Evidence for the existence of regulatory sites for Ca^{2+} on the $\text{Na}^+/\text{Ca}^{2+}$ carrier of cardiac mitochondria

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The Na^+ -induced efflux of Ca^{2+} catalysed by the $\text{Na}^+/\text{Ca}^{2+}$ carrier of cardiac mitochondria is strongly inhibited by extramitochondrial Ca^{2+} . The nature of this inhibition was investigated as follows. (a) The apparent association of external Na^+ and the Ca^{2+} analogue Sr^{2+} with substrate-binding sites (i.e. those sites involved in cation translocation) is promoted markedly by K^+ . The inhibition of $\text{Na}^+/\text{Ca}^{2+}$ exchange by external Ca^{2+} is affected little by K^+ . (b) There is a competitive relationship between the binding of external Na^+ and external Ca^{2+} to substrate-binding sites, whereas at low concentrations ($<4\mu\text{M}$) extramitochondrial Ca^{2+} is a partial non-competitive inhibitor with respect to external Na^+ . (c) This inhibition by external Ca^{2+} is characterized by a maximal decrease of about 70% in the V_{max} of $\text{Na}^+/\text{Ca}^{2+}$ exchange and by co-operative binding of external Ca^{2+} to sites that are half saturated by $0.7\text{--}0.8\mu\text{M}$ free Ca^{2+} . The binding of Ca^{2+} and Sr^{2+} to substrate-binding sites shows no co-operativity. These criteria suggest that the $\text{Na}^+/\text{Ca}^{2+}$ carrier may contain regulatory sites that render the carrier sensitive to changes in extramitochondrial $[\text{Ca}^{2+}]$ within the physiological range.

There is evidence that a number of intramitochondrial enzymes involved in oxidative metabolism may be regulated by changes in free Ca^{2+} over the range $0.1\text{--}10\mu\text{M}$ (Denton & McCormack, 1980; Yamada *et al.*, 1980). This aspect of mitochondrial metabolism stresses the importance of the Ca^{2+} -transport systems of the mitochondrial inner membrane, since these systems determine the concentration of intramitochondrial free Ca^{2+} . The Ca^{2+} -transport systems in mitochondria of heart and some other tissues operate as elements of a transport cycle that catalyses the recycling of Ca^{2+} , Na^+ and H^+ across the inner membrane (Crompton *et al.*, 1976b; Crompton & Heid, 1978; Nicholls & Crompton, 1980; Denton & McCormack, 1980). Evidence *in vitro* indicates that this cycle is able to set the intramitochondrial free $[\text{Ca}^{2+}]$ within the limits required for control of intramitochondrial enzymes (Denton & McCormack, 1980; Kessar & Crompton, 1981).

It is therefore important to establish the factors that control the cycle in order to bring about the appropriate changes in intramitochondrial free $[\text{Ca}^{2+}]$. In this respect, the regulatory properties

Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; HEDTA, *N*-(2-hydroxyethyl)ethylenediamine-*NN'*-triacetic acid.

of the two key carriers of the cycle are of particular interest, i.e. the Ca^{2+} uniporter and the $\text{Na}^+/\text{Ca}^{2+}$ antiporter, which catalyse influx and efflux respectively. Previous work indicates that the promotion of Ca^{2+} influx into mitochondria by α -adrenergic agonists (Taylor *et al.*, 1980) reflects an activation of the Ca^{2+} uniporter (Kessar & Crompton, 1981). Since adrenergic stimulation of heart may lead to increased intracellular Ca^{2+} (Reuter & Scholz, 1977, and references therein), it is important to consider also the consequences of increased cytoplasmic Ca^{2+} . The present paper examines the effects of extramitochondrial Ca^{2+} on the activity of the $\text{Na}^+/\text{Ca}^{2+}$ antiporter of cardiac mitochondria. The data reported suggest that the antiporter may contain regulatory sites which confer a high sensitivity to cytoplasmic Ca^{2+} .

Methods

Mitochondrial preparation

Rat heart mitochondria were prepared generally as described previously (Crompton *et al.*, 1976b), except that 0.05% (w/v) bovine serum albumin (fatty acid-free) was included in the initial homogenization medium. The inclusion of bovine serum albumin is advantageous, since it yielded mito-

chondria that exhibit an extremely low basal rate of Ca^{2+} efflux (defined below). Mitochondria prepared in this way contained 10–16 nmol of endogenous Ca^{2+} /mg of protein.

In the experiments of Figs. 2 and 9, which required mitochondria with little endogenous Ca^{2+} , the hearts were homogenized in medium containing 210 mM-mannitol, 70 mM-sucrose, 10 mM-Tris/HCl (pH 7.3), 1 mM-EGTA and 0.05% bovine serum albumin. The first mitochondrial pellet was washed in the same medium without bovine serum albumin. The second mitochondrial pellet was washed in the same medium without bovine serum albumin and EGTA. Mitochondria purified in this way (Ca^{2+} -depleted mitochondria) contained 2–3 nmol of endogenous Ca^{2+} /mg of protein.

The protein contents of the mitochondrial preparations were determined by a modified biuret method (Kröger & Klingenberg, 1966), with bovine plasma albumin (Sigma Chemical Co., St. Louis, MO, U.S.A.) as standard.

Assay of the $\text{Na}^+/\text{Ca}^{2+}$ antiporter

The activity of the $\text{Na}^+/\text{Ca}^{2+}$ antiporter was measured at 25°C by monitoring the fluxes of Ca^{2+} or Sr^{2+} according to the exchange reaction studied. Mitochondria were preloaded with either Ca^{2+} or Sr^{2+} (Ca^{2+} -depleted mitochondria) in a reaction medium containing 120 mM-KCl plus choline chloride (as stated in the Figure legends), 5 mM-Tris/Hepes (pH 7.0), 0.5–2.0 mg of mitochondrial protein/ml, 1 μg of rotenone/mg of mitochondrial protein and either 7–15 nmol of CaCl_2 or 25 nmol of SrCl_2 /mg of protein. The accumulation of Ca^{2+} or Sr^{2+} was started by the addition of 5 mM-succinate (Tris salt) as respiratory substrate, and it was essentially complete after 2 min. Further Ca^{2+} or Sr^{2+} fluxes via the uniporter were blocked by the addition of 1 nmol of Ruthenium Red/mg of protein. The exchange reactions were started 1 min later by introducing Na^+ , Ca^{2+} or Sr^{2+} as described below. All flux rates were corrected for the slow basal rates of Ca^{2+} or Sr^{2+} flux (0–0.5 nmol/min per mg of protein) that occurred in the absence of added Na^+ , Ca^{2+} or Sr^{2+} . The exchange reactions were assayed as follows.

(a) The initial rates of Na^+ -induced efflux of Ca^{2+} from Ca^{2+} -preloaded mitochondria were measured with the Ca^{2+} -indicator arsenazo III and a Perkin-Elmer model 356 double-beam spectrophotometer operating at 675–685 nm (Scarpa *et al.*, 1978). Arsenazo III (100 μM) was added to the reaction medium at the beginning of the preincubation period.

The extramitochondrial $[\text{Ca}^{2+}]$ was decreased to about 1 μM at the end of the preloading period, and the rates of Na^+ -induced efflux of Ca^{2+} at extramitochondrial $[\text{Ca}^{2+}]$ greater and less than this value

(but at constant intramitochondrial Ca^{2+}) were obtained by adding the appropriate amounts of CaCl_2 or EGTA (2–5 μM) immediately before efflux was started with Na^+ . The extramitochondrial free $[\text{Ca}^{2+}]$ was determined by calibrations with Ca^{2+} /HEDTA buffers as described previously (Crompton *et al.*, 1976a).

The initial rates of Na^+ -induced Ca^{2+} efflux at 'zero' extramitochondrial free Ca^{2+} (<0.1 μM) were measured in the presence of 50 μM -EGTA by the La^{3+} -stop procedure with mitochondria preloaded with $^{45}\text{Ca}^{2+}$ of known specific radioactivity as described previously (Crompton *et al.*, 1977). The time-courses of Na^+ -induced efflux of $^{45}\text{Ca}^{2+}$ in the presence and absence of 2 mM-EGTA (Fig. 4) were measured similarly, except that the reactions were stopped by centrifugation as described by Crompton *et al.* (1977).

(b) The Sr^{2+} -induced efflux of Ca^{2+} from Ca^{2+} -pre-loaded mitochondria was measured with arsenazo III as described in (a) above, except that the wavelengths were 653–634 nm. The difference between the spectra of the Ca^{2+} -arsenazo III and Sr^{2+} -arsenazo III complexes permits measurement of Ca^{2+} with little interference from Sr^{2+} at this wavelength pair, as shown in Fig. 1; the absorbance change caused by Ca^{2+} is about 20-fold greater than that caused by Sr^{2+} .

In general, there was a constant 'initial' rate until about 3 nmol of Ca^{2+} had effluxed. In practice it was not possible to obtain accurate initial rates with additions of Sr^{2+} less than about 10 μM . Calibrations were made by adding standard amounts of CaCl_2 to the system containing the relevant $[\text{Sr}^{2+}]$.

(c) Ca^{2+} uptake by Sr^{2+} -loaded mitochondria (Sr^{2+} -induced uptake of Ca^{2+}) was measured with arsenazo III at 653–634 nm as stated above (b). The mitochondria were Ca^{2+} -depleted during preparation as described under 'Mitochondrial preparation'. Representative traces are reported in Fig. 2.

Fig. 1. Sr^{2+} -induced efflux of Ca^{2+} . Heart mitochondria (1.5 mg of protein) were preloaded with 30 nmol of Ca^{2+} and inhibited with 2 nmol of Ruthenium Red in 3 ml of medium containing 120 mM-KCl as described in the Methods section. The efflux of Ca^{2+} induced by the addition of SrCl_2 (13 μM) was monitored with arsenazo III.

Fig. 2. Sr^{2+} -induced influx of Ca^{2+}

In curves (a) and (b), Ca^{2+} -depleted heart mitochondria (3 mg of protein) were preloaded with 75 nmol of Sr^{2+} and inhibited with 3 nmol of Ruthenium Red in a medium containing 120 mM-KCl as described in the Methods section. At the point indicated, either $10 \mu\text{M}$ - CaCl_2 (curve a) or $10 \mu\text{M}$ - CaCl_2 plus 2 mM- NaCl (curve b) was added. Changes in extramitochondrial $[\text{Ca}^{2+}]$ were followed with arsenazo III. In curve (c), $10 \mu\text{M}$ - CaCl_2 was added to mitochondria which were not preloaded with SrCl_2 . These mitochondria were pretreated in exactly the same way as in curves (a) and (b), except that SrCl_2 was omitted. Curves (b) and (c) are displaced slightly to allow the initial rates to be seen clearly.

The addition of Ca^{2+} to Sr^{2+} -preloaded mitochondria results in a partial reuptake of the added Ca^{2+} (curve a). Mitochondria that are not preloaded with Sr^{2+} take up added Ca^{2+} very slowly (about 0.5 nmol of Ca^{2+} /min per mg of protein), which may be due to incomplete inhibition of the uniporter by Ruthenium Red, and all Ca^{2+} influxes were corrected for the Sr^{2+} -independent Ca^{2+} influx.

The inhibition by Na^+ of the initial rate of Sr^{2+} -induced Ca^{2+} influx (Fig. 2) is considered in the Results section. In the presence of Na^+ some Na^+ -induced efflux of Sr^{2+} occurs. It is important to note that the Na^+ -induced loss of Sr^{2+} (<1 nmol of Sr^{2+} /min per mg of protein) during the initial-rate

Fig. 3. Ca^{2+} -induced efflux of $^{45}\text{Ca}^{2+}$
The Ca^{2+} -induced efflux of $^{45}\text{Ca}^{2+}$ was determined by the EGTA-stop procedure in a medium containing KCl as described in the Methods section. Symbols: O, $1.3 \mu\text{M}$ external Ca^{2+} ; ●, $11 \mu\text{M}$ external Ca^{2+} .

measurements decreased the intramitochondrial Sr^{2+} contents by less than 4%.

(d) The Ca^{2+} -induced efflux of $^{45}\text{Ca}^{2+}$ was measured with mitochondria preloaded with about 50 nCi of $^{45}\text{Ca}^{2+}$ /mg of protein. $^{45}\text{Ca}^{2+}$ efflux was started by the addition of Ca^{2+} buffers containing $100 \mu\text{M}$ -HEDTA and sufficient Ca^{2+} to give the free $[\text{Ca}^{2+}]$ stated, as described in detail by Crompton *et al.*, (1977). Ca^{2+} -induced $^{45}\text{Ca}^{2+}$ efflux was stopped after 10 s by the addition of 1 mM-EGTA. The mitochondria were sedimented and $^{45}\text{Ca}^{2+}$ efflux was determined by established procedures (Crompton *et al.*, 1977). Each determination was accompanied by a control, in which the Ca^{2+} buffer was not added, and which enabled the basal efflux of $^{45}\text{Ca}^{2+}$ to be measured. Fig. 3 shows the time courses of Ca^{2+} -induced $^{45}\text{Ca}^{2+}$ efflux in the presence of $1.3 \mu\text{M}$ and $11 \mu\text{M}$ extramitochondrial Ca^{2+} obtained with the EGTA-stop technique. The initial rates were constant, within experimental error, for at least 10 s.

Analysis of kinetic data

Previous studies (Crompton *et al.*, 1977, 1980; Crompton, 1980) have indicated that the following exchanges are catalysed by the $\text{Na}^+/\text{Ca}^{2+}$ antiporter: $n \text{ Na}^+/\text{Ca}^{2+}$, $\text{Ca}^{2+}/\text{Sr}^{2+}$ and $\text{Ca}^{2+}/\text{Ca}^{2+}$ (stoichiometry 1:1). Na^+ and Ca^{2+} are therefore alternative extramitochondrial substrates for exchange with the intramitochondrial cation, and their binding to

external sites on the antiporter (E) may be considered as follows:

For the sake of simplicity it is assumed that mixed complexes containing both external Na^+ and external Ca^{2+} bound to sites involved in cation translocation (i.e. substrate-binding sites) are negligible (the data of Fig. 9, which show a competitive interrelation between the binding of Na^+ and Ca^{2+} to substrate-binding sites, agree with this assumption). In addition, the binding of internal substrates was not considered; in all experiments it was established that the concentration of internal bivalent cation (Ca^{2+} or Sr^{2+} , depending on the exchange measured) was sufficient to saturate the carrier. With these assumptions a rapid-equilibrium treatment gives the equations stated below.

(a) The initial rate of Na^+ -induced efflux of Ca^{2+} (v) was analysed according to the following relation, which assumes that only the fully Na^+ -loaded carrier ($E \cdot Na_n^+$) is significantly active:

$$\frac{v}{V_{max.}} = \left(\frac{[Na^+]}{K_s^{Na} + [Na^+]} \right)^n$$

or, in reciprocal form:

$$\frac{1}{v^{1/n}} = \frac{K_s^{Na}}{V_{max.}^{1/n}} \cdot \frac{1}{[Na^+]} + \frac{1}{V_{max.}^{1/n}} \quad (1)$$

(b) The initial rate of Ca^{2+} (external)/ Sr^{2+} (internal) exchange (v) in the presence of varied external Na^+ was analysed according to the following:

$$\frac{v}{V_{max.}} = \frac{[Ca^{2+}]/K_s^{Ca}}{\left(1 + \frac{[Na^+]}{K_i^{Na}} \right)^n + \frac{[Ca^{2+}]}{K_s^{Ca}}}$$

or, in reciprocal form:

$$\frac{1}{v} = \frac{K_s^{Ca}}{V_{max.}} \cdot \left(1 + \frac{[Na^+]}{K_i^{Na}} \right)^n \cdot \frac{1}{[Ca^{2+}]} + \frac{1}{V_{max.}} \quad (2)$$

where K_i^{Na} , the inhibition constant for Na^+ , equals K_s^{Na} . The slopes of the double-reciprocal plots [from eqn. (2)] were used to calculate the K_i^{Na} value as follows:

$$\text{Slope}^{1/n} = \left(\frac{K_s^{Ca}}{V_{max.}} \right)^{1/n} \cdot \frac{[Na^+]}{K_i^{Na}} + \left(\frac{K_s^{Ca}}{V_{max.}} \right)^{1/n} \quad (3)$$

Results

Inhibition of the Na^+ -induced efflux of Ca^{2+} by extramitochondrial Ca^{2+}

The effect of extramitochondrial Ca^{2+} on the time course of Na^+ -induced efflux of Ca^{2+} is reported in Fig. 4. In these experiments, the preloading phase

Fig. 4. Na^+ -induced efflux of $^{45}Ca^{2+}$ in the presence and absence of extramitochondrial free Ca^{2+}

Mitochondria (1.5 mg) were preloaded with 18 nmol of $^{45}Ca^{2+}$ /mg of protein in a medium containing 120 mM-KCl. The preloading period was stopped by the addition of 1.5 nmol of Ruthenium Red when the extramitochondrial $[^{45}Ca^{2+}]$ has been decreased to $7 \mu M$. The following additions were made 1 min later (zero time): ●, 10 mM-NaCl; ○, 10 mM-NaCl plus 2 mM-EGTA; ▲, 2 mM-EGTA. The ordinate scale on the right gives the extramitochondrial $[^{45}Ca^{2+}]$ in the absence of EGTA (●).

with Ca^{2+} was stopped before it had attained completion, so that, in the absence of EGTA, the extramitochondrial $[Ca^{2+}]$ increased from about $7 \mu M$ to $15 \mu M$ during Na^+ -induced efflux. It is evident that the Na^+ -induced efflux of Ca^{2+} was promoted by the presence of EGTA. However, EGTA induced no net efflux of Ca^{2+} when Na^+ was absent. These data indicate that Na^+ -induced Ca^{2+} efflux was inhibited significantly by 7 – $15 \mu M$ extramitochondrial Ca^{2+} .

The inhibitory action of extramitochondrial Ca^{2+} was investigated further, as shown in Fig. 5, which reports the initial rates of Na^+ -induced efflux of Ca^{2+} at various concentrations of extramitochondrial Ca^{2+} and with 1 – 10 mM- Na^+ . All the data were obtained with the same intramitochondrial Ca^{2+} content. Ca^{2+} efflux was promoted by the substrate Na^+ and was inhibited by external Ca^{2+} . Ca^{2+} inhibited markedly to about $3 \mu M$, but increase in $[Ca^{2+}]$ above $3 \mu M$ caused negligible further inhibition. Essentially the same pattern of inhibition occurred at all the concentrations of Na^+ tested, e.g. the rates with $1 \mu M$ and $4 \mu M$ extramitochondrial Ca^{2+} were respectively 59 – 62% and 31 – 35% of the rates in the absence of external Ca^{2+} , irrespective of the $[Na^+]$.

It was clearly important to account for the nature of the Ca^{2+} -inhibition curves. It has been reported that heart may contain two types of mitochondria,

Fig. 5. Inhibition of the Na^+ -induced efflux of Ca^{2+} by extramitochondrial Ca^{2+}

The initial rate of Na^+ -induced efflux of Ca^{2+} (v , nmol of Ca^{2+} /min per mg of protein) at 25°C and the extramitochondrial concentrations of free Ca^{2+} were measured in a medium containing 120 mM-KCl as described in the Methods section. All data were obtained with 18–20 nmol of intramitochondrial Ca^{2+} /mg of protein. Symbols: ●, 10 mM- Na^+ ; ○, 5 mM- Na^+ ; ▲, 2 mM- Na^+ ; △, 1 mM- Na^+ .

although Polytron treatment, as used here in the homogenization procedure, released only one type (Palmer *et al.*, 1977; McMillin-Wood *et al.*, 1980). Nevertheless, the possibility was examined that the inhibition curves may reflect the presence of two mitochondrial populations, of which one contains an antiporter that was inhibited strongly by external Ca^{2+} and the other contains an antiporter that was insensitive to extramitochondrial Ca^{2+} . If this were true, the strongly inhibited system would be inhibited completely in the experiment of Fig. 4 that was conducted in the absence of EGTA, since the extramitochondrial $[\text{Ca}^{2+}]$ was always above $7\text{ }\mu\text{M}$. In this case, Ca^{2+} efflux would occur only in the weakly inhibited population, and the difference between the Ca^{2+} contents in the presence and absence of EGTA at 1.5 min (about 8 nmol of Ca^{2+} /mg of protein) would represent the Ca^{2+} content of the Ca^{2+} -inhibited population. Accordingly, efflux from the mixed population would be incomplete, and about 8 nmol of residual Ca^{2+} /mg of protein would be expected after prolonged incubation.

However, this behaviour was not observed. In the absence of EGTA, Ca^{2+} efflux continued after 1.5 min of incubation (at about 3.7 nmol of Ca^{2+} /min per mg of protein) until essentially all the Ca^{2+} was released. Therefore, the pattern of inhibition of the $\text{Na}^+/\text{Ca}^{2+}$ exchange by external Ca^{2+} (Fig. 5) cannot be accounted for by assuming that the mitochondrial preparation contained a mixed population.

In addition, it was necessary to take account of the fact that extramitochondrial Ca^{2+} is a product of $\text{Na}^+/\text{Ca}^{2+}$ exchange and, at appropriate concentrations, must inhibit the exchange. However, the strong inhibition by Ca^{2+} was partial, being essentially complete at $3\text{--}4\text{ }\mu\text{M}$ - Ca^{2+} , and it was not affected detectably by a 10-fold change in substrate (Na^+) concentration. These characteristics suggest that the inhibition may not have arisen by product inhibition (mass action) alone. Nevertheless, it was essential to clarify the extent to which the observed inhibition was due to simple product inhibition. This question was approached by comparing some features of the interaction of external Ca^{2+} with the antiporter as a substrate/product and as an inhibitor. These investigations are reported in the following sections, in which substrate binding refers to the binding of Ca^{2+} and Na^+ to those sites on the antiporter involved in cation translocation, and inhibitor binding refers to the site occupied by external Ca^{2+} when it inhibits the $\text{Na}^+/\text{Ca}^{2+}$ exchange.

Apparent binding of Na^+ and Ca^{2+} as substrates and Ca^{2+} as an inhibitor in the presence and absence of K^+

The catalysis of both the $\text{Na}^+/\text{Ca}^{2+}$ and $\text{Ca}^{2+}/\text{Ca}^{2+}$ exchanges by the $\text{Na}^+/\text{Ca}^{2+}$ antiporter is greatly activated by the presence of K^+ (Crompton, 1980; Crompton *et al.*, 1980). In this section, the characteristics of K^+ activation are compared with the effects of K^+ on the Ca^{2+} inhibition of antiporter activity.

The activation by K^+ of the exchange between internal and external bivalent cations was investigated as reported in Figs. 1 and 6. In practice, it was not possible to obtain accurate kinetic data on the effects of K^+ on the $\text{Ca}^{2+}/\text{Ca}^{2+}$ exchange, since the progress of the reaction cannot be followed continuously. Instead, the analogue Sr^{2+} was used, and the Sr^{2+} -induced efflux of Ca^{2+} was monitored as shown in Fig. 1. Fig. 6 reports double-reciprocal plots of the effects of K^+ on the dependence of the Sr^{2+} -induced Ca^{2+} efflux on the external $[\text{Sr}^{2+}]$. K^+ causes a 9-fold decrease in the K_s value for Sr^{2+} , i.e. the K_s^{Sr} values with zero K^+ , 5 mM- K^+ and 120 mM- K^+ are about $40\text{ }\mu\text{M}$, $20\text{ }\mu\text{M}$ and $4.5\text{ }\mu\text{M}$ respectively. K^+ also brings about a relatively small increase in V_{max} (1.6-fold).

The activation by K^+ of the $\text{Na}^+/\text{Ca}^{2+}$ exchange was investigated as shown in Fig. 7. The initial rate of exchange exhibits a sigmoidal dependence on $[\text{Na}^+]$ in both the presence and absence of K^+ (Fig. 7a). The sigmoidicity may reflect the involvement of more than 1 Na^+ ion per cycle of exchange, as discussed previously (Crompton *et al.*, 1976b, 1977).

Fig. 6. Effects of K^+ on the Sr^{2+} -induced efflux of Ca^{2+} . The initial rates of Sr^{2+} -induced efflux of Ca^{2+} (v , nmol of Ca/min per mg of protein) at $25^\circ C$ were measured as described in the Methods section and indicated in Fig. 1. Symbols: $\bullet$, 120 mM-KCl; Δ , 5 mM-KCl, 115 mM-choline chloride; $\circ$, zero KCl, 120 mM-choline chloride.

Fig. 7. Effects of K^+ on the Na^+ -induced efflux of Ca^{2+} . The initial rates of Na^+ -induced efflux of Ca^{2+} (v , nmol of Ca^{2+} /min per mg of protein) were measured at $25^\circ C$ as described in the Methods section. The extramitochondrial $[Ca^{2+}]$ varied between 4 and $6 \mu M$. In Fig. 7(b), the data are plotted according to eqn. (1) (see the Methods section) with $n=3$. Symbols: $\bullet$, 120 mM-KCl, zero choline chloride; Δ , 5 mM-KCl, 115 mM-choline chloride; $\circ$, zero KCl, 120 mM-choline chloride.

In order to facilitate the interpretation of the effects of K^+ on the Na^+/Ca^{2+} exchange, relations were sought that took into account the exchange of more than 1 Na^+ ion per Ca^{2+} ion and expressed the data in linear form, as indicated in the Methods section. It is apparent from the lowermost plot of Fig. 7(b) that the data conform very closely to eqn. (1) when $n=3$ (correlation coefficient 0.9998; with $n=2$, the same data yield a correlation coefficient of 0.991).

Fig. 7(b) shows that, in the absence of K^+ , the apparent K_s^{Na} value is increased about 3-fold, i.e. the K_s^{Na} values with zero K^+ , 5 mM- K^+ and 120 mM- K^+ are about 10.5 mM, 6.4 mM and 3.4 mM respectively. K^+ also causes a small increase in V_{max} (1.2-fold). The data do not preclude the possibility that K^+ preferentially promotes the binding of only one (or two) Na^+ ion, since differences between the K_s^{Na} values for individual sites may be revealed only by more extensive analysis. In the event that this is true, however, the conclusion that K^+ affects

the binding of Na^+ would not be invalidated, but merely refined.

The effect of K^+ on the inhibition of Na^+/Ca^{2+} exchange by external Ca^{2+} is shown in Fig. 8. Since K^+ activates the Na^+/Ca^{2+} exchange in the absence of external Ca^{2+} , for ease of comparison the data are expressed as a percentage of the rates observed in the absence of external Ca^{2+} (the rates with zero external Ca^{2+} are reported in the legend of Fig. 8). K^+ (120 mM) changes the inhibitory potency of external Ca^{2+} to a small extent only, e.g. the $[Ca^{2+}]$ required for 40% inhibition is increased 1.3-fold in the absence of K^+ .

Interactions between the binding of Ca^{2+} and Na^+ to the antiporter

The interactions between the binding of Ca^{2+} and Na^+ (as substrates), and between the binding of Ca^{2+} (as an inhibitor) and Na^+ , were compared.

Substrate interactions were investigated by the effect of Na^+ on the Sr^{2+} -induced uptake of Ca^{2+} .

Fig. 8. Effect of K^+ on the inhibition by extramitochondrial Ca^{2+} of the Na^+ -induced efflux of Ca^{2+} . The methods used are described in the legend of Fig. 5. The initial rates of Na^+ -induced Ca^{2+} efflux (v) are expressed as a percentage of the rates determined at zero extramitochondrial Ca^{2+} . Symbols: ●, 120 mM-KCl, zero choline chloride, 10 mM- Na^+ (v at zero external Ca^{2+} , 14.5 nmol of Ca^{2+} /min per mg); ▲, 120 mM-KCl, zero choline chloride, 2 mM- Na^+ (v at zero external Ca^{2+} , 2.5 nmol of Ca^{2+} /min per mg); ○, zero KCl, 120 mM-choline chloride, 10 mM- Na^+ (v at zero external Ca^{2+} , 3.8 nmol of Ca^{2+} /min per mg).

Fig. 9. Inhibition by Na^+ of the Sr^{2+} -induced uptake of Ca^{2+} .

The initial rates of Ca^{2+} uptake by Sr^{2+} -loaded mitochondria (v , nmol of Ca^{2+} /min per mg of protein) were measured in a medium containing 120 mM-KCl as described in the legend of Fig. 2. In Fig. 9(a) the data are plotted according to eqn. 2 (Methods section). Symbols: ●, zero Na^+ ; ○, 1 mM- Na^+ ; ▲, 2 mM- Na^+ ; △, 3 mM- Na^+ . In Fig. 9(b), the slopes of the plots of Fig. 9(a) are replotted according to eqn. (3) (Methods section).

The Sr^{2+} -induced uptake of Ca^{2+} was investigated as reported in Fig. 2, which shows that the initial rate of this reaction is inhibited by Na^+ . In Fig. 9(a) the inhibition of Na^+ is analysed with respect to the extramitochondrial $[\text{Ca}^{2+}]$. The plots are interpreted best in terms of a competitive interrelation between Na^+ and Ca^{2+} . The apparent K_s^{Ca} values with zero, 1 mM-, 2 mM- and 3 mM- Na^+ are 4 μM , 11 μM , 20 μM and 43 μM respectively.

The slopes of the plots in Fig. 9(a) may be used to calculate the apparent K_i value for Na^+ according to eqn. (3). The replot of the slopes is shown in Fig. 9(b), and it agrees reasonably with the predicted linear relation when $n=3$. The intercept on the abscissa of the replot ($-K_i^{\text{Na}}$) yields a value for K_i^{Na} of about 2.5 mM. This value is in good agreement with the K_s^{Na} values calculated from the $[\text{Na}^+]$ -dependence of the $\text{Na}^+/\text{Ca}^{2+}$ exchange (2.3–3.4 mM; Figs. 7b and 10), and therefore the data are consistent with a competitive interrelation between the binding of external Na^+ and external Ca^{2+} as substrates of the antiporter. A competitive interrelation is implicit in an antiport mechanism in which Na^+ and Ca^{2+} are alternative external substrates for exchange with the internal cation.

The interaction between the binding of Ca^{2+} (as an inhibitor) and Na^+ was examined as reported

in Fig. 10. The $[\text{Na}^+]$ -dependence of the initial rates of $\text{Na}^+/\text{Ca}^{2+}$ exchange were analysed as a function of the external $[\text{Ca}^{2+}]$. An increase in external $[\text{Ca}^{2+}]$ over the range 0–4 μM caused a progressive decrease in V_{max} of $\text{Na}^+/\text{Ca}^{2+}$ exchange (from 27.5 to 9.5 nmol of Ca^{2+} /min per mg of protein), but the K_s^{Na} value was not changed significantly (2.3–2.6 mM).

Fig. 10 (inset) shows the decrease in V_{max} (ΔV_{max}) of the $\text{Na}^+/\text{Ca}^{2+}$ exchange as a function of external $[\text{Ca}^{2+}]$. Double-reciprocal plots of these data (not shown) indicate that the maximal decrease in V_{max} ($\Delta V_{\text{max}}^{\text{max}}$) by external Ca^{2+} is 19.5 nmol of Ca^{2+} /min per mg of protein, which is equivalent to 70% of the V_{max} observed in the absence of external Ca^{2+} (27.5 nmol of Ca^{2+} /min per mg of protein). In other words, external Ca^{2+} decreases the V_{max} of $\text{Na}^+/\text{Ca}^{2+}$ exchange by a maximum of 70%.

In summary, the analysis of Fig. 10 indicates that external Ca^{2+} (<4 μM) is a partial non-competitive inhibitor with respect to Na^+ , whereas Fig. 9 indicates that there is a competitive relation between the binding of Ca^{2+} and Na^+ as substrates. This discrepancy suggests that the strong inhibition by Ca^{2+} is due to occupation of a site(s) that is not the

Fig. 10. Analysis of the inhibition by Ca^{2+} of the initial rate of Na^+ -induced efflux of Ca^{2+} . The initial rates of Na^+ -induced Ca^{2+} efflux (v , nmol of Ca^{2+} /min per mg of protein) were determined in a medium containing 120 mM-KCl at 25°C as described in the Methods section. The data are plotted according to eqn. (1) (Methods section), with $n = 3$. The extramitochondrial free $[\text{Ca}^{2+}]$ was as follows: (a), zero ($<0.1 \mu\text{M}$); (b), $0.6 \mu\text{M}$; (c) $0.8 \mu\text{M}$; (d) $1 \mu\text{M}$; (e), $1.5 \mu\text{M}$; (f), $2 \mu\text{M}$; (g), $4 \mu\text{M}$. The inset shows the relation between ΔV_{max} and the extramitochondrial $[\text{Ca}^{2+}]$, where $\Delta V_{\text{max}} = V_{\text{max}}$ (with zero external Ca^{2+}) $- V_{\text{max}}$ (with external Ca^{2+} present).

Fig. 11. Hill plots of the binding of external Ca^{2+} and Sr^{2+} as substrates and of external Ca^{2+} as an inhibitor of the antiporter

The initial rates (v) of Ca^{2+} -induced efflux of $^{45}\text{Ca}^{2+}$ (O) and Sr^{2+} -induced efflux of Ca^{2+} (Δ) were measured in a medium containing 120 mM-KCl as described in the Methods section. The data for the decrease in V_{max} of the $\text{Na}^+/\text{Ca}^{2+}$ exchange (ΔV_{max}) by external Ca^{2+} (●) are those of Fig. 10 (inset); ΔV_{max} is the maximal decrease in ΔV_{max} by external Ca^{2+} (as described in the text.).

substrate-binding site for Ca^{2+} , and that occupation of this site(s) decreases $\text{Na}^+/\text{Ca}^{2+}$ exchange activity by 70%.

Co-operative interactions between the inhibitory binding sites for Ca^{2+}

The decrease in $V_{\max}$ (i.e. $\Delta V_{\max}$) of the $\text{Na}^+/\text{Ca}^{2+}$ exchange by external Ca^{2+} provides a measure of the occupancy of the inhibitory binding sites for Ca^{2+} . In Fig. 11, the $\Delta V_{\max}$ values are reported as a Hill plot. The slope of the plot (2.0) indicates that Ca^{2+} inhibits by binding to two or more sites and that co-operative interactions occur between these sites. Half-maximal inhibition is attained with $0.7 \mu\text{M}-\text{Ca}^{2+}$.

For comparison, Fig. 11 also shows plots of the dependence of the initial rates of the $\text{Ca}^{2+}/^{45}\text{Ca}^{2+}$ and $\text{Sr}^{2+}/\text{Ca}^{2+}$ exchanges on the extramitochondrial $[\text{Ca}^{2+}]$ and $[\text{Sr}^{2+}]$. The best fit of both plots yields a slope of 0.97, which suggests that the antiporter contains one external substrate site for bivalent cation. The K_s values calculated from the plots are about $2 \mu\text{M}$ (Ca^{2+}) and $5 \mu\text{M}$ (Sr^{2+}).

Discussion

It has been shown (Fig. 4) that low concentrations of extramitochondrial Ca^{2+} inhibit the $\text{Na}^+/\text{Ca}^{2+}$ exchange, and the essential question raised by this observation is whether the inhibition is caused by occupation of sites involved in Ca^{2+} translocation (i.e. substrate/product sites) or whether it reflects the existence of other Ca^{2+} -binding sites on the antiporter. One approach used to investigate this question involves the $\text{Na}^+/\text{Ca}^{2+}$ exchange, for which it is necessary to take account of multiple binding sites for Na^+ . Regarding the number of such sites, direct measurements have shown that the stoichiometry of the reaction catalysed by the $\text{Na}^+/\text{Ca}^{2+}$ carrier of cardiac sarcolemma is $3\text{Na}^+ : 1\text{Ca}^{2+}$ (Pitts, 1979), and the electrogenic nature of the exchange agrees with this stoichiometry (Reeves & Sutko, 1980; Philipson & Nishimoto, 1980; Caroni *et al.*, 1980). On the other hand, Affolter & Carafoli (1980) detected no net flux of charge during $\text{Na}^+/\text{Ca}^{2+}$ exchange in mitochondria, although any charge imbalance in the reaction may have been compensated by the respiratory chain, which was active in these experiments. In the present work, both the $[\text{Na}^+]$ -dependence of the rate of $\text{Na}^+/\text{Ca}^{2+}$ exchange (Fig. 7b) and the inhibition by Na^+ of the $\text{Ca}^{2+}/\text{Sr}^{2+}$ exchange (Fig. 9) are adequately accounted for in terms of three independent binding sites for Na^+ , and the K_s^{Na} values obtained from the two sets of data are comparable (2.3–3.4 mM). However, the relations that describe the kinetic behaviour of the mitochondria antiporter are not

used primarily to diagnose the stoichiometry, but merely as an empirical means to interpret the data within the context of the question raised above.

There arise four lines of evidence from the data reported that the inhibition of the $\text{Na}^+/\text{Ca}^{2+}$ exchange by external Ca^{2+} is not due to occupation of substrate/product sites for Ca^{2+} .

(a) Occupation of inhibitory sites yields a maximal inhibition of 70% (Fig. 10), whereas it would be predicted that occupation of external substrate sites by Ca^{2+} would cause a maximal inhibition of 100%.

(b) The binding of Ca^{2+} to the inhibitor sites is non-competitive with respect to Na^+ (Fig. 10), but the binding of Ca^{2+} to the substrate sites is competitive with Na^+ (Fig. 9). Quantitatively, about $0.7\text{--}0.8 \mu\text{M}-\text{Ca}^{2+}$ is sufficient to inhibit half-maximally the $\text{Na}^+/\text{Ca}^{2+}$ exchange irrespective of $[\text{Na}^+]$ (Figs. 5 and 11), whereas, for example, the apparent K_s^{Ca} value, $2\text{--}4 \mu\text{M}$ (in the absence of Na^+), is increased about 10-fold by $3 \text{ mM}-\text{Na}^+$ (Fig. 9).

(c) The binding of the external substrates, Na^+ and Sr^{2+} , appears to be greatly promoted by K^+ (Figs. 6 and 7), but the binding of Ca^{2+} to inhibitor sites is changed only slightly by K^+ (Fig. 8).

(d) The data indicate that co-operative binding of two or more Ca^{2+} ions to inhibitor sites occurs, whereas there is no evidence for the involvement of more than one external site when Ca^{2+} and Sr^{2+} bind as substrates (Fig. 11).

The evidence outlined above suggests that the $\text{Na}^+/\text{Ca}^{2+}$ antiporter of cardiac mitochondria contains regulatory sites on the external face of the inner membrane, which confer Ca^{2+} -sensitivity to the carrier and are distinct from the substrate/product sites for Ca^{2+} . The putative regulatory sites respond to changes in $[\text{Ca}^{2+}]$ within the physiological range and show co-operative interactions. These features may render the antiporter highly sensitive to changes in extramitochondrial free $[\text{Ca}^{2+}]$.

Another consequence of these findings may be mentioned, namely that the $\text{Na}^+/\text{Ca}^{2+}$ antiporter operates faster than previously stated. Earlier rate measurements (Crompton *et al.*, 1976b, 1977) were conducted generally within the range $3\text{--}6 \mu\text{M}$ external Ca^{2+} , when the Ca^{2+} -inhibition is almost fully expressed, and yielded $V_{\max}$ values of $12\text{--}15 \text{ nmol of } \text{Ca}^{2+}/\text{min per mg of protein at } 25^\circ\text{C}$. The present data indicate that the $V_{\max}$ of $\text{Na}^+/\text{Ca}^{2+}$ exchange is about $27 \text{ nmol/min per mg}$ (at $<0.1 \mu\text{M}$ external Ca^{2+}).

The possible physiological significance of the regulation of $\text{Na}^+/\text{Ca}^{2+}$ exchange by extramitochondrial Ca^{2+} is considered best within the context of the transport cycle that establishes the distribution of free Ca^{2+} across the inner membrane. The two key carriers of this cycle are the Ca^{2+} uniporter,

which catalyses unidirectional Ca^{2+} influx, and the $\text{Na}^+/\text{Ca}^{2+}$ antiporter, which catalyses active Ca^{2+} extrusion. In the presence of Mg^{2+} , the activity of the Ca^{2+} uniporter shows a sigmoidal dependence on the external $[\text{Ca}^{2+}]$, and about $20\text{--}30\text{ }\mu\text{M}$ Ca^{2+} is required for half-maximal activity (Crompton *et al.*, 1976a). It is therefore reasonable to predict that an increase in sarcoplasmic $[\text{Ca}^{2+}]$ will cause both an increase in unidirectional influx (owing to increased occupancy of the uniporter) and a decrease in the rate of Ca^{2+} extrusion (owing to Ca^{2+} inhibition of the antiporter), so that net Ca^{2+} influx will be promoted.

The capacity of the $\text{Na}^+/\text{Ca}^{2+}$ cycle to relay changes in cytoplasmic $[\text{Ca}^{2+}]$ to the mitochondrial matrix may be limited by its slow response relative to the rapidity of the beat-to-beat changes in cytoplasmic $[\text{Ca}^{2+}]$, and it seems likely that the cycle may respond to the time-average cytoplasmic free $[\text{Ca}^{2+}]$, as discussed previously (Crompton, 1980; McCormack & Denton, 1981; Kessar & Crompton, 1981). Evidence that a long-term increase in sarcoplasmic Ca^{2+} is transmitted via the cycle into the mitochondrial matrix has been reported (Hiraoka *et al.*, 1980; McCormack & Denton, 1981). Studies with isolated heart mitochondria have shown that, at physiological concentrations of extramitochondrial Ca^{2+} , the cycle sets the intramitochondrial free Ca^{2+} within the range required for control of the Ca^{2+} -sensitive dehydrogenases in the mitochondrial matrix (Denton *et al.*, 1980; Kessar and Crompton, 1981). Thus the passive relay of Ca^{2+} changes from cytoplasm to matrix may be one factor that allows oxidative metabolism to keep pace with contractile activity, as discussed by Denton & McCormack (1980). The concerted action of cytoplasmic Ca^{2+} on the uniporter and antiporter suggests that this relay mechanism may respond to changes in cytoplasmic Ca^{2+} in a very sensitive manner.

It would be predicted that a passive response of the cycle to an increase in cytoplasmic Ca^{2+} might occur during adrenergic stimulation of heart, since β -adrenergic agonists activate the Ca^{2+} channel of the sarcolemma and allow increased influx of extracellular Ca^{2+} (Reuter & Scholz, 1977, and references therein). In this case the effects of cytoplasmic Ca^{2+} on the antiporter and uniporter may be reinforced by the selective α -adrenergic-mediated activation of uniporter conductance (Kessar & Crompton, 1981; Crompton & Kessar, 1981). On these grounds one may speculate that the combined action of these three factors may exert a critical role during the adrenergic stimulation of cardiac respiration (Williamson, 1964, 1966) by producing an increased intramitochondrial free $[\text{Ca}^{2+}]$ with consequent activation of the tricarboxylate cycle.

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