Effects of Adrenergic Agonists and Mitochondrial Energy State on the Ca^{2+} Transport Systems of Mitochondria†

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ABSTRACT: This study investigates the effects of adrenergic agonists and mitochondrial energy state on the activities of the Ca^{2+} transport systems of female rat liver mitochondria. Tissue perfusion with the α-adrenergic agonist phenylephrine and with adrenaline, but not with the β-adrenergic agonist isoprenaline, induced significant activation of the uniporter and the respiratory chain. Uniporter activation was evident under two sets of experimental conditions that excluded influences of $\Delta\psi$, i.e., at high $\Delta\psi$, where uniporter activity was $\Delta\psi$ independent, and at low $\Delta\psi$, where uniporter conductance was measured. Preincubation of mitochondria with extracts from phenylephrine-perfused tissue quantitatively reproduced uniporter activation when comparison was made with mitochondria treated similarly with extracts from tissue perfused without agonist. Similar, but more extensive, data were obtained with heart mitochondria pretreated with extracts from hearts perfused with the α-adrenergic agonist methoxamine. Phenylephrine did not affect Ca^{2+} efflux mediated by the Na^+–Ca^{2+} carrier or the Na^+-independent system. In contrast, the liver mitochondrial Na^+–Ca^{2+} carrier was activated by tissue perfusion with isoprenaline; the Na^+-independent system was unaffected. Na^+–Ca^{2+} carrier activation was not associated with any change in a number of basic bioenergetic parameters. It is concluded that the Ca^{2+} transport systems of liver mitochondria may be controlled in an opposing manner by α-adrenergic agonists (promotion of Ca^{2+} influx) and β-adrenergic agonists (promotion of Ca^{2+} efflux). At $\Delta\psi$ values >110 mV, the Na^+-independent system was activated by increase in $\Delta\psi$; the uniporter and Na^+–Ca^{2+} carrier activities were insensitive to $\Delta\psi$ changes in this range. In consequence, decreased $\Delta\psi$ induced net Ca^{2+} uptake during steady-state Ca^{2+} cycling. This offers a further potential mechanism whereby liver mitochondrial Ca^{2+} and Ca^{2+}-sensitive oxidative metabolism may be autoregulated by the mitochondrial energy state.

It is now accepted that Ca^{2+} enters and leaves mitochondria by separate transport systems that mediate continuous Ca^{2+} cycling and establish a steady-state distribution of Ca^{2+} across the inner membrane (Crompton, et al., 1976; Puskin et al., 1976). The Ca^{2+} cycle comprises a single influx system (uniporter) and one (Na^+–Ca^{2+} carrier) or more (Na^+-independent system) transport systems for Ca^{2+} efflux. All current evidence indicates that the uniporter mediates passive Ca^{2+} flux down the Ca^{2+} electrochemical gradient and that the Na^+–Ca^{2+} carrier mediates Na^+/Ca^{2+} exchange [for a review, see Crompton (1985)]. These two transport processes may be resolved by means of their sensitivity to ruthenium red (uniporter), Ca^{2+} antagonists (Na^+–Ca^{2+} carrier; Vaghy et al., 1982), and lanthanides (Crompton et al., 1979). In contrast, the Na^+-independent system is poorly characterized (Gunter et al., 1983). Nevertheless, unlike mitochondria from heart and many other tissues in which Na^+-independent Ca^{2+} efflux activity is negligible (Crompton, et al., 1978), mitochondria from liver apparently contain comparable activities of the two efflux systems (Haworth et al., 1980; Goldstone & Crompton, 1982; Nedergaard, 1984) which raises the question of why two efflux systems are necessary. As a working hypothesis, it seems conceivable that the two efflux systems may not carry out precisely the same function and, by extension, that they may be independently regulated to permit mitochondrial Ca^{2+} to adjust to differing cellular constraints. In line with this, β-adrenergic agonists and glucagon activate the Na^+–Ca^{2+} carrier, but not the Na^+-independent system, of liver mitochondria (Goldstone et al., 1983).

The role of the cycle in Ca^{2+} metabolism is controversial. A function in the regulation of cytosolic Ca^{2+} concentration has been proposed (Nicholls, 1978; Becker, 1980; Reinhart et al., 1984; Nedergaard, 1984), but an alternative case can now be made that in general the cycle may control intramitochondrial free Ca^{2+} concentration according to the regulatory requirements of key, Ca^{2+}-sensitive dehydrogenases of the mitochondrial matrix, i.e., pyruvate dehydrogenase (via Ca^{2+} activation of pyruvate dehydrogenase phosphatase), NAD-linked isocitrate dehydrogenase, and α-oxoglutarate dehydrogenase [see Denton & McCormack (1980) and references cited therein]. According to the latter concept, increases in cytosolic free Ca^{2+} concentration associated generally with cell activation may be relayed via the cycle to produce increases in matrix free Ca^{2+} concentration with consequent promotion of oxidative metabolism and ATP production. Observations consistent with the relay concept have been obtained with mitochondria from heart, skeletal muscle, brain, and adipose tissue (Denton et al., 1980; Hansford, 1981; Ashour & Hansford, 1983; Hansford & Castro, 1985; Marshall et al., 1984) and from the effects of inotropic agents on mitochondrial Ca^{2+} and pyruvate dehydrogenase activity in perfused heart (Crompton et al., 1983; McCormack & England, 1984). The same enzymes from liver are also activated by Ca^{2+}, and the relay concept has recently been extended to this tissue (McCormack, 1985) in connection with the stimulation of oxidative metabolism by α-adrenergic agonists and glucagon (Reinhart et al., 1982; Blackmore et al., 1983; Binet & Claret, 1983; Sugano et al., 1980).

The present paper examines the effects of energy state and adrenergic agonists on the properties of the Ca^{2+} transport

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systems of liver mitochondria. The administration of adrenaline to liver elicits stable changes in several of the properties of isolated mitochondria (see Discussion), and this assortment of responses prompts the question of their interdependence and whether they are expressions of a more fundamental change. Halestrap et al. (1985) proposed that one such change may be in matrix volume and that increases in this parameter may underlie hormone-induced stimulation of respiration and other properties. Siess et al. (1981) suggested that the hormone-induced effects may be artifactual and introduced during the mitochondrial isolation procedure, although this has been contested on a number of grounds (Jansen et al., 1983; Allen et al., 1983). It becomes important, therefore, to examine whether any observed change in Ca^{2+} transport is merely the consequence of an already established change and to address the question of artifacts. In approaching this question, use is made of the increased stability of heart mitochondria with respect to liver mitochondria. In addition, the relay concept (above) may be considered to be one aspect of the question of whether mitochondrial Ca^{2+} concentration in general is controlled according to the cellular requirement for ATP. Examination of these issues leads to the novel conclusion that the liver mitochondrial Ca^{2+} transport systems may be controlled in an opposing manner by α_1-adrenergic and β-adrenergic agonists and, quite independently, by the mitochondrial energy state.

Materials and Methods

Preparation of Mitochondria. Mitochondria were prepared from female, Sprague-Dawley rat livers and hearts and their protein contents determined as described before (Goldstone et al., 1983; Crompton et al., 1983). All livers were perfused before homogenization as described previously (Goldstone et al., 1983) with Krebs–Henseleit medium containing 120 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.3 mM $CaCl_2$, 25.3 mM $NaHCO_3$, 10 mM tris(hydroxymethyl)aminomethane (Tris)[1] lactate, and 1 mM Tris/pyruvate. The perfusion medium (37 °C) was gassed continuously with O_2/CO_2 (19:1). After perfusion for 30 min, the right lobe was clamped off, removed, and homogenized. The remaining lobes were perfused for a further 2 min with either 1 μM adrenaline, 1 μM isoprenaline, or 10 μM phenylephrine introduced into the perfusion medium. The treated lobes were then homogenized immediately.

Measurement of Ca^{2+} Fluxes. Ca^{2+} fluxes were monitored with the indicator arsenazo III using a Perkin-Elmer, Model 356, dual-wavelength spectrophotometer operating at 375–385 nm. Mitochondria (containing 2–3 mg of protein) were preincubated in 3 mL of standard reaction medium (pH 7.0, 25 °C) containing 120 mM KCl, 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (K^+ salt), 60 μM arsenazo III, 0.5 mM KH_2PO_4, 1 mM $MgCl_2$, and 1 μg of rotenone/mg of protein. After 6–10 min, when all endogenous Ca^{2+} had effluxed (checked as described previously; Goldstone et al., 1983), $CaCl_2$ was added so that the total amount of Ca^{2+} in the control and test incubations was exactly the same (7–10 nmol of Ca^{2+}/mg of protein). CCCP and 10 μM TPP^+ were introduced at this stage as indicated in the legends.

Ca^{2+} influx into liver mitochondria was measured after addition of a Ca^{2+} pulse to preenergized mitochondria; this procedure was adopted to allow full development of $\Delta\psi$ before measurement of influx (which is relatively fast in liver mitochondria); 6 mM succinate and 200 μM ATP (K^+ salt) were added to the standard incubation, and when the uptakes of endogenous Ca^{2+} and TPP^+ were complete (approximately 1 min), a Ca^{2+} pulse (10 nmol of Ca^{2+}/mg of protein) was introduced (as in Figure 1). Following uptake, 2 nmol of ruthenium red/mg of protein was added to block the uniporter and permit measurement of the Na^+-independent rate of Ca^{2+} efflux used to correct influx measurements (Figure 1). Unless otherwise stated, uniporter activities were calculated from the time taken to decrease extramitochondrial Ca^{2+} concentration from 3 to 2 μM. Ca^{2+} efflux from liver mitochondria via the Na^+-independent system and the Na^+–Ca^{2+} carrier was measured after addition of 2 nmol of ruthenium red/mg of protein ± 6 mM NaCl after accumulation of 7–10 nmol of Ca^{2+}/mg of protein, ensuring that the total Ca^{2+} concentration of test and control incubations were equal (above). Na^+–Ca^{2+} carrier activity was calculated from the increment in efflux rate induced by Na^+; this procedure has been justified previously (Goldstone et al., 1983).

Ca^{2+} fluxes in heart mitochondria were measured similarly except that influx was determined after addition of 6 mM succinate plus 20 μM ATP (K^+ salts) and a Ca^{2+} pulse was not added (as in Figure 6). Efflux was measured with 20 μM ATP in place of 200 μM ATP.

Measurement of $\Delta\psi$, ΔpH, Matrix Volume, and Respiration. $\Delta\psi$ was determined from the accumulation of TPP^+ by using a selective electrode as described before (Crompton et al., 1983). In practice, Ca^{2+} fluxes and $\Delta\psi$ were determined in parallel by transferring 1-mL portions of the incubations to the TPP^+ electrode chamber (as in Figure 1). Values of $\Delta\psi$ were calculated by taking into account membrane binding of TPP^+ as described by Rottenberg (1984); in this treatment, the partitioning of TPP^+ into the membrane from the matrix is effectively equivalent to an increase in matrix volume of 8 μL/mg of protein in liver mitochondria. The same value was assumed for heart mitochondria, although this may have overestimated somewhat the values of $\Delta\psi$ in view of the greater inner membrane surface area. Matrix volumes were determined with [U-^{14}C]sucrose and ^3H_2O [as described in Crompton et al. (1983)]. ΔpH was measured by using [U-^{14}C]acetate (0.2 mM) and ^3H_2O (Nicholls, 1974). Mitochondrial respiration was measured polarographically with a Clark-type O_2 electrode.

Preparation of Extracts from Perfused Hearts and Livers. Rat hearts were perfused by the Langendorff technique as described before (Crompton et al., 1983) in Krebs–Henseleit medium (see Preparation of Mitochondria) minus lactate and pyruvate. Two hearts were perfused in parallel. After 15-min perfusion, 20 μM methoxamine was introduced into the perfusion medium of one of the hearts, and 2 min later, the heart was freeze-clamped in aluminum tongs precooled in liquid N_2. The remaining heart, without methoxamine, was freeze-clamped immediately afterward. Each sample of frozen tissue was scraped into a precooled mortar, ground to a powder under liquid N_2, and then transferred to a tared vessel containing 10 mL of ice-cold 0.6 N $HClO_4$. The two suspensions were stirred in ice for 15 min and then weighed. The weight of tissue extracted was obtained by difference. Insoluble material was removed by centrifugation. The supernatant fluids were each added to 0.7 mL of 50 mM Tris/4-(2-hydroxyethyl)-1-piperazineethanesulfonate (pH 7.2), and the pH was adjusted to 7.2 with 2 M KOH in ice. The solutions were left on ice for about 1 h after which the precipitated $KClO_4$ was removed by centrifugation at 2 °C. The solutions were freeze-dried and the residues dissolved in 210 mM mannitol/70 mM su-

[1] Abbreviations: TPP^+, tetraphenylphosphonium; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; P_i, inorganic phosphate; ΔpH, inner membrane pH gradient; $\Delta\psi$, inner membrane potential; Tris, tris(hydroxymethyl)aminomethane.

crose corresponding to 4 mL/g of heart extracted. The extracts were stored at −9 °C. Rat livers were perfused as stated previously (see Preparation of Mitochondria) in the presence and absence of 10 μM phenylephrine. The control and test lobes were freeze-clamped and extracted as described for heart.

Pretreatment of Mitochondria. Heart mitochondria isolated as described (see Preparation of Mitochondria) were divided into two portions. One portion was treated with control heart extract and the other with extract from methoxamine-perfused heart (test extract). The control and test extracts used with each mitochondrial preparation were derived from hearts perfused and extracted in parallel.

Pretreatment with extracts was carried out as follows: Mitochondria (10 mg of protein) were incubated at 25 °C in 3.2 mL of medium containing 2 mL of heart extract, 0.2 mM $MgCl_2$, 50 μM atractylate, 1 mM ATP, and 10 μg of rotenone. After 2 min, 30 mL of ice-cold 210 mM mannitol/70 mM sucrose/10 mM Tris-HCl (pH 7.2) was added. The mitochondria were sedimented (46000*g*-min; r_{av}, 8.2 cm) and then washed twice in the same medium by resuspension and sedimentation. In preliminary experiments (Table III), heart mitochondria were pretreated in the same way except that extract was replaced with 2 mL of 210 mM mannitol/70 mM sucrose/10 mM Tris-HCl (pH 7.2). Atractylate was included to prevent ATP-dependent Ca^{2+} accumulation during pretreatment. Liver mitochondrial preparations were divided and treated similarly with control and test extracts from the same liver, except that atractylate was replaced by oligomycin (5 μg/mg of protein) since atractylate adversely affected liver mitochondrial stability.

Statistical Analysis. The effects of agonists on a single variable were analyzed by the Student's paired *t* test. The regression slopes of velocity/$\Delta\psi$ plots (Table II, column d) were compared as follows (Armitage, 1971): The residual variance for each set of control (c) and hormone (h) data was estimated from $S_c^2(n-2) = (\sum_v{}^2 - n\bar{v}^2) - [(\sum v\Delta\psi - n\bar{v}\Delta\bar{\psi})^2/(\sum\Delta\psi^2 - n\Delta\bar{\psi}^2)]$ where the velocities (v), mean $\bar{v}$, $\Delta\psi$, mean $\Delta\bar{\psi}$, and the number of observations (n) refer to control data. S_h was estimated similarly. The pooled variance was obtained, $S^2 = [S_c^2(n_c-2) + S_h^2(n_h-2)]/(n_c+n_h-4)$, and the difference was tested by

$$t = \frac{\text{slope}_h - \text{slope}_c}{\left\{S^2\left[\left(\frac{1}{\sum\Delta\psi^2 - n\Delta\bar{\psi}^2}\right)_c + \left(\frac{1}{\sum\Delta\psi^2 - n\Delta\bar{\psi}^2}\right)_h\right]\right\}^{1/2}}$$

on $n_h + n_c - 4$ degrees of fredom.

RESULTS

Influence of $\Delta\psi$ on Ca^{2+} Transport by Liver Mitochondria. Initial experiments derived conditions under which uniporter activities in different mitochondrial preparations might be compared unambiguously. Simple measurements of the rates of Ca^{2+} accumulation are inadequate in this respect, first, since net Ca^{2+} uptake is the result of uniporter-mediated influx and Na^+-independent efflux and, second, since the influence of $\Delta\psi$ must also be considered. Na^+-independent Ca^{2+} efflux may be measured after inhibition of the uniporter with ruthenium red as shown in Figure 1A. In the present study, all rates of Ca^{2+} uptake designated "uniporter activities" were corrected for the rate of Na^+-independent Ca^{2+} efflux over the same range of intra- and extramitochondrial Ca^{2+} concentrations used to calculate uniporter activities and at the same value of $\Delta\psi$ (considered below).

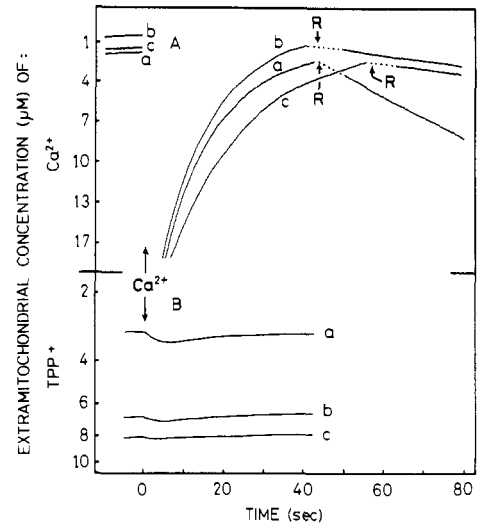

FIGURE 1: Effects of the uncoupler CCCP on the uptake and Na^+-independent release of Ca^{2+} and on TPP^+ accumulation by liver mitochondria. Mitochondria were incubated as described under Materials and Methods with succinate and ATP until the uptakes of endogenous Ca^{2+} and of TPP^+ were complete. A Ca^{2+} pulse (10 nmol/mg of protein) was added at zero time to parallel incubations measuring $[Ca^{2+}]$ (A) and $[TPP^+]$ (B). Ruthenium red (R, 2 nmol/mg of protein) was added as indicated. Curves a, no CCCP; curves b, +45 pmol of CCCP/mg of protein; curves c, +60 pmol of CCCP/mg of protein.

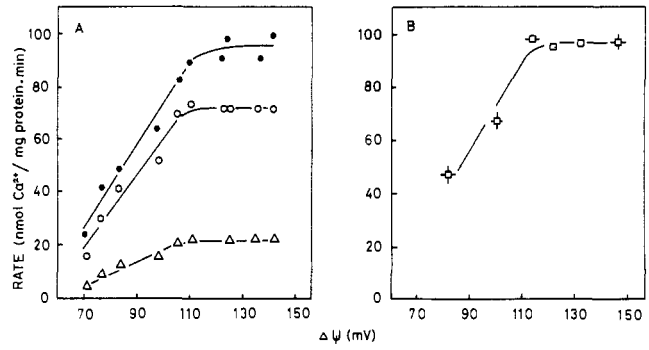

FIGURE 2: Dependence of uniporter activity on $\Delta\psi$ in liver mitochondria. Assays were conducted as in Figure 1; $\Delta\psi$ was varied with 0–95 pmol of CCCP/mg of protein. (Panel A) Uniporter activities of a single preparation measured over the following ranges of extramitochondrial free $[Ca^{2+}]$: (●) 5.5–6 μM; (○) 4.5–5 μM; (△) 2.0–2.5 μM. (Panel B) Collated data from five separate mitochondrial preparations, each assayed over the range 5.5–6 μM external Ca^{2+} concentration at six values of $\Delta\psi$. Bars indicate SEM (not indicated when less than symbol size).

Variations in $\Delta\psi$ occurred inevitably between different mitochondrial preparations and also during Ca^{2+} uptake by any particular preparation. This problem was minimized by introducing Ca^{2+} pulses to preenergized mitochondria in the presence of 0.5 mM P_i and 0.2 mM ATP; lower concentrations of these components lead to considerably greater variations in $\Delta\psi$ (data not shown). A typical depolarization during Ca^{2+} uptake under the present assay conditions, evident from the transient loss of TPP^+ to the medium, is shown in Figure 1B, trace a. The question of whether such a small depolarization (146–141 mV in this case) would introduce significant error into uniporter assays was assessed as reported in Figure 2A. It is evident that the relation between liver mitochondrial uniporter activity and $\Delta\psi$ consisted of two domains, a linear ohmic domain below 110 mV and a sharply defined plateau above this value. The same relation was observed irrespective of external free $[Ca^{2+}]$. This behavior was displayed consistently as shown in Figure 2B, which collates data from five separate mitochondrial preparations. The existence of two

Table I: Effect of Liver Perfusion with Adrenergic Agonists on Uniporter Activity ($\Delta\psi$) and Respiration of Isolated Mitochondria[a]

parameter	control value of parameter	% change caused by			
		adrenaline	phenylephrine	isoprenaline	sham
uniporter act. [nmol of Ca^{2+} (mg of protein)$^{-1}$ min^{-1}]	21.3 ± 0.7 (35)	+26 ± 6[c] (13)	+22 ± 2[b] (13)	+10 ± 8 (5)	−2 ± 3 (4)
$\Delta\psi$ (mV)	151 ± 4 (42)	+1 ± 1 (13)	+1 ± 2 (13)	+1 + 1 (12)	−2 ± 4 (4)
respiration (+CCCP) [ng-atom of O (mg of protein)$^{-1}$ min^{-1}]	94 ± 2 (50)	+29 ± 12[d] (13)	+34 ± 13[d] (13)	+6 ± 3 (20)	+5 ± 12 (4)
respiration (−CCCP) [ng-atom of O (mg of protein)$^{-1}$ min^{-1}]	29 ± 1 (46)	+7 ± 4 (13)	+12 ± 14 (13)	−8 ± 4 (16)	+9 ± 8 (4)

[a] All measurements were made under identical conditions except that CCCP was added for uncoupled respiration. The percent changes were obtained by comparison of control and test mitochondria from lobes of the same livers. The data are given as means ± SEM (number of experiments). [b] $P < 0.002$. [c] $P < 0.01$. [d] $P < 0.05$.

domains of $\Delta\psi$ dependence has not been reported previously.

At high $\Delta\psi$, therefore, a small depolarization during Ca^{2+} uptake (e.g., 5 mV, as in trace a of Figure 1) would not influence uniporter activity provided that $\Delta\psi$ was always maintained above 110 mV. On the other hand, even small depolarizations would introduce significant errors at low $\Delta\psi$; for example, a depolarization from 105 to 103 mV (as in trace b, Figure 1B) would lead to an 11% underestimate of uniporter activity (calculated from the relation of Figure 2A). Although such low values of $\Delta\psi$ were not encountered under standard assay conditions (minus CCCP), omission of ATP or P_i did lead to Ca^{2+}-induced depolarizations below the critical value of 110 mV.

Figure 1 reveals that a decrease in $\Delta\psi$ from 145 mV (trace a) to 105 mV (trace b) inhibited Ca^{2+} efflux via the Na^+-independent system; a further decrease in $\Delta\psi$ to 86 mV (trace c) produced no further inhibition. A complete analysis of the dependence of Na^+-independent Ca^{2+} efflux activity on $\Delta\psi$ (Figure 3) shows that the system was markedly activated by an increase in $\Delta\psi$ above 110 mV but that it was independent of $\Delta\psi$ below this value. It appears then that the Na^+-independent system and the uniporter display converse dependencies on $\Delta\psi$ with a cutoff at about the same value (110 mV). Neither dependence is attributable to changes in matrix volume, since this parameter was constant within the effective range of $\Delta\psi$ (Figure 3).

Analogous measurement of Ca^{2+} efflux via the Na^+–Ca^{2+} carrier revealed no detectable effect of $\Delta\psi$ above 90 mV (data not shown). Dissipation of $\Delta\psi$ to very low values (40–50 mV) did lead to Na^+–Ca^{2+} carrier inhibition (50–60%), which is in line with Na^+–Ca^{2+} carrier inhibition upon block of the respiratory chain with antimycin a (Crompton et al., 1977). It may be concluded that of the two Ca^{2+} efflux systems, only the Na^+-independent system is potentially subject to variations in $\Delta\psi$ in the physiological range.

It would be predicted that the steady-state distribution of Ca^{2+} across the inner membrane, when influx via the uniporter and efflux via the Na^+-independent system are equal, would reflect the $\Delta\psi$ dependencies of the two processes such that depolarization to any value of $\Delta\psi$ above about 110 mV would result in net Ca^{2+} accumulation in the new steady state. Comparison of the steady-state distribution attained in Figure 1A before Ca^{2+} pulse addition, i.e., with 10 nmol of total endogenous Ca^{2+}/mg of protein, confirms this prediction (analogous results were obtained for steady-state distributions after Ca^{2+} pulses). The steady-state extramitochondrial free Ca^{2+} concentration in the presence of CCCP (0.5 μM; $\Delta\psi$ = 106 mV; trace b) was markedly lower than that in the absence of CCCP (1.5 μM; $\Delta\psi$ = 146 mV; trace a). The 3-fold decrease in extramitochondrial free Ca^{2+} concentration is about that expected from the decrease in the Na^+-independent rate of Ca^{2+} efflux (3.6-fold) and a negligible effect of $\Delta\psi$ on uniporter activity. A further decrease in $\Delta\psi$ (to 86 mV, trace c) increased steady-state extramitochondrial Ca^{2+} concentration in line with decreased uniporter activity.

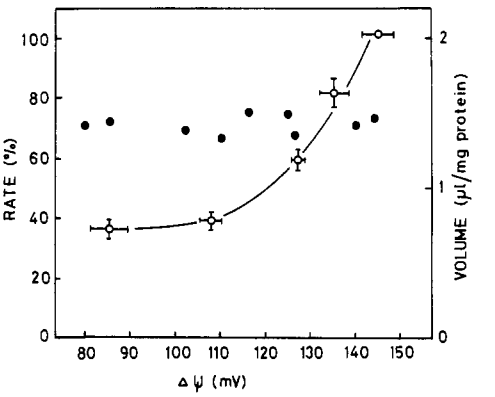

FIGURE 3: Influence of $\Delta\psi$ on Na^+-independent Ca^{2+} efflux and matrix volume in rat liver mitochondria. (Open symbols) Na^+-independent Ca^{2+} efflux activities of five separate mitochondrial preparations (means ± SEM) each determined at five values of $\Delta\psi$ manipulated by 0–100 pmol of CCCP/mg of protein. The values are expressed as a percent of the rates in the absence of CCCP in each case. (Closed symbols) Matrix volumes under the same assay conditions from a single mitochondrial preparation.

Influence of α_1- and β-Adrenergic Agonists on Uniporter Activity of Liver Mitochondria. Table I compares the effects of adrenergic agonists on uniporter activity of isolated liver mitochondria. A small, but highly significant, activation was induced by liver perfusion with adrenaline and with the α_1-adrenergic agonist phenylephrine. The β-adrenergic agonist isoprenaline had no significant effect. The sham data confirm that no systematic error was present in the procedure. Under the same reaction conditions, $\Delta\psi$ was unchanged by any agonist, and the value, 151–152 mV, was well above the critical point (110 mV) below which $\Delta\psi$ influenced uniporter activity. In addition, the matrix volume (1.31 ± 0.07 μL/mg of protein in control mitochondria) was not significantly changed by either phenylephrine (−8 ± 8%) or adrenaline (0 ± 12%).

Analogous experiments were performed over the region in which uniporter activity is sensitive to changes in $\Delta\psi$. Figure 4 reports the $\Delta\psi$ dependencies of uniporter activities in mitochondria isolated from control and phenylephrine-perfused lobes of the same liver. The regression slope of the relation between uniporter activity and $\Delta\psi$, which represents uniporter conductance, was clearly increased by phenylephrine. In this particular experiment, the "phenylephrine" and control curves did not extrapolate to the same abscissal value (+17 and −7 mV, respectively). Although this behavior was sometimes observed, overall there was no significant difference between the control and agonist-extrapolated abscissal values with phenylephrine (+3 ± 3 mV; nine experiments) or with adrenaline (+1 ± 1 mV; five experiments).

This type of experiment was repeated with paired mitochondrial preparations from nine livers (Table II). In each of the nine experiments, a null hypothesis that the two regression slopes were identical was constructed. A test statistic to determine the significance level at which this hypothesis could be rejected was calculated (Materials and Methods).

Table II: Effect of Adrenergic Agonists on Uniporter Conductance of Rat Liver Mitochondria[a]

(a) agonist	(b) control conductance [pmol of Ca^{2+} (mg of protein)$^{-1}$ mV^{-1}]	(c) Δ conductance (%) induced by agonist	(d) significance of each Δ conductance, no. of expt with P <			
			0.002	0.01	0.05	NS
phenylephrine	173 ± 14	+28 ± 5 (<0.002; 9)	5	2	2	0
adrenaline	198 ± 31	+19 ± 2 (<0.02; 5)	4	0	1	0
isoprenaline	188 ± 37	+12 ± 15 (NS; 5)	0	0	2	3
sham	177 ± 16	0 ± 2 (NS; 4)	0	0	0	4

[a] Each experiment was conducted as in Figure 4 with six to nine points for each control and test plot. Data are expressed as follows: columns b and c, means ± SEM (P; number of experiments); column d, the differences in regression slopes were tested as described under Materials and Methods. NS, not significant.

Table III: Effect of Pretreatment of Heart Mitochondria on Δψ and Ca^{2+} Transport[a]

pretreatment condn	Δψ (mV)	Na^+-independent Ca^{2+} efflux [nmol of Ca^{2+} (mg of protein)$^{-1}$ min^{-1}]	uniporter act. [nmol of Ca^{2+} (mg of protein)$^{-1}$ min^{-1}]
(a) none	154 ± 3 (5)	0.5 ± 0.2 (5)	8.6 ± 0.7 (5)
(b) rotenone, Mg^{2+}, ATP, atractylate	146 ± 3 (5)	0.7 ± 0.2 (5)	6.4 ± 0.9 (5)
(c) rotenone, Mg^{2+}, ATP, atractylate, control extract	145 ± 3 (9)	0.6 ± 0.2 (9)	6.6 ± 0.7 (9)

[a] Heart mitochondria pretreated in the presence (c) and absence (b) of control heart extracts were compared with untreated mitochondria (a). Uniporter activities were measured over the range 8–6 μM extramitochondrial Ca^{2+}.

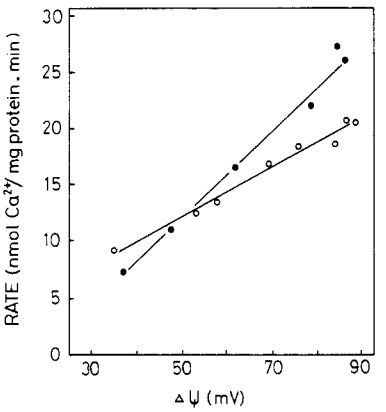

FIGURE 4: Uniporter activities vs. Δψ in mitochondria from lobes of the same liver perfused in the presence and absence of phenylephrine. (Open symbols) Control. (Closed symbols) Phenylephrine treated. Δψ was varied with 100–160 pmol of CCCP/mg of protein.

In all nine cases, the slopes of the phenylephrine curves were significantly greater than the control curves (column d, Table II). The average increase in Ca^{2+} conductance over the nine experiments with phenylephrine amounted to 28 ± 5% (mean ± SEM; column c, Table II).

Likewise, in similar experiments with adrenaline as agonist, all five "adrenaline" conductances were significantly greater than their respective controls, and the average increase amounted to 19%. However, with isoprenaline as agonist, only two of the five test conductances differed significantly from their corresponding controls, and the significance levels in these cases were low ($P < 0.05$). Overall, isoprenaline yielded no significant change in uniporter conductance. Sham experiments confirmed the absence of any systematic error in the procedure (Table II).

In summary, both assay procedures indicated that the Ca^{2+} uniporter was activated by an α_1-adrenergic mechanism in a manner quite independent of Δψ. Under similar experimental conditions, phenylephrine and adrenaline, but not isoprenaline, also increased maximal respiratory capacity (uncoupled respiration; Table I). The parallel effects of these agonists on respiratory chain and uniporter activities underline the necessity of assay procedures that permit changes in uniporter activity to be resolved from possible Δψ-mediated influences of respiration.

α_1-Adrenergic Activation of the Uniporter in Vitro in Liver and Heart Mitochondria. It became important (see Discussion) to examine the feasibility of reproducing α_1-adrenergic activation of the uniporter via in vitro incubations with isolated mitochondria. Experiments with the potential effectors inositol 1,4,5-triphosphate (2 μM), inositol 1,4-diphosphate (2 μM), and inositol 1-phosphate (10 μM) yielded no significant activation. The question was then approached by investigating the capacity of aqueous tissue extracts of agonist-treated tissue to induce activation.

In each experiment, mitochondria from a single stock were treated with extracts derived from agonist-perfused and control lobes of the same liver (Materials and Methods). In six such experiments, the uniporter activity of the mitochondria pretreated with extracts of phenylephrine-perfused liver was increased by 42 ± 14% (mean ± SEM; $P < 0.05$) with respect to the same mitochondria pretreated with control extracts. Under the same assay conditions, the Δψ values of the test and control mitochondria did not differ significantly (147 ± 6 mV and 144 ± 5 mV, respectively).

However, investigations with liver mitochondria were limited by the instability of some preparations after pretreatment. Such preparations were stable for about 1 h, but thereafter, the capacity for Δψ generation diminished with time. While this would not negate the validity of the above experiments, since uniporter activities and Δψ were determined immediately after preparation and simultaneously, it nevertheless compromised more extensive assays at varied Δψ. The question was pursued more systematically with heart mitochondria, in which the uniporter also appears to be activated by α_1-adrenergic mechanism (Crompton et al., 1983) and which, after treatment with extracts, invariably maintained all relevant parameters (Δψ, uniporter activity, and Na^+-independent efflux) constant for several hours after preparation. In experiments with heart, methoxamine was used as the α_1-adrenergic agonist since phenylephrine also exhibits β-adrenergic effects at >1 μM (Scholz, 1980).

Initial experiments examined the tolerance of heart mitochondria to pretreatment with extracts (Table III). Pretreatment with extract caused some loss in uniporter activity (about 23%) and in developed Δψ (rows a and c). A similar change occurred when the mitochondria were pretreated similarly but without extract (row b). Thus, the small decrease

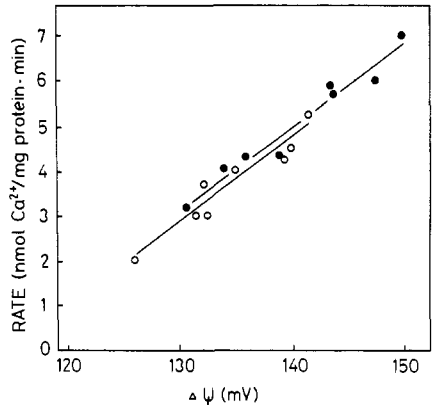

FIGURE 5: Effect of pretreating heart mitochondria on uniporter activity at varied $\Delta\psi$. Heart mitochondria were pretreated with control tissue extract in the presence of ATP, Mg^{2+}, and atractylate (Materials and Methods). Mitochondria thus treated (open symbols) were compared with untreated mitochondria of the same stock (closed symbols). $\Delta\psi$ was varied with 0–40 pmol of CCCP/mg of protein.

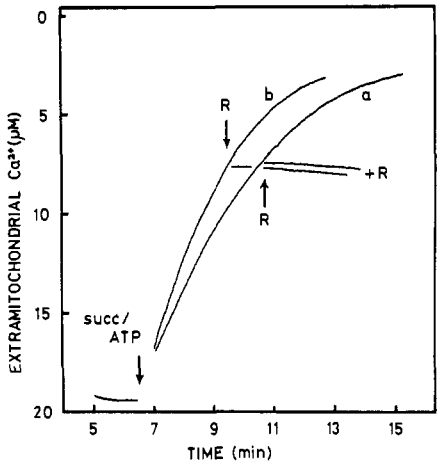

FIGURE 6: Influx and Na^+-independent efflux of Ca^{2+} from heart mitochondria pretreated with extracts of hearts perfused in the presence and absence of methoxamine. After preincubation, Ca^{2+} uptake was started with 6 mM succinate + 20 μM ATP (succ/ATP); ruthenium red (R, 2 nmol/mg of protein) was introduced to separate incubations as indicated. Trace a, control; trace b, methoxamine treated. The two traces during preincubation were superimposable. The values of $\Delta\psi$ determined when extramitochondrial Ca^{2+} concentration was 7 μM were 143 mV (control) and 144 mV (methoxamine).

in both parameters is attributable to pretreatment per se, rather than the presence of extract. Moreover, the decrease of uniporter activity after pretreatment may be accounted for by the decrease in $\Delta\psi$ (Figure 5); i.e., no loss was incurred when comparison is made at equipotential points.

Figure 6 reports typical traces of Ca^{2+} uptake by heart mitochondria of the same stock pretreated with extracts from hearts perfused in the presence (test) and absence (control) of methoxamine. The test mitochondria accumulated Ca^{2+} more rapidly than the controls, but the very slow, Na^+-independent rates of Ca^{2+} efflux did not differ detectably, and in this particular experiment, the $\Delta\psi$ values were about the same (legend). However, in general, although there was no consistent difference between the $\Delta\psi$ values of control and test mitochondria, any particular pair of control and test mitochondria from the same stock rarely developed the same $\Delta\psi$. In addition, the relation between uniporter activity and $\Delta\psi$ in heart mitochondria displayed no plateau region (Figure 5). Therefore, assays were conducted also at lowered $\Delta\psi$ (+CCCP) to permit comparison of rates at equipotential points (Figure 7). Comparison at the highest equipotential point

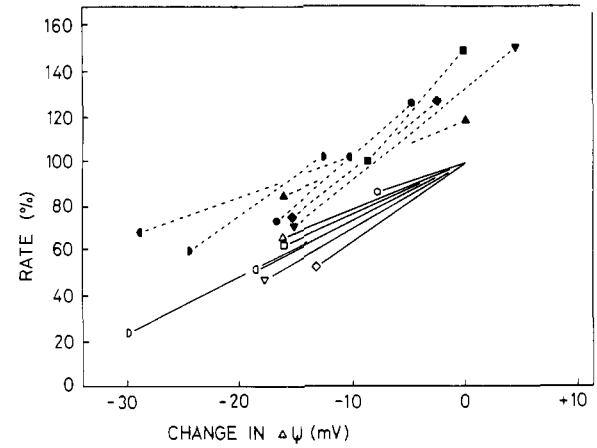

FIGURE 7: Uniporter activities at varied $\Delta\psi$ in mitochondria pretreated with extracts from control and methoxamine-perfused hearts. Mitochondria from seven separate preparations were each treated with extracts of heart perfused in the presence (closed symbols) and absence (open symbols) of methoxamine; seven control extracts and seven test (methoxamine) extracts were used in total, one of each with each mitochondrial preparation. Test and control data derived from the same mitochondrial stock are indicated by closed and open symbols of the same type (e.g., ▲ and △). Assays were conducted in the presence and absence of CCCP in each case. To facilitate data comparison, the control values of uniporter activity and $\Delta\psi$ in the absence of CCCP are standardized to values of 100% and 0 mV, respectively [absolute values 6.9 ± 0.6 nmol of Ca^{2+} (mg of protein)$^{-1}$ min^{-1} and 144 ± 3.1 mV; means ± SEM in the seven experiments]. The other values (i.e., control + CCCP, test ± CCCP) are given with respect to the standardized values in each case. For clarity, test data are joined by dashed lines.

for each pair of lines (test and control) yields a methoxamine-induced activation of 37 ± 4% ($P < 0.001$); comparison at the lowest equipotential points gives an activation of 41 ± 6% ($P < 0.002$). These data would seem to provide firm indications that uniporter activation in heart and also in liver is mediated by a H_2O-soluble metabolite. The relevance of these observations is considered further under Discussion.

Influence of α_1- and β-Adrenergic Agonists on Ca^{2+} Efflux from Liver Mitochondria. In agreement with previous reports (Goldstone & Crompton, 1982; Goldstone et al., 1983), liver perfusion with phenylephrine did not significantly affect the activity of the Na^+-independent system or the Na^+–Ca^{2+} carrier (data not shown). Isoprenaline, however, stimulated Na^+–Ca^{2+} carrier activity by about 150% but had no effect on the Na^+-independent system (Table IV). The β-adrenergic specificity of the Na^+–Ca^{2+} carrier contrasts with the α_1-adrenergic specificity of the uniporter. Na^+–Ca^{2+} carrier activation was not associated with any significant change in a number of basic bioenergetic parameters, i.e., $\Delta\psi$, ΔpH, respiratory chain activity, or matrix volume (Table IV). The respiratory control ratio was also unaffected by isoprenaline (control, 4.9 ± 0.36; isoprenaline, 5.06 ± 0.42; means ± SEM, 10 separate experiments). It may be concluded that β-adrenergic activation of the Na^+–Ca^{2+} carrier is independent of respiration and derived parameters. Analogous experiments with heart mitochondria did not detect any change in the activities of the Na^+–Ca^{2+} carrier or the Na^+-independent system by either isoprenaline (1 μM, 2 min) or methoxamine (10 μM, 2 min).

Discussion

The experiments reported indicate that the liver mitochondrial Ca^{2+} uniporter is activated by tissue perfusion with α_1-adrenergic agonists. Although this stimulation coexists with enhanced respiratory chain activity, the assay procedures employed appear firmly to rule out a causal relationship mediated

Table IV: Effect of Isoprenaline on Na^+–Ca^{2+} Carrier Activity and Other Parameters[a]

parameter	control value of parameter	% change by isoprenaline in (a) parameter	% change by isoprenaline in (b) Na^+–Ca^{2+} carrier act.
$\Delta\psi$ (mV)	145 ± 3 (7)	+3 ± 2	+140 ± 15
ΔpH (units)	0.4 ± 0.5 (4)	−9 ± 18	+162 ± 26
respiration (+CCCP) [ng-atom of O (mg of protein)$^{-1}$ min^{-1}]	114 ± 10 (10)	+8 ± 6	+150 ± 13
matrix volume (μL/mg of protein)	0.74 ± 0.12 (9)	+11 ± 12	+149 ± 15
Na^+-independent Ca^{2+} efflux [nmol of Ca^{2+} (mg of protein)$^{-1}$ min^{-1}]	0.84 ± 0.09 (11)	+9 ± 6	+151 ± 13

[a] The percent changes were obtained by comparing mitochondria isolated from lobes of the same livers. Each row of data was obtained with the same mitochondrial preparations and under identical experimental conditions (except for the presence of CCCP in respiration measurements). Data are given as means ± SEM (number of experiments). The control value of Na^+–Ca^{2+} carrier activity was 0.33 ± 0.04 nmol of Ca^{2+} (mg of protein)$^{-1}$ min^{-1} (13).

by $\Delta\psi$, the only recognized parameter that connects the two. Promotion of Ca^{2+} uptake by phenylephrine was noted previously (Taylor et al., 1980), but neither a strict correlation with $\Delta\psi$ nor corrections for Na^+-independent Ca^{2+} efflux were applied, and the effect was considered to be secondary to enhanced respiration. The distinction between $\Delta\psi$-mediated enhancement of Ca^{2+} uptake and a more direct modification of the uniporter, proposed here, is fundamental, not only mechanistically but also with regard to significance, since the former would not apply at physiological values of $\Delta\psi$, when the uniporter does not respond to changes in this parameter.

Inattention to these same issues may have led also to erroneous conclusions concerning the effects of glucagon (Hughes & Barritt, 1978; Yamazaki et al., 1980; Andea-Waltenbaugh et al., 1981). Wingrove et al. (1984), however, observed no significant effect of glucagon on uniporter activity when comparisons were made at constant $\Delta\psi$, which substantiates the conclusion drawn here (from uniporter insensitivity to isoprenaline) that the uniporter responds specifically to α_1-adrenergic, and not cAMP-mediated, stimulation. In complete contrast, cAMP may well mediate Na^+–Ca^{2+} carrier activation in liver, since this was sensitive to glucagon and to isoprenaline (Goldstone et al., 1983; this study), which increases cAMP in female rat livers (Blair et al., 1979; Studer & Borle, 1982). The contrast is sharpened by the fact that, unlike the uniporter, the Na^+–Ca^{2+} carrier was unaffected by phenylephrine.

The present study, although not definitive, nevertheless provides evidence against artifacts introduced during mitochondrial preparation. Thus, uniporter activation was quantitatively reproduced by incubation of already isolated mitochondria with aqueous tissue extracts under conditions (in heart, at least) where neither the presence of extract (Table III) nor the pretreatment procedure (Figure 5) appeared deleterious to uniporter activity at constant $\Delta\psi$. α-Adrenergic stimulation leads to the rapid appearance of inositol phosphates of which inositol 1,4,5-triphosphate exerts a critical role in mobilizing Ca^{2+} from an endoplasmic reticulum fraction in liver and other tissues [for a review, see Berridge (1984)]. None of the inositol phosphates tested affected the uniporter, but other metabolites have been recognized [e.g., inositol 1,3,4-triphosphate; Irvine et al., 1984) which merit further investigation in this respect. The β-adrenergic stimulation of the Na^+–Ca^{2+} carrier occurred without detectable change in respiration, $\Delta\psi$, ΔpH, or matrix volume. If a change in a basic mitochondrial property, artifactual or otherwise, were responsible in turn for changes in respiration and other functions, including Na^+–Ca^{2+} carrier activity, then a parallel activation of such functions would be anticipated. Since none was found, the data suggest a more direct modification of the Na^+–Ca^{2+} carrier. In addition, in all cases so far investigated, the liver mitochondrial functions enhanced by glucagon are similarly affected by α_1-adrenergic stimulation. These functions include pyruvate carboxylation, succinate oxidation, $\Delta\psi$, ΔpH, uncoupler-stimulated ATPase, and Ca^{2+} retention time (Garrison & Hayes, 1975; Titheradge & Coore, 1976; Taylor et al., 1980; Titheradge et al., 1979). The specific responses of the uniporter (α_1–adrenergic) and Na^+–Ca^{2+} carrier (β-adrenergic, glucagon) of liver mitochondria are the only known cases in which this general observation does not apply, and this would further suggest independent control of these functions.

Mitochondrial Ca^{2+} concentration will be governed by the relative kinetic properties of the influx and efflux systems and the level of cystolic free Ca^{2+} concentration [for a review, see Crompton (1985)]. The present study indicates that the kinetic properties may respond to two sets of conditions: first to the hormonal state of the tissue and second to the energy state of the mitochondria themselves.

The present data suggest a complex hormonal response in which the steady-state behavior of the cycle in liver mitochondria may be regulated in an opposing manner by α_1-adrenergic and β-adrenergic stimulation, the former promoting influx (uniporter activation) and the latter promoting efflux (Na^+–Ca^{2+} carrier activation). It may be noted that although the degrees of retained activation of the uniporter and the Na^+–Ca^{2+} carrier are quite disproportionate (22–28% and 150%, respectively), Na^+–Ca^{2+} carrier-mediated efflux accounts for only a part of the total efflux capacity, and if the total (Na^+ dependent plus Na^+ independent) is considered, then a β-adrenergic-induced increase of 40% is indicated (from the data of Table IV).

α_1-Adrenergic agonists increase cytosolic free Ca^{2+} concentration in liver (Charest et al., 1983; Thomas et al., 1984; Berthon et al., 1984), initially via release from an endoplasmic reticulum fraction (Joseph et al., 1984; Dawson et al., 1986) and, possibly, via subsequent influx across the plasma membrane (Barritt et al., 1981). It was considered earlier that mitochondrial Ca^{2+} may be mobilized (Exton, 1980; Reinhart et al., 1984); recent studies, however, are more consistent with increased mitochondrial Ca^{2+} concentration. Thus, after α_1-adrenergic stimulation, the Ca^{2+}-sensitive enzymes pyruvate dehydrogenase and α-oxoglutarate dehydrogenase are activated, and TCA cycle flux is increased (Assimacopoulos-Jeannet et al., 1983; Siess et al., 1983; Oviasu & Whitton, 1984; McCormack, 1985; Taylor et al., 1986). Under the same conditions, mitochondrial $^{45}Ca^{2+}$ concentration of hepatocytes is also increased (Foden & Randle, 1978; Althaus-Saltzman et al., 1980). The present data reinforce this concept in indicating that mitochondrial Ca^{2+} concentration may be increased not only by raised cytosolic free Ca^{2+} concentration but also via activation of the uniporter.

Analogous studies with β-adrenergic agonists have not been reported. Glucagon has been quite widely investigated in this respect, but no clear consensus has emerged. Several groups

have noted activation of pyruvate dehydrogenase and α-oxoglutarate dehydrogenase following administration of glucagon to hepatocytes in glucose-containing medium or to rats (Assimacopoulos-Jeannet et al., 1983; Oviasu & Whitton, 1984; McCormack, 1985). On the other hand, others have detected no change in pyruvate dehydrogenase activity of hepatocytes or perfused liver by glucagon, under conditions where phenylephrine produced activation (Claus & Pilkis, 1977; Sies et al., 1983); it is perhaps significant that these studies employed media containing lactate/pyruvate in place of glucose (as in the present study). Recently, Cheng and Larner (1985) observed opposing actions of α_1-adrenergic (stimulatory) and β-adrenergic (inhibiting) agonists on pyruvate dehydrogenase of adipocytes and interpreted the latter effect in terms of decreased mitochondrial Ca^{2+} but possible inhibition by acetyl-CoA was not considered. Of particular relevance are the hepatocyte studies of Foden and Randle (1978; also with lactate) that showed not only a phenylephrine-induced increase in mitochondrial Ca^{2+} concentration (mentioned above) but also a decrease in mitochondrial Ca^{2+} concentration after administration of glucagon (2 nM, 5–15 min). This action of glucagon was abolished by insulin. On the other hand, McCormack (1985) observed enhanced activities of pyruvate dehydrogenase and isocitrate dehydrogenase in mitochondria isolated from glucagon-treated rats and that this enhancement was abolished by Ca^{2+} depletion of the mitochondria, which suggests that under these conditions mitochondrial Ca^{2+} concentration increased in response to glucagon.

An aspect of Ca^{2+} cycle behavior that should not be overlooked is that in the absence of carrier regulation, matrix free Ca^{2+} concentration would be fixed by cytosolic free Ca^{2+} concentration and the latter therefore would be the essential determinant of Ca^{2+} regulation of oxidative metabolism. Hormonal modification of the transport systems (above) may be one means of modulating this otherwise strict dependence. However, it would seem advantageous that mitochondria themselves exert some control over the influence of cytosolic Ca^{2+} concentration on their own metabolism. The $\Delta\psi$ dependence of the Na^+-independent system together with the insensitivity of the uniporter to physiological changes in $\Delta\psi$ may provide such a mechanism. Steady-state mitochondrial Ca^{2+} concentration is determined by the kinetic properties of the transport systems, rather than thermodynamically, and although Ca^{2+} accumulation is energy dependent, a decrease in $\Delta\psi$ in the physiological range nevertheless produced a new steady-state distribution with increased mitochondrial Ca^{2+} concentration. Such an increase in turn would activate oxidative metabolism and promote restoration of the high energy state. If this mechanism operates in vivo, it may allow matrix free Ca^{2+} concentration to adjust to the mitochondrial energy state in the absence of external factors and complement other recognized regulators of oxidative metabolism (ATP/ADP, NADH/NAD) in rendering mitochondria self-regulatory to some degree in energy state. Further studies are needed to elucidate whether the Na^+-independent system responds directly to $\Delta\psi$ or to some dependent parameter and whether such a parameter changes sufficiently in vivo. Finally, the present work provides perhaps a clue to why liver mitochondria possess two distinct transport systems for Ca^{2+} efflux, since it appears that whereas the Na^+-independent system responds to energy state, the Na^+–Ca^{2+} carrier is insensitive to this parameter but is responsive to hormonal intervention.

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Registry No. Ca, 7440-70-2; phenylephrine, 59-42-7; adrenalin, 51-43-4; methoxamine, 390-28-3.

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Thermal Expansion of a Protein

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ABSTRACT: The thermal expansion of a protein, metmyoglobin, was investigated by analysis of the refined X-ray crystal structures at 80 and 255-300 K. On heating from 80 to 300 K, the volume occupied by myoglobin increases by approximately 3%. The linear thermal expansion coefficient is estimated to be 115 $\times 10^{-6}$ K^{-1}. This value is more than twice as large as that of liquid water but less than that of benzene. As the temperature is raised, the internal volume change does not come from the large, atom-sized internal cavities in the structure but from an increase in the small, subatomic free volumes between atoms. The largest expansion occurs in the region of the CD and GH corners; both these regions move away from the center of the protein. The remainder of the expansion results from the lengthening of contacts between segments of secondary structure.

In any condensed medium, there is a dynamic balance between the cohesive forces holding atoms or molecules together and the thermally driven random motion of these atoms. At higher temperatures, the availability of more thermal energy implies greater motion, which usually increases the average distance between atoms. In a protein, unlike a simple atomic solid or liquid, both structure and motion are highly irregular.[1]

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[1] Proteins are dynamic systems, and their function depends not only on their average structures but also on fluctuations about that average structure. General reviews of protein dynamics are given by Careri et al. (1975), Gurd and Rothgeb (1979), Debrunner and Frauenfelder (1982), Karplus and McCammon (1983, 1986), and Petsko and Ringe (1984).

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