Ca^{2+}-dependent inhibition by trifluoperazine of the Na^{+}-Ca^{2+} carrier in mitoplasts derived from heart mitochondria

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The interaction of trifluoperazine and extramitochondrial Ca^{2+} with the heart mitochondrial Na^{+}-Ca^{2+} carrier has been investigated. External Ca^{2+} inhibits the carrier equally in mitochondria and mitoplasts in which the outer membrane is lysed. Sensitivity to Ca^{2+} is not removed by washing mitoplasts under varied conditions. Trifluoperazine is a potent inhibitor of the carrier in mitoplasts but not in mitochondria. Trifluoperazine inhibition in mitoplasts depends markedly on the presence of extramitochondrial Ca^{2+} (2 μM)

Heart *Mitochondria* *Calmodulin* *Ca^{2+}* *Na^{+}* *Phenothiazine*

1. INTRODUCTION

It is now generally accepted that intramitochondrial Ca^{2+} in heart is established by steady-state cycling via the uniporter (influx) and the Na^{+}-Ca^{2+} carrier (efflux) [1]. A plausible function of this cycle in heart is to control intramitochondrial Ca^{2+} at levels appropriate for regulation of certain Ca^{2+} sensitive dehydrogenases of the mitochondrial matrix [2–5]. Ca^{2+} cycling means that the level of matrix Ca^{2+} will be determined by the kinetic properties of the two transport systems in relation to the periodicity and amplitude of th cytosolic Ca^{2+} transients (review, [6]). In this regard previous studies revealed that increase in extramitochondrial [Ca^{2+}] over the range (0–2 μM) occurring during contraction and relaxation markedly inhibited Na^{+}-Ca^{2+} carrier activity [7]. Kinetic data were consistent with the presence of external regulatory sites for Ca^{2+} on the carrier and a role for these in the control of matrix Ca^{2+} was proposed.

Ca^{2+} sensitivity of cytosolic reactions is typically conferred by calmodulin, and in many (but not all) cases calmodulin associates reversibly with the target protein in a manner sensitive to calmodulin antagonists, e.g. trifluoperazine and other phenothiazine drugs (review, [8]). However, there is no indication that calmodulin or a related protein is implicated in any of the Ca^{2+}-sensitive enzyme or transport processes of mitochondria. One possible exception is the report [9] that low concentrations of trifluorperazine inhibit activation of phosphate-dependent Ca^{2+} efflux from liver mitochondria by a non-dialysable cytosolic factor. In view of the importance of Ca^{2+} regulation of the Na^{+}-Ca^{2+} carrier, we examined the process and its sensitivity to trifluoperazine in heart mitoplasts, in which the outer membrane is broken, thereby allowing for possible dissociation of the Ca^{2+} regulatory sites. The data suggest a close interrelation between non-dissociable sites conferring Ca^{2+} sensitivity and sites with a high intrinsic affinity for trifluoperazine.

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2. MATERIALS AND METHODS

2.1. *Preparation of heart mitochondria and mitoplasts*

Rat heart mitochondria were prepared as described previously [7]. Mitoplasts (inner membrane plus matrix fraction) were prepared by the digitonin technique [10] according to Pedersen et al. [11]. The mitoplasts were washed twice in ice-cold 120 mM KCl containing 10 mM Tris-Hepes (pH 7.0) by resuspension (approx. 10 mg mitoplast protein/50 ml) and resedimentation. The mitoplasts were suspended finally in the same medium plus 0.06% (w/v) bovine serum albumin.

Outer membrane lysis was assessed by the loss from the particulate fraction of creatine phosphokinase, an enzyme located in the intermembrane space of whole mitochondria [12]. Creatine phosphokinase was assayed at 25°C according to Forster et al. [13]. In 3 separate preparations, the creatine phosphokinase activity of the original mitochondria (14.2 ± 0.7 μmol/min per mg mitochondrial protein; mean ± SE) was quantitatively recovered in the soluble fraction (14.2 ± 2.7) with negligible activity in the mitoplasts (0.3 ± 0.2).

Cytochrome ($a + a_3$) contents of mitochondria and mitoplasts were determined as described in [5].

2.2. *Measurement of Ca^{2+} fluxes*

Spectrophotometric assays were carried out at 25°C with a Perkin–Elmer dual-wavelength spectrophotometer operating at 675 minus 685 nm as described [7]. The basic reaction medium (3 ml) contained 120 mM KCl, 10 mM Tris-Hepes (pH 7.0), 0.5 mM KH_2PO_4, 50 μM cytochrome *c*, 3 μg rotenone and mitochondria or mitoplasts (approx. 2 nmol cyt $a + a_3$). Arsenazo III (70 μM) was included together with other additions as stated.

Assays with $^{45}Ca^{2+}$ were carried out by preloading the mitochondria or mitoplasts with approx. 0.1 μCi $^{45}Ca^{2+}$/nmol cyt $a + a_3$ in basic reaction medium (above; 25°C) plus 4 mM succinate (K^+ salt). Total mitoplast or mitochondrial Ca^{2+} was determined (8–12 mol/mol cyt $a + a_3$) as in [14] so that the specific activity of intramitochondrial Ca^{2+} was known. Portions of the incubate were transferred to 4 Eppendorf tubes. Ruthenium red (2 mol/mol cyt $a + a_3$) was added to each and, immediately afterwards, the following additions were made simultaneously to the tubes indicated: none (1), 5 mM NaCl (2), 2 mM EGTA (3), and 5 mM NaCl plus 2 mM EGTA (4). The tubes were centrifuged 15 s later in an Eppendorf bench centrifuge for 1 min. The $^{45}Ca^{2+}$ contents of the supernatants were determined [14]. Na^+-induced efflux of Ca^{2+} in the presence of EGTA was calculated from the difference between the supernatant $^{45}Ca^{2+}$ contents of tubes 3 and 4. Na^+-induced efflux in the absence of EGTA was given by tubes 1 and 2. Residual incubate (without ruthenium red) was also centrifuged to give external Ca^{2+} at the beginning of efflux. From this value and that of tube 2, the mean external free [Ca^{2+}] during efflux in the absence of EGTA was calculated. This was routinely in the range 2–3 μM. In the experiments of fig.2c and d, external free Ca^{2+} was changed above and below this range by adding respectively either $^{45}Ca^{2+}$ or 12 μM $^{45}Ca^{2+}$ plus EGTA to yield the free external [Ca^{2+}] stated. In all cases the $^{45}Ca^{2+}$ added was the same specific activity as that loaded. This precaution was taken to prevent any Ca^{2+}-Ca^{2+} exchange by the Na^+-Ca^{2+} carrier (stoichiometry 1:1 [14]) causing net $^{45}Ca^{2+}$ efflux.

2.3. *Measurement of inner membrane potential ($\Delta\psi$)*

$\Delta\psi$ in mitoplasts and mitochondria was measured as described [5] in basic reaction medium (5 ml) containing 5 μM tetraphenylphosphonium chloride, 5 mM NaCl, 7 μM ruthenium red, 4 mM succinate, 0.5 μCi [U-^{14}C]sucrose and 2 μCi [^{3}H]H_2O.

3. RESULTS AND DISCUSSION

Initial experiments assessed the capacity of mitoplasts to accumulate and release Ca^{2+}. Fig.1 compares the uptake and release of Ca^{2+} by mitoplasts and the parent mitochondria from which they were derived. Ca^{2+} uptake occurred more slowly in mitoplasts; in 12 such experiments the rate of Ca^{2+} influx at 5–6 μM external Ca^{2+} amounted to 48 ± 7% (mean ± SE) of that in parent mitochondria. It was essential to include cytochrome *c* in the mitoplast assays. in its absence, respiration-dependent Ca^{2+} accumulation was barely detectable (<1 mol Ca^{2+}/mol cyt $a + a_3$). Cytochrome *c* is known to be lost on rupture of the outer membrane and washing in saline media [15], as in the mitoplast preparation procedure. Maximal stimulation of Ca^{2+} uptake was attained with 50 μM

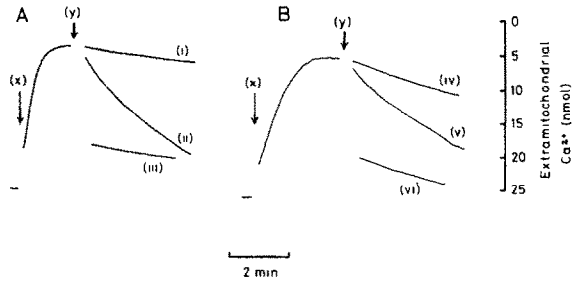

Fig.1. Comparison of Ca^{2+} influx and efflux in mitochondria and mitoplasts Ca^{2+} fluxes in mitochondria (A) and mitoplasts (B) were measured spectrophotometrically. When efflux of endogenous Ca^{2+} was complete (10 min) $CaCl_2$ was added to give a total of 26 nmol. Ca^{2+} uptake was started with 5 mM succinate (x). Efflux was induced by addition (y) of the following: 4 nmol ruthenium red (i and iv); 4 nmol ruthenium red plus 5 mM NaCl (ii and v); 4 nmol ruthenium red plus 15 nmol $CaCl_2$ (iii and vi).

cytochrome *c* (standard conditions).

The rate of Ca^{2+} release in the presence of ruthenium red alone (Na^+-independent release; curves i and iv) was increased in mitoplasts (210 ± 31%; mean ± SE, 12 experiments), whereas the rate of Ca^{2+} efflux in the presence of Na^+ was decreased (curves ii and v). Evidence based on relative sensitivities to lanthanides [16] and hormones [17] indicates that the Na^+-independent process does not reflect a residual capacity of the Na^+-Ca^{2+} carrier to operate in the absence of Na^+, but is attributable to a distinct system. This is substantiated by the relative sensitivities to trifluoperazine reported here. Accordingly, Na^+-Ca^{2+} carrier activity was always calculated from the difference between the rates of Ca^{2+} efflux in the presence and absence of Na^+. In 12 experiments, Na^+-Ca^{2+} carrier activity amounted to 33 ± 4% (mean ± SE) of that in parent mitochondria.

Since uniporter inhibition by ruthenium red may be due to inhibitor binding to a glycoprotein subunit that may be partially lost on rupture of the outer membrane [18], it was essential to check whether ruthenium red effectively prevented Ca^{2+} uptake in mitoplasts. This was done by measuring the Na^+-independent release of Ca^{2+} in the presence of increased extramitochondrial free Ca^{2+} (curve vi). The rate of Ca^{2+} release was the same as that at low extramitochondrial Ca^{2+} (curve iv) confirming that reuptake of Ca^{2+} was not occurring. Similar conclusions are applicable to mitochondria (curves i and iii).

In summary, the gross features of mitochondrial Ca^{2+} transport are retained in mitoplasts. The differences that do occur, namely decreased uniporter and Na^+-Ca^{2+} carrier activities and stimulation of Na^+-independent Ca^{2+} efflux, may be due to some impairment in energy transduction since comparable effects are seen in whole mitochondria on decrease in $\Delta\psi$ [1]. Measurements of $\Delta\psi$ in mitoplasts (under conditions on curve iv) yielded values of 136 ± 7 mV, compared with 162 ± mV in mitochondria (means ± SE; 3 determinations).

3.1. *The effects of trifluoperazine and extramitochondrial Ca^{2+} on Ca^{2+} efflux from mitoplasts and mitochondria*

The influence of trifluoperazine on Na^+-Ca^{2+} carrier activity is shown in fig.2a and b. Trifluoperazine inhibited carrier activity under all conditions tested. In the absence of extramitochondrial free Ca^{2+} (plus EGTA) about 70 μM trifluoperazine yielded 50% inhibition in both mitochondria and mitoplasts. A similar low sensitivity was observed in mitochondria with 2–3 μM external Ca^{2+} (fig.2a). In contrast, the presence of 2–3 μM extramitochondrial Ca^{2+} increased the sensitivity of mitoplasts to trifluoperazine very markedly, and about 5 μM trifluoperazine sufficed for 50% inhibition (fig.2b). As stated above, Na^+-Ca^{2+} carrier activity is known to be energy-dependent [1]. However, 10 μM trifluoperazine had no detectable effect on $\Delta\psi$ of mitoplasts under these conditions; in 2 experiments, $\Delta\psi$ (138 mV, 134 mV) was decreased by 2 mV and 0 mV. Therefore, a direct action of the inhibitor on the transport process is indicated.

Typical traces showing the effect of 5 μM trifluoperazine on Ca^{2+} efflux from mitoplasts are given in fig.3. Whereas the Na^+-induced increment in Ca^{2+} efflux is inhibited, the Na^+-independent rate of Ca^{2+} efflux is quite unaffected. It appears therefore, that of the two efflux processes in heart mitoplasts, only the Na^+-Ca^{2+} carrier is acutely sensitive to trifluoperazine. Further experiments showed that increasing extramitochondrial Ca^{2+} from 2–3 μM to 10–11 μM did not increase inhibition by 5 μM trifluoperazine, i.e. trifluoperazine

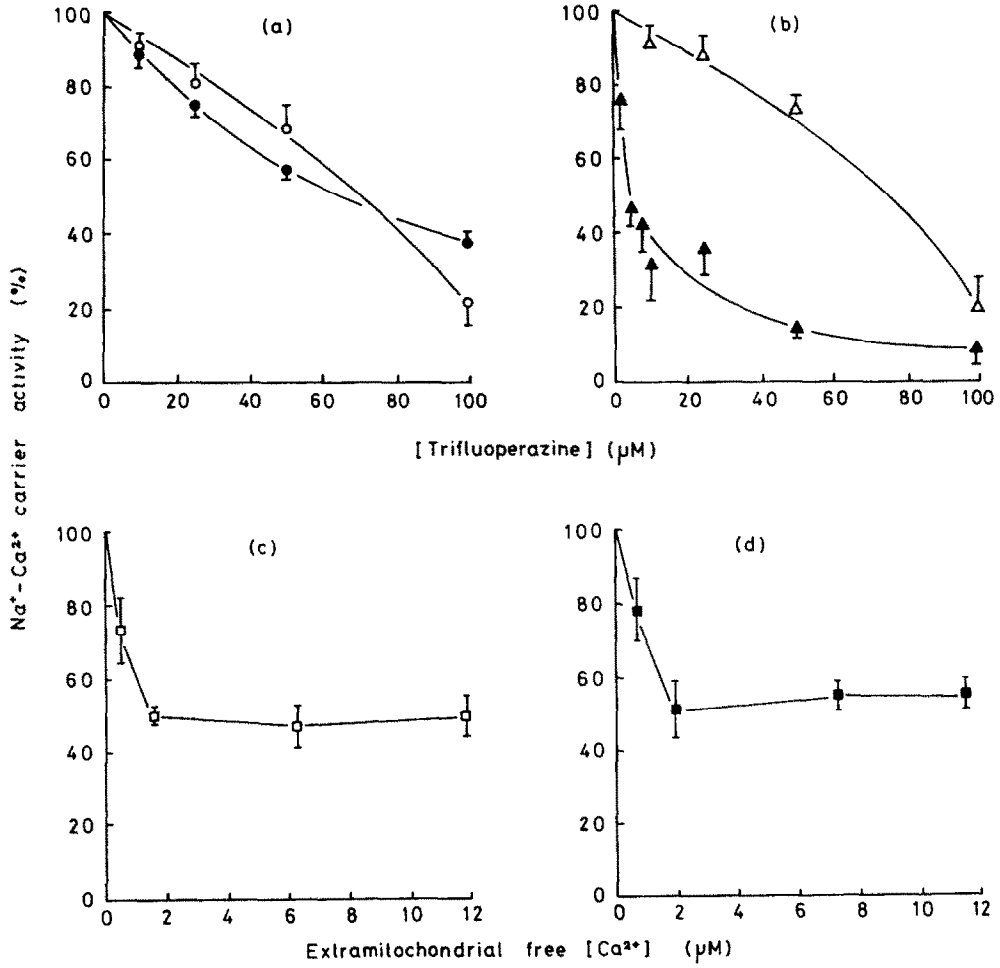

Fig.2. inhibition of $Na^+ \cdot Ca^{2+}$ carrier activity in mitochondria and mitoplasts by trifluoperazine and extramitochondrial Ca^{2+}. The effects of trifluoperazine on mitochondria (a) and mitoplasts (b) were measured with $^{45}Ca^{2+}$ in the presence of 2 mM EGTA (○, △) and in the absence of EGTA (2–3 μM extramitochondrial free Ca^{2+}) as in fig.1 (●, ▲). The effects of extramitochondrial free Ca^{2+} on mitochondria (c) and mitoplasts (d) were determined with $^{45}Ca^{2+}$ as stated in section 2. Na^+-Ca^{2+} carrier activities are expressed as a percentage of the activities in the absence of trifluoperazine (a,b) or external free Ca^{2+} (c,d) in each case. In all cases, Na^+-Ca^{2+} carrier activities were measured with 9–12 mol internal Ca^{2+}/mol cyt $a+a_3$, and were corrected for the Na^+-independent rates of Ca^{2+} efflux. Bars indicate SE for 3 (c,d) or 4 (a,b) separate experiments.

sensitivity was fully expressed with 2–3 μM external Ca^{2+}.

The data reported above indicate that occupation of high-affinity binding sites by external Ca^{2+} is required for high trifluoperazine sensitivity of the Na^+-Ca^{2+} carrier in mitoplasts. As stated in section 1, there is evidence for the presence of external Ca^{2+}-binding sites on the Na^+-Ca^{2+} carrier, quite distinct from those that mediate the actual translocation of Ca^{2+} across the inner membrane [7]. Ca^{2+} interacts over the range 0–2 μM with these sites to produce partial inhibition of Na^+-Ca^{2+} carrier activity. If these sites are responsible for conferring high trifluoperazine sensitivity a minimal requirement is the they are present in mitoplasts.

Fig.2c and d compare the effects of external Ca^{2+} on Na^+-Ca^{2+} carrier activity in mitochondria and mitoplasts. The pattern of inhibition is the same in both cases, i.e., partial, with a maximal in-

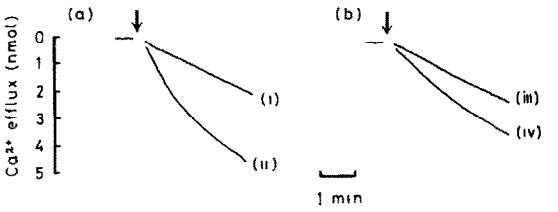

Fig.3. The effects of trifluoperazine on Ca^{2+} efflux from mitoplasts. Mitoplasts (1.7 nmol cyt $a+a_3$) were preloaded with 10 nmol Ca^{2+} as in fig.1. Efflux was started at the arrows by addition of either 4 nmol ruthenium red (i, iii) or 4 nmol ruthenium red plus 5 mM NaCl (ii, iv). (a) Trifluoperazine absent; (b) 5 μM trifluoperazine present.

hibition of about 50% at $<2\,\mu$M external Ca^{2+}. It may be concluded that the component containing these sites is not dissociable into the intermembrane space of mitochondria, but is sufficiently tightly bound to the inner membrane to be fully retained in mitoplasts. In fact other procedures in which mitoplasts were washed in medium (section 2) containing either 2 mM EGTA or 10 μM trifluoperazine also failed to dislocate these sites as judged by the fact that Ca^{2+} produced a similar pattern of inhibition with mitoplasts washed in this way. In principle, therefore, these sites may be involved in the modification of trifluoperazine sensitivity. it is clear however that the differing trifluoperazine sensitivity observed in mitochondria and mitoplasts in the presence of external Ca^{2+} cannot be attributed to a change in the Ca^{2+}-binding characteristics of these sites, since this is not detectably different in mitochondria and mitoplasts. Rather, it appears that the sensitivity of the Ca^{2+} (external) form of the carrier to trifluoperazine is greatly increased in mitoplasts. A possible explanation is that access of trifluoperazine to its binding site(s) is restricted in mitochondria, but that some perturbation of the inner membrane by digitonin leads to exposure of these sites in mitoplasts. In any event, trifluoperazine binds to the relevant sites in mitoplasts with an affinity comparable to that with calmodulin ($K_D \sim 1\,\mu$M [8]).

In conclusion, the present studies with heart mitochondrial mitoplasts indicate the existence of a component of the Na^+-Ca^{2+} carrier with a high affinity for trifluoperazine. The requirement for low concentrations of Ca^{2+} for high trifluoperazine sensitivity is particularly significant since trifluoperazine binding to several proteins is Ca^{2+}-dependent, occurring with the Ca^{2+} form of the protein (calmodulin, troponin C and S-100 [19–21]). In the case of the Na^+-Ca^{2+} carrier this dependence suggests that the components conferring sensitivity of Ca^{2+} and to trifluoperazine are closely interactive, if not the same.

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