Modification by Calcium Ions of Adenine Nucleotide Translocation in Rat Liver Mitochondria

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1. Added Ca^{2+} stimulates the translocation of ATP by isolated rat liver mitochondria. 2. The apparent K_m for added Ca^{2+} in stimulating the translocation of $200\text{ }\mu\text{M}$ -ATP is approx. $160\text{ }\mu\text{M}$ ($75\text{ }\mu\text{M}$ 'free' Ca^{2+}). 3. The greatest stimulation of ATP translocation by Ca^{2+} occurs at the lower concentrations of ATP. 4. Sr^{2+} (and to a lesser extent Ba^{2+}) can replace Ca^{2+} whereas Mg^{2+} and Mn^{2+} have only little ability to stimulate ATP translocation. 5. Translocation of dATP is also stimulated by Ca^{2+} whereas that of ADP is stimulated to only a relatively small degree. 6. Studies with metabolic inhibitors and uncouplers provide evidence that stimulation by Ca^{2+} and by uncouplers is additive and that the mechanism of Ca^{2+} stimulation does not seem to involve the high-energy intermediate of oxidative phosphorylation. 7. In the presence of Ca^{2+} , ATP is able to effectively compete with ADP for translocation. 8. Added K^+ further enhances the ability of Ca^{2+} to stimulate ATP translocation. 9. These findings are discussed in relation to the potential involvement of Ca^{2+} in modifying enzymic reactions involved in the regulation of cell metabolism.

There is substantial evidence for the existence in mammalian mitochondria of an enzyme complex responsible for the translocation of adenine nucleotides into and out of these organelles (Brierley & O'Brien, 1965; Bruni *et al.*, 1965; Chappell & Crofts, 1965, 1966; Duee & Vignais, 1965; Pfaff & Klingenberg, 1968; Pfaff *et al.*, 1965, 1969; Winkler *et al.*, 1968). Investigations of this adenine nucleotide translocase have revealed several interesting features, among which are the absolute specificity for adenine nucleotides, the involvement of an obligatory 'one-for-one' exchange, the very high affinity for adenine nucleotides and the inhibition by low concentrations of atractyloside. The translocase probably plays a major role in overall cell-metabolism through its ability to establish a phosphorylation potential in the mitochondria different from that in the cytosol (Klingenberg *et al.*, 1969). These and other properties of the reaction have been reviewed (Klingenberg & Pfaff, 1968).

An additional feature of the translocase is the stimulation of ATP translocation by low concentrations of Ca^{2+} (Spencer & Bygrave, 1971). In the present communication we present a more detailed account of this finding. Kinetic experiments have been carried out which describe the general properties of the Ca^{2+} -stimulated translocation.

Experimental

Materials

[8- ^{14}C]ATP (40 mCi/mmol), [8- ^3H]ATP (23.4 Ci/mmol), [8- ^{14}C]ADP (50 mCi/mmol) and [2- ^3H]ADP

(5.9 Ci/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Hexokinase (EC 2.7.1.1), glucose 6-phosphate dehydrogenase (EC 1.1.1.49), pyruvate kinase (EC 2.7.1.40), adenylate kinase (EC 2.7.4.3), lactate dehydrogenase (EC 1.1.1.27) and all nucleotides were purchased from C. F. Boehringer und Soehne, G.m.b.H., Mannheim, Germany. Other chemicals were obtained as follows: rotenone, antimycin A, oligomycin, gramicidin D and 1-(*N*-2-hydroxyethyl)piperazine-*N'*-yl)ethanesulphonic acid (hepes) from Sigma Chemical Co., St. Louis, Mo., U.S.A.; potassium atractylate (atractyloside) and valinomycin from Calbiochem, Los Angeles, Calif., U.S.A.; Soluene and 5-(4-biphenyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole from Packard Instrument Co., Downers Grove, Ill., U.S.A. All other reagents were of analytical grade.

The concentrations of 'free' Ca^{2+} and 'free' ATP were calculated from the known stability constants of the CaATP^{2-} and CaHATP^- species (Sillen & Martell, 1964; Taqui Khan & Martell, 1966).

Animals

Wistar strain albino rats of approx. 200g body weight were used in all experiments. They were maintained on a normal diet.

Methods

Isolation of mitochondria. Animals were stunned and then killed by decapitation. The livers were rapidly removed and placed in ice-cold isolation medium containing 250 mM-sucrose, 2 mM-hepes and 1 mM-EDTA buffer adjusted to pH 7.4 with KOH

(final concentration approx. 1 mM). The liver was minced with scissors and homogenized with a glass-Teflon tissue disintegrator (Arthur H. Thomas Co., Philadelphia, Pa., U.S.A.; size C) which was motor-driven at 900 rev./min for 45 s. The resulting suspension was made up to 100 ml with isolation medium and centrifuged at 2500 rev./min (600g) for 5 min in a Sorvall RC-2B refrigerated centrifuge (Ivan Sorvall Inc., Newtown, Conn., U.S.A.) fitted with the SS-34 rotor. The supernatant was centrifuged at 8000g for 10 min and the mitochondrial pellet resuspended by hand in 70 ml of EDTA-free 250 mM-sucrose-2 mM-hepes-KOH buffer, pH 7.4. The above two centrifugation steps were then repeated, and the final mitochondrial pellet was washed with EDTA-free isolation medium and resuspended at a concentration of 30 mg of mitochondrial protein/ml.

Preparation of mitochondria for 'back-exchange' experiments. [For theory see Pfaff & Klingenberg (1968).] Samples of the mitochondrial suspension, containing 100 mg of protein, were incubated with 2 μ Ci of [14 C]ATP or [14 C]ADP or with 5 μ Ci of [3 H]ATP or [3 H]ADP for either 30 min at 4°C or 90 min at 0°C. These mitochondria were then centrifuged (8000g for 10 min in the Sorvall centrifuge) and washed twice by centrifugation in EDTA-free isolation medium and finally resuspended to the original protein concentration.

Incubation conditions. All experiments were performed in a medium consisting of 200 mM-sucrose containing 2 mM-hepes (pH 7.4) and at a temperature of 4°C with 1-2 mg of mitochondrial protein/ml present. Other additions were made as indicated in the legends to figures and tables.

Incubation methods. Three different types of incubation system were used. (a) Time-course determinations, using the 'back-exchange' technique, were done in a thermostatically-controlled water-jacketed glass incubation vessel (vol. 1.5 ml). Stirring was accomplished by means of a small magnet. Mitochondria were introduced to the reaction medium 1 min before starting the reaction unless otherwise indicated. The reaction to be studied was initiated by the addition of non-radioactive adenine nucleotides with or without Ca^{2+} as indicated. At predetermined time-intervals, portions of the incubation mixture were removed, added to an Eppendorf Microfuge tube containing 50 μ M-atractyloside and the tube contents mixed on a Vortex-Genie mixer (Scientific Industries Inc., Springfield, Mass., U.S.A.). Approx. 10 s before initiating the reaction, a sample was removed to determine the 'unspecific' leakage of endogenous labelled adenine nucleotides. This was used in calculating the zero-time control. The shortest reaction time obtainable by this method was 5 s.

(b) Determination of the initial rates of reactions were performed in Eppendorf Microfuge tubes. The reaction was initiated by the addition of a portion

of the mitochondrial suspension. The final volume was 250 μ l. The reaction was stopped, usually after 10 s, by the addition of 50 μ M-atractyloside. Mixing, both on the addition of mitochondria and of atractyloside, was performed by gentle agitation on a Vortex-Genie mixer. The tubes were temperature-equilibrated by insertion in a specially designed Perspex rack placed in a water bath. In both of these methods the mitochondria were separated from the incubation mixture by centrifugation in an Eppendorf bench centrifuge for 2 $\frac{1}{2}$ min. Methods (a) and (b) were used only for back-exchange reactions. Control experiments indicated that both methods gave quantitatively similar results.

(c) Forward-exchange experiments were performed as in (a) or (b) above with [14 C]ATP or [14 C]ADP. The reaction was stopped in the usual way with atractyloside and the mitochondria were separated by aspiration with a water-driven vacuum pump. In dual-labelling experiments (see the Results section) a sample of the supernatant was retained and counted for radioactivity. The sediment was then washed with 0.5 ml of the basic incubation medium and recentrifuged. The supernatant was removed and the pellet dissolved in 0.1 ml of Soluene.

Radioactivity measurements. Radioactivity was determined by counting in a sample of the supernatant (back-exchange experiments) or the solubilized mitochondrial pellet (forward-exchange experiments). The samples were dissolved in 10 ml of scintillation fluid (6 g of scintillant, 400 ml of ethoxyethanol and 600 ml of toluene) and counted to 1% error in a Packard Tri-Carb scintillation spectrometer on appropriate channel settings. All c.p.m. were converted into d.p.m. by using an external standard.

Specific activities. Data were analysed as indicated by Pfaff & Klingenberg (1968) and Pfaff *et al.* (1969). To do the analyses, the size of the readily exchangeable endogenous adenine nucleotide pool present in mitochondria had to be determined. The percentage exchange of endogenous adenine nucleotides or, in more absolute terms, the activity of the translocase expressed in nmol of adenine nucleotide translocated/min per mg of mitochondrial protein could then be calculated.

Analytical methods. Protein was determined by a modified biuret method with bovine albumin fraction V as standard. Mitochondria were solubilized with 2% sodium deoxycholate. ATP was assayed by the method of Lamprecht & Trautshold (1965) and ADP and AMP by the method of Adam (1965). Radioactive adenine nucleotides were analysed for radiochemical purity by chromatography on DEAE-cellulose paper (Morrison, 1968).

Results

Fig. 1 shows the influence of different added Ca^{2+} concentrations on the exchange of 200 μ M-ATP by

Fig. 1. Effect of Ca^{2+} on the rate of ATP translocation

Mitochondria were incubated by method (a) for the times shown, in the basic medium supplemented with $200\mu\text{M}$ -ATP. o, No addition; ■, $100\mu\text{M}$ - Ca^{2+} ; ▲, $200\mu\text{M}$ - Ca^{2+} ; ●, $400\mu\text{M}$ - Ca^{2+} .

isolated rat liver mitochondria. The rate of exchange is markedly stimulated in the presence of the added Ca^{2+} . The zero-order rate constants for these time-courses vary from 5.4 to $16.1\text{ nmol/min per mg}$ of protein between zero and $400\mu\text{M}$ - Ca^{2+} respectively. Other experiments have shown that measurements involving the forward-exchange technique give quantitatively similar results to those obtained with the back-exchange technique (see for example, Table 7). The rate of leakage of labelled adenine nucleotides from the mitochondria remained low and constant for up to 15 min in the absence and presence of Ca^{2+} . These findings indicate that Ca^{2+} was causing little membrane rupture during the period of incubation.

Effect of Ca^{2+} concentration on the translocation of ATP

Data in Fig. 2 show the effect of increasing the added Ca^{2+} concentrations on the exchange (hereafter referred to as translocation) of $200\mu\text{M}$ -ATP. The slight deviation from the hyperbolic curve seen at the lower Ca^{2+} concentrations is highly reproducible and has also been seen by Meisner (1971). The

Fig. 2. Effect of Ca^{2+} concentration on the translocation of ATP

Mitochondria were incubated by method (b) in the basic medium supplemented with $200\mu\text{M}$ -ATP and the Ca^{2+} concentrations shown.

apparent K_m for added Ca^{2+} is approx. $150\mu\text{M}$. This is equivalent to $75\mu\text{M}$ 'free' Ca^{2+} . The maximal stimulation attained in this experiment was 270%. The rate of ATP translocation in the absence of added Ca^{2+} was $6.0\text{ nmol/min per mg}$ of protein.

The data in Table 1 show the kinetic constants obtained when the influence of Ca^{2+} concentration on the translocation of ATP at different ATP concentrations is examined.

It was ascertained that, under the experimental conditions employed, no detectable breakdown of exogenous ATP took place. Two things are evident from these data. First, the greatest relative stimulation of the translocase by Ca^{2+} occurs at the lower ATP concentrations. Similar effects were observed by Meisner (1971) with a K^+ -stimulated system. In the present system the maximal stimulation decreases from 520 to 270% over the range of ATP concentrations of 5 to $200\mu\text{M}$. Secondly, the apparent K_m values for Ca^{2+} , as a function of added cation, increase as the ATP concentration is increased; these varied in the range 80 – $160\mu\text{M}$. However, when the K_m values are determined as a function of 'free' Ca^{2+} , the apparent affinities of the system for Ca^{2+} seem to be equivalent at approx. $80\mu\text{M}$. This value is similar to that of the mitochondrial low-affinity binding sites for Ca^{2+} (Scarpa & Azzi, 1968). The large exchange-activity at low ATP and high Ca^{2+} concentrations, when the concentration of free ATP is negligible, would suggest that the CaATP^{2-} complex itself is a substrate for the adenine nucleotide translocase.

Table 1. *Kinetic constants for Ca^{2+} -stimulated translocation of ATP at several added ATP concentrations*

Mitochondria were incubated by method (b) with the ATP and Ca^{2+} concentrations shown. The kinetic data were determined by using double-reciprocal plots. The basal rate of translocation was 4.8 nmol/min per mg of protein.

ATP concentration (μM)	$V_{\text{max.}}$ (maximal relative activation)	Apparent K_m for Ca^{2+} (μM)	
		'Added'	'Free'
5	6.2	80	78
10	5.0	90	85
50	4.1	105	84
100	3.9	130	83
200	3.7	160	75

Table 2. *Specificity of the bivalent metal ions in stimulating ATP translocation*

Mitochondria were incubated by method (b) in the basic medium supplemented with 200 μM -ATP and various concentrations of the metal ion to be tested. The kinetic data were determined as indicated in the legend to Table 1. The rate in the absence of added metal ions was 5.0 nmol/min per mg of protein.

Metal ion tested	$V_{\text{max.}}$ (maximal relative activation)	Apparent K_m for metal ion (μM)	
		'Added'	'Free'
Ca^{2+}	3.8	160	75
Sr^{2+}	3.9	155	93
Mg^{2+}	2.3	690	540
Ba^{2+}	2.0	290	215
Mn^{2+}	1.2	*	

* Not determined.

Bivalent metal ion specificity for stimulation of the translocase

Table 2 shows the specificity of the stimulated translocation for bivalent metal ions, expressed in terms of apparent K_m values, both for added and 'free' metal ion, as well as the $V_{\text{max.}}$. Sr^{2+} appears to mimic Ca^{2+} in having both a similar K_m and $V_{\text{max.}}$. Mg^{2+} , however, has a much lower affinity for the stimulation of ATP translocation with the K_m being about fourfold higher than that for either Ca^{2+} or Sr^{2+} . This decreased affinity is accompanied by a decreased maximal stimulation which is approximately one-half of that for Ca^{2+} . Ba^{2+} seems to be intermediate between the $\text{Ca}^{2+}/\text{Sr}^{2+}$ pair and Mg^{2+} in enhancing the translocation of ATP, at least when expressed in terms of the K_m . However, the maximal relative activation is less than that for Ca^{2+} , Sr^{2+} or Mg^{2+} . Of the other bivalent cations tested only Mn^{2+} tends to show any stimulatory effects. In this case the maximal activation is only 1.2 at up to 6.4 mM added metal ion. No accurate value for the K_m can be ascribed to this cation. The effect of Fe^{2+} was negligible.

Influence of metabolic inhibitors on Ca^{2+} -stimulated ATP translocation

Carafoli *et al.* (1965) observed an uptake of adenine nucleotides during uptake of limited amounts of Ca^{2+} in the presence of added P_i . To distinguish further the properties of the Ca^{2+} -stimulated translocation of ATP described herein, from the effects observed by Carafoli *et al.* (1965), the rates of ATP translocation were examined in the presence of a variety of respiratory inhibitors and uncouplers.

Antimycin A only slightly stimulates the translocase in either the absence or presence of Ca^{2+} (Table 3). Oligomycin by itself has virtually no effect on the translocation of ATP either in the absence or presence of Ca^{2+} and of antimycin A. Carbonyl cyanide *m*-chlorophenylhydrazone increases the translocation rate in both the absence and presence of Ca^{2+} . Pfaff & Klingenberg (1968) observed that uncouplers of oxidative phosphorylation stimulate the translocation of ATP but not that of ADP. The data in Table 3 indicate that Ca^{2+} is not acting as an uncoupler but has a stimulatory action distinctly different from that of carbonyl cyanide *m*-

chlorophenylhydrazine. Indeed, their interactions in promoting the translocation of ATP are additive. The extra addition of any or a combination of the other respiratory inhibitors compensates, to a small degree, for the stimulatory effect of carbonyl cyanide *m*-chlorophenylhydrazine. The decrease in translocation activity is about 20–25% less than the maximal rate observed when only carbonyl cyanide *m*-chlorophenylhydrazine and Ca^{2+} are present together.

The influence of the polypeptide antibiotics gramicidin and valinomycin was also tested. As

shown in Table 4, the addition of either antibiotic slightly increased the translocation of ATP by about 30% in the absence of Ca^{2+} , confirming previous independent studies (Pfaff & Klingenberg, 1968). With Ca^{2+} , however, the situation is a little more complex. The stimulation of ATP translocation produced by Ca^{2+} is negated when valinomycin, but not when gramicidin, is present. The addition of either of these antibiotics to an uncoupler-stimulated system reverses this stimulation. This is compatible with the findings of Klingenberg & Pfaff (1968).

Table 3. *Effect of metabolic inhibitors on the Ca^{2+} -stimulated ATP translocation*

Mitochondria were incubated by method (a) in a basic medium supplemented with $200\mu\text{M}$ -ATP. Other additions were: $200\mu\text{M}$ - Ca^{2+} , $0.225\mu\text{M}$ -antimycin A, $4\mu\text{g}$ of oligomycin and $5\mu\text{M}$ -carbonyl cyanide *m*-chlorophenylhydrazine (CCCP). The rates of translocation were calculated as shown by Pfaff *et al.* (1969). The preincubation time was 6 min.

Inhibitor added	Rate of translocation (nmol/min per mg of protein)			
	Ca^{2+} absent		Ca^{2+} present	
		Relative activation		Relative activation
None	5.9	1.0	12.8	2.2
Antimycin A	6.2	1.1	14.7	2.5
Oligomycin	5.8	1.0	12.4	2.1
Antimycin A + oligomycin	6.5	1.1	15.1	2.6
CCCP	12.5	2.1	23.6	4.0
CCCP+antimycin A	12.0	2.0	22.1	3.7
CCCP+oligomycin	11.4	1.9	20.5	3.5
CCCP+antimycin A + oligomycin	10.2	1.7	18.1	3.1

Table 4. *Effect of ion-transporting antibiotics on the Ca^{2+} -stimulated translocation of ATP*

Mitochondria were incubated by method (a) in the basic medium supplemented with $200\mu\text{M}$ -ATP. Other additions were: $200\mu\text{M}$ - Ca^{2+} , $2\mu\text{M}$ -gramicidin, $2\mu\text{g}$ of valinomycin and $5\mu\text{M}$ -carbonyl cyanide *m*-chlorophenylhydrazine (CCCP). The rates of translocation were calculated as indicated in Table 3. The preincubation time was 6 min.

Inhibitor added	Rate of translocation (nmol/min per mg of protein)			
	Ca^{2+} absent		Ca^{2+} present	
		Relative activation		Relative activation
None	5.7	1.0	11.8	2.1
CCCP	10.5	1.8	20.0	3.5
Gramicidin	7.6	1.3	14.6	2.6
Gramicidin+CCCP	9.4	1.7	15.4	2.7
Valinomycin	7.3	1.3	8.7	1.5
Valinomycin+CCCP	7.7	1.4	11.3	2.0

Table 5. Nucleotide specificity of Ca^{2+} -stimulated translocation

Mitochondria were incubated by method (b) in a basic medium supplemented with the nucleotides (present at $200\text{ }\mu\text{M}$) and various concentrations of Ca^{2+} . Values in parentheses indicate the nmol of adenine nucleotide translocated/min per mg of protein when Ca^{2+} was not added.

Nucleotide tested	V_{max} (maximal relative activation)	Apparent K_m for Ca^{2+} (μM)	
		'Added'	'Free'
ATP	3.8 (6.0)	160	75
ADP	1.5 (16.4)	90	79
dATP	4.0 (1.3)	155	73

Fig. 3. Effect of Ca^{2+} concentration on the translocation of adenine nucleotides

Mitochondria were incubated by method (b) in the basic reaction medium supplemented with ATP, ADP (each at $200\text{ }\mu\text{M}$) and Ca^{2+} as indicated. (a) $\circ$, No addition; $\bullet$, $200\text{ }\mu\text{M}$ - Ca^{2+} . (b) $\square$, No addition; $\blacksquare$, $200\text{ }\mu\text{M}$ - Ca^{2+} .

Nucleotide specificity in Ca^{2+} -stimulated translocation

Table 5 shows the maximal relative activation of the translocation of $200\text{ }\mu\text{M}$ -adenine nucleotide and the concentrations of Ca^{2+} , both 'free' and 'added', necessary to achieve half this level of stimulation. The V_{max} for ATP and dATP is approximately the same. This may be compared with that for ADP, which is about sixfold less. Since 'free' Ca^{2+} is probably the species involved in the stimulatory effect, it is not surprising that the K_m values obtained for all three nucleotides are all of the order of $75\text{ }\mu\text{M}$ 'free' Ca^{2+} . Other nucleotides tested included AMP, GTP, ITP, CTP, UTP and CDP. Their rates of translocation were minimal either in the presence or absence of Ca^{2+} . The specificity of the translocase for ADP and ATP is well documented (Winkler *et al.*, 1968; Pfaff *et al.*, 1965).

Effect of Ca^{2+} on adenine nucleotide translocation as a function of adenine nucleotide concentration

The effect of a constant concentration of added Ca^{2+} ($200\text{ }\mu\text{M}$) on the translocation of ATP measured

as a function of ATP concentration is shown in Fig. 3(a). At all concentrations of ATP examined there is a significant stimulation of translocation activity. The degree of stimulation is greatest at the lower ATP concentrations (cf. data in Table 1). A similar type of experiment is shown in Fig. 3(b) but for this the activity was examined and is expressed as a function of ADP concentration. Again, the degree of stimulation is greatest at the lower nucleotide concentrations. A more direct comparison between ATP and ADP may be made when these data are replotted in a double-reciprocal form to compare the K_m and V_{max} values (see Figs. 4a and 4b). On addition of $200\text{ }\mu\text{M}$ - Ca^{2+} the apparent affinity for ATP increases threefold from 37 to $12.5\text{ }\mu\text{M}$ whilst that for ADP increases approx. twofold from 16.5 to $8.5\text{ }\mu\text{M}$. Concomitantly, values for the V_{max} increase by 45% from 8.7 to 12.5 and by 15% from 14.3 to $16.4\text{ nmol/min per mg of protein}$ for ATP and ADP respectively.

The data in Table 6 show the effect of various added Ca^{2+} concentrations on the affinity and maximal translocation rate of ATP. As the concentration of Ca^{2+} is increased to 0.6 mM the affinity for ATP

Fig. 4. Double-reciprocal plots of the data in Fig. 3

(a) ○, No addition; ●, 200 μM-Ca²⁺. (b) □, No addition; ■, 200 μM-Ca²⁺.

Table 6. Influence of several concentrations of added Ca²⁺ on the apparent K_m and V_{max.} for ATP and ADP translocation

Mitochondria were incubated by method (a) in the basic medium supplemented with Ca²⁺ as indicated and various concentrations of ATP or ADP. V_{max.} and K_m values were determined by double-reciprocal plots. Values in parentheses were obtained when ADP was present instead of ATP.

[Ca ²⁺] (μM)	V _{max.} (nmol/min per mg of protein)	Apparent K _m for ATP (μM)
0	9.3	33
50	11.8	23
100	13.0	17
200	13.6	15
400	14.2	12.5
600	14.9	10.5
(0)	(14.9)	(18)

also increases by approx. threefold, from 33 to 10.5 μM. This may be compared with an affinity for ADP, without added Ca²⁺, of 18 μM. Under the same conditions the V_{max.} values increased by 60% to 14.9 nmol/min per mg of protein. This increased V_{max.} value for ATP translocation is the same as that for ADP.

Effect of Ca²⁺ on the competitive effects between ADP and ATP for translocation

By using a dual-labelling technique it is possible to determine the effect of either ADP or ATP on the translocation of the other nucleotide (see Pfaff & Klingenberg, 1968). By adopting this type of experiment and including Ca²⁺, one is able to obtain con-

ditions under which the addition of an equimolar concentration of ADP does not appreciably alter the ability of a particular concentration of ATP to be translocated. This effect is particularly noticeable at the lower concentrations of adenine nucleotide. Thus (Table 7) the addition of 50 μM-ADP and 200 μM-Ca²⁺ makes little difference to the rate of translocation of 50 μM-ATP when compared with that rate observed in the absence of these additives. However, the addition of ADP did increase by approx. four-fold the overall translocation of adenine nucleotides as determined by the release of endogenous ³H radioactivity into the incubation medium. Conditions can also be obtained such that the addition of ATP and Ca²⁺ together results in a more marked diminution of the ADP translocation rate than is caused by ATP alone.

Table 7. *Effect of Ca^{2+} on the simultaneous translocation of ATP and ADP*

Mitochondria were prelabelled with [^3H]ATP as indicated in the Experimental section. The inward and outward rates of adenine nucleotide translocation were determined by the method of Pfaff & Klingenberg (1968). Ca^{2+} ($200\ \mu\text{M}$) and adenine nucleotide ($50\ \mu\text{M}$) were present as shown below. The values are in nmol of adenine nucleotide translocated/min per mg of protein. The difference values reflect the difference in rates between the forward translocation of [^{14}C]ADP and the backward translocation of [^3H]adenine nucleotides.

Reactants present	Translocation		
	Forward (^{14}C)	Backward (^3H)	Difference
[^{14}C]ATP	2.9	2.7	—
[^{14}C]ATP + Ca^{2+}	7.9	7.7	—
[^{14}C]ATP + ADP	0.9	8.5	7.6
[^{14}C]ATP + ADP + Ca^{2+}	3.0	11.8	8.8
[^{14}C]ADP	7.8	7.7	—
[^{14}C]ADP + Ca^{2+}	9.3	9.7	—
[^{14}C]ADP + ATP	7.1	8.1	1.0
[^{14}C]ADP + ATP + Ca^{2+}	7.4	11.0	3.6

Fig. 5. *Influence of Ca^{2+} on K^{+} -stimulated translocation of ATP*

Mitochondria were incubated by method (b) in a basic medium supplemented with $200\ \mu\text{M}$ -ATP and various concentrations of K^{+} as indicated. o, No added Ca^{2+} ; ●, $200\ \mu\text{M}$ - Ca^{2+} present.

Influence of Ca^{2+} on K^{+} -stimulated translocation of ATP

Meisner (1971) has shown that added K^{+} enhances the translocation of both ADP and ATP by isolated heart and liver mitochondria. The data of Fig. 5 confirm this observation for ATP translocation. They also show that the addition of $200\ \mu\text{M}$ - Ca^{2+} both

further stimulates the translocation of ATP and appears to increase the affinity of the system for K^{+} . The respective interactions of K^{+} and of Ca^{2+} with the translocase appear to be additive.

Influence of K^{+} on Ca^{2+} -stimulated translocation of ATP

Addition of $20\ \text{mM}$ - K^{+} enhances the Ca^{2+} -stimulated ATP translocation over the entire Ca^{2+} concentration-range examined (Fig. 6a). The added K^{+} also appears to increase the apparent affinity of ATP translocation for Ca^{2+} . This was confirmed in an experiment (Fig. 6b) in which a number of Ca^{2+} concentration curves were determined in the presence of different amounts of K^{+} . As the K^{+} concentration was increased from 0 to $100\ \text{mM}$, the apparent K_m for Ca^{2+} decreased from 160 to $50\ \mu\text{M}$. The maximal effect was achieved by $20\ \text{mM}$ - K^{+} with a half-maximal effect at about $4\ \text{mM}$ - K^{+} . These values are close to those found by Meisner (1971) for K^{+} stimulation of adenine nucleotide translocation by isolated mitochondria.

As both K^{+} and Ca^{2+} are independently capable of increasing the apparent affinity of ATP and ADP for translocation, it was of interest to examine their combined effects. The influence of K^{+} , of Ca^{2+} and of K^{+} plus Ca^{2+} on the kinetic parameters associated with adenine nucleotide translocation was determined (Table 8). In agreement with Meisner (1971), K^{+} increases the affinity of both ADP and ATP for translocation by over 100%. In contrast with Meisner (1971) we consistently found an increase in the V_{max} of about 30% in the presence of $20\ \text{mM}$ - K^{+} . The very large increase in affinity for ATP in the presence of Ca^{2+} , and the correspondingly slight increase for ADP, is consistent with other data (see Figs. 3 and 4). The

Fig. 6. Influence of K⁺ on the apparent affinity of Ca²⁺ for stimulating ATP translocation

Mitochondria were incubated by method (b) in a basic medium supplemented with 200 μM-ATP and various concentrations of Ca²⁺ or K⁺ as indicated. The solute concentration of the medium in the absence of added K⁺ or Ca²⁺ was 200 milliosmolar. (a) o, No K⁺ added; ●, 20mM-K⁺ present. (b) The apparent K_m values for Ca²⁺ were calculated from double-reciprocal plots redrawn from a series of Ca²⁺ concentration curves as in (a).

Table 8. Influence of K⁺ and Ca²⁺ on the apparent K_m and V_{max.} for ATP and ADP translocation

Mitochondria were incubated by method (b) in the basic medium supplemented with KCl and/or Ca²⁺ as indicated with various concentrations of ATP or ADP. V_{max.} and K_m values were determined from double-reciprocal plots of the data.

Metal ion added	ATP		ADP	
	K _m (μM)	V _{max.} (nmol/min per mg of protein)	K _m (μM)	V _{max.} (nmol/min per mg of protein)
None	90	7.2	50	17
KCl (20mM)	42	10.0	21	21
Ca ²⁺ (200 μM)	25	14.0	37	18.5
KCl (20mM) + Ca ²⁺ (200mM)	14	17.5	19	24.5

most remarkable feature of the results, however, is that in the presence of both K⁺ and Ca²⁺ the apparent affinity for ATP is increased even further and, in this experiment (Table 8), is as great as that for ADP. The V_{max.} for ATP increases by 140% to 17.5 nmol/min per mg of protein whereas that for ADP increases by only 45% to 24.5 nmol/min per mg of protein. The relatively high values for the apparent K_m for ATP and ADP, obtained in the absence of added metal ions, reflect the higher concentrations of mitochondria used in this particular experiment. Pfaff & Klingenberg (1968) have shown that the apparent affinity for adenine nucleotides can be considerably increased if very low concentrations of mitochondria are employed in the reaction mixture.

In other control experiments employing a combination of ³H₂O, [¹⁴C]sucrose and the light-scattering technique, no swelling of the mitochondria occurred under the conditions of the translocase assay in the absence or presence of KCl.

Discussion

Any enzyme or enzyme complex that functionally links one cell compartment with another must play a central role in cell metabolism. In this respect the adenine nucleotide translocase is especially important since it performs two interrelated functions. First, it is able to control the relative concentrations of ATP and ADP, perhaps the most important chemical

species in the cell, in the cytoplasm and mitochondrial matrix. These two compounds are involved in many aspects of cell metabolism, if not as substrates for a reaction then certainly as chemical effectors or modifiers of numerous enzymic reactions. Secondly, the translocase is responsible for the transport of ADP and ATP into and out of the mitochondria. In this way it acts as a channel in the energy metabolism of the cell by 'assisting' in the production of ATP, the universal energy donor. Thus any physiological substance that can alter the activity of this and other controlling enzyme systems must, in itself, be involved in the control of cell metabolism.

Our studies have clearly shown that low concentrations of Ca^{2+} markedly enhance the activity of the adenine nucleotide translocase. Meisner (1971) has made a similar observation. The following are some of the more outstanding features of the stimulatory effect. First, the affinity for Ca^{2+} is high, with values for the K_m being well within the physiological concentration range for this ion (Thiers & Vallee, 1957). Secondly, Ca^{2+} stimulates the translocation of ATP to a much greater extent than that of ADP. The importance of this point is demonstrated by the observation that, in the presence of Ca^{2+} , ATP is able to compete with ADP for translocation (Table 7). It has been argued that the ability of ADP to be translocated more effectively than ATP (in the absence of Ca^{2+}) leads to the attainment of the respective phosphorylation potentials found in the cytoplasm and mitochondria (Pfaff & Klingenberg, 1968; Klingenberg *et al.*, 1969). We have shown that Ca^{2+} is probably capable of influencing these potentials. The third important aspect of the Ca^{2+} stimulation is the specificity for the bivalent metal ions. Our results clearly indicate that Mg^{2+} and Mn^{2+} , each of which form metal-ATP complexes more readily than Ca^{2+} (see, for example, Sillen & Martell, 1964), have relatively little effect on ATP translocation particularly at those concentrations where Ca^{2+} produces near-maximal stimulation (Table 2).

Analysis of the kinetic data shows that both the affinity for adenine nucleotides and the maximal velocity of the translocase are increased by Ca^{2+} ; this is especially true for ATP (Figs. 3 and 4). This suggests that Ca^{2+} may be producing two effects on the translocase. First, it could be stimulating the translocation by binding to the mitochondrial membrane in the vicinity of the translocase (see below). Secondly, the CaATP^{2-} species, which as mentioned above seems to be a substrate for the translocase, may also be influencing the kinetic characteristics of the translocase. This would in part explain the specificity of the stimulation for ATP; the stability constant for CaADP^- is more than an order of magnitude less than that for CaATP^{2-} (Sillen & Martell, 1964).

The very high affinity of Ca^{2+} -stimulated ATP

translocation for Ca^{2+} , particularly in the presence of K^+ , is noteworthy. The values obtained for the affinity constants lie within the range observed for other parameters of oxidative phosphorylation (see Chance, 1963; Bygrave & Lehninger, 1967). They are higher than the values reported by Pfaff *et al.* (1969) obtained from experiments with low concentrations of mitochondria. Presumably the values obtained in the present work would have been even lower if lower concentrations of mitochondria had been used in the reaction mixtures.

The means by which Ca^{2+} stimulates the translocation of ATP cannot be elucidated solely from the data in this paper. Some tentative conclusions, however, can be reached. It appears unlikely that accumulation of Ca^{2+} is obligatory for stimulation of ATP translocation. Information from experiments with the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone indicates that Ca^{2+} itself is not acting as an uncoupler (see Lehninger, 1964) and thereby directly influencing the proton gradient across the inner mitochondrial membrane (cf. Klingenberg *et al.*, 1969). This is supported by the additive nature of the Ca^{2+} - and uncoupler-stimulated exchanges (Table 3). Stimulation of ATP translocation by K^+ was also shown to be additive with that of uncouplers (Meisner, 1971) and we have confirmed this (T. Spencer & F. L. Bygrave, unpublished work). Similarly oligomycin, which inhibits ATP-supported Ca^{2+} accumulation, had little effect on Ca^{2+} -stimulated ATP translocation. Further, the bivalent metal ion specificity for stimulation of ATP translocation bears little relation to the specificity for accumulation of bivalent ions. The results of Carafoli (1965) would suggest that the apparent order for accumulation by isolated rat liver mitochondria is $\text{Ca}^{2+} > \text{Sr}^{2+} \gg \text{Mn}^{2+} = \text{Ba}^{2+}$. Mg^{2+} is not accumulated by these mitochondria to any large degree (Lehninger *et al.*, 1967).

Three independent pieces of information raise the possibility that the enhanced enzymic activity results from an interaction of Ca^{2+} with phospholipids located in the environment of the translocase. First, the affinity of the Ca^{2+} -stimulated translocation for Ca^{2+} is of the order found for the low-affinity Ca^{2+} -binding sites; these are probably lipid in nature (Scarpa & Azzi, 1968). Interaction with phospholipids is also consistent with the bivalent ion specificity for K^+ self-diffusion across phosphatidylserine bilayers (Papahadjopoulos & Ohki, 1970). The other point is that valinomycin, which is thought to interact with membrane phospholipids (see for example Pressman, 1965), prevents to a considerable extent the ability of Ca^{2+} to stimulate ATP translocation (Table 4). The results of further studies on this aspect of the work will be of interest.

The present findings suggest that Ca^{2+} stimulation of the adenine translocase may have a physiological role in the cell. We envisage a type of 'feed-forward'

Scheme 1. Envisaged link through Ca^{2+} between Ca^{2+} permease and adenine nucleotide translocase

The dashed line indicates an interaction of Ca^{2+} with the translocase. This interaction does not necessarily involve concomitant translocation of Ca^{2+} with the ATP. ~ is the energy pressure necessary for Ca^{2+} accumulation

mechanism (see Scheme 1) in which the increased ATP translocated in the presence of Ca^{2+} would be available for hydrolysis by the adenosine triphosphatase (ATPase). The energy produced during ATP breakdown would enable Ca^{2+} to be accumulated via the Ca^{2+} permease. Thus the accumulation of Ca^{2+} by mitochondria would be controlled to a certain extent by its own concentration outside the mitochondria. Consistent with this argument is the finding that the accumulation of Ca^{2+} by rat liver mitochondria as a function of Ca^{2+} concentration follows sigmoidal saturation-kinetics (Bygrave *et al.*, 1971, 1972).

This study, like some others (Bygrave, 1967; Meli & Bygrave, 1972; J. B. Roberts & F. L. Bygrave, unpublished work), again illustrates the importance of the ionic environment in modifying the activity of key enzymic processes sensitive to metal ions. Further studies of this nature will no doubt shed further light on the ways in which fluctuations in cellular cation concentrations exert their influence on cell metabolism (Bygrave, 1967; Rasmussen, 1970).

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