Pyruvate utilization by synaptosomes is independent of calcium

Risto A. Kauppinen and David G. Nicholls

Neurosciences Research Group, Department of Psychiatry, Ninewells Medical School, University of Dundee, Dundee DD1 9SY, Scotland

Received 27 February 1986

The significance of Ca^{2+} is assessed for the activation of pyruvate oxidation by intact nerve terminals (synaptosomes). Titration of glucose-depleted synaptosomes with pyruvate in the presence of either veratridine or uncoupler stimulates respiration in a Ca^{2+}-independent manner. Additionally, the ability of exogenous pyruvate to support the mitochondrial membrane potential in situ is independent of Ca^{2+}. It is concluded that Ca^{2+} does not regulate pyruvate oxidation in intact synaptosomes.

Pyruvate *Synaptosome* *Ca^{2+}*

1. INTRODUCTION

When isolated, uncoupled mitochondria from a variety of tissues, including brain, are incubated in the presence of ATP and a substrate other than pyruvate, the proportion of pyruvate dehydrogenase in the active, dephosphorylated form (PDH_A) can be modulated by varying the matrix free Ca^{2+} concentration ($[Ca^{2+}]_m$) in the range 0.1–1 μM (reviews [1,2]). This has led to the concept that a primary role of mitochondrial Ca^{2+} transport is to regulate $[Ca^{2+}]_m$ and hence to control the activity of the complex [1]. However, the importance of this regulatory mechanism in the brain is currently debatable (review [2]).

Isolated nerve terminals (synaptosomes) retain high respiratory control [3], ATP/ADP ratios [4] and plasma and mitochondrial membrane potentials ($\Delta\psi_p$ and $\Delta\psi_m$) [3]. They also extrude Ca^{2+} [5] and maintain sub-micromolar cytosolic free Ca^{2+} ($[Ca^{2+}]_c$) [6]. Pyruvate, either generated endogenously [7] or added [7,8], is the predominant substrate for the intra-synaptosomal mitochondria. Since synaptosomes can be rapidly and extensively depleted of Ca^{2+} by excess EGTA [5,9], this allows the effect of Ca^{2+} on pyruvate oxidation to be determined for mitochondria in a closely physiological environment. Additionally, we have shown that the value of $\Delta\psi_m$ can vary by up to 20 mV, dependent upon pyruvate availability and oxidation [7]. Thus, monitoring the effect of Ca^{2+} on pyruvate-supported synaptosomal potentials provides a second, independent approach.

Here, we test the hypothesis that Ca^{2+} facilitates the ability of intact synaptosomes to utilize exogenous pyruvate (i) to respire under conditions where the intra-synaptosomal mitochondria are close to 'State 3'; and (ii) to maintain $\Delta\psi_m$ under control, near 'State 4' conditions. Our results do not support the hypothesis, and we conclude that the Ca^{2+} regulation of PDH_A which can be seen with both the isolated enzymes [10] and isolated mitochondria [11] including brain [6,12,13] is not significant under the conditions pertaining in the intact terminal.

Abbreviations: $\Delta\psi_p$, plasma membrane potential; $\Delta\psi_m$, in situ mitochondrial membrane potential; $[Ca^{2+}]_c$, cytosolic free Ca^{2+} concentration; $[Ca^{2+}]_m$, mitochondrial free Ca^{2+} concentration; PDH_A, PDH_B, pyruvate dehydrogenase A and B

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/86/$3.50

2. MATERIALS AND METHODS

Cerebrocortical synaptosomes were prepared from Dunkin Hartley strain guinea-pigs as described [14], and where indicated were resuspended in glucose-free, Ca^{2+}-free incubation medium and recentrifuged to remove glucose contaminating the sucrose preparation medium [7].

Incubation media contained 122 mM NaCl, 3.1 mM KCl, 0.4 mM KH_2PO_4, 5 mM $NaHCO_3$, 20 mM Na-Tes, 1.2 mM $MgCl_2$, 16 μM bovine serum albumin (pH 7.4, 30°C). 1.3 mM $CaCl_2$ or 1.3 mM Na-EGTA was added at 5 min. Except where veratridine was added, the EGTA incubations contained additionally 1 μg/ml of tetrodotoxin (TTx) to inhibit enhanced Na^+ cycling across the plasma membrane [15]. Incubations were continued for 10 min before further additions to deplete residual glucose [7] and Ca^{2+} [5,9] as appropriate. Respiration was determined in Hansa-Tech oxygen electrode chambers (Kings Lynn, England). The accumulation of tetraphenylphosphonium (TPP) was used to monitor changes in $\Delta\psi_m$ or $\Delta\psi_p$ [16].

Ficoll was obtained from Pharmacia (Uppsala) and exhaustively dialysed against water. TPP bromide was from Fluorochem (Glossop, England). Veratridine, TTx and other reagents were from Sigma (Poole, England).

3. RESULTS

We have previously shown that synaptosomal glycolysis is absolutely dependent upon exogenous glucose [7]. In the absence of glucose, endogenous substrates are utilized [17] but their capacity to support uncontrolled respiration is very restricted, and may be restored by the additions either of glucose or pyruvate [7]. Although synaptosomal preparations contain some 'free' brain mitochondria these do not contribute significantly to the respiration or TPP uptake under the present conditions [7].

Freshly prepared synaptosomes contain less than 0.6 nmol Ca^{2+} per mg synaptosomal protein [5]. In media containing 1.3 mM Ca^{2+} this increases to 5 nmol Ca^{2+} per mg protein, of which at least 40% is intra-mitochondrial, corresponding to about 20 nmol Ca^{2+} per mg mitochondrial protein [5]. $[Ca^{2+}]_c$ stabilizes at about 0.2 μM [6]. EGTA depletes synaptosomal Ca^{2+} with a $t_{1/2}$ of 3 min [5], thus 10–30 min exposure of fresh synaptosomes to EGTA will cause an extreme depletion of Ca^{2+}.

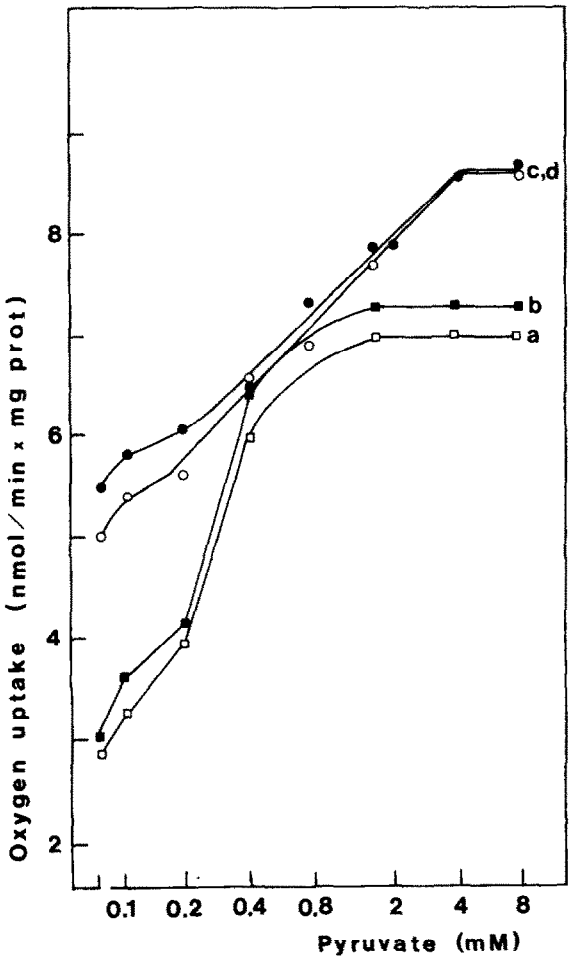

Fig.1. The effect of exogenous pyruvate on synaptosomal respiration in the presence of veratridine or FCCP. Influence of Ca^{2+} and EGTA. Substrate-depleted synaptosomes (2 mg protein/ml incubation) were incubated in the absence of glucose, with the addition at 5 min of 1.3 mM $CaCl_2$ (○, □), 1.3 mM Na-EGTA (■) or 1.3 mM EGTA plus 1 μg/ml TTx (●). At 15 min 1 μM FCCP (●, ○) or 100 μM veratridine (■, □) were added followed by the indicated concentration of Na pyruvate.

Veratridine depolarizes the plasma membrane [3,18], increases ATP turnover by inducing dissipative Na^+ cycling [3], and indirectly activates Ca^{2+} entry [5], raising $[Ca^{2+}]_c$ to 0.8 μM [6]. All

effects except the increase in $[Ca^{2+}]_c$ also occur in the presence of EGTA. It is thus possible to examine separately the role of Ca^{2+} and of increased energy demand upon the ability of synaptosomes to utilize pyruvate. Fig.1 shows the effect of added pyruvate upon respiration in the presence of 50 nmol/mg protein veratridine. With 1.3 mM Ca^{2+} present (trace a) respiration increases maximally by 147% in the presence of 2 mM pyruvate. The titration of pyruvate with Ca^{2+}-depleted synaptosomes (trace b) is identical, 142% stimulation again being attained by 2 mM pyruvate. The facility with which exogenous pyruvate supports respiration is thus completely independent of Ca^{2+}.

Fig.1 also shows pyruvate titrations in the presence of the minimum concentration of FCCP needed to achieve uncontrolled respiration. Again there is no difference between Ca^{2+}-loaded and -depleted terminals (traces c,d).

In the presence of Ca^{2+}, the indicated $\Delta\psi_m$, determined by TPP accumulation after correcting for $\Delta\psi_p$ [3], can vary by up to 20 mV depending upon the supply of pyruvate for the intrasynaptosomal mitochondria [7]. Thus, fig.2 shows the 50% decrease in steady-state TPP accumula-

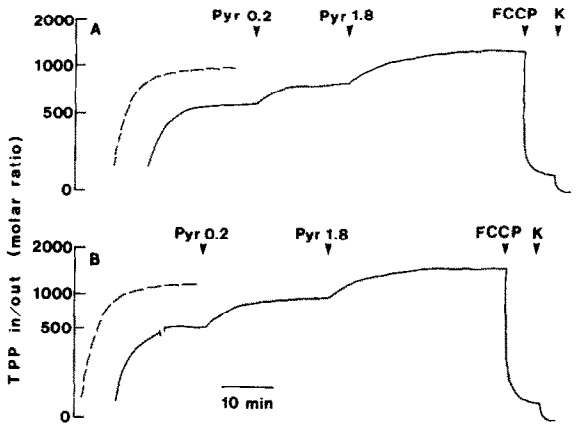

Fig.2. The effect of exogenous pyruvate on synaptosomal TPP accumulation. Influence of Ca^{2+} and EGTA. Substrate-depleted synaptosomes (2 mg protein/ml incubation) were incubated in the presence (– – –) or absence (——) of 10 mM glucose. At 5 min 1 μM TPP together with 1.3 mM $CaCl_2$ (A) or 1.3 mM Na-EGTA plus 1 μg/ml TTx (B) were added. Further additions were made where indicated of 0.2 mM pyruvate (Pyr 0.2), 1.8 mM pyruvate (Pyr 1.8), 4 μM FCCP and 30 mM KCl (K).

Table 1

Synaptosomal TPP accumulation: influence of glucose, pyruvate, Ca^{2+} and EGTA

Additions	TPP_i^+%	
	+ 1.3 mM Ca^{2+}	+ 1.3 mM EGTA + TTx
10 mM glucose	1054 ± 25 (4)	1217 ± 67 (4)
Glucose absent	541 ± 31 (5)	494 ± 39 (4)
+ 0.2 mM pyruvate	938 ± 84 (4)	926 ± 66 (3)
+ 2 mM pyruvate	1715 ± 105 (5)	1636 ± 69 (4)

Synaptosomes were incubated exactly as described in fig.2. Values are means ± SE for the number of independent experiments given in parentheses

tion of glucose-deprived synaptosomes relative to controls. Addition of 0.2 mM pyruvate raises the TPP accumulation, and the hyperpolarization was maximal with 2 mM added pyruvate. When pyruvate transport or oxidation is inhibited (by α-cyano-4-hydroxycinnamate and arsenite, respectively) no effect on TPP accumulation is seen [7]. 'Free' brain mitochondria failed to accumulate significant TPP in either the presence or absence of Ca^{2+} [7]. This, together with the characteristic slow equilibration of TPP (fig.2), indicates that the increased TPP uptake is due to intact synaptosomes, rather than contaminating free brain mitochondria.

The response to pyruvate in the absence of Ca^{2+} (fig.2B) was virtually identical to that of the Ca^{2+}-replete terminals. Thus even in the absence of an increased energy demand synaptosomes utilize pyruvate in a Ca^{2+}-independent manner. Table 1 summarizes the collected results of a number of experiments. We have previously shown that $\Delta\psi_p$ does not change significantly on addition of pyruvate [7], thus the increased TPP accumulation can be ascribed to an increase in $\Delta\psi_m$ of up to 30 mV on addition of 2 mM pyruvate.

4. DISCUSSION

The Ca^{2+} dependency of the interconversion of PDH_A and PDH_B in isolated brain mitochondria has been intensively investigated [6,12,13]. The enzyme is largely activated [12,13] although Hansford and Castro [6] could modulate the

PDH_A content from 25 to 75% of total activity by varying Ca^{2+} from 0.1 to 1 μM in the absence of pyruvate and the presence of 1 mM ATP. In the more physiological environment of the intact synaptosome, the proportion of PDH_A is also high, but is remarkably independent of the Ca^{2+} concentration in the synaptosomal incubation [6,8]. Schaffer and Olson [8] observed a decrease in PDH_A from 91 to 83% with EGTA, while Hansford and Castro [6] found that PDH_A remained constant at 68% even after 5 min exposure of synaptosomes to excess EGTA.

Hansford and Castro [6] showed that veratridine caused a significant increase in the proportion of PDH_A in rat synaptosomes. This did not occur if ouabain was additionally present, indicating that the activation was associated with increased ATP turnover rather than depolarization-induced Ca^{2+} entry. The authors found in contrast that veratridine increased PDH_A in the presence of oligomycin; however, this combination of enhanced energy demand and inhibited oxidative phosphorylation leads to a massive fall in ATP [3]. 5 min incubation with EGTA had no effect whatsoever on basal levels of PDH_A [6], indicating that the high activation cannot be ascribed to the relatively high Ca^{2+} content of synaptosomes. Hansford and Castro [6] did observe that EGTA lowered the proportion of PDH_A after veratridine from 81 to 73%, however our results show that this slight change does not detectably alter the ability of the synaptosomes to utilize pyruvate.

In synaptosomes metabolizing glucose, pyruvate dehydrogenase is exposed to physiological concentrations of its substrate pyruvate, a potent inhibitor of PDH kinase [19,20] which strongly protects PDH_A against inactivation in isolated brain mitochondria [13]. Schaffer and Olson [8] reported that exogenous pyruvate produced little or no activation of pyruvate dehydrogenase in synaptosomes metabolizing glucose. However, these authors failed to take account of the pyruvate generated by glycolysis. Their results, and those of Hansford and Castro [6] therefore suggest that the physiological concentration of pyruvate within the terminals is sufficient to maintain pyruvate dehydrogenase in a highly activated state, masking any effect of Ca^{2+} seen in isolated mitochondria in unphysiological absence of its substrate.

Our results demonstrate that in the intact terminal the ability of exogenous pyruvate to support uncontrolled respiration and to maintain the membrane potential of intra-synaptosomal mitochondria shows no dependency upon Ca^{2+}. Our preparations of synaptosomes maintain overall ATP/ADP ratios of 5:1 [7], $\Delta\psi_p$ and $\Delta\psi_m$ of 65 and 150 mV, respectively [15], respiratory control ratios of 5 [7] and a 10-fold uncoupler stimulation of glycolysis [7]. Evidently, the preparation shows a very high degree of bioenergetic integrity. Therefore the regulatory role proposed for $[Ca^{2+}]_m$, which can be seen with isolated mitochondria when PDH_A is low due to the absence of pyruvate and the addition of ATP to the incubation to elevate matrix ATP/ADP ratios [6,11–13], does not operate in the more closely physiological preparation of the intact terminal.

ACKNOWLEDGEMENTS

The expert technical assistance of Mr C. Adam is gratefully acknowledged. The work was supported by a grant from the UK Medical Research Council. R.A.K. was on leave of absence from the Dept of Clinical Neurophysiology, University Hospital of Kuopio, Finland, and was supported by an EMBO Fellowship.

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