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Role of Ca^{2+} ions in the regulation of intramitochondrial metabolism in rat heart

Evidence from studies with isolated mitochondria that adrenaline activates the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes by increasing the intramitochondrial concentration of Ca^{2+}

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1. Increases in the amount of active, non-phosphorylated, pyruvate dehydrogenase which result from the perfusion of rat hearts with adrenaline were still evident during the preparation of mitochondria in sucrose-based media containing EGTA (at 0°C) and their subsequent incubation at 30°C in Na^+-free KCl-based media containing respiratory substrates and EGTA. The differences from control values gradually diminished with time of incubation, but were still present after 8min. Similar increases resulting from an increase in the concentration of Ca^{2+} in the perfusing medium also persisted. However, similar increases caused by 5mM-pyruvate were only maintained during the preparation of mitochondria, not their incubation. 2. Parallel increases, within incubated mitochondria, were found in the activity of the 2-oxoglutarate dehydrogenase complex assayed at a non-saturating concentration of 2-oxoglutarate. 3. The enhancement of the activities of both of these Ca^{2+}-sensitive enzymes within incubated mitochondria as a result of perfusion with adrenaline or a raised concentration of Ca^{2+} in the medium could be abolished within 1min by the presence of 10mM-NaCl. This effect of Na^+ was blocked by 300μM-diltiazem, which has been shown to inhibit Na^+-induced egress of Ca^{2+} from rabbit heart mitochondria [Vághy, Johnson, Matlib, Wang & Schwartz (1982) *J. Biol. Chem.* **257**, 6000–6002]. 4. The enhancements could also be abolished by increasing the extramitochondrial concentration of Ca^{2+} to a value where it caused maximal activation of the enzymes within control mitochondria. 5. The results are consistent with the hypothesis that adrenaline activates rat heart pyruvate dehydrogenase by increasing the intramitochondrial concentration of Ca^{2+} and that this increase persists through to incubated mitochondria. 6. Support for this conclusion was obtained by the yielding of a similar set of results from parallel experiments performed on control mitochondria that had firstly been preincubated (under conditions of steady-state Ca^{2+} cycling across the inner membrane) with sufficient proportions of Ca–EGTA buffers to achieve a similar degree of Ca^{2+}-activation of pyruvate dehydrogenase (as caused by adrenaline) and had then undergone the isolation procedure again.

Previous work from this laboratory has shown that the activities of three mammalian enzymes that are exclusively intramitochondrial can be stimulated up to about 3–4-fold by increases in the concentration of free Ca^{2+} within the range 0.1–10μM. These are pyruvate dehydrogenase phosphate phosphatase (Denton *et al.*, 1972), NAD^+–isocitrate dehydrogenase (Denton *et al.*, 1978) and the 2-oxoglutarate dehydrogenase complex (McCormack & Denton, 1979). Phosphatase activation leads to increased amounts of PDH_a; Ca^{2+} activation of the other two enzymes results in decreased K_m values for their respective substrates

Abbreviation used: PDH_a, the active, non-phosphorylated, form of the pyruvate dehydrogenase complex.

threo-D_s-isocitrate and 2-oxoglutarate. These three enzymes are generally regarded to be important sites in the regulation of intramitochondrial oxidative metabolism in mammals and thus Denton & McCormack (1980, 1981) have proposed that Ca^{2+} may act as a means by which extrinsic agents such as hormones could affect this vital process.

There is indirect evidence to support this proposal. In studies with intact coupled mitochondria from rat heart incubated with Na^+ and Mg^{2+}, both the amount of PDH_a and the rate of utilization of non-saturating concentrations of 2-oxoglutarate were found to be increased up to 3–4-fold by rises in the extramitochondrial concentration of Ca^{2+} in the incubation medium within the expected physiological range [about 0.05–5 μM; see, e.g., Rasmussen & Goodman (1977)] (Hansford & Cohen, 1978; Denton *et al.*, 1980; Hansford, 1981; Hansford & Castro, 1981). The tricarboxylate carrier of heart mitochondria has a low activity (Chappell & Robinson, 1968), and as yet a means of monitoring the activity of NAD^+-isocitrate dehydrogenase within these mitochondria has not been found. However, Marshall *et al.* (1984) have reported similar findings for this enzyme (and the other two) within intact coupled mitochondria from rat epididymal adipose tissue.

A rise in the extramitochondrial concentration of Ca^{2+} within the range mentioned above has been measured in the cytoplasm of intact ferret heart cells as a result of their exposure to adrenaline (this is due to increased Ca^{2+} concentration in systole, and is regarded as being 'time-averaged') (Marban *et al.*, 1980). This hormone results in 3–4-fold increases in PDH_a in the perfused rat heart (Hiraoka *et al.*, 1980; McCormack & Denton, 1981); similar increases in PDH_a are also found in hearts perfused with medium containing high concentrations of Ca^{2+}. Furthermore, these increases in PDH_a can be prevented by perfusing with Ruthenium Red (McCormack & England, 1983), which is a potent inhibitor of Ca^{2+} uptake into isolated mitochondria (Moore, 1971; Vasington *et al.*, 1972). However, others have argued that the concentration of Ca^{2+} in mitochondria of the rat heart and of other mammalian tissues may always be too large for changes in this parameter to be relevant to the regulation of the dehydrogenases (Williamson & Cooper, 1980; Coll *et al.*, 1982; Joseph *et al.*, 1983).

The present study was undertaken to try to test more directly the hypothesis that adrenaline activates rat heart pyruvate dehydrogenase via a rise in the intramitochondrial concentration of Ca^{2+}, by examining the persistence of this effect during the preparation of mitochondria and their incubation under conditions designed to assess the role of intramitochondrial Ca^{2+} in this persistence.

Experimental

Sources and preparation of mitochondria

Hearts from male or female Wistar rats (220–280g) were used.

Hearts were perfused by the Langendorff technique (drip-through without recirculation) at 37°C and 7kPa, with gassed (O_2/CO_2, 19:1) bicarbonate-buffered medium (after Krebs & Henseleit, 1932); this contained 1.5mM-$CaCl_2$, 0.25mM-EGTA, 0.2mM-potassium phosphate and 10mM-glucose, with further additions as indicated in Figure and Table legends.

Mitochondria were prepared from perfused hearts by disrupting them with a pre-cooled Polytron probe as described by Denton *et al.* (1980). The lower portion (approximately half) of each heart was rapidly excised, briefly blotted and quickly plunged into 8ml of ice-cold isolation medium [250mM-sucrose / 20mM-Tris / HCl (pH7.3)/2mM-EGTA/1% albumin (fatty acid depleted)] and then homogenized within 5s. [When required, the remaining tissue was freeze-clamped by another worker immediately after the excision.] A further 35ml of isolation medium was then added, and mitochondria were prepared as described by Kerbey *et al.* (1976), except that mitochondria were only sedimented once (i.e. the wash was omitted). When required, a sample (1ml) of the diluted homogenate was taken before mitochondrial preparation and spun at 10000*g* for 25s; the resulting pellet was immediately frozen for subsequent assay of pyruvate dehydrogenase activity. On the basis of total pyruvate dehydrogenase activity, the yield of mitochondria after isolation compared to the original homogenate was 30–50%; none of the treatments used affected the recovery of mitochondria in the final preparation. Mitochondria from non-perfused hearts were prepared as described by Denton *et al.* (1980). The isolated mitochondria were resuspended in the same sucrose-based medium as described above, but without the albumin, to about 20mg of protein/ml.

Incubation of mitochondria and assay of intramitochondrial Ca^{2+}-sensitive enzymes

Mitochondria were incubated (at 0.5–1mg of protein/ml unless stated) in a basic medium consisting of 125mM-KCl, 20mM-Tris/HCl (pH7.3) and 5mM-potassium phosphate under the conditions described, and with additions as indicated, in the Figure and Table legends. It should be noted that, unless 10mM-NaCl was added, an extra 10mM-KCl was present. Potassium salts were used unless otherwise stated. Incubation volume was 1ml unless otherwise stated. Samples in which pyruvate dehydrogenase was to be assayed were

sedimented (10000*g* for 20s) and rapidly frozen unless otherwise stated.

Frozen tissue and mitochondrial samples were extracted and assayed for both the amount of PDH_a and the total amount of pyruvate dehydrogenase present, as described by McCormack *et al.* (1982). Results are given as the percentage of total enzyme existing as PDH_a. 2-Oxoglutarate-dependent oxygen uptake (assayed with an oxygen electrode) and $NAD(P)^+$ reduction (assayed on a double-beam spectrophotometer) were monitored as described by McCormack & Denton (1980) and McCormack *et al.* (1982) respectively. Results are given as the percentages of the maximal 2-oxoglutarate-dependent rate of oxidation or extent of $NAD(P)^+$ reduction (i.e. measured at saturating 2-oxoglutarate concentrations) which were evident at suitable non-saturating concentrations of 2-oxoglutarate; the actual concentrations used are given in the Figure and Table legends. The total amount of pyruvate dehydrogenase and the maximal 2-oxoglutarate-dependent rate of oxidation and extent of $NAD(P)^+$ reduction were all unaffected by any of the treatments used when assessed on the basis of mitochondrial protein. Values obtained for these parameters were very similar to those published previously by Denton *et al.* (1980) and McCormack *et al.* (1982). It should be noted in time-course studies involving measurements made in sedimented mitochondria that the time of sedimentation has been included as part of the time of incubation.

Loading of mitochondria with ^{45}Ca and assay of ^{45}Ca content

Mitochondria (approx. 4mg of protein/ml) were preincubated at 30°C in 3–5ml of KCl-based media (see above) containing 10mM-2-oxoglutarate, 0.2mM-malate and 0.1–0.5μCi of ^{45}Ca/ml and in the presence of sufficient $CaCl_2$ (usually about 80μM) to give near-maximal Ca^{2+}-dependent increases in PDH_a content. This amount of $CaCl_2$ was determined in each experiment by monitoring the Ca^{2+}-dependent increases in the rate of oxidation of a non-saturating concentration of 2-oxoglutarate by mitochondria incubated at approx. 4mg of protein/ml (as described above), since the sensitivity of 2-oxoglutarate oxidation to extramitochondrial Ca^{2+} is very similar to that of pyruvate dehydrogenase (Denton *et al.*, 1980). In this manner the specific radioactivity of free $^{45}Ca^{2+}$ could be kept high, and variations in the EGTA added with the mitochondrial suspension (equivalent to a final concentration of approx. 0.4mM) and the endogenous Ca content of solutions (usually about 20μM; see McCormack & Denton, 1979) could be allowed for without the use of Ca–EGTA buffers. In this way an increase in PDH_a similar to that caused by adrenaline action on hearts could be achieved. After preincubation for 4min as described above, a large excess (at least 6 vol.) of ice-cold sucrose-based isolation medium was added, and the mitochondria were sedimented and resuspended as described above; on the basis of total pyruvate dehydrogenase activity, the recovery of mitochondria after re-preparation was over 95%. Mitochondria were then incubated as described in the legend of Fig. 3, after which they were sedimented by centrifuging at 10000*g* for 20s. The supernatant was then rapidly removed and the walls of the tube were quickly wiped with tissue paper held in forceps. The pellet was suspended in 300μl of water and then deproteinized by the addition of $HClO_4$ (final concn. 2%, w/v). After centrifugation for 1min at 10000*g*, a sample of the resultant supernatant was monitored for ^{45}Ca content as described by Severson *et al.* (1976). Recovery of [^{3}H]sucrose added to the preincubation medium as an extramitochondrial-space marker in the final mitochondrial pellets was insignificant, and so no correction for ^{45}Ca originally present in the extramitochondrial compartment at the end of the preincubation period was necessary.

Use of Ca–EGTA buffers, assay of mitochondrial protein and ATP, and statistical tests

Ca–EGTA buffers were prepared and used as described by Denton *et al.* (1978) and McCormack *et al.* (1982). Mitochondrial protein was measured by the method of Gornall *et al.* (1949), and mitochondrial ATP content was measured by the method of Stanley & Williams (1969) in samples prepared as described by McCormack & Denton (1980). Statistical significance was assessed by Student's *t* test.

Chemicals, biochemicals and radiochemicals

All chemicals and biochemicals were obtained from either Boehringer Corp., Lewes, East Sussex, U.K., or BDH Chemicals, Poole, Dorset, U.K., with the exceptions of isoprenaline (Kodak, Kirkby, Liverpool, U.K.), Ruthenium Red (Sigma, Poole, Dorset, U.K.) and diltiazem {*cis*-(+)-3-(acetyloxy)-5-[2-(dimethylamino)ethyl]-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4(5*H*)-one}, which was kindly given by Dr. G. Satzinger of Goedecke A.G., 78 Freiburg, Germany. Radiochemicals were obtained from either Amersham International, Amersham, Bucks., U.K. or New England Nuclear, 6072 Dreieich, Germany.

Results and discussion

Persistence of the effects of adrenaline on rat heart PDH_a during the preparation and subsequent incubation of mitochondria in Na^+-free media

Increases in the amount of PDH_a as a result of the perfusion of rat hearts with adrenaline (Hiraoka *et al.*, 1980; McCormack & Denton, 1981) remain evident during the preparation of mitochondria and their subsequent incubation at 30°C with respiratory substrates and EGTA for up to 8 min (Fig. 1). Beyond this time of incubation, the ATP content of mitochondria began to fall. A rise in the intramitochondrial concentration of Ca^{2+} as a result of adrenaline action has been proposed as the mechanism for this effect on PDH_a in perfused hearts (Hiraoka *et al.*, 1980; McCormack & Denton, 1981; McCormack *et al.*, 1982; McCormack & England, 1983). A rise in perfusing-medium Ca^{2+} concentration from 1.5 to 6 mM leads to a rise in the cytoplasmic concentration of Ca^{2+} and thus, independently of cyclic AMP, stimulates contraction and the conversion of phosphorylase from the *b* into the *a* form (see McCormack & England, 1983). This treatment also results in a similar increase in PDH_a to that caused by adrenaline (McCormack & Denton, 1981), and furthermore gave results similar to those shown in Fig. 1 for adrenaline (not shown in full; see Table 1).

The remainder of this paper is concerned with testing the above hypothesis by establishing whether or not the persistence of these effects into incubated mitochondria is due to elevated intramitochondrial Ca^{2+} concentrations.

Effects of EGTA, Na^+ and diltiazem on the egress of Ca^{2+} from mitochondria pre-loaded with Ca^{2+} in vitro

Increases in the concentration of Ca^{2+} within intact coupled mitochondria from rat heart in response to an increase in the extramitochondrial concentration of Ca^{2+} can be readily monitored by assaying for Ca^{2+}-dependent increases in either the

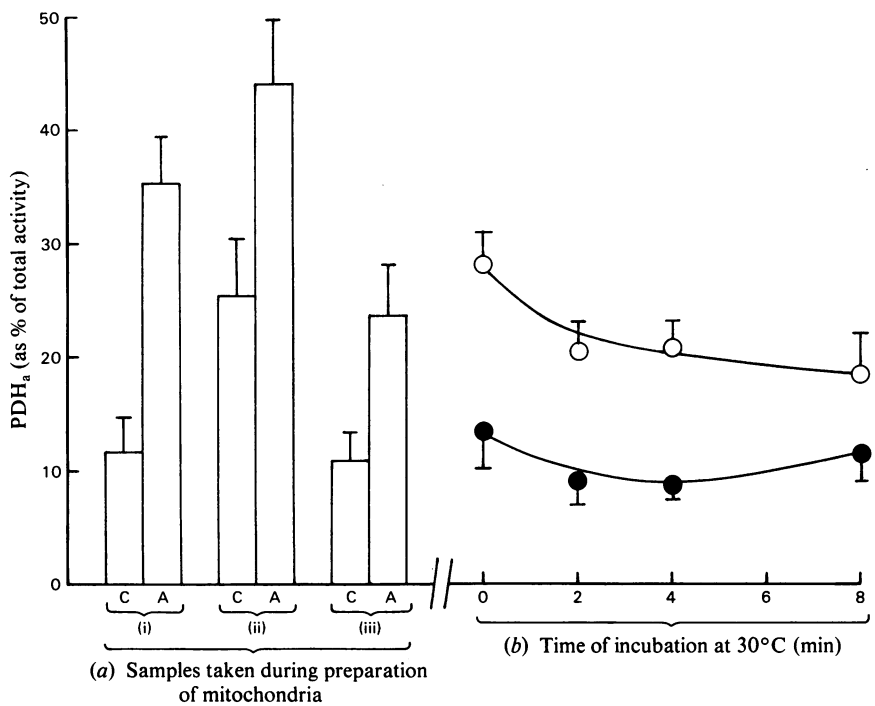

Fig. 1. *Persistence of the increases in PDH_a caused by the perfusion of rat hearts with medium containing adrenaline during the preparation of mitochondria and their subsequent incubation at 30°C with respiratory substrates in Na-free KCl-based media containing EGTA*

Hearts were perfused under control conditions for 11 min (C; ●) or for 10 min, with the subsequent addition of 2 μM-adrenaline for 1 min (A; ○). (*a*) During the preparation of mitochondria (as described in the Experimental section) samples were taken for the assay of PDH_a at the following stages: (i) freeze-clamped heart, (ii) homogenate of fresh unfrozen tissue used in the preparation of mitochondria, and (iii) freshly isolated mitochondria (at 0°C). (*b*) Mitochondria were incubated at 30°C in KCl-based media (see the Experimental section) containing 10 mM-2-oxoglutarate, 0.2 mM-malate and 2 mM-EGTA, except for zero-time samples, which were added directly to extraction medium. Results are given as means ± S.E.M. (bars) for at least four different preparations of mitochondria.

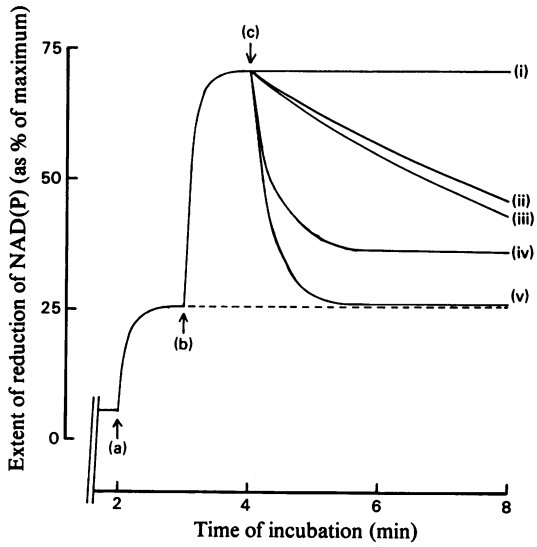

Fig. 2. *Effects of EGTA, Na^+ and diltiazem on an increased extent of 2-oxoglutarate-dependent reduction of mitochondrial NAD(P)$^+$ resulting from Ca^{2+} action*

Mitochondria prepared from non-perfused hearts were incubated at 30°C in 2ml of KCl-based media (see the Experimental section) containing 0.5mM-EGTA and 1mM-ADP. The ADP allowed rapid oxidation of endogenous substrate; this was complete within 1min, and the extent of reduction at this point was taken to be 0%. Further ADP-stimulated oxidation was then blocked by the addition of 2μM-antimycin; this resulted in a small amount of NAD(P)$^+$ reduction, which was complete within 30s to give a new steady-state level of NAD(P)$^+$ reduction. Antimycin also allowed new steady-state levels of NAD(P)$^+$ reduction induced by 2-oxoglutarate to be achieved more rapidly (within 1min). Then at point (a) 0.5mM-2-oxoglutarate was added, followed at point (b) by 0.25mM-$CaCl_2$ plus 0.25mM-EGTA (resultant free $[Ca^{2+}] \simeq 30$nM; this $[Ca^{2+}]$ results in approx. 70% of the maximal Ca^{2+} effect) in the absence (continuous line) or presence (broken line) of 1μM-Ruthenium Red. At point (c) the following additions were made: (i) 10mM-KCl (control); (ii) 5mM-EGTA (resultant free $[Ca^{2+}] \simeq 2$nM); (iii) 10mM-NaCl plus 300μM-diltiazem; (iv) 10mM-NaCl; (v) 10mM-NaCl plus 5mM-EGTA. It should be noted that none of the additions at point (c) had any effect if they were added at point (b) instead of 0.25mM-$CaCl_2$ plus 0.25mM-EGTA, nor did the addition of Ca–EGTA buffer at point (b) to give the resultant $[Ca^{2+}]$ of (ii). Results from a typical experiment are shown. Absorption owing to addition of antimycin and 2-oxoglutarate in the absence of mitochondria (see McCormack *et al.*, 1982) has been omitted for clarity of presentation. The 100% reduction refers to that achieved with 2mM-2-oxoglutarate in the presence of 2mM-EGTA plus 1mM-$CaCl_2$ (free $[Ca^{2+}] \simeq 50$nM); no further reduction could be achieved by adding further 2-oxoglutarate. This maximal reduction was also evident if other substrates were used (see McCormack *et al.*, 1982).

amount of PDH_a (Denton *et al.*, 1980; Hansford, 1981) or the rate of utilization of non-saturating concentrations of 2-oxoglutarate (Denton *et al.*, 1980; Hansford & Castro, 1981). An example of this is shown in Fig. 2. In this experiment, the activity of the 2-oxoglutarate dehydrogenase complex was continuously followed by observing changes in the extent of absorption due to NAD(P)H within rat heart mitochondria incubated with a non-saturating concentration of 2-oxoglutarate (Hansford & Castro, 1981; McCormack *et al.*, 1982).

An increase in the extramitochondrial Ca^{2+} concentration (to about 30nM) caused a marked increase in the absorption, corresponding to the activation of the 2-oxoglutarate dehydrogenase complex by the resulting increase in intramitochondrial Ca^{2+} concentration, as found in previous studies (Hansford & Castro, 1981; McCormack *et al.*, 1982). The extramitochondrial Ca^{2+} concentration was then greatly and instantaneously decreased by the addition of EGTA to give a final free Ca^{2+} concentration of about 2nM; however, the absorption only decreased slowly over the next 8min.

In contrast, if Na^+ was also added with EGTA, then the decrease in NAD(P)H absorption and thus the intramitochondrial concentration of Ca^{2+} was very rapid, and appeared complete within 1min [Fig. 2(*c*), trace (v)]. This is consistent with the operation of the Na/Ca antiporter of heart mitochondria characterized by Crompton *et al.* (1977, 1978), who reported that Na^+ stimulates the egress of Ca^{2+} several-fold. Vághy *et al.* (1982) have demonstrated that Na/Ca exchange in rabbit heart mitochondria can be inhibited by several drugs which are more commonly used as Ca-channel blockers in intact heart preparations (see Fleckenstein, 1977). Diltiazem was found to be the most potent of these, with a half-maximal effective concentration of about 7μM and a saturating effect at about 300μM; concentrations higher than about 500μM appeared to damage the mitochondria. Fig. 2 shows that 300μM-diltiazem blocked the effects of Na^+. The addition of diltiazem after Na^+ could reverse its effects only if the extramitochondrial concentration of Ca^{2+} was maintained [i.e. in Fig. 2, trace (iv) but not trace (v)].

The results of Fig. 2 and similar experiments in other studies (Denton *et al.*, 1980; Hansford, 1981; Hansford & Castro, 1981) suggest that in the absence of Na^+ an increased intramitochondrial concentration of Ca^{2+} can persist for several minutes even when the extramitochondrial concentration of Ca^{2+} is kept low by the presence of EGTA. Under these conditions, net egress of Ca^{2+} will no doubt be taking place, but nevertheless it appears that the rate of fall of the concentration of intramitochondrial Ca^{2+} is sufficiently slow at 30°C to suggest that the assay of intramitochondrial Ca^{2+}-sensitive en-

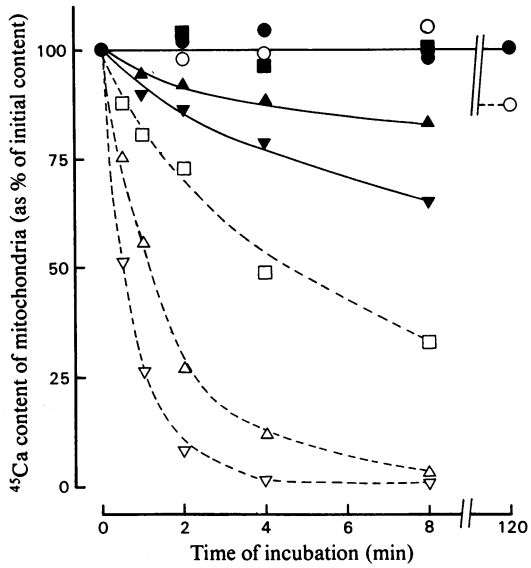

Fig. 3. *Effects of Na^+ on the egress of ^{45}Ca from pre-loaded mitochondria incubated at different temperatures*

Mitochondria from non-perfused hearts were pre-loaded with ^{45}Ca and re-isolated as described in the Experimental section. Mitochondria were incubated at 0°C (●, ○), 20°C (■, □), 30°C (▲, △) or 37°C (▼, ▽) in KCl-based media (see the Experimental section) containing 10mM-2-oxoglutarate, 0.2mM-malate and 2mM-EGTA and in the absence (closed symbols, continuous lines) or presence (open symbols, broken lines) of 10mM-NaCl as indicated. The initial content refers to that measured in freshly prepared mitochondria at 0°C (with the assumption that no loss occurs during the sedimentation of mitochondria at this temperature; only one symbol is shown here for clarity); this was calculated to correspond to approx. 3–4nmol of Ca/mg of mitochondrial protein on the basis of specific radioactivity of the media, which agrees well with the values obtained by Hansford & Castro (1981) for the mitochondrial Ca content when a near-maximal Ca^{2+}-activation of the 2-oxoglutarate dehydrogenase complex was evident. Results shown are means of values obtained from at least three different mitochondrial preparations; S.E.M. values on the points given were less than 15% of the values shown. Similar results were obtained if increases in ^{45}Ca in the media were monitored. The K_m value for the effect of Na^+ at 30°C was approx. 1mM, which is similar to values published previously (Denton *et al.*, 1980; Hansford & Castro, 1981). It should be noted that the activities of both pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes had returned to Ca^{2+}-unstimulated values when approx. 10–25% of the ^{45}Ca remained in the mitochondria.

zymes within freshly prepared mitochondria (which requires incubation at temperatures within the range 20–37°C) could be used to identify changes in the intramitochondrial concentration of Ca^{2+}.

Further support for this view was obtained from experiments in which the retention of ^{45}Ca by pre-loaded rat heart mitochondria was studied (Fig. 3). These mitochondria were preincubated with medium containing enough $CaCl_2$, under conditions of steady-state Ca^{2+} cycling across the mitochondrial inner membrane, to cause near-maximal Ca^{2+}-dependent activation of both the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes (see the Experimental section). Previous studies have shown that an extramitochondrial concentration of free Ca^{2+} of about 30–50nM is required to achieve this degree of activation in the absence of added Na^+ and Mg^{2+} (Denton *et al.*, 1980).

In the absence of added Na^+, the rate of loss of ^{45}Ca from the pre-loaded mitochondria on their incubation in medium containing EGTA and oxidizable substrates (oxoglutarate and malate) was very slow. Even at 37°C only about 30% was lost in 8min. On addition of Na^+, the rate of ^{45}Ca egress was much greater at 20, 30 and 37°C. These effects of Na^+ could be prevented by the addition of 300μM-diltiazem (results not shown). Diltiazem, but not Ruthenium Red, also blocked the stimulated release of ^{45}Ca from mitochondria induced by the presence of Ca–EGTA buffers to give a free $[Ca^{2+}] \simeq 50$nM; this was approx. 50% of the rates shown in Fig. 3 for Na^+-induced release (results not shown), suggesting that diltiazem also blocks Ca/Ca exchange on the Na/Ca antiporter (see Crompton *et al.*, 1977).

There was little or no loss of ^{45}Ca from mitochondria incubated at 0°C for up to 2h, even in the presence of Na^+ (Fig. 3). This was also the case if the KCl-based incubation media were replaced with the sucrose-based media used for the isolation of mitochondria (results not shown).

These studies indicated that the loss of Ca^{2+} from rat heart mitochondria might be extremely slow during their preparation and subsequent incubation in medium containing EGTA but lacking Na^+. It thus seems plausible that, if adrenaline were to act on pyruvate dehydrogenase via an increase in the intramitochondrial concentration of Ca^{2+}, then the increase should persist throughout the isolation of mitochondria. Furthermore, it could be predicted that the addition of Na^+ to incubation media should cause a rapid loss of Ca^{2+} from the mitochondria and that this loss could be blocked by diltiazem. The feasibility of these views was further strengthened by studying the effects of Na^+ and diltiazem on PDH_a in mitochondria that had previously been preincubated with medium containing Ca^{2+} under conditions very similar to those employed to load the mitochondria with ^{45}Ca.

Persistence of the effects of a preincubation of mitochondria with sufficient Ca^{2+} to cause a 3–4-fold increase in PDH_a, during the re-preparation and subse-

quent incubation of mitochondria with or without Na^+ and/or diltiazem

The effects on PDH_a owing to preincubation of mitochondria with Ca^{2+} were found to persist during the re-preparation and subsequent incubation of mitochondria (Fig. 4). The inclusion of Na^+, diltiazem or Ruthenium Red in the cold isolation medium had no appreciable effect on this persistence. This is consistent with the results of Fig. 3, and is of particular relevance because there will be some carry-over of Na^+ from heart perfusions.

As predicted, the persistent effect on PDH_a within incubated mitochondria as a result of preincubation with Ca^{2+} could be abolished within 1 min by the addition of Na^+, and moreover, this effect of Na^+ could be blocked by diltiazem (Fig. 4).

Comparison of (a) the effects of a preincubation of mitochondria with Ca^{2+} with (b, c) the effects of the perfusion of rat hearts with medium containing (b) adrenaline or (c) increased Ca^{2+} concentration on PDH_a in subsequently isolated mitochondria incubated under a variety of conditions

Table 1 shows that treatments (*a*), (*b*) and (*c*) result in very similar responses of PDH_a to a variety of different incubation conditions. In particular, the persistent effect caused by perfusion of hearts with (*b*) adrenaline or (*c*) a raised concentration of Ca^{2+}

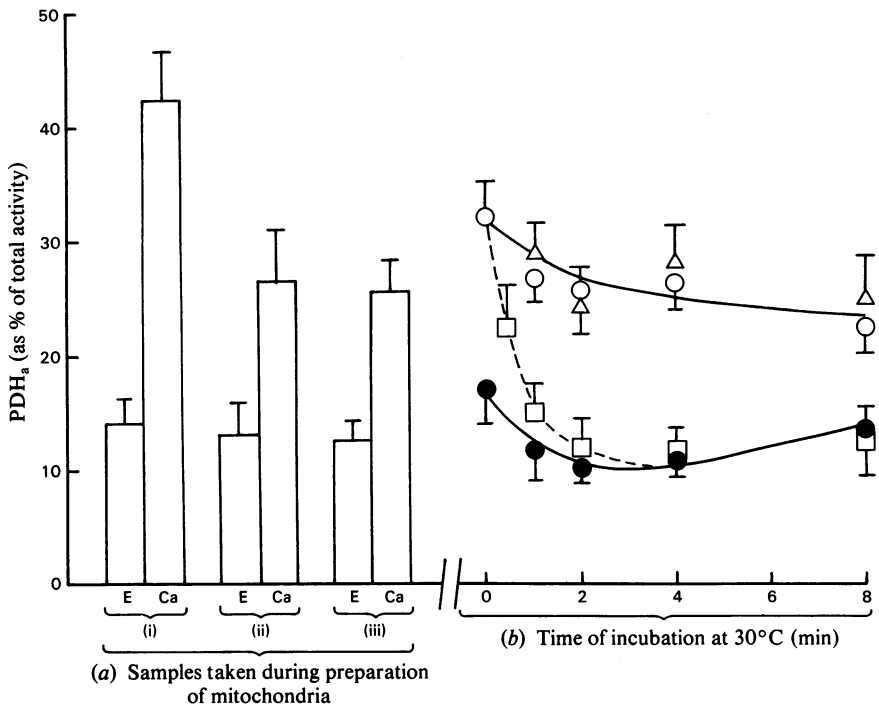

Fig. 4. *Effects of Na^+ and diltiazem on the persistence of the increases in PDH_a caused by the preincubation of mitochondria with Ca^{2+} during the re-isolation of mitochondria and their incubation in KCl-based media containing respiratory substrates and EGTA*

Mitochondria from non-perfused hearts were preincubated and re-isolated as described for Fig. 3 in the Experimental section, except that in the preincubation ^{45}Ca was omitted and either 2mM-EGTA (E; ●) or 2mM-EGTA plus 1mM-$CaCl_2$ (free $[Ca^{2+}] \simeq 50$nM) (Ca; ○, □, △) were present. In (*a*) samples were taken (i) at the end of the preincubation, (ii) after the addition of excess cold isolation media and (iii) after re-isolation of the mitochondria (at 0°C). In (*b*) mitochondria were incubated as given in the legend of Fig. 1 with no further additions (○, ●) or with 10mM-NaCl in the absence (□, broken line) or presence (△) of 300μM-diltiazem. NaCl and/or diltiazem addition did not affect values in incubations of control mitochondria (i.e. ●). Results are given as in Fig. 1. The presence of 2μM-Ruthenium Red, 10mM-NaCl, 300μM-diltiazem or combinations thereof in the sucrose-based isolation medium had no appreciable effects on the PDH_a values. However, if warm isolation medium (30°C) was used, then the effects of preincubation with Ca^{2+} shown in (*a*) (iii) and (*b*) were largely abolished, and were completely abolished if 10mM-NaCl was added to the warm isolation medium. Also, similar results were obtained if mitochondria were loaded with Ca^{2+} by preincubating them in the presence of 10mM-NaCl and 1mM-$MgCl_2$; however, under these conditions 5mM-EGTA plus 4.8mM-$CaCl_2$ (free $[Ca^{2+}] \simeq 1.3\mu$M) had to be added to achieve a similar degree of activation of pyruvate dehydrogenase to that shown.

in the medium can be abolished by Na^+, and the effect of Na^+ can be blocked by diltiazem under several different incubation conditions.

Several of the results of Table 1 are worthy of further comment. Mg^{2+} is an effector of the Ca^{2+}-transport system of the mitochondrial inner membrane; however, as it inhibits uptake (Crompton *et al.*, 1976), it will only be effective when extramitochondrial Ca^{2+} plays a role in determining intramitochondrial Ca^{2+} concentration, and thus has no effect on the results obtained in the presence of EGTA. McCormack *et al.* (1982) demonstrated that the effects of Ca^{2+} on PDH_a within incubated mitochondria could be amplified by the presence of pyruvate, presumably because it slows down the operation of the phosphorylation–dephosphorylation cycle of the pyruvate dehydrogenase complex (Kerbey *et al.*, 1976) by inhibiting the kinase reaction (Linn *et al.*, 1969). The effects of treatments (*a*), (*b*) and (*c*) on PDH_a also appear to be amplified in the presence of pyruvate. The effects of Na^+ in abolishing the persistent effects on PDH_a appear to take about three times as long with pyruvate present as in its absence (not shown in full: Table 1); this is again consistent with a partially inhibited kinase activity. Similar effects to those of pyruvate could also be observed with ADP and oligomycin, presumably for the same reasons (results not shown) (see McCormack *et al.*, 1982). If, as proposed, an increased intramitochondrial Ca^{2+} concentration is responsible for the persistent effects on PDH_a resulting from treatments (*a*), (*b*) or (*c*), then it would be predicted that differences from control values could be abolished by causing the concentration of Ca^{2+} within mitochondria from both control and stimulated conditions to be raised so that a saturating concentration of intramitochondrial Ca^{2+} for its effects on PDH_a is achieved; this was found to be the case (Table 1). A change in respiratory substrate did not alter the responses of PDH_a (Table 1).

The amount of PDH_a present within mitochondria is the result of the relative activities of pyruvate dehydrogenase kinase and pyruvate dehydrogenase phosphate phosphatase. The results presented so far strongly suggest that the persistent increases in PDH_a caused by treatments (*a*), (*b*) and (*c*) are the result of an increased concentration of intramitochondrial Ca^{2+} compared with controls, leading to the Ca^{2+}-dependent stimulation of phosphatase activity, though Ca^{2+} may also inhibit the kinase (Cooper *et al.*, 1974). They also suggest that it is very unlikely that the increases in PDH_a caused by treatments (*a*), (*b*) and (*c*) persist into incubated mitochondria simply because under these conditions both the kinase and phosphatase are inactive. The effects of Na^+ in particular indicate that the phosphorylation–dephosphorylation cycle must be active in the incubated mitochondria. This is supported by the observations by Sale & Randle (1980, 1982), who measured the incorporation of $[^{32}P]_i$ into pyruvate dehydrogenase, and its reversal, in mitochondria incubated under conditions similar to those used in the present study.

Finally, experiments were performed (results not shown) in which PDH_a was increased to about 30–40% of total activity by either preincubation of mitochondria with 0.5mM-potassium pyruvate or perfusion of hearts with 5mM-sodium pyruvate (Kerbey *et al.*, 1976; McCormack *et al.*, 1982). These increases caused by pyruvate persisted during the preparation of mitochondria, but were rapidly abolished by the subsequent incubation of mitochondria in the absence of Na^+ under the basic conditions described in the legend for Table 1(i). Furthermore, pre-exposure to pyruvate did not affect 2-oxoglutarate dehydrogenase complex activity within mitochondria incubated under any of the conditions used (see below).

It should be noted that treatments (*a*), (*b*) and (*c*) did not have any appreciable effects on the coupling ratios (Chance & Williams, 1956) of subsequently isolated mitochondria (assessed with several different respiratory substrates) or their content of ATP after a 4min incubation under the basic conditions described in the legend for Table 1(i), with or without Na^+ (results not shown); values obtained for these parameters were very similar to those published previously (Denton *et al.*, 1980).

Effects of (a) a preincubation of mitochondria with Ca^{2+} and (b, c) the perfusion of rat hearts with medium containing (b) adrenaline or (c) increased Ca^{2+} concentration on the activity of the 2-oxoglutarate dehydrogenase complex within subsequently isolated and incubated mitochondria

The proposal that the persistent increases in PDH_a described above are due to raised concentrations of intramitochondrial Ca^{2+} were substantiated by parallel studies on the activity of the 2-oxoglutarate dehydrogenase complex within mitochondria. Treatments (*a*), (*b*) and (*c*) all resulted in significant decreases in the apparent K_m value of the 2-oxoglutarate dehydrogenase complex for 2-oxoglutarate when the enzyme was assayed by two different techniques within subsequently isolated mitochondria incubated in Na^+-free media containing EGTA. Again, Na^+ abolished differences from control values, whereas diltiazem prevented this action of Na^+. Also, again as shown in Table 1 for PDH_a, these differences could be abolished by increasing the extramitochondrial concentration of Ca^{2+} to a value where it caused a saturating Ca^{2+}-dependent activation of the 2-oxoglutarate

Table 1. *Comparison of (a) the effects of a preincubation of mitochondria with Ca^{2+} with (b, c) the effects of the perfusion of rat hearts with medium containing (b) adrenaline or (c) increased Ca^{2+} concentration, on PDH_a in subsequently incubated mitochondria*

Mitochondria were prepared as in Fig. 4 [for (*a*)] and Fig. 1 [for (*b*) and (*c*)], except that in (*c*) hearts were perfused with an extra 5mM-$CaCl_2$ and not adrenaline after the pre-perfusion period. In (i) mitochondria were incubated at 30°C in KCl-based media (see the Experimental section) for 4min in the presence of 10mM-2-oxoglutarate, 0.2mM-malate and 2mM-EGTA, with further additions or changes as indicated; in (ii) the 2-oxoglutarate and malate were replaced as indicated. Results are given as means ± S.E.M. for the numbers of different mitochondrial preparations in parentheses. $*P \leqslant 0.05$, $**P \leqslant 0.01$ and $***P \leqslant 0.001$ for the effects of (*a*), (*b*) or (*c*) compared with the appropriate control value (i.e. across rows); $\dagger P \leqslant 0.05$, $\dagger\dagger P \leqslant 0.01$ and $\dagger\dagger\dagger P \leqslant 0.001$ for the effects of Na^+ compared with the appropriate value obtained in its absence (i.e. down columns). Abbreviation: N.D., not done.

		PDH_a (as % of total activity) in incubated mitochondria prepared from:				
		mitochondria preincubated with:		hearts perfused with medium containing:		
		2mM-EGTA (control)	(*a*) 2mM-EGTA plus 1mM-$CaCl_2$ (free $[Ca^{2+}] \simeq 50$nM)	No additions (control)	(*b*) Adrenaline	(*c*) Increased Ca^{2+} concentration
(i) Additions or changes to incubation medium	None	10.8±1.1 (8)	26.1±2.5 (8)***	8.2±1.2 (10)	20.4±1.8 (10)***	18.8±2.0 (10)***
	10mM-NaCl	11.3±1.6 (8)	12.2±1.5 (8)†††	8.0±1.2 (10)	7.2±1.4 (10)†††	7.5±1.0 (10)†††
	300μM-diltiazem	12.2±0.9 (3)	24.6±2.0 (3)**	9.3±1.4 (3)	18.1±2.0 (3)*	20.5±2.1 (3)*
	300μM-diltiazem plus 10mM-NaCl	13.0±1.4 (4)	28.1±2.6 (4)**	7.6±0.9 (5)	19.7±2.3 (4)**	18.2±2.9 (4)**
	1mM-$MgCl_2$	11.8±1.5 (3)	29.4±3.0 (3)**	9.0±2.0 (3)	19.2±1.4 (3)*	N.D.
	1mM-$MgCl_2$ plus 10mM-NaCl	12.6±1.7 (3)	14.0±2.1 (3)†	8.6±1.6 (3)	7.0±1.1 (3)††	N.D.
	pH changed to 7.6	21.3±2.6 (3)	39.4±3.4 (3)*	13.4±2.0 (3)	25.1±3.4 (3)*	23.3±1.8 (3)*
	pH7.6 plus 10mM-NaCl	21.0±1.7 (3)	20.9±2.1 (3)††	12.6±2.0 (3)	11.7±1.9 (3)†	10.8±2.0 (3)†††
	0.5mM-potassium pyruvate	18.7±1.8 (5)	49.3±4.1 (5)***	22.6±3.5 (6)	40.6±4.6 (5)*	39.2±4.2 (5)*
	0.5mM-potassium pyruvate plus 10mM-NaCl	17.7±2.3 (5)	23.3±2.9 (5)†††	21.8±3.0 (6)	24.8±3.4 (5)†	20.7±3.0 (5)††
	0.5mM-$CaCl_2$ (free $[Ca^{2+}] \simeq 20$nM)	16.9±2.8 (3)	29.8±3.3 (3)*	21.7±2.8 (3)	37.4±4.8 (3)*	N.D.
	0.75mM-$CaCl_2$ (free $[Ca^{2+}] \simeq 30$nM)	24.6±3.2 (3)	36.6±2.9 (3)*	N.D.	N.D.	N.D.
	1mM-$CaCl_2$ (free $[Ca^{2+}] \simeq 50$nM)	36.4±2.7 (3)	44.4±2.7 (3)	40.4±3.2 (3)	46.5±4.1 (3)	42.9±4.1 (3)
	1.5mM-$CaCl_2$ (free $[Ca^{2+}] \simeq 150$nM)	48.5±3.2 (3)	49.8±4.6 (3)	45.1±4.2 (3)	47.1±3.8 (3)	44.2±4.0 (3)
	1.75mM-$CaCl_2$ (free $[Ca^{2+}] \simeq 350$nM)	49.1±4.1 (3)	48.6±3.5 (3)	N.D.	N.D.	N.D.
	1mM-$CaCl_2$ plus 10mM-NaCl	12.4±1.0 (3)††	13.6±2.1 (3)††	7.9±1.9 (3)†††	6.7±1.1 (3)†††	7.9±0.6 (3)†
(ii) Alternative substrates	5mM-succinate	13.3±1.8 (3)	35.4±1.8 (3)***	10.1±2.1 (3)	22.1±3.2 (3)*	N.D.
	As above plus 10mM-NaCl	12.8±1.8 (3)	16.2±2.1 (3)††	9.5±1.3 (3)	10.4±0.9 (3)†	N.D.
	5mM-glutamate plus 1mM-malate	15.4±2.6 (3)	33.4±3.3 (3)*	13.0±1.1 (3)	26.9±3.0 (3)*	N.D.
	As above plus 10mM-NaCl	15.6±1.8 (3)	15.2±2.0 (3)††	13.2±1.4 (3)	12.5±2.0 (3)†	N.D.

dehydrogenase complex within control mitochondria (results not shown). Similar results to those given for 2-oxoglutarate oxidation [Table 2(i)] could be obtained by monitoring the evolution of $^{14}CO_2$ from 2-oxo[1-^{14}C]glutarate (results not shown). The differences from control values in the activity of this Ca^{2+}-sensitive enzyme as a result of treatments (*a*), (*b*) and (*c*) did not persist for as long as those noted (Figs. 1 and 4) for PDH_a and appeared to be largely abolished by about 5 min. This was probably the result of the incubation period before 2-oxoglutarate addition (see the legend of Table 2), as it was found that these conditions (which were necessary to achieve steady-state conditions) led to an approximate doubling of the rate of ^{45}Ca egress from ^{45}Ca-loaded mitochondria compared with the conditions used for the measurement of PDH_a (which were similar to those described in Fig. 3). It should be noted that treatments (*a*), (*b*) or (*c*) had no effect on the rate of oxidation of non-saturating or saturating concentrations of either succinate alone or glutamate in the presence of malate (added instead of 2-oxoglutarate) by mitochondria incubated in the absence or presence of Na^+ as described in Table 2(i).

Effect of the perfusion of rat hearts with medium containing isoprenaline, and effects of the injection of rats in vivo with adrenaline, on the activities of the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes in subsequently isolated and incubated mitochondria

Some studies were also performed on mitochondria prepared from rat hearts perfused with medium containing 0.5 μM-DL-isoprenaline or from rat hearts removed from anaesthetized rats which had been injected *in vivo* with adrenaline as described by McCormack & Denton (1981). Essentially similar results to those given above as a consequence of the perfusion of rat hearts with medium containing adrenaline were obtained (results not shown). In particular, the former treatments resulted in persistently increased activities of both of the Ca^{2+}-sensitive enzymes in mitochondria incubated in media containing EGTA under the basic conditions described for Fig. 1 and Tables 1(i) and 2. Furthermore, differences from control values could again be abolished by Na^+, and also the effects of Na^+ could be blocked by diltiazem.

General conclusions

The studies reported in this paper appear to represent the most direct evidence to date that adrenaline action on the rat heart results in an increase in the intramitochondrial concentration of Ca^{2+}, and that this in turn increases PDH_a and the activity of the 2-oxoglutarate dehydrogenase complex, plus presumably also that of NAD^+–isocitrate dehydrogenase. The studies have demonstrated that the efflux of Ca^{2+} from rat heart mitochondria is sufficiently slow under appropriate conditions for alterations in intramitochondrial Ca^{2+} occurring in the intact heart to persist during the preparation of mitochondria and thus to have effects on the Ca^{2+}-sensitive intramitochondrial dehydrogenases during subsequent incubations. It is important to emphasize that alterations in PDH_a and the apparent K_m of 2-oxoglutarate dehydrogenase for oxoglutarate similar to those associated with an increase in Ca^{2+} can be brought about, at least in part, by intramitochondrial changes in the ADP/ATP and NAD^+/NADH concentration ratios or in H^+ concentration (see McCormack & Denton, 1979; Denton & McCormack, 1980). However, these can be eliminated in favour of an increase in intramitochondrial Ca^{2+} as the basis of the persistent changes in PDH_a and 2-oxoglutarate dehydrogenase activity that have been described, for the following reasons. Most important is the demonstration that the persistent increases are rapidly reversed by addition of Na^+ to mitochondrial incubation media and that this reversal of the effects of hormone treatment is blocked by diltiazem, an inhibitor of the Na^+-dependent system for egress of Ca^{2+} from heart mitochondria (Vághy *et al.*, 1982). Other evidence includes the loss of the persistent changes on incubation of mitochondria in medium free of Na^+ but containing sufficient Ca^{2+} to cause maximum increases in both PDH_a and 2-oxoglutarate dehydrogenase complex activity, and the lack of persistence of changes in PDH_a after perfusion of hearts with medium containing pyruvate. Finally, further strong support is afforded by the very striking similarity in the changes in PDH_a and 2-oxoglutarate dehydrogenase activity seen in mitochondria under a variety of conditions from hearts perfused with medium containing adrenaline to those observed both in mitochondria from hearts perfused with medium containing an increased concentration of Ca^{2+} and in mitochondria that had been loaded with Ca^{2+} during a preincubation procedure.

The gradient of Ca^{2+} across the mitochondrial inner membrane is determined by a cycle made up of the uptake of Ca^{2+} into mitochondria on a uniporter driven by the membrane potential plus the efflux of Ca^{2+} by a charge-compensated mechanism; in the intact heart, efflux presumably occurs very largely by the Na/Ca antiporter. This cycle will dissipate energy, but this is probably only a very small fraction of that produced by mitochondria (for reviews, see Carafoli, 1979; Nicholls & Crompton, 1980). Little is known about the mech-

Table 2. *Comparison of (a) the effects of a preincubation of mitochondria with Ca^{2+} with (b, c) the effects of perfusion of rat hearts with medium containing (b) adrenaline or (c) increased Ca^{2+} concentration, on (i) the initial rate of oxidation of, and (ii) the extent of NAD(P)$^+$ reduction induced by, a non-saturating concentration of 2-oxoglutarate*

Mitochondria were prepared as for Table 1 and were incubated at 30°C in KCl-based media (see Experimental section) containing 2mM-EGTA and other additions as indicated and with the further additional presence of either (i) 2mM-ADP plus 1mM-malate or (ii) 2mM-ADP alone. In (i) mitochondria were preincubated until a linear base rate of oxygen uptake was achieved (after about 1min); 0.5mM-2-oxoglutarate was added after 90s to give a new linear rate within 30s and was then followed 2min later by a saturating 2-oxoglutarate concentration (20mM). In (ii) mitochondria were preincubated (including antimycin addition after 1min) as in Fig. 2 to give a basal steady-state extent of reduction by 90s, at which point 0.5mM-2-oxoglutarate was added; this resulted in a new steady-state level of reduction within 90s and was then followed 2min later by 2mM-2-oxoglutarate together with 2mM-$CaCl_2$ plus 2mM-EGTA (resultant free $[Ca^{2+}] \simeq 50$nM), which gave a saturating level of reduction (see Fig. 2 legend). The treatments used did not affect the basal or saturating rates and extents. Results are expressed as the percentage of the saturating responses which were evident at 0.5mM-2-oxoglutarate, and are given as means ± S.E.M. for the numbers of different mitochondrial preparations in parentheses. $*P \leqslant 0.05$ and $**P \leqslant 0.01$ for the effects of (*a*), (*b*) or (*c*) compared with the appropriate control value (i.e. across rows); $\dagger P \leqslant 0.05$, $\dagger\dagger P \leqslant 0.01$ and $\dagger\dagger\dagger P \leqslant 0.001$ for the effects of Na^+ compared with the appropriate value obtained in its absence (i.e. down columns). Abbreviation: N.D., not done.

Additions	(i) Rate of oxidation of 0.5mM-2-oxoglutarate (as % of maximum rate of 2-oxoglutarate oxidation), and (ii) extent of NAD(P)$^+$ reduction induced by 0.5mM-2-oxoglutarate (as % of maximum reduction), in incubated mitochondria prepared from:				
	mitochondria preincubated with:		hearts perfused with medium containing:		
	2mM-EGTA (control)	(*a*) 2mM-EGTA plus 1mM-$CaCl_2$ (free $[Ca^{2+}] \simeq 50$nM)	No additions (control)	(*b*) Adrenaline	(*c*) Increased Ca^{2+} concentration
(i) None	19.9 ± 1.6 (12)	35.4 ± 3.3 (12)**	23.4 ± 2.1 (10)	34.6 ± 2.6 (8)**	32.0 ± 3.6 (6)*
10mM-NaCl	18.8 ± 1.1 (10)	17.9 ± 1.0 (10)†††	25.0 ± 1.3 (10)	23.5 ± 2.5 (8)††	22.2 ± 1.8 (6)†
300μM-diltiazem	21.7 ± 1.2 (3)	33.6 ± 3.9 (3)*	24.2 ± 2.0 (4)	32.2 ± 2.6 (4)*	N.D.
300μM-diltiazem plus 10mM-NaCl	18.3 ± 2.6 (3)	32.4 ± 4.0 (3)*	23.4 ± 2.3 (6)	32.4 ± 2.0 (6)*	31.7 ± 2.5 (4)*
(ii) None	33.6 ± 3.1 (3)	60.8 ± 3.2 (3)**	24.7 ± 2.3 (3)	46.7 ± 4.1 (3)*	46.8 ± 3.2 (3)**
10mM-NaCl	31.4 ± 3.5 (3)	28.4 ± 2.5 (3)††	30.4 ± 3.1 (3)	28.4 ± 2.6 (3)†	33.6 ± 3.3 (3)†
300μM-diltiazem plus 10mM-NaCl	34.6 ± 4.0 (3)	55.5 ± 3.6 (3)*	30.4 ± 2.9 (3)	43.4 ± 2.9 (3)*	47.1 ± 4.0 (3)*

anism whereby Ca^{2+} is able to leave heart, or indeed other, mitochondria in the absence of Na^+. The results of the present study suggest that it is quite separate from the Na^+-dependent pathway, as it appears insensitive to diltiazem. We cannot offer an entirely satisfactory explanation for the slow efflux of Ca from heart mitochondria incubated with medium containing EGTA.

The addition of Ca^{2+} to mitochondria incubated in the absence of Na^+ and Mg^{2+} leads to prompt increases in both PDH_a and 2-oxoglutarate dehydrogenase complex activity (see, e.g., Fig. 2; Denton *et al.*, 1980). For instance, half-maximal effects of Ca^{2+} require a free concentration of about 20nM under these conditions. At this concentration the increases in both enzymes are complete within 1–2min and are maintained for at least 5min or, with PDH_a, 10min. It seems reasonable to conclude that this is the result of a steady-state intramitochondrial concentration of Ca^{2+} of about 1 μM (see also Coll *et al.*, 1982) being reached within 1–2min and that the rate of Ca^{2+} efflux matches the rate of Ca^{2+} uptake. It is therefore surprising that on addition of EGTA the rate of Ca^{2+} efflux should be so low. Further work, including detailed studies into the kinetic properties of the Na^+-independent efflux pathway and into the bound forms of Ca^{2+} within mitochondria, is required to clarify this point.

There now seems to be overwhelming evidence that the increase in oxidative metabolism in hearts exposed to adrenaline involves activation of three key dehydrogenases by an increase in intramitochondrial Ca^{2+}. A rise in the concentration of Ca^{2+} in the cytoplasm of heart cells in systole is, of course, the means by which this hormone stimulates contraction in the heart, and we have suggested that as a result of this the mitochondria are exposed to a 'time-averaged' increase in the concentration of Ca^{2+} in the cytoplasm, which in turn leads to an increase in intramitochondrial Ca^{2+} (see Denton & McCormack, 1980, 1981).

There are many other instances where a rise in the concentration of Ca^{2+} in the cytoplasm of cells is thought to form an important part of the mechanism by which a hormone or neurotransmitter brings about its response (for reviews, see, e.g., Cohen, 1978; Kretsinger, 1978; Michell, 1979; Exton, 1980; Rasmussen & Goodman, 1977; Wolff & Brostrom, 1979). In addition, many of these responses involve energy-requiring processes, and thus we have suggested in more general terms (Denton & McCormack, 1980, 1981), that a resultant rise in the intramitochondrial concentration of Ca^{2+} may be an important means by which energy production could be stimulated in these situations. This mechanism is attractive because a common messenger would be used for both cytoplasmic and intramitochondrial effects and would allow oxidative metabolism to be stimulated without the need for decreases in ATP or NADH.

It is to be hoped that the approach used in the present paper, together with the use of Ruthenium Red on intact cell preparations (McCormack & England, 1983), will allow the role of changes in intramitochondrial Ca^{2+} to be explored in a wide variety of situations.

Note added in proof (received 2 December 1983)

Crompton *et al.* (1983) have reported that the addition of 1 μM-adrenaline to the perfusion media of rat hearts for 2min results in an increase in the total Ca content of subsequently isolated mitochondria from a control value of about 1.5 to about 4nmol/mg of mitochondrial protein. Since both Hansford & Castro (1981) and Coll *et al.* (1982) have shown in earlier studies with isolated rat heart mitochondria that the activation of 2-oxoglutarate dehydrogenase by intramitochondrial free Ca^{2+} is observed when the total Ca content rises within the range 0.5–4nmol/mg of mitochondrial protein, the findings of Crompton *et al.* (1983) add further evidence in support of our contention that adrenaline increases the concentration of free Ca^{2+} within rat heart mitochondria and that this leads to activation of the three dehydrogenases.

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