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STEREOSPECIFIC ACTION OF DILTIAZEM ON THE MITOCHONDRIAL Na–Ca EXCHANGE SYSTEM AND ON SARCOLEMMAL Ca-CHANNELS

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Abstract—The benzothiazepine diltiazem is a potent Ca-channel blocker, which also inhibits the Na-dependent Ca-efflux from heart mitochondria. In this study, the action of the 4 stereoisomers of diltiazem has been investigated using guinea-pig heart and liver mitochondria. The rate of the Na-dependent Ca-efflux from liver mitochondria has been found to be 10 times smaller than in heart mitochondria. Otherwise, the exchange systems from the two tissues have been found to be pharmacologically indistinguishable. Both the (+)-optical isomers of the *cis*- and *trans*-forms of diltiazem inhibit Na–Ca exchange activity with comparable potency (IC_{50} of 10–20 μM), while the (−)-optical isomers are ineffective ($IC_{50} > 200\ \mu M$). Radioligand binding experiments have revealed that only one stereoisomer of diltiazem, the (+)-*cis* form, interacts with high affinity with the Ca-channel receptors of guinea-pig heart sarcolemma preparations ($K_D = 120$ nM).

The results have shown that the Ca-channel of plasma membranes and the mitochondrial Na–Ca exchanger have different stereospecific requirements for the binding of diltiazem.

Isolated mitochondria accumulate large amounts of Ca under energized conditions, and buffer the free Ca concentration in the medium down to a specific set point [1, 2]. Ca is assumed to enter the matrix space through a carrier-mediated process driven by the electrical component of the proton electrochemical potential that links respiration to ATP synthesis. Ca leaves the matrix space down its concentration gradient through a mechanism involving exchange with Na ions. The Na-linked pathway is particularly developed in mitochondria from heart, brain and excitable tissues, but only minimally active in those from liver [3–5]. In the latter, Ca-efflux occurs mainly via a Ca/H^+ exchanger [6]. The external set point of the mitochondrial Ca-buffering system depends on the respective rates of these transport mechanisms and is thus modified by their selective activation or inhibition.

Some classes of chemical compounds (among them amiloride derivatives [7], benzothiazepines [8], benzodiazepines [9]) have been reported to inhibit the Na–Ca exchange system in heart mitochondria. However, none of the compounds tested has so far proven sufficiently potent and selective to be used in the investigation of the physiological significance of the mitochondrial Na–Ca route in the intracellular Ca-homeostasis. The Ca-channel blocker diltiazem is among the most potent inhibitors of the mitochondrial Na–Ca exchangers so far reported [8], but is effective in the 10^{-5} M range, i.e. a concentration far above that required to inhibit the Ca-influx across the Ca-channels of the plasma membrane [10]. Of the 4 isomeric forms of diltiazem known to exist (the (+)- and (−)-isomers of the *cis*- and *trans*-forms), the (+) and (−) forms of the *cis*-stereoisomer were analyzed in a recent study [9], where it was found that only the (+) *cis* diltiazem was active against the Na-linked route of heart mitochondria. This report presents the results of experiments on the effect of the 4 diltiazem stereoisomers on the activity of the heart and liver mitochondrial Na–Ca exchange and compares them with their ability to displace tritiated methoxyverapamil from its binding site on cardiac Ca-channel receptors. The findings have shown that the mitochondrial Na-Ca exchange and the plasma membrane Ca-channel respond differently to the various stereoisomers of diltiazem.

MATERIALS AND METHODS

Chemicals. (−)-[*N*-methyl-^{3}H] desmethoxyverapamil (84 Ci/mmol) was obtained from Amersham (Bucks, U.K.). All drugs used in this study were supplied by the Chemistry Department of Ciba-Geigy (Basel, Switzerland).

The synthesis of the racemic *trans* isomer of diltiazem, the *trans*-3-(acetyl-oxy)-5-[2-(dimethylamino)-ethyl]-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4(5H)-one, was effected essentially according to the procedures published by Kugita *et al.* [11, 12] and Hashiyama *et al.* [13].

To obtain the (+)-*trans* and (−)-*trans* -enantiomers, the racemic erythro-2-hydroxy-3-(2-amino-phenylthiol)-3-(4-methoxy-phenyl)-propionic acid was cleaved with cinchonidine. The resultant (+) or (−)-rotatory acid was converted in the same way as the racemic acid to give the end-products.

The (+)-erythro-acid yielded the (−)-*trans*-3-(acetyloxy) - 5 - [2 - (dimethyl-amino) - ethyl] - 2,3-dihydro- 2- (4-methoxyphenyl)- 1,5- benzothiazepin-4(5H)-one (F: 194–196°) and the (−)-erythro-acid the (+) *trans*-rotatory enantiomer (F: 193.5–193.5°C). The hydrochloride of the (−)-*trans* enantiomer has an $[\alpha]_D^{20°} = -564.7° \pm 1°$. The hydrochloride of the (+)-*trans* enantiomer has an $[\alpha]_D^{20°} = +566.8° \pm 1°$. The rotational values were calculated on the basis of the anhydrous hydrochloride. All other chemicals were of the best quality commercially available.

Biological material. Pirbright white male guinea-pigs (Ciba-Geigy Animal Breeding Unit, Stein, Switzerland) were sacrificed by cervical dislocation and hearts or livers were removed and cleaned of connective tissue.

Sarcolemmal membranes were prepared as described [14] with the exception that $MgCl_2$, EDTA* and phenylmethylsulfonyl-fluoride were omitted in the buffers used. The membranes were frozen in liquid nitrogen and stored at −80°. Heart mitochondria were isolated as previously described [4]. The hearts were homogenized with a Polytron homogenizer in a medium containing 210 mM mannitol, 70 mM sucrose, 10 mM Tris–Cl, 0.2 mM EGTA, and 0.05% BSA, final pH 7.4. Washings of the mitochondrial pellets were carried out without BSA and EGTA.

Liver mitochondria were prepared by the method of Chan *et al.* [15] using the homogenization medium described above with 2 mM EGTA and 0.5% BSA.

Assays. Ca-fluxes across heart mitochondria were measured at 37° with the metallochromic Ca-indicator arsenazo III (Sigma, St. Louis, MO) using a dual wavelength spectrophotometer (Shimadzu UV-3000) with a wavelength pair set at 675–685 nm. The medium was composed of 120 mM KCl, 20 mM Tris–Cl pH 7.2, 50 μM arsenazo, 5 μM rotenone and about 1 mg mitochondria/ml. Additions were made as indicated in the legends to the figures. When required, inhibitors of the Na–Ca exchanger were added at the beginning of the experiment.

Liver mitochondria were investigated in a similar way with 0.1 μM RR and 200 μM arsenazo.

Mitochondrial respiratory activity was monitored with an oxygen electrode of the Clark-Type (Yellow Springs Instruments Co., OH) as previously described [16].

Determination of free Ca concentrations during the Ca-fluxes experiments was carried out with an internal calibration procedure using high concentrations of EGTA (5 mM) and Ca (1–5 mM). The computer program and the dissociation constants used were the same as in [17].

(−) - [*N* - methyl - ^{3}H]desmethoxyverapamil was used as ligand in the binding assays. Membranes (protein 30–60 μg) were incubated for 60 min at 25° with the radioligand and competitors in a final volume of 1 ml of 50 mM Tris-Cl, pH 7.4. Kinetic experiments showed that under these conditions equilibrium was reached. After incubation, samples were rapidly diluted with 10 ml of ice-cold 50 mM Tris–HCl, pH 7.4, and filtered under vacuum through Whatman GF/C filters which had been pretreated for two hours with polyethyleneimine (0.3%). The filters were washed twice with the same buffer and radioactivity was measured by liquid scintillation counting. Binding in the presence of 10^{-5} M verapamil was defined as nonspecific (15–20% of total binding when using 2 nM(−)[^{3}H]desmethoxyverapamil). Dose–response curves were analyzed by non-linear regression [18]. Protein concentrations were determined according to Lowry *et al.* [19] using BSA as standard.

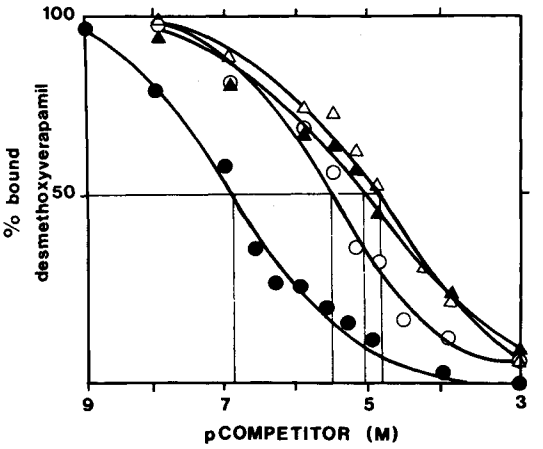

Fig. 1. Inhibition of binding of (−)-[^{3}H]-desmethoxyverapamil to guinea-pig heart membranes by the four stereoisomers of diltiazem. Inhibition curves were determined in the presence of 2 nM (−)-[^{3}H]-desmethoxyverapamil. The data given are representative of 3–4 experiments, each assayed in duplicate. The following IC_{50} values were obtained: (+)-*cis* (●) 0.11 μM, (−)-*cis* (○) 4.8 μM, (+)-*trans* (▲) 6.4 μM, (−)-*trans* (△) 14 μM.

RESULTS

The fact that all known inhibitors of the mitochondrial Na–Ca exchange system are also active on other biological mechanisms limits their use in integrated systems such as whole cells or tissues; bepridil, diltiazem, and several dihydropyridines are orders of magnitude more potent as Ca-entry blockers than as inhibitors of the mitochondrial exchange [8, 10, 20]. In an effort to identify more selective inhibitors of the Na–Ca exchanger of mitochondria, the four stereoisomers of diltiazem were thus synthesized and studied comparatively on two different systems, the Ca-channel of heart sarcolemma and the Na–Ca exchanger of mitochondria.

Binding of the diltiazem stereoisomers to the Ca-channel receptors of heart sarcolemmal membranes

Figure 1 shows that the four diltiazem stereoisomers displace [^{3}H]-desmethoxyverapamil from its high affinity binding sites on Ca-channel receptors with the following IC_{50} values: (+) *cis*, 0.11 μM; (−) *cis*, 4.8 μM; (+) *trans*, 6.4 μM; and (−) *trans*, 14 μM. This order in potency of the isomers correlates well with their ability to produce coronary dilatation: it

* *Abbreviations used:* EDTA, ethylenediaminetetraacetic acid; EGTA; ethylene glycol bis(β-aminoethyl ether); *N,N,N',N'*-tetraacetic acid; BSA, bovine serum albumin; RR, Ruthenium red.

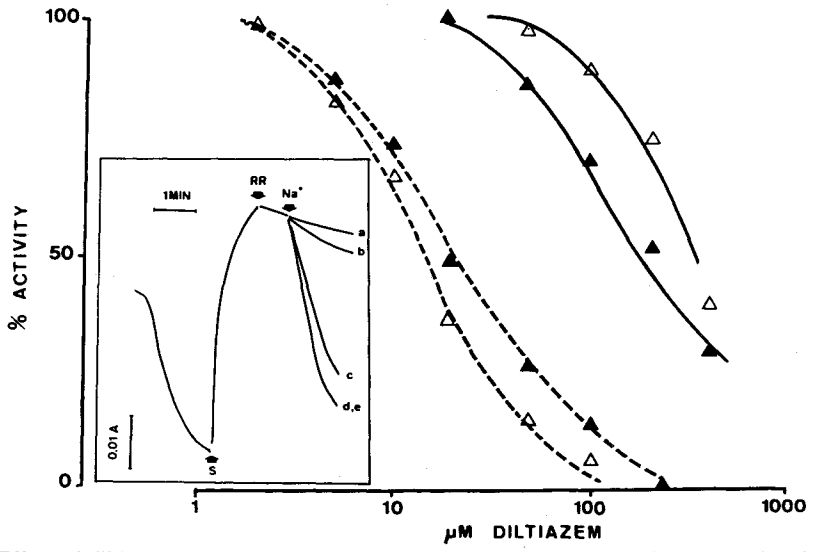

Fig. 2. Effect of diltiazem stereoisomers on the Na–Ca exchange system of heart mitochondria. The experimental conditions were the same as described for the inset of Fig. 2. The activity of the exchanger was measured as initial rate of Ca-efflux induced by Na after subtraction of the basal Na-independent rate. Continuous lines were obtained in the presence of the (−)-forms of diltiazem; dashed lines, in the presence of the (+)-optical forms. (△), *cis*-diltiazem; (▲) *trans*-diltiazem. Inset: Na-induced Ca-release from heart mitochondria. Heart mitochondria (about 1 mg/ml) were added to a medium containing 120 mM KCl, 20 mM Tris–Cl pH 7.2, and 50 μM arsenazo III at 37°. 5 μM rotenone and 100 μM diltiazem derivatives (a, (+)*cis*; b, (+)*trans*; c, (−)*trans*; d, (−)*cis*; e, control) were added. 5 mM succinate (S), 1 μM Ruthenium red (RR) and 20 mM NaCl (Na^+) were added where indicated in the figure.

is known that diltiazem [(+), *cis*] is 10 times more potent than its (−), *cis* stereoisomer and 30 times more potent than its (+) or (−), *trans* stereoisomers when injected directly into the coronary arteries in the anesthetized dogs [21].

Effect of the diltiazem stereoisomers on the Na–Ca *exchanger of heart and liver mitochondria*

Isolated heart mitochondria invariably contain endogenous Ca which can be released to the assay medium by the addition of rotenone, which blocks the oxidation of endogenous NAD-linked substrates. In the presence of a substrate such as succinate, mitochondria restore their *trans*-membrane potential and reaccumulate the lost Ca in the matrix space (Fig. 2). Thus, the kinetics of Na-dependent Ca-efflux from mitochondria is best studied after blockade of the carrier-mediated uptake process with the selective inhibitor Ruthenium red. Under these conditions, addition of Na to heart mitochondria after succinate-induced accumulation of the lost Ca induces rapid efflux. Maximal efflux rates at saturating Na concentrations (i.e. 20 mM) were 20–25 nmol/mg·min with heart mitochondria. Figure 2 shows that the Na–Ca exchanger was strongly inhibited by 100 μM (+), *cis*- and (+), *trans*-diltiazem, while the (−) optical isomers were ineffective. Oxidative phosphorylation and active Ca-uptake were not affected by all 4 stereoisomers up to a concentration of 0.5 mM (not shown).

The inhibition of the exchanger was concentration-dependent. From the experiments shown in Fig. 2 it can be calculated that the IC_{50} for the (+), *cis*- and (+), *trans*-forms of diltiazem was 17 μM and 25 μM, respectively. Higher concentrations of the (−) isomers also produced inhibition. However, the IC_{50} of the (−), *cis*- and (−), *trans*-stereoisomers was 300 μM and 250 μM, respectively.

The Na–Ca exchange system is known to be only poorly active in liver mitochondria. Stimulation by Na ions of the Ca-efflux rate is best observed in liver mitochondria under specific conditions [22–25], i.e. by perfusing liver with β-agonists [24], or by using lower concentrations of RR to inhibit active Ca reuptake [25]. Under the experimental conditions used in this study (see legend to Fig. 3), Na induces a clear stimulation of the Ca-efflux. Half-maximal effects were achieved with 7–9 mM Na (not shown, see also [22–25]), in agreement with what was observed on heart or brain mitochondria. The maximal efflux rates, however, were only 1.5–2.5 nmol Ca/mg·min. Even considering a higher degree of contamination of the preparation by non-mitochondrial membranes than in heart, these rates are one order of magnitude lower than in heart mitochondria. Since the stimulation of Ca-efflux by Na in liver mitochondria does not necessarily imply a Na–Ca exchanger, the effect of known inhibitors of the exchanger of heart mitochondria was tested. Diltiazem, clonazepam and bepridil inhibited the Na-stimulation of Ca-efflux also in liver mitochondria with IC_{50}s almost identical to those obtained in heart (not shown). Figure 3 shows that the Na-dependent Ca-release was inhibited only by the (+) optical isomers of the *cis*- and *trans*-forms of diltiazem also in liver mitochondria repeating the strict steric requirement typical of the heart mitochondria exchanger (see Fig. 2).

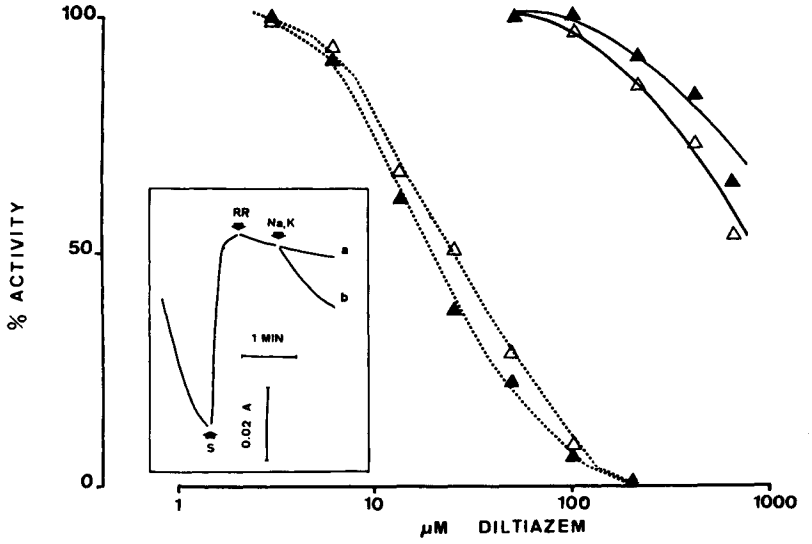

Fig. 3. Effect of diltiazem stereoisomers on the Na-stimulated Ca-efflux from liver mitochondria. Initial rates of Na-induced Ca-release are plotted against various concentrations of the (+) optical forms of diltiazem (▲, *trans*; △, *cis*-stereoisomers) and the (−) optical forms of diltiazem (▲, *trans*-, △; *cis*-stereoisomers) as described for Fig. 2 but using liver mitochondria. Inset: Na-induced Ca release from liver mitochondria. The experiments were carried out basically as described in the legend to Fig. 2 with about 1 mg liver mitochondria/ml and 200 μM arsenazo in the uptake buffer. 5 mM succinate, 0.1 μM Ruthenium red, and 20 mM KCl (curve a) or 20 mM NaCl (curve b) were added where indicated (arrows).

Experiments on isolated heart mitochondria [4] and chemically-skinned heart myocytes [26] have shown that the addition of physiological intracellular concentrations of Na induced a shift of the cytosolic free Ca to a higher set-point level. Thus, at least in heart, the Ca-buffering capacity of mitochondria could in principle be modified *in vivo* by fluctuations of the intracellular Na-concentration or, conversely, by inhibition of the mitochondrial Na–Ca exchanger. This is shown in Fig. 4, where the ability of heart mitochondria to lower the external concentration of ionized Ca was severely impaired by the presence of 10 mM Na^+. By contrast, Na^+ had no appreciable effect on the set-point level of ionized Ca in the case of liver mitochondria. In the presence of an Na–Ca exchange inhibitor, such as clonazepam, heart mitochondria optimally buffer free Ca down to the levels observed in the absence of Na.

DISCUSSION

Although the ability of mitochondria isolated from most tissues to accumulate large amounts of Ca ions has been recognized for more than two decades [27], their role in the regulation of the free Ca concentration in the cytosol is still a matter for debate. In heart, the apparent affinity of the mitochondrial electrophoretic Ca-uptake system (10–20 μM) measured in the presence of physiological electrolyte concentrations [28] seems to be too low for an important role in the precise and rapid Ca regulation in the sub-μM range. However, more than 30% of heart cells volume is occupied by mitochondria, and it could be shown that their Ca-buffering capacity is in principle adequate to lower the free Ca concentration after a Ca spike to the μM range within a fraction of a second [29]. Thus, even if the set-point of mitochondria for external Ca is definitely too high to account in full for the relaxation of the myofibrils, it is possible that a sizeable portion of the excitatory Ca is transiently accumulated by mitochondria during relaxation. The activity of the Na–Ca exchange system causes a shift in the set-point of extramitochondrial Ca, with the result that the ability of mitochondria to buffer Ca varies as a function of the cytosolic Na concentration. In heart cells, the intracellular free Na concentration approaches the Na EC_{50} value of the Na–Ca exchange system, presumably rendering the Ca buffering capacity of mitochondria suboptimal under normal conditions [4, 29] (Fig. 4) and further limited during Na-overload. Thus, to assess the contribution of heart mitochondria to the cellular regulation of Ca in heart and to clarify the physiological importance of the mitochondrial Na–Ca exchange system it becomes worthwhile to experimentally shift the mitochondrial set-point for external Ca by selectively modifying the rate of the Na-induced Ca-release pathway. Under conditions which induce Na-overload, a large increase of cytosolic free Ca levels has been reported [30]. One problem here is that, in addition to the mitochondrial exchanger, other biological systems involved in Ca-homeostasis also respond to fluctuations of cytosolic Na. Thus, a more direct way to study the problem is by selectively inhibiting the mitochondrial Na–Ca exchanger. The experiment shown in Fig. 4 is indeed indicative of the ability of a Na–Ca exchange inhibitor in shifting the set-point of heart mitochondria for external Ca. The improvement in Ca-buffering ability in the presence of clonazepam would have been even more dramatic in the presence of higher Na concentrations in the incubation medium (not shown).

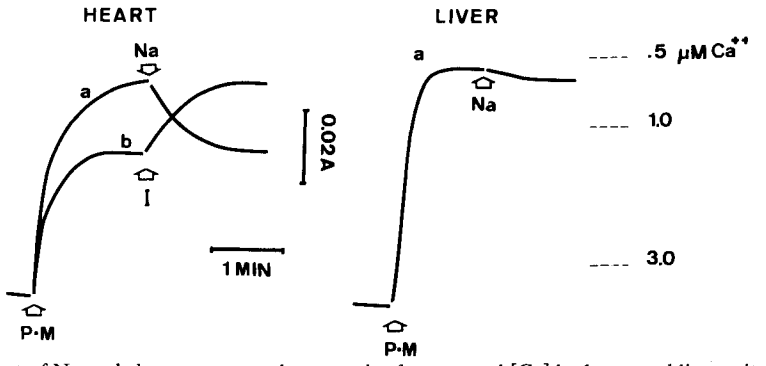

Fig. 4. Effect of Na and clonazepam on the set-point for external [Ca] by heart and liver mitochondria. Cardiac or liver mitochondria were incubated at 37° in a medium composed of 120 mK KCl, 20 mM Tris–Cl, pH 7.2, and 0 mM NaCl (curves a) or 10 mM NaCl (curve b). At the time indicated, 5 mM pyruvate and 2.5 mM malate were added (P–M). Where indicated, 10 mM NaCl or 60 μM clonazepam (I) were added. Approximative values of free Ca concentrations during the experiment were calculated as described in the Methods section.

Several classes of compounds have been described possessing inhibitory activity on the exchange system. Their use for physiological studies is limited by their moderate potency (IC_{50} in the 10^{-5} M range) and especially by their lack of inhibiting selectivity on the exchanger. Actually, all inhibitors so far described affect other biological systems also involved in Ca-homeostasis with higher potency. Therefore, in the absence of ideal inhibitors, it is worthwhile to improve the selectivity of the known ones in favor of the mitochondrial exchanger. Along these lines, the potency of the stereoisomers of the Ca-antagonist diltiazem has been investigated. To determine their activity on the Ca-channel, binding studies using tritiated desmethoxyverapamil are convenient [31–33, 35] even if some evidence has been presented for a non-competitive action between diltiazem and verapamil-like compounds [34]. Tritiated desmethoxyverapamil has been preferred to [^{3}H] diltiazem since the latter has low affinity for its binding site and is thus unsuitable with membranes with a low receptor density, such as cardiac sarcolemma. Since the binding data and the pharmacological effects of the diltiazem isomers correlate well, it is justified to relate their effects to the Ca-channel, irrespective of whether they are due to a competitive interaction or to allosteric effects. The results presented here have shown that the (+), *trans*-stereoisomer of diltiazem, while maintaining a high potency of inhibition of the mitochondrial Na–Ca exchanger, has 60-fold lower affinity for the receptor sites on the Ca-channels of cardiac sarcolemma than the (+), *cis*-stereoisomer, thus improving the selectivity ratio in favor of the exchanger about 60 times. Of interest is also the finding that both the Na–Ca exchanger of mitochondria and the sarcolemmal Ca-channels have strict but distinct steric requirements for diltiazem-binding.

The Na–Ca exchanger is probably present, although variously active, in mitochondria from most tissues. Liver mitochondria contain a slow Ca-efflux pathway which is stimulated by Na. In this study, its properties have been investigated and it has been found that they are very similar to those of the exchanger of heart mitochondria. The exchange systems in the two tissues are pharmacologically indistinguishable since they respond in the same way to the various classes of inhibitors and, in particular, since they display identical steric requirements for the (+) optical isomers of diltiazem. Thus, the Na–Ca exchanger in heart and in liver mitochondria are probably identical. In liver, however, the maximal velocity of the exchanger is far lower than that of heart mitochondria. It is likely that the system does not contribute significantly to the set-point for external Ca *in situ* (Fig. 4).

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