionophore. Ca^{2+} uptake was then started by the addition of 2 mM Tris-succinate. The Ca^{2+} bound by mitochondria in the absence of ionophore was determined for each La^{3+} or ruthenium red concentration, and subtracted from the value measured in the presence of ionophore.

Table 2. Initial rates of Ca^{2+} uptake by energized rat liver mitochondria, in the presence of the neutral synthetic ionophore, and of varying amounts of $LaCl_3$ or ruthenium red

Inhibitor	Initial rate of uptake (nmoles Ca^{2+}/mg protein min)
5 µM La^{3+}	12
10 µM La^{3+}	8.8
20 µM La^{3+}	10
30 µM La^{3+}	10
35 µM La^{3+}	3
2 µM RR	8.6
3 µM RR	8.3
5 µM RR	8.1
6 µM RR	7.4
7 µM RR	5.3
8 µM RR	11.2
10 µM RR	9

Ca^{2+} uptake was started with Tris-succinate under the same conditions as described for the Ca^{2+} accumulation measurements (table 1), and terminated 30 sec later by the addition of 20 mM KCl. The latter binds to the synthetic ionophore, and completely inhibits the further uptake of Ca^{2+}. The Ca^{2+} bound by mitochondria in the absence of ionophore was determined for each La^{3+} or ruthenium red concentration and subtracted from the value measured in the presence of ionophore.

the initial rate of Ca^{2+} uptake decreased markedly. In the absence of ionophore and inhibitors of the natural Ca^{2+} carrier, the mitochondria accumulated about 40 nmoles Ca^{2+}/mg protein/min with an initial rate of approximately 100 nmoles Ca^{2+}/mg protein min.

From these data, it is clear that La^{3+}, up to a concentration of 40 µM, or ruthenium red, up to a concentration of 10 µM, do not inhibit the energy-linked uptake of Ca^{2+} by mitochondria mediated by a neutral Ca^{2+} ionophore. If non-specific surface charge effects, induced by the interaction of the inhibitors with the mitochondrial membrane, were important, one would have expected the ionophore-mediated uptake to be inhibited at the concentrations of La^{3+} and ruthenium red that block the natural uptake process. The 2 inhibitors, therefore, most likely interact with a specific site on the natural Ca^{2+} transport system of the membrane. At higher concentrations, La^{3+} inhibits also the ionophore-mediated Ca^{2+} transport. This effect is probably due to unspecific charge effects at the membrane surface (or, possibly, also to competitive binding of La^{3+} to the ionophore), but it is most likely unrelated to the 'normal' inhibitory effect of the cation.

Abbreviations: BSA, bovine serum albumine; RR, ruthenium red; HEPES, N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic acid.

1 Acknowledgments. The authors are indebted to Prof. W. Simon, ETH Zurich, for having provided samples of the synthetic neutral Ca^{2+} ligand, and to M. Mattenberger for the valuable technical assistence. The work was supported by a grant of the Swiss Nationalfonds (grant No. 3.1720.75).

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Action of β-(4-chlorophenyl-GABA) on uptake and metabolism of GABA in different subcellular fractions of rat brain

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Summary. β-(4-chlorophenyl)-GABA, a GABA mimetic compound, acts as an inhibitor of GABA metabolism in both synaptosomal and extrasynaptosomal compartments. It has no significant action on GABA or Glu uptake by synaptosomes.

β-p-chlorophenyl-γ-aminobutyric acid (β-p-CPG, Lioresal) has shown considerable potential in the control of spasticity, and has been extensively studied from a pharmacological point of view[1-7]. The drug is structurally related to the central inhibitory transmitter γ-aminobutyric acid (GABA) and is apparently able to penetrate the blood-brain barrier on systemic administration.

We undertook a study of the biochemical aspect of β-p-CPG activity, that is to say, its possible action on the 2 enzymes responsible for the synthesis and degradation of GABA: Glutamate decarboxylase (GAD) and GABA transaminase (GABA-T). β-p-CPG appears to have some GABA-ergic properties and could possibly inhibit GABA-T like amino-oxyacetic acid (AOAA) which enhances brain GABA levels in this way. The action of β-p-CPG on the enzymes was studied in the 2 sites implicated in GABA metabolism: synaptosomal GAD, a cytosolic enzyme, and extrasynaptosomal and synaptosomal GABA-T, a mitochondrial enzyme.

Material and methods. Preparation of synaptosomes. Male Sprague-Dawley rats (180–200 g) were decapitated and synaptosomes were prepared from the mesencephalon by the method of Gray and Whittaker[8] as modified by Israel and Frachon Mastour[9]. Preparation of extrasynaptosomal mitochondria. Extrasynaptosomal mitochondria were prepared by the method of Gray and Whittaker[8] as modified

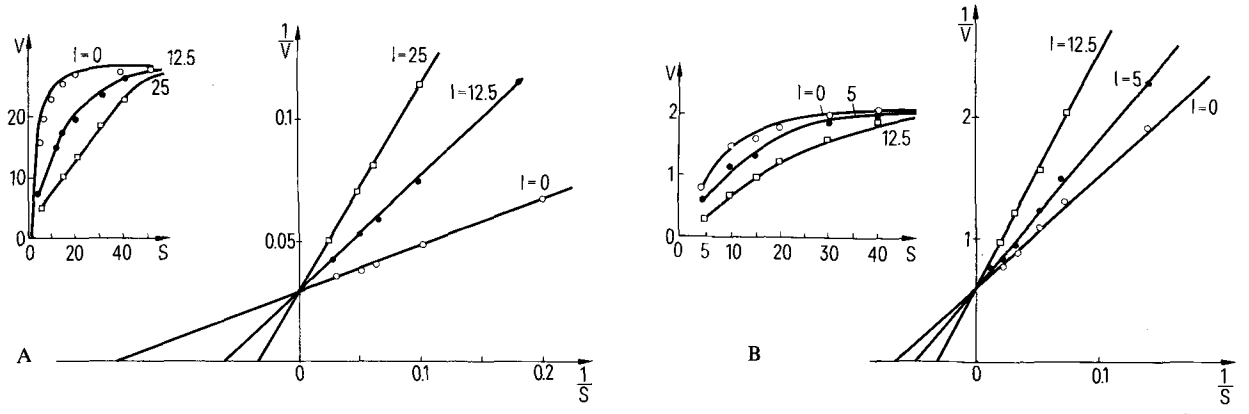

Fig. 1. Inhibition of *A* extrasynaptosomal and *B* synaptosomal $GABA_T$ by Lioresal. Reciprocal plot (according to Lineweaver-Burk) of 1/v against $(GABA)^{-1}$ concentration at 2 concentrations of Lioresal and a fixed concentration of α-ketoglutarate (25 mM). Velocity is expressed in µM of succinyl semialdehyde formed in 1 h, by 1 ml of enzyme solution.