Ca^{2+} Ions and the Output of Acetylcoenzyme A from Brain Mitochondria

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Abstract. Purified rat brain mitochondria were incubated in the presence of pyruvate or (1–^{14}C)pyruvate and the output of the pyruvate-generated acetylcoenzyme A (acetyl-CoA) from the mitochondria into the medium was measured and compared with the rate of (1–^{14}C)pyruvate decarboxylation. When $CaCl_2$ (1 mmol-/l) was added to the incubation medium, the output of acetyl-CoA from the mitochondria was increased 3.8—6 times; at the same time, the rate of pyruvate decarboxylation (and of the intramitochondrial acetyl-CoA production) increased only 1.3 times. After repeated freezing and thawing, the output of acetyl-CoA into the medium was higher, but the stimulatory effect of Ca^{2+} ions was considerably diminished. It is concluded that Ca^{2+} ions increase the output of acetyl-CoA from the mitochondria by altering the permeability of mitochondrial membranes rather than by increasing the activity of the pyruvate dehydrogenase complex. Possible physiological role of the observed effect of Ca^{2+} ions in the control of acetylcholine synthesis in the nerve terminals is discussed.

Key words: Acetylcoenzyme A — Mitochondria — Brain — Acetylcholine — Calcium

Introduction

The main sources of acetylcoenzyme A (acetyl-CoA) in mammalian cells are pyruvate and fatty acids. The enzymes responsible for the synthesis of acetyl-CoA from these substrates, i.e., the pyruvate dehydrogenase complex and the enzymes of the fatty acid β-oxidation cycle, are localized in the mitochondrial matrix; as a result, most acetyl-CoA is being produced in this compartment of the cell, surrounded by the rather impermeable inner mitochondrial membrane. The way in which the acetyl groups of the intramitochondrial acetyl-CoA become available for chemical reactions occurring outside the mitochondria has not been fully clarified. It is generally accepted that, in non-nervous tissues, the acetyl-CoA produced in the inner mitochondrial space is transformed to some other compound (citrate, acetylcarnitine, acetate) which passes through the inner mitochondrial membrane and is converted back to acetyl-CoA either in the outer mitochondrial space or in the cytosol (Greville 1969).

In the brain, the topic of acetyl group transfer from the mitochondria has been studied mainly in association with the synthesis of acetylcholine (ACh) (for reviews see Quastel 1978; Tuček 1978, 1983; Jope 1979). There is no doubt that most acetyl groups in brain ACh originate from glucose (Browning and Schulman 1968; Tuček and Cheng 1970, 1974) and it seems likely that the supply of the glucose-derived acetyl groups from the mitochondria for the extramitochondrial synthesis of ACh proceeds in several parallel ways: via citrate (Sollenberg and Sörbo 1970; Sterling and O'Neil 1978; Szutowicz et al. 1981; Doležal and Tuček 1981; Tuček et al. 1981; Říčný and Tuček 1982), acetylcarnitine (Sterri and Fonnum 1980; Doležal and Tuček 1981) and other intermediates, and possibly also via a direct passage of acetyl-CoA across the inner mitochondrial membrane (Tuček 1967, 1970, 1978; Benjamin and Quastel 1981).

Although it is generally assumed that the inner mitochondrial membrane is very little (if at all) permeable to acetyl-CoA (Greville 1969), it has been noted by Tuček (1967) that the output of acetyl-CoA from brain mitochondria incubated *in vitro* can be greatly increased by Ca^{2+} ions. Subsequently, Polak et al. (1978) reported that Ca^{2+} promoted the release of a precursor of acetyl-CoA from the mitochondria, and the supposed precursor was later identified as acetyl-CoA itself (Polak, personal communication). Ca^{2+}-mediated stimulation of acetyl-CoA output from the mitochondria has been confirmed by Benjamin and Quastel (1981).

Ca^{2+} ions could enhance the release of acetyl-CoA from the mitochondria in one of two ways: (1) by increasing the permeability of mitochondrial membranes, or (2) by raising the activity of the pyruvate dehydrogenase complex, mainly through activation of pyruvate dehydrogenase phosphatase (Denton et al. 1975; Hansford 1981; McCormack et al. 1982). In the present experiments, an attempt has been made to distinguish between these two possibilities by comparing the effect of Ca^{2+} ions on the output of acetyl-CoA from brain mitochondria incubated in the presence of pyruvate with their effect on the rate of the reaction catalysed by pyruvate dehydrogenase, measured by the rate of decarboxylation of (1–^{14}C)pyruvate.

Methods

Material. Ficoll 400 was from Pharmacia (Uppsala, Sweden), HEPES (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid) from Serva (Heidelberg, F.R.G.), thiamine pyrophosphate, EDTA, EGTA and Tris from Sigma (St. Louis, U.S.A.), CoA from Boehringer (Mannheim, F.R.G.) and dichloracetate from Fluka (Buchs, Switzerland). (1–^{14}C)pyruvate was from Interkommerz (Berlin G.D.R.); it was purified by thin layer chromatography on Polygram 400 (Macherey and Nagel, Düren, F.R.G.) cellulose plates with ethylether-formic acid-water (7:2:1) as solvent.

Choline acetyltransferase (ChAT, EC 2.3.1.6) was partly purified from bovine cadate nuclei according to Fonnum (1976) up to the stage of CM-Sephadex chromatography. One unit of the enzyme is defined as the amount of ChAT catalysing the formation of 1 μmol of ACh per min at 38°C.

Isolation of mitochondria. Male Wistar rats of 170—220 g body mass were killed by cervical dislocation and decapitation and mitochondria were prepared from their forebrains according to the Method I of Lai and Clark (1979). The method involves homogenization in 0.25 mol/l sucrose containing 0.5 mmol/l EDTA and 10 mmol/l Tris-HCl buffer (pH 7.4), sedimentation of a crude mitochondrial pellet, its resuspending in a solution containing 3% (w/v) Ficoll 400, 120 mmol/l mannitol, 30 mmol/l sucrose, 0.025 mmol/l EDTA, and 5 mmol/l Tris-HCl (pH 7.4), and sedimentation of the mitochondria by centrifugation through a more concentrated solution containing 6% (w/v) Ficoll 400, 240 mmol/l mannitol, 60 mmol/l sucrose, 0.05 mmol/l EDTA, and 10 mmol/l Tris (pH 7.4). The pellet of purified mitochondria was resuspended in 120 mmol/l KCl with 30 mmol/l HEPES buffer (pH 7.4).

Disruption of mitochondrial membranes was achieved by repeated (3 times) freezing of the mitochondrial suspension in a mixture of dry CO_2 and ethanol followed by thawing and, in the end, by 3 times 15 s homogenization in an all-glass Potter-Elvehjem homogenizer.

Incubation of the mitochondria and measurement of the acetyl-CoA output. The acetyl-CoA released from the mitochondria into the medium was measured as ACh, to which it had been converted during incubations of the mitochondria in the presence of an excess of choline and ChAT. Samples of 100 µl of mitochondrial suspensions were incubated in a final volume of 600 µl of incubation medium of the following final composition: 10 mmol/l HEPES (pH 7.4), 5 mmol/l potassium phosphate (pH 7.4), 120 mmol/l KCl, 1.25 mmol/l $MgCl_2$, 10 mmol/l choline chloride, 0.05 mmol/l eserine sulphate, 5 mmol/l sodium pyruvate, 1 mmol/l sodium malate, 1 mmol/l NAD^+, 1.2 mmol/l thiamine pyrophosphate, 0.1 mmol/l CoA, 50 milliunits of ChAT per incubation vial, and additions as indicated. The incubation medium was saturated with O_2 and the incubations were performed under an atmosphere of O_2; they were at 38°C and lasted 30 min. At the end of incubation, the medium was separated from the tissue by centrifugation (45 s, 10 000 g). The supernatant solution was kept frozen at −20°C and its content of ACh was determined by bioassay on guinea-pig ileum within 3 days.

Measurement of the rate of the decarboxylation of pyruvate. Mitochondria were incubated under the same conditions as described for measurements of the acetyl-CoA output, except that ChAT was not included in the incubation medium, and that (1-^{14}C)pyruvate (specific radioactivity: 7 MBq · mol^{-1}) was used instead of non-radioactive pyruvate. The incubation vials contained a small tube with inserted filtration paper. At the end of incubation, 0.4 ml of hyamine hydroxide were injected through the plastic cover of the incubation vial into the tube with the filtration paper, and 0.2 ml of 15% (w/v) of perchloric acid were injected into the incubation mixture. The vials were then kept overnight at room temperature and on the next day the filtration papers containing the absorbed $^{14}CO_2$ were transferred to a scintillation vial with Bray's solution and their radioactivity was counted.

Protein concentration was determined according to Peterson (1977) using human serum albumin as standard.

Results

In the experiment summarized in Fig. 1, mitochondria incubated in the control medium (with no Ca^{2+} and no EGTA added) released (mean ± S.E.) 7.20 ± 0.24 nmol acetyl-CoA . h^{-1} · (mg protein)$^{-1}$. The amount of acetyl-CoA released was increased by 31%, 190% and 283% when Ca^{2+} (0.1, 0.5, and 1.0 mmol/l, respectively) was added to the incubation medium.

The effects of Ca^{2+} on the release of acetyl-CoA and on the decarboxylation of

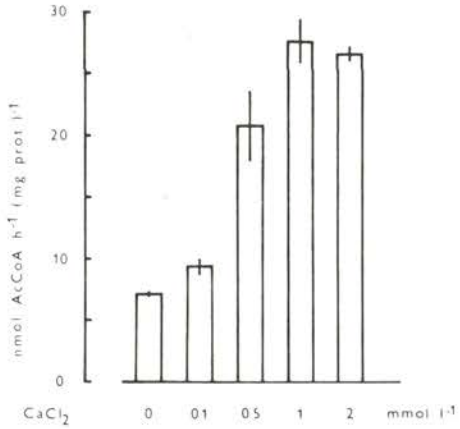

Fig. 1. Acetyl-CoA output (nmol.h^{-1}./(mg protein)$^{-1}$) from rat brain mitochondria incubated in the medium described under Methods and containing different concentrations of $CaCl_2$. Columns are means (± S. E.) of 5 observations.

pyruvate were compared in experiments in which Ca^{2+}-EGTA buffers were used (Figs. 2A and 2B). The concentration of Ca^{2+} ions actually present in the incubation medium after the addition of varying amounts of $CaCl_2$ and EGTA was estimated according to Portzehl et al. (1964) from their equation (1)

$$K'_{MeL_{total}} = [MeL_{total}] / [Me^{2+}][L_{total}]$$

where $[MeL_{total}]$ corresponds to the total concentration of Ca-EGTA complexes and $[L_{total}]$ to the concentration of not complexed EGTA; the combined apparent association constant $K'_{MeL_{total}}$ had been calculated to correspond (at pH 7.4) to 2.984×10^7 l/mol. The estimates of free Ca^{2+} concentrations after the additions of different amounts of $CaCl_2$ and EGTA are indicated in the legend to Fig. 2A; the estimate that in the absence of any addition of $CaCl_2$ and EGTA the concentration of Ca^{2+} in the medium is of the order of 10^{-9} mol/l (Ca^{2+} originating from impurities in water and reagents) has been taken from Lüttgau and Spiecker (1979).

In the experiment summarized in Fig. 2A, the amount of acetyl-CoA released into the medium (nmol.h^{-1}.(mg protein)$^{-1}$) was 7.07 ± 1.52 in the absence of added $CaCl_2$ or EGTA and 7.33 ± 2.49 in the presence of 2 mmol/l EGTA; it was 6 or 7-fold higher after the addition of 1 mmol/l $CaCl_2$ or of 2 mmol/l EGTA + 3 mmol/l $CaCl_2$, respectively. In a parallel experiment (performed, however, on a different batch of mitochondria), the rate of (1–^{14}C)pyruvate decarboxylation (Fig. 2B) was much less affected by variations of Ca^{2+} concentration than the output of acetyl-CoA. The amount of CO_2 formed by the mitochon-

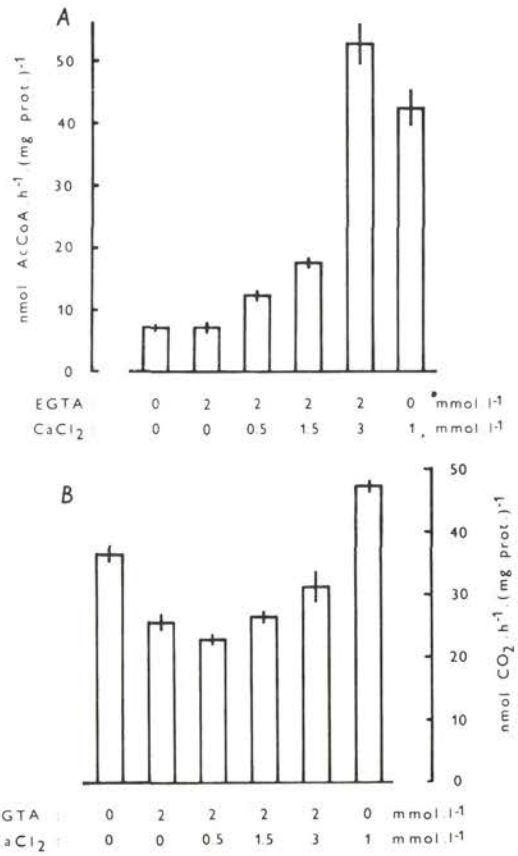

Fig. 2A. Acetyl-CoA output (nmol, h^{-1}./(mg protein)$^{-1}$) from rat brain mitochondria incubated in the medium described under Methods and containing different concentrations of $CaCl_2$ and EGTA. The concentration of Ca^{2+} ions estimated as described under Results was of the order, from left to right: 10^{-9}, 10^{-14}, 10^{-8}, 10^{-7}, 10^{-3}, 10^{-3} mol/l. Data are means (±S.E.) of 5—12 experiments.

Fig. 2B. The rate of pyruvate decarboxylation (measured as the formation of $^{14}CO_2$ from (1–^{14}C) pyruvate present in the incubation medium) by rat brain mitochondria incubated as in Fig. 2A. Data are means (±S.E.) from 3—10 incubations.

dria was 36.34 ± 1.25 nmol.h^{-1}.(mg protein)$^{-1}$ in the absence of added $CaCl_2$ and EGTA and 25.80 ± 1.37 after the addition of 2 mmol/l EGTA. In the presence of 1 mmol/l $CaCl_2$, the rate of CO_2 formation was 29% higher than in the corresponding control (without $CaCl_2$ and EGTA), and in the presence of 2 mmol/l EGTA + 3 mmol/l $CaCl_2$ the rate of decarboxylation was 20% higher than in the presence of 2 mmol/l EGTA alone.

Repeated freezing and thawing (Fig. 3) increased the rate of acetyl-CoA release from the mitochondria by 78%, but the effect of 1 mmol/l $CaCl_2$ on the rate of acetyl-CoA output from the disrupted mitochondria was much smaller

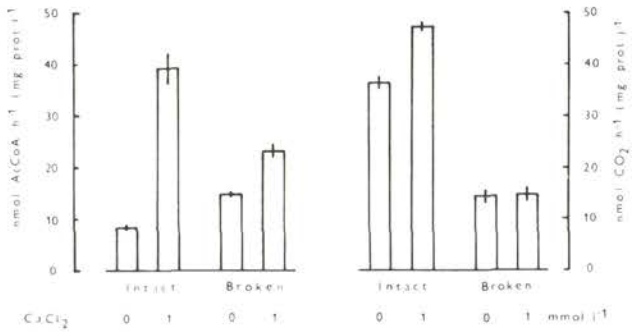

Fig. 3. The effect of $CaCl_2$ (1 mmol/l) added to the incubation medium on the release of acetyl-CoA (left) and on the pyruvate decarboxylation (right) by intact brain mitochondria ("Intact") and by mitochondria damaged by 3-fold freezing and thawing ("Broken"). Data are means (±S. E.) of 4—5 experiments.

(57% increase) than from controls (370% increase). Freezing and thawing considerably reduced the rate of CO_2 formation, both in the absence and in the presence of Ca^{2+}.

An attempt has been made to increase the output of acetyl-CoA and the rate of CO_2 formation by adding dichloracetate (1 mmol/l) into the incubation medium (Fig. 4). Dichloracetate is known to inhibit the activity of pyruvate dehydrogenase kinase and thus to increase the activity of pyruvate dehydrogenase (Whitehouse et al. 1974). Under our experimental conditions, no stimulation of acetyl-CoA output and of CO_2 formation from pyruvate was achieved by dichloracetate, either in the absence or in the presence of added $CaCl_2$.

Discussion

Ca^{2+} ions are known to activate the enzyme pyruvate dehydrogenase phosphatase. This way, they assist the dephosphorylation and thereby the activation of pyruvate dehydrogenase (Denton et al. 1975; Hansford 1981; McCormack et al. 1982). In view of this action of Ca^{2+} ions, one could assume that the increase in the release of acetyl-CoA from the mitochondria, as observed in the presence of Ca^{2+} in the medium (Tuček 1967; Polak et al. 1978; Benjamin and Quastel 1981) is a consequence of an increased formation of acetyl-CoA in the mitochondria. However, it may be seen from the present results that the effects of Ca^{2+} on the output of acetyl-CoA are not accompanied by similar effects on the rate of the formation of CO_2 from pyruvate, which may be taken as indicator of the rate of the formation of acetyl-CoA. Therefore, it appears likely that Ca^{2+} acts by altering the release of acetyl-CoA from the mitochondria (by increasing the permeability of the

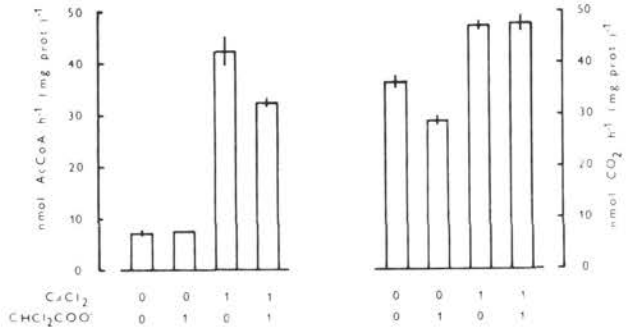

Fig. 4. The output of acetyl-CoA from the mitochondria into the medium (left) and pyruvate decarboxylation (right) during incubations of mitochondria in the absence or presence of $CaCl_2$ (1 mol/l) and of dichloroacetate (1 mol/l) in the incubation medium. Data are means (±S. E.) of 4—5 experiments.

inner mitochondrial membrane) rather than by changing the production of acetyl-CoA in the mitochondria.

This conclusion is supported by several observations. In earlier experiments, Ca^{2+} lost its activity after treatment of the mitochondria with ether (Tuček 1967) or with Triton X–100 (Benjamin and Quastel 1981); both of these agents apparently removed the permeability barrier preventing acetyl-CoA from leaving the mitochondria, as indicated by the increased output of acetyl-CoA into the medium. In the present experiments, repeated freezing and thawing also increased the output of acetyl-CoA into the medium and at the same time it diminished the effect of Ca^{2+} on the output. The fact that the effect of Ca^{2+} on the output of acetyl-CoA was not fully abolished after 3-fold repeated freezing and thawing seems to be best explained on assumption that the procedure used for mitochondrial disruption was not sufficiently effective and that some of the mitochondria survived with undamaged or little damaged membranes.

The near-inability of Ca^{2+} to increase the rate of pyruvate decarboxylation as observed in the present work may be due to the fact that, under the conditions of our experiments, most or all pyruvate dehydrogenase was in its active (dephosphorylated) form. The activity of pyruvate dehydrogenase changes in inverse relation with the ratios of acetyl-CoA/CoA and NADH/NAD^+ (Batenburg and Olson 1975; Petit et al. 1975; Hansford 1976; Kerbey et al. 1976, 1977; Roche and Cate 1976). Since the incubation medium contained high concentrations of CoA and NAD^+, and these coenzymes could very probably enter the mitochondria after their membranes have became more permeable in the presence of Ca^{2+}, the low acetyl-CoA/CoA and NADH/NAD^+ ratios were likely to support the active (non-phosphorylated) state of pyruvate dehydrogenase. The presence of a high

concentration of pyruvate in the medium probably acted in the same direction (Taylor et al. 1975; Kerbey et al. 1976; McCormack et al. 1982). The observation that the rate of pyruvate decarboxylation was unaltered by dichloracetate, supports the view that most of pyruvate dehydrogenase was in a non-phosphorylated state during the present incubations.

Different batches of mitochondria prepared by the procedure of Lai and Clark (1979) varied in some of their properties (rate of pyruvate decarboxylation, extent of Ca^{2+} effect on acetyl-CoA output) for reasons we cannot define. For technical reasons, measurements of CO_2 formation had to be performed on different batches of mitochondria than the measurements of the output of acetyl-CoA. The results of the two parallel measurements were therefore not always in perfect quantitative agreement. In spite of this qualification, it is apparent that the release of acetyl-CoA from mitochondria incubated without the addition of $CaCl_2$ represented less than one fifth of the total acetyl-CoA formed inside the mitochondria by the decarboxylation of pyruvate. It is likely that most of the acetyl-CoA formed in the mitochondria was utilized in the Krebs cycle. In the presence of 1 mmol/l Ca^{2+}, the amount of acetyl-CoA released into the medium was roughly equal with that formed by the decarboxylation of pyruvate, suggesting that the Krebs cycle became inoperative, probably as a consequence of the losses of coenzymes and intermediates into the medium.

Physiological significance of the observed Ca^{2+}-induced increase in the output of acetyl-CoA from the mitochondria remains uncertain. The concentrations of Ca^{2+} used in the present and previous (Tuček 1967; Polak et al. 1978; Benjamin and Quastel 1981) experiments appear too high to be physiologically relevant, since the concentrations of Ca^{2+} in the living cells are of the order of 10^{-7} mol/l (review Baker 1976). The activity of presynaptic nerve terminals is associated with an influx of Ca^{2+} ions into the terminals and with an increase of their concentration in the terminals (Llinás et al. 1972; Llinás 1977; Dunant et al. 1980; review Stinnnakre 1977), but the level to which the concentration of Ca^{2+} rises during intense synaptic activity remains to be established (Blaustein et al. 1978). It is noteworthy that the influx of Na^+ ions into the nerve terminals might be capable of promoting the release of Ca^{2+} from the intraterminal mitochondria (Carafoli 1979), thus opening a second pathway leading to an increased concentration of Ca^{2+} in the nerve-ending axoplasm. The observed effects of Ca^{2+} on mitochondrial permeability to acetyl-CoA suggest an interesting possibility how the increased concentration of Ca^{2+} during synaptic activity might automatically improve the supply of one of the substrates for the synthesis of ACh in the nerve terminals. To evaluate this possibility, it will be necessary to know more about the concentration of Ca^{2+} actually occurring in the presynaptic axoplasm during activity, and about the sensitivity of mitochondrial membranes to Ca^{2+} ions when the mitochondria are in their natural intracellular milieu, rather than in an artificial incubation medium.

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