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Role of Ca^{2+} in pyruvate dehydrogenase interconversion in brain mitochondria and synaptosomes

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1. The steady-state content of active (dephospho) pyruvate dehydrogenase (PDH_A) of suspensions of coupled rat brain mitochondria oxidizing succinate was found to be markedly increased with increasing free Ca^{2+} ion concentration of the medium, with a half-maximal effect at $10^{-6.43}$ M Ca^{2+}. Other ions were present in these studies at concentrations appropriate for the cytosol. 2. Depolarization of the plasma membrane of synaptosomes caused an increase in the steady-state content of PDH_A, with veratridine giving a larger increase than depolarization by 33 mM-KCl. Values were $68 \pm 1\%$ ($n = 13$) and $81 \pm 1\%$ ($n = 19$) of maximal activity, for control incubations and incubations in the presence of 30 μM-veratridine, respectively. 3. Measurements of cytosolic free Ca^{2+} concentrations ($[Ca^{2+}]_{cyt.}$) in these suspensions of synaptosomes, with the use of the fluorescent Ca^{2+}-indicator Quin-2, indicated an increase on depolarization, with the change due to 30 μM-veratridine being larger in extent than that due to 33 mM-KCl. Values were 217 ± 21 nM ($n = 15$), 544 ± 48 nM ($n = 15$) and 783 ± 75 nM ($n = 14$) for control, KCl-depolarized and veratridine-depolarized synaptosomes respectively. 4. Experiments in which synaptosomes were treated with Ruthenium Red, an inhibitor of mitochondrial Ca^{2+} uptake, gave much lower resting contents of PDH_A ($42 \pm 2\%$ of maximal), but failed to prevent totally an increase on depolarization. 5. Addition of an excess of EGTA to the synaptosomal suspension just before the addition of veratridine resulted in a partial diminution in the response of PDH_A content. Parallel studies with Quin-2 indicated no increase in $[Ca^{2+}]_{cyt.}$ on addition of veratridine, under these conditions. 6. Thus an increase in $[Ca^{2+}]_{cyt.}$ forms only a part of the mechanism whereby pyruvate dehydrogenase interconversion responds to depolarization. A decrease in the ATP/ADP ratio may also be important, as inferred from the results of experiments with ouabain, which inhibits the $Na^+ + K^+$-dependent ATPase.

There is an increasing body of evidence favouring the idea that the Ca^{2+} ion may be important in the control of substrate oxidation in central nervous tissue. Thus it is known that the fraction of pyruvate dehydrogenase existing in the active (dephospho) form (PDH_A) is increased when hippocampal slices are subjected to repetitive electrical stimulation (Browning *et al.*, 1979, 1981), and is decreased when the hippocampus is denervated (Baudry *et al.*, 1982). Similarly, it is known that depolarization of cortical slices (Kovachich & Haugaard, 1977) and of synaptosomes (Schaffer & Olson, 1980) by high concentrations of K^+ leads to an increase in the content of PDH_A.

It is also known that the depolarization of presynaptic terminals of central nervous tissue from higher animals results in an influx of Ca^{2+} ions, and that this is part of the mechanism leading to the release of neurotransmitters (Katz & Miledi, 1967; Blaustein, 1975; Raiteri & Levi, 1978). More recently, it has been confirmed that the free Ca^{2+} concentration of the cytosol ($[Ca^{2+}]_{cyt.}$) increases as a consequence of this influx, in experiments with synaptosomes (pinched-off presynaptic nerve terminals) and either the metallochromic indicator Arsenazo III (Åkerman & Heinonen, 1983) or the fluorescent Ca^{2+}-indicator Quin-2 (Ashley *et*

Abbreviation used: PDH_A, active (dephospho) pyruvate dehydrogenase.

al., 1984; Richards *et al.*, 1984). Similarly, studies with Quin-2 have shown transient increases in $[Ca^{2+}]_{cyt.}$ associated with the challenge of adrenal medullary cells with acetylcholine (Knight & Kesteven, 1983).

The question arises as to whether the increase in $[Ca^{2+}]_{cyt.}$ is the signal to the pyruvate dehydrogenase interconversion system, in view of the known sensitivity of the pyruvate dehydrogenase phosphatase reaction to micromolar concentrations of Ca^{2+} (Denton *et al.*, 1972; Pettit *et al.*, 1972) and of the demonstrated increase in PDH_A content of intact, respiring, heart muscle mitochondria that occurs when the Ca^{2+} concentration of the medium is increased from 10^{-7} to 10^{-6}M (Hansford & Cohen, 1978; Denton *et al.*, 1980; Hansford, 1981). The other plausible signals, on the basis of our current knowledge of pyruvate dehydrogenase interconversion, are decreases in ATP/ADP and NADH/NAD^+ ratios (for reviews see Hansford, 1980; Reed, 1981). These might be expected to follow from increased activity of the $Na^+ + K^+$-dependent ATPase and the interaction of ADP with oxidative phosphorylation.

Some distinction between the effects of these various signals has been achieved in studying the response of heart pyruvate dehydrogenase to positive inotropic interventions (McCormack & England, 1983). Those authors showed that Ruthenium Red, an inhibitor of the mitochondrial uptake of Ca^{2+} (Moore, 1971; Vasington *et al.*, 1972), totally prevented an increase in PDH_A content in response to adrenaline (epinephrine) or to an elevation of perfusion fluid Ca^{2+} concentration, without preventing the response of force production or of phosphorylase *a* content. Pyruvate dehydrogenase is an intramitochondrial enzyme and senses the intramitochondrial Ca^{2+} concentration, whereas force production and phosphorylase interconversion react to changes in $[Ca^{2+}]_{cyt.}$: thus this result strongly implicates the transport of Ca^{2+} into the mitochondrion as part of the mechanism of the response to positive inotropic agents.

The purpose of the present work was to evaluate the role of these different effectors in central nervous tissue. Experiments were carried out at two levels. First, the response of coupled, respiring, mitochondria to changes in Ca^{2+} concentration in the medium was evaluated, in analogy to experiments previously carried out with heart mitochondria (Hansford & Cohen, 1978; Denton *et al.*, 1980; Hansford, 1981). This was necessary, as there is no information available on the response of nervous-tissue mitochondria to plausible cytosolic values of Ca^{2+} concentration, i.e. 10^{-6}–10^{-7}M, although there is earlier work showing a response of pyruvate dehydrogenase interconversion in intact mitochondria to chelating agents and Ruthenium Red (Jope & Blass, 1975) and to millimolar concentrations of Mg^{2+} and Ca^{2+} in the presence of the bivalent ionophore A23187 (Booth & Clark, 1978). Secondly, experiments were carried out with synaptosomes, in an extension of the work of Schaffer & Olson (1980). These experiments used specific inhibitors to dissect apart effects of Ca^{2+} from those of adenine nucleotides, as in the approach used by McCormack & England (1983) with the heart. Further, we took advantage of the newly available Ca^{2+}-indicator Quin-2 (Tsien, 1980, 1981; Tsien *et al.*, 1982) to measure synaptosomal $[Ca^{2+}]_{cyt.}$ under the conditions used in the studies on pyruvate dehydrogenase interconversion.

Experimental

Preparation of mitochondria and synaptosomes

Mitochondria were prepared from cerebral cortex of two male Wistar rats, essentially as described by Nicholls (1978), but with a medium comprising 0.3M-sucrose, 10mM-Hepes/KOH and 1mM-EGTA, pH7.4. Synaptosomes were prepared from cerebral cortex of two or three male Sprague–Dawley rats, of weight 350–450g, essentially as described by Nicholls (1978) and Scott & Nicholls (1980). The homogenization medium comprised 0.32M-sucrose, 10mM-Hepes/KOH and 0.5mM-EGTA, pH7.4; the final washing step and resuspension of the synaptosomes used a medium omitting EGTA.

For measurements of PDH_A content and of $[Ca^{2+}]_{cyt.}$, synaptosomes were incubated at 37°C in a medium ('incubation medium') comprising 0.13M-NaCl, 4mM-KCl, 1mM-$CaCl_2$, 1mM-$MgSO_4$, 20mM-Hepes/NaOH, 1.2mM-sodium phosphate and 10mM-D-glucose, of final pH7.4.

Measurement of PDH_A content of mitochondria and synaptosomes

Pyruvate dehydrogenase interconversion was quenched by adding 50μl portions of mitochondrial or synaptosomal incubations to 250μl portions of an ice-cold medium comprising 50mM-Hepes/KOH, pH7.1, 3mM-EGTA, 25mM-NaF, 0.1% Triton X-100, 1mM-dichloroacetate and 1mM-dithiothreitol. After mixing, 0.2ml of the quenched mitochondria or synaptosomes was added to 0.8ml of pyruvate dehydrogenase assay mixture, comprising 50mM-Hepes/KOH, 1.1mM-$MgSO_4$, 4mM-dithiothreitol, 2.5μM-rotenone, 2mM-NAD^+, 0.18mM-thiamin pyrophosphate, 0.08mM-CoA, 0.08mM-EGTA, 16.7mM-L-lactate and 2.5units of lactate dehydrogenase/ml. The temperature was 25°C and the final pH was 7.1. Increase in absorbance was measured at 340nm.

This method of assay of pyruvate dehydrogenase depends on maintenance of equilibrium of the lactate dehydrogenase reaction, and is therefore unaffected by additional lactate dehydrogenase from the tissue preparations.

Measurement of $[Ca^{2+}]_{cyt.}$ of synaptosomes with the use of Quin-2

Synaptosomes (10mg of protein) were added to 4ml of 'incubation medium' of the composition indicated above, to which were added D-glucose to 10mM and Quin-2/AM, dissolved in dimethyl sulphoxide, to 100μM. The suspension was shaken under an atmosphere of O_2 for 1h at 37°C. At this point, it was centrifuged for 2min in a Beckman Microfuge, the pellet and walls of the tube were rinsed, and the synaptosomes were resuspended to 4ml by using a Teflon Dounce homogenizer.

The Quin-2-loaded synaptosomes were stored at 37°C under O_2, and used during the next 1.5h. Other batches of synaptosomes were loaded at appropriate intervals. Control experiments were carried out with unloaded suspensions.

Measurement of $[Ca^{2+}]_{cyt.}$ was based on the fluorescence of intracellular Quin-2, as further described in the Results and discussion section. Fluorescence was measured with an Aminco–Chance DW2 dual-wavelength spectrophotometer, fitted with a total-fluorescence attachment. Excitation was at 339nm, with a 10nm bandpass, and emitted light was filtered through Farrand Optical glass filters nos. 4-70 and 3-73, to cut out the exciting light. The sample was maintained at 37°C and stirred mechanically.

Materials

Quin-2/AM was from Calbiochem; oligomycin and veratridine were from Sigma Chemical Co.; Ruthenium Red was from Polysciences; carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was from Pierce Chemical Co.; digitonin was from Fisher Scientific Co., and was recrystallized twice and dissolved in ethanol. All other reagents were of the highest grade available commercially.

Results and discussion

Effect of extramitochondrial Ca^{2+} concentration on PDH_A content of brain mitochondria

Fig. 1 shows that increasing the free Ca^{2+} concentration of the incubation medium over the range 10^{-7}–10^{-6}M results in a 3-fold increase in the PDH_A content of respiring, coupled, brain mitochondria. The Ca^{2+} concentration required for 50% activation was found to be $10^{-6.43}$M. Sampling was carried out after a 10min incubation in these studies, at which point enzyme interconversion had reached a steady state (results not shown).

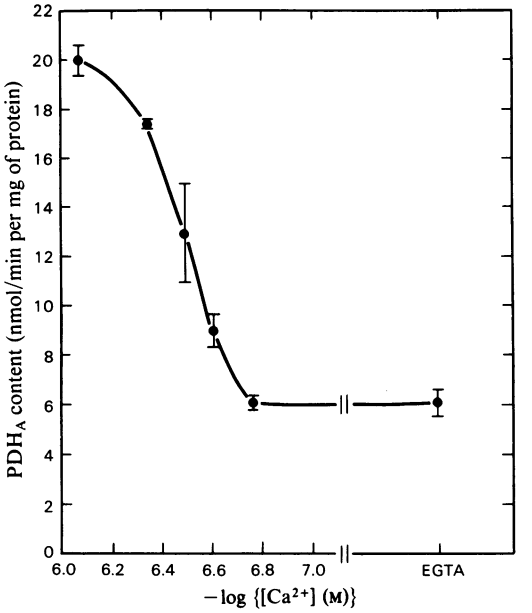

Fig. 1. *Effect of extramitochondrial free Ca^{2+} concentration on the PDH_A content of rat brain mitochondria*
Mitochondria (0.5mg of protein) were added to 0.5ml of incubation medium comprising 0.13M-KCl, 20mM-Hepes/KOH, 5mM-potassium phosphate, 10mM-NaCl, 1mM-$MgSO_4$, 5mM-Tris succinate, 1mM-ATP (sodium salt), 2mg of fat-free bovine serum albumin/ml and 2mM-Ca^{2+}/EGTA buffer giving the indicated value of free Ca^{2+} concentration. The pH was 7.2 and the temperature was 25°C. Then 10min later, 50μl portions were removed in duplicate, quenched to prevent further pyruvate dehydrogenase interconversion and assayed for PDH_A under conditions generating $V_{max.}$. Further details are given in the Experimental section. Each point is the mean for four experiments, each involving a separate mitochondrial preparation. The error bars represent the S.E.M.

Total pyruvate dehydrogenase activity was found to be 23.4 ± 0.3nmol/min per mg of protein (4) (mean ± S.E.M., *n* in parentheses). This result was obtained on adding dichloroacetate (1mM) to incubation mixtures containing $10^{-6.48}$M-Ca^{2+}; dichloroacetate is an inhibitor of pyruvate dehydrogenase kinase (Whitehouse *et al.*, 1974) and has been shown to have some activity in central nervous tissue (Abemayor *et al.*, 1984). The PDH_A content shown in Fig. 1 therefore varies from 25% to 75% of total activity, as a function of Ca^{2+} concentration. Absolute values are lower than those measured in some previous work (Jope & Blass, 1975; Booth & Clark, 1978), though part of the difference is accounted for by temperature, which was 25°C in the present study. Other work gives values comparable with those shown here (Evans, 1983).

This study (Fig. 1) demonstrates a greater range of PDH_A contents than had previously been achieved with brain mitochondria, which generally have been found to maintain a very high percentage of the enzyme in the form PDH_A (Jope & Blass, 1975), unless inhibitors of phosphatase activity or ionophores are added (Booth & Clark, 1978). The present study uses a medium similar to cytosol, and avoids the use of inhibitors, and is thus closer to physiology. The degree of inactivation achieved at low ambient Ca^{2+} concentrations is dependent partly on the presence of ATP in these studies (results not shown), and partly on a fairly lengthy incubation in the presence of 10mM-Na^+, which potentiates Ca^{2+} efflux from brain mitochondria via Na^+/Ca^{2+} exchange (Crompton *et al.*, 1978; Nicholls, 1978). Thus the mitochondria were probably more successfully depleted of Ca^{2+} in the studies of Fig. 1 than in previous work, except where the ionophore A23187 and EGTA were used in combination (Booth & Clark, 1978).

Effect of depolarization of synaptosomes on the interconversion of pyruvate dehydrogenase

A pioneering study by Schaffer & Olson (1980) showed that the fraction of pyruvate dehydrogenase existing as PDH_A increased from approximately 90% to 100% when synaptosomes were depolarized by high concentrations of K^+. The purpose of the present study was to evaluate the role of Ca^{2+} ions in signalling the depolarization event to the pyruvate dehydrogenase interconversion system. In early experiments, it was found that an increase in the K^+ concentration of the medium from 4mM to 33mM resulted in an increase in the PDH_A content of synaptosomes from $60.2 \pm 2.7\%$(9) of total activity to $69.2 \pm 2.0\%$(9) when the interconversion was quenched 5min after addition of KCl. This change was statistically significant ($P < 0.05$), albeit small. However, it was found that the alkaloid veratridine, which is considered to depolarize neuronal preparations by holding open Na^+ channels (Ohta *et al.*, 1973; Blaustein & Goldring, 1975), gave rise to a greater change in PDH_A content, and this protocol was therefore adopted in further studies.

Table 1 shows that, in experiments involving 24 preparations of synaptosomes, 30μM-veratridine caused a highly significant increase in PDH_A content, from 68 to 81% of total activity. Higher concentrations of veratridine (100μM) gave no greater effect (results not shown).

This response was only partially dependent on the presence of extracellular Ca^{2+}. Diminution of extracellular free Ca^{2+} concentration to approx. 0.33mM, by the inclusion of EGTA from the beginning of the experiment, did not affect the response to depolarization (cf. lines 1–4). However, the addition of a molar excess of EGTA over the Ca^{2+} in the medium immediately before the addition of veratridine resulted in some blunting of the response to depolarization (cf. lines 1 and 7).

Table 1. *Response of pyruvate dehydrogenase to depolarization of synaptosomes with veratridine*

Synaptosomes (1.25mg of protein) were added to 0.3ml of 'incubation medium', of the composition given in the Experimental section, and incubated for 20min at 37°C, under an atmosphere of O_2. In Expts. 3 and 4, 0.67mM-EGTA was also present during this preincubation phase, this being sub-stoichiometric with respect to the Ca^{2+}: in Expts. 11 and 12, 20μM-Ruthenium Red was present. At 20min, veratridine was added to give 30μM final concentration, except in the control incubation (1), in which no further addition was made. Where indicated, ouabain, EGTA and oligomycin were added 5s before veratridine, to give final concentrations of 0.45mM, 1.3mM and 2μg/ml respectively. In all cases 50μl portions were removed at 25min, quenched, and sampled for PDH_A content as described in the Experimental section. Results are mean values ±S.E.M. with the numbers of experiments in parentheses. The maximal activity of pyruvate dehydrogenase was 7.04 ± 0.14 nmol/min per mg of protein at 25°C, in 24 preparations of synaptosomes. Abbreviation: N.S., not significant.

Expt. no.	Preincubation	Addition	PDH_A (% of total PDH)
1	—	—	68 ± 1 (13)
2	—	Veratridine (30μM)	81 ± 1 (19) $P < 0.001$ versus 1
3	EGTA (0.67mM)	—	69 ± 1 (6)
4	EGTA (0.67mM)	Veratridine (30μM)	80 ± 3 (6) $P < 0.001$ versus 1
5	—	Veratridine (30μM)+ouabain (0.45mM)	74 ± 1 (13) $P < 0.005$ versus 2; N.S. versus 6
6	—	Ouabain (0.45mM)	71 ± 1 (9) $P < 0.05$ versus 1
7	—	Veratridine (30μM)+EGTA (1.3mM)	73 ± 1 (18) $P < 0.005$; $P < 0.001$ versus 2
8	—	EGTA (1.3mM)	69 ± 1 (4)
9	—	Veratridine (30μM)+oligomycin (2μg/ml)	81 ± 2 (5) $P < 0.001$ versus 1
10	—	Oligomycin	68 ± 1 (5)
11	Ruthenium Red (20μM)	—	42 ± 2 (7) $P < 0.001$ versus 1
12	Ruthenium Red (20μM)	Veratridine (30μM)	53 ± 1 (9) $P < 0.001$ versus 11

The decreased response to veratridine is highly statistically significant ($P<0.001$). Thus there is evidence that extracellular Ca^{2+} is required for a full response to depolarization, but that 0.33mM is sufficient. A major, but less than maximal, response persists in the absence of extracellular Ca^{2+}.

Incubation of the synaptosomes with Ruthenium Red, an inhibitor of the mitochondrial Ca^{2+}-uptake pathway (Moore, 1971; Vasington *et al.*, 1972), from the beginning of the experiment resulted in a very pronounced decrease in PDH_A content (cf. lines 1 and 11). However, the Ruthenium Red did not abolish the response to depolarization (cf. lines 11 and 12). Although Ruthenium Red does not seem a likely penetrant of the plasma membrane, being a sexivalent cation (Luft, 1971), the experiments of McCormack & England (1983) strongly suggest that it does enter cardiac myocytes during heart perfusion. The basis of this statement is the effect of Ruthenium Red in preventing the activation of pyruvate dehydrogenase, an intramitochondrial enzyme, by raised perfusion-fluid Ca^{2+} concentration, but not preventing the activation of phosphorylase and of contractility, these by contrast being directly sensitive to $[Ca^{2+}]_{cyt.}$. Similarly, the present results are most simply explained by an entry of the stain into the synaptosomes and inhibition of the mitochondrial Ca^{2+} uniport. At a given value of $[Ca^{2+}]_{cyt.}$, this would result in a lowering of the value of intramitochondrial free Ca^{2+} concentration, owing to the existence of an uninhibited Ca^{2+}/Na^+ exchange catalysing Ca^{2+} efflux from the mitochondrion (for a review see Nicholls & Åkerman, 1982). The lowered PDH_A content is then a plausible consequence of the lowered mitochondrial free Ca^{2+} concentration. Although the Ruthenium Red does not abolish the response of pyruvate dehydrogenase to depolarization, the point to emphasize is the small magnitude of the response in view of the poise of the interconversion system at the level of approx. 50% PDH_A. Thus these experiments lend support to a role of Ca^{2+} in mediating the response to depolarization.

A similar conclusion emerges from the elevation of PDH_A content by the cardiac glycoside ouabain, which inhibits the $Na^+ + K^+$-dependent ATPase (Escueta & Appel, 1969); cf. lines 1 and 6. This leads to a slight, progressive, increase in $[Ca^{2+}]_{cyt.}$ over a 5min period, as revealed by experiments with Quin-2 of the type described below. The mechanism of this is probably a combination of the activation of voltage-sensitive Ca^{2+} channels and a reversal of the Na^+/Ca^+ exchange reaction, due to a decrease in both chemical and electrical components of the Na^+ electrochemical gradient (Coutinho *et al.*, 1984).

However, Ca^{2+} is not the only mediator of the depolarization-induced changes in PDH_A content. Thus ouabain diminishes the response to veratridine (cf. lines 2 and 5), indicative of a role of $Na^+ + K^+$-dependent ATPase in the response. As mentioned above, the baseline activity before depolarization is somewhat raised by the ouabain, minimizing the scope for further increases due to veratridine. It is most likely that depolarization activates $Na^+ + K^+$-dependent ATPase through an elevation of cytosolic Na^+ concentration, and that the availability of ADP to the mitochondria increases as a consequence of increased ATPase activity. This would decrease the mitochondrial ATP/ADP ratio and could potentially activate pyruvate dehydrogenase (for reviews see Hansford, 1976, 1980; Reed, 1981). Ouabain would prevent these effects.

The fact that oligomycin, an inhibitor of the mitochondrial ATP synthetase (see Slater, 1967), fails to prevent veratridine-induced increases in PDH_A content (cf. lines 9 and 10) strongly suggests that changes in the mitochondrial NADH/NAD^+ ratio are not necessary for this response. This provides a contrast with the situation in heart mitochondria, where decreases in NADH/NAD^+ have a potent activatory effect (Hansford & Cohen, 1978).

Measurement of $[Ca^{2+}]_{cyt.}$ in synaptosomes with the use of Quin-2

It seemed important actually to measure $[Ca^{2+}]_{cyt.}$ under the conditions of relevance to pyruvate dehydrogenase regulation. Further, at the initiation of the present study, there were no reports of $[Ca^{2+}]_{cyt.}$ in synaptosomes under resting or depolarized conditions, and so such measurements would contribute to our knowledge of $[Ca^{2+}]_{cyt.}$ homoeostasis in nervous tissue. The fluorescent chelating agent Quin-2 developed by Tsien (1980, 1981) offered a straightforward approach to this problem.

As shown in Fig. 2, the fluorescence of a suspension of synaptosomes that had been loaded with Quin-2/AM for 1h, as described in the Experimental section, was found to increase very rapidly on addition of KCl to give 25mM. The artifact obtained by addition of KCl to a suspension of synaptosomes not loaded with Quin-2 was small (results not shown). This increase in fluorescence shown in Fig. 2 is indicative of an increased saturation of Quin-2 with Ca^{2+}. Absolute values of $[Ca^{2+}]_{cyt.}$ in the resting state and on KCl addition can be calculated provided that fluorescence minima ($F_{min.}$) and maxima ($F_{max.}$) are obtained, corresponding to zero binding of Ca^{2+} and to saturation of Quin-2 with Ca^{2+} respectively, and that the K_d for Ca^{2+} binding to Quin-2 is known.

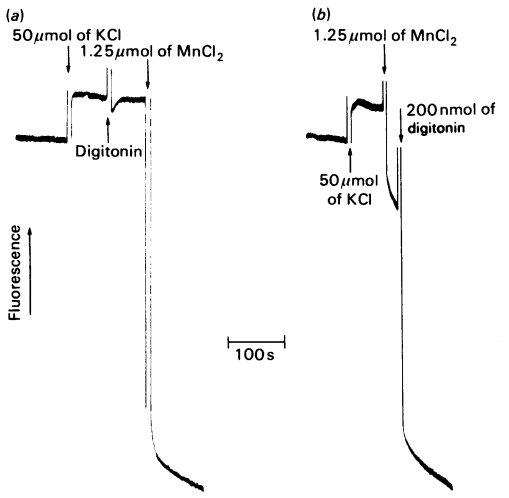

Fig. 2. *Response of synaptosomal* $[Ca^{2+}]_{cyt.}$ *to depolarization with KCl*

The fluorescence of synaptosomes loaded with Quin-2 was followed as described in the Experimental section. A 1 ml portion of a suspension of synaptosomes (2.5 mg of protein) was added to 1.5 ml of incubation medium (see the Experimental section) containing 10 mM-D-glucose. Then 10 min later the recording shown was begun. An increase in fluorescence corresponds to an increase in $[Ca^{2+}]_{cyt.}$. In these experiments an addition of $MnCl_2$ before digitonin was used to nullify a contribution of extrasynaptosomal Quin-2 to the total fluorescence signal; addition of $MnCl_2$ in the presence of digitonin (*a*) nullified the contribution of intrasynaptosomal Quin-2 as well.

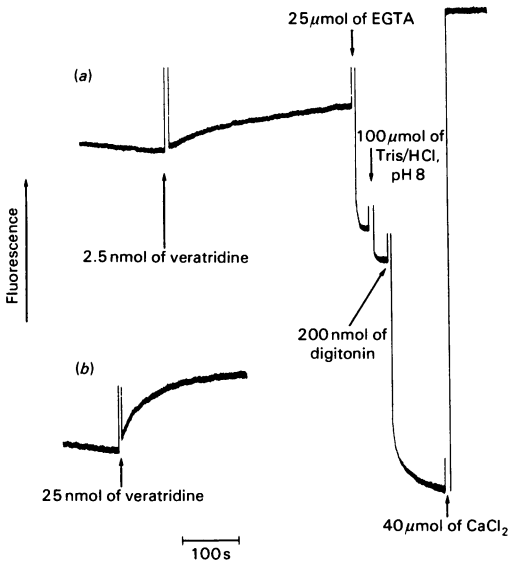

Fig. 3. *Response of synaptosomal* $[Ca^{2+}]_{cyt.}$ *to depolarization with veratridine*

Partial depolarization was achieved in (*a*) by the addition of 2.5 nmol of veratridine, giving a final concentration of 1 μM. More-complete depolarization was achieved in (*b*) by the addition of 25 nmol of veratridine. Calibration of the fluorescence recording was achieved by using EGTA to generate $F_{min.}$, and a subsequent molar excess of $CaCl_2$ to generate $F_{max.}$.

The approach is described in detail and with clarity by Tsien *et al.* (1982) in a study of $[Ca^{2+}]_{cyt.}$ of lymphocytes. In the present study, $F_{max.}$ was obtained by the addition of digitonin, and the exposure of intrasynaptosomal Quin-2 to the 1 mM-Ca^{2+} of the incubation medium. Careful correction was made for a significant apparent decrease in fluorescence that occurs when digitonin is added to unloaded synaptosomes: this artifact is the explanation of the apparent failure of digitonin to increase fluorescence in Fig. 2(*a*). Generation of $F_{min.}$ was by addition of $MnCl_2$, as described by Hesketh *et al.* (1983), or by addition of a 10-fold molar excess of EGTA over Ca^{2+}, at pH 8. Allowance was made for a component of extrasynaptosomal Quin-2, which was revealed by the addition of either $MnCl_2$ (Fig. 2*b*) or EGTA (Fig. 3*a*) before the addition of digitonin, and which would erroneously raise the calculated values of $[Ca^{2+}]_{cyt.}$ if not corrected for.

On the basis of a K_d of 115 nM (Tsien *et al.*, 1982), and the protocol of Mn^{2+} addition, experiments with nine different preparations of synaptosomes gave values of 217 ± 21 nM(15), 544 ± 48 nM(15) and 783 ± 75 nM(15) for $[Ca^{2+}]_{cyt.}$ under conditions of rest, KCl-induced depolarization and veratridine-induced depolarization respectively. This resting value is somewhat higher than that obtained by Ashley *et al.* (1984) and Richards *et al.* (1984), using media of comparable Ca^{2+} concentration. The reason for the discrepancy is not clear. The rapid increase in $[Ca^{2+}]_{cyt.}$ caused by addition of KCl is similar to that seen by Ashley *et al.* (1984), Richards *et al.* (1984) and Meldolesi *et al.* (1984).

The response to 30 μM-veratridine was quite crucial for the interpretation of the PDH_A results, and was found to be larger than the response to KCl (data given above), though the kinetics were much slower. The time course of the response of $[Ca^{2+}]_{cyt.}$ to veratridine is given in Figs. 3(*a*) and 3(*b*), where the effect of 10 μM alkaloid (*b*) is contrasted with that of 1 μM (*a*). The large response of $[Ca^{2+}]_{cyt.}$ to 30 μM- (and, indeed, to 10 μM) veratridine is quite different from the rather small effects noted by Ashley *et al.* (1984) in another study of synaptosomes with Quin-2. It is, however, quite consistent with the larger effect of veratridine on PDH_A content (Table 1 and the text), when compared with the addition of KCl.

It should be pointed out, however, that the correlation between a greater elevation by veratridine of $[Ca^{2+}]_{cyt.}$ and a greater activation of

pyruvate dehydrogenase does not establish that Ca^{2+} is the signal to enzyme interconversion. The reason is that the rate of O_2 uptake by synaptosomes oxidizing glucose is also elevated to a greater extent by veratridine [$\times 3.5 \pm 0.6(3)$] than by KCl [$\times 2.04 \pm 0.06(6)$]. Thus there is probably a larger change in the ATP/ADP ratio too. However, the results with Quin-2 are certainly consistent with such a role for Ca^{2+}.

Fig. 4(*a*) shows that addition of 3.3μmol of EGTA to a suspension of synaptosomes in a medium containing 2.5μmol of Ca^{2+} completely prevents an increase in $[Ca^{2+}]_{cyt.}$ when veratridine is added a few seconds later. Thus the partial activation of pyruvate dehydrogenase that occurs under these conditions (Table 1) cannot be attributed to a rise in $[Ca^{2+}]_{cyt.}$, but must instead be due to other factors, and most plausibly a decrease in the ATP/ADP ratio.

When Ruthenium Red was present from the beginning of an incubation, the increase in $[Ca^{2+}]_{cyt.}$ due to KCl and to veratridine was in no way curtailed (Fig. 4*b*), and in several instances was indeed heightened. This apparent enhancement, however, did not achieve statistical significance. Thus it is quite clear that the large effect that Ruthenium Red has in diminishing values of PDH_A content (Table 1) cannot be attributed to a decline in $[Ca^{2+}]_{cyt.}$, but more probably reflects an inhibition at the level of mitochondrial Ca^{2+} transport, in keeping with its known role (Moore, 1971; Vasington *et al.*, 1972).

General conclusions

We have established that isolated brain mitochondria respond to changes in the Ca^{2+} concentration of their environment with a 3-fold increase in the content of PDH_A, when the Ca^{2+} concentration is raised from approx. 200nM to 500nM (Fig. 1). We have further established via direct measurement with Quin-2 that these values correspond to resting $[Ca^{2+}]_{cyt.}$ and that achieved on KCl-induced depolarization respectively. Veratridine gives somewhat higher values than does KCl. Thus it is reasonable to suppose that control of enzyme interconversion by Ca^{2+} ions functions *in vivo*. This argument does depend, however, on the successful reconstruction of the cytosolic milieu in the cuvette, and it is possible that effectors of mitochondrial Ca^{2+} transport exist that are yet unrecognized.

Experiments with synaptosomes avoid this criticism, and point only to a partial role of Ca^{2+} in mediating depolarization-induced increases in PDH_A content (Table 1). The best evidence for this is the demonstration that addition of excess EGTA before veratridine totally prevents an increase in $[Ca^{2+}]_{cyt.}$ (Fig. 4*a*) (and see also Richards *et al.*, 1984) but only partially curtails the increase in

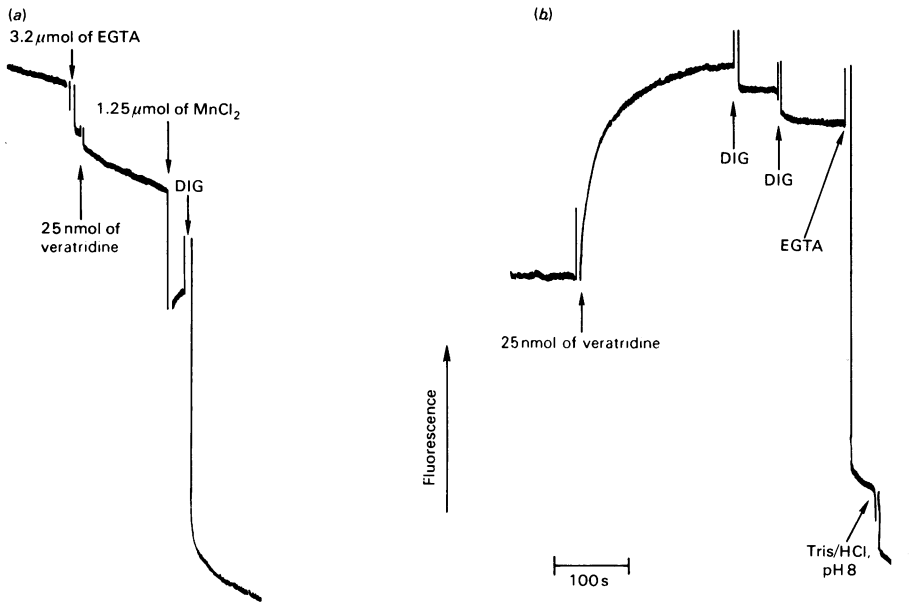

Fig. 4. *Response of synaptosomal* $[Ca^{2+}]_{cyt.}$ *to depolarization with veratridine* (*a*) *in the absence of extracellular* Ca^{2+} *and* (*b*) *in the presence of Ruthenium Red*

In (*a*) 3.2μmol of EGTA was added 15s before the veratridine, to an incubation of volume 2.5ml and containing 2.5μmol of $CaCl_2$. In (*b*) the synaptosomes were incubated with 10nmol of Ruthenium Red from the beginning of the experiment. Other conditions are given in the Experimental section and in the text. Abbreviation: DIG, digitonin.

PDH_A content (Table 2). It is understood that this conclusion applies to these specific experimental conditions and that, in particular, depolarization by veratridine may differ from depolarization by KCl in the effect that an elevated cytosolic Na^+ concentration has on the mitochondrial Ca^{2+} efflux pathway (see Åkerman & Nicholls, 1981). Changes in PDH_A content induced by KCl addition were smaller than those due to veratridine, similar in magnitude to the changes described by Schaffer & Olson (1980), and would not readily permit the studies with inhibitors that were important in the present work.

We are interested in the effect of Ca^{2+} on pyruvate dehydrogenase interconversion in brain in view of the importance of the pyruvate dehydrogenase complex in providing acetyl groups for acetylcholine synthesis (Gibson *et al.*, 1975), as well as its central role in catabolic metabolism in brain. Further, the steady-state content of PDH_A may be of particular importance in acetyl group provision, as we have shown previously that heart mitochondria generate very high ratios of acetyl-CoA/CoA when the content of PDH_A rises in response to activation of the phosphatase by Ca^{2+} and respiration is limited by availability of ADP (Hansford & Cohen, 1978). Thus an increase in $[Ca^{2+}]_{cyt.}$ in response to depolarization not only may stimulate the release of the neurotransmitter acetylcholine, as has been documented (for reviews see Katz, 1969; Rubin, 1970; Douglas, 1974), but may also potentiate neurotransmitter synthesis, through an increase in PDH_A content and the acetyl-CoA/CoA ratio. In this context, the changes in synaptosomal PDH_A content described in the present study are fairly modest (Table 1). In that the effect of Ca^{2+} on isolated mitochondria is substantially larger (Fig. 1), it may be that the synaptosomal preparations tend to be overloaded with Ca^{2+}, and this would warrant additional studies.

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