Biochem. J. (1989) 262, 377–380 (Printed in Great Britain)

Evidence for the regulatory function of synaptoplasmic acetyl-CoA in acetylcholine synthesis in nerve endings

Hanna BIELARCZYK and Andrzej SZUTOWICZ
Department of Clinical Biochemistry, Medical Academy, ul. Dębinki 7, 80–211 Gdańsk, Poland

Isolated synaptosomes maintained a relatively stable level of acetyl-CoA during their incubation in the presence of 30 mM-KCl. Addition of Ca^{2+} resulted in inhibition of pyruvate oxidation and slight activation of acetylcholine synthesis. The cation decreased acetyl-CoA in intrasynaptosomal mitochondria, but did not alter its content in synaptoplasm. Verapamil did not affect pyruvate oxidation, but decreased acetyl-CoA in synaptoplasm and inhibited acetylcholine synthesis in synaptosomes. It indicates that Ca^{2+} might regulate acetylcholine synthesis through changes in the direct transfer of acetyl-CoA from mitochondria to synaptoplasm.

INTRODUCTION

It is known that acetylcholine (ACh) content in brain is maintained at a stable level irrespective of activity of cholinergic neurons [1,2]. To explain it, one assumes that activity of choline acetyltransferase in cytoplasm of cholinergic endings is high enough to supplement quickly losses of ACh resulting from its release [2]. On the other hand the rate of ACh synthesis has to depend on concentrations of its precursors, since they are present in brain at levels much below those saturating this enzyme [3–5]. Concentrations of acetyl-CoA and choline in synaptoplasm are thought to be determined by their supply from the intramitochondrial and the extracellular compartment respectively [2,6]. Oxidative decarboxylation of pyruvate in intrasynaptosomal mitochondria is a principal source of acetyl-CoA, which is by intermediary pathways transferred to the site of ACh synthesis [1,2,6]. However, no information on the level of acetyl-CoA in synaptoplasm is available. Therefore we investigated relationships between acetyl-CoA levels in subsynaptosomal compartments and the rate of ACh synthesis in synaptosomes utilizing pyruvate. Preliminary results of this work have been presented [7].

MATERIALS AND METHODS

Ficoll was obtained from Pharmacia (Uppsala, Sweden) and purified by dialysis and freeze-drying. Sucrose for density-gradient centrifugation and $CaCl_2$ were from Merck (Darmstadt, Germany). The lactone of (−)-hydroxycitric acid was given by Dr. Y. S. Lewis (Mysore, India), and verapamil was provided by Knoll A.G. (Ludwigshafen, Germany). All other biochemicals were from Sigma Chem. Co. (St. Louis, MO, U.S.A.), and inorganic reagents were from P.O.Ch. (Gliwice, Poland). [2-^{14}C]Pyruvate was from O.P.D.I. (Swierk, Poland) and [1-^{14}C]acetylcholine from The Radiochemical Centre (Amersham, Bucks., U.K.).

Preparation of synaptosomes and mitochondria

White Wistar male rats weighing 200–250 g were killed by decapitation, and their brains were immediately placed in ice-cold 320 mM-sucrose in 5 mM-Tris/HCl buffer (pH 7.4)/0.1 mM-EDTA. Forebrains were homogenized in the same medium in a Teflon/glass homogenizer (clearance 0.25 mm) for 1 min at 600 rev./min. Synaptosomal and mitochondrial fractions were isolated by differential centrifugation and flotation in Ficoll density gradient [8].

Incubations and metabolite determinations

Synaptosomes (3.5 mg of protein) were incubated in a final volume of 2 ml of medium containing: 15 mM-Na-Hepes, 1.5 mM-sodium phosphate buffer (final pH of the medium 7.54), 85 mM-NaCl, 30 mM-KCl, 2.5 mM-sodium pyruvate, 2.5 mM-sodium L-malate, 1.0 mM-choline chloride, 0.2 mM-eserine salicylate and 32 mM-sucrose. Incubations were started by addition of 0.2 ml of synaptosomal suspension and carried out for 30 min at 37 °C with shaking at 100 cycles/min. At the end of incubation, 1.5 ml of the suspension was centrifuged for 3 min at 10000 *g*, and the resulting supernatant and pellet were used for pyruvate and acetyl-CoA (CoA) determinations respectively [8,9].

The remaining 0.5 ml of the suspension was mixed with an equal volume of solution containing 125 mM-KCl, 20 mM-Na-Hepes buffer, pH 7.4, 3 mM-EDTA and 0.7 mg of digitonin. After 30 s the particulate fraction was separated from synaptoplasm by centrifugation for 30 s through 0.5 ml of a mixture of silicone oils (AR-20 and Versilube F-50; 1:1, v/v) into 0.1 ml of 400 mM-$HClO_4$ and used for determination of acetyl-CoA [9]. To study [^{14}C]ACh synthesis parallel incubations were run, in which [2-^{14}C]pyruvate (47 μCi/sample) was used and formation of [^{14}C]ACh was measured as described elsewhere [10].

Enzyme assays

Activities of lactate dehydrogenase and glutamate dehydrogenase were determined by spectrophotometric methods [11,12].

Protein assay

Protein was determined by the method of Bradford [13].

Abbreviation used: ACh, acetylcholine.

Table 1. Effect of digitonin on rat brain synaptosomes and mitochondria

For enzyme assays digitonin-treated synaptosomes or mitochondria were centrifuged through the layer of silicone oil into 320 mM-sucrose, and for acetyl-CoA/CoA measurements into 400 mM-$HClO_4$. Results are means ± S.E.M. for four experiments; n.a., not applicable.

Parameter/treatment	Synaptosomes	Mitochondria
Protein (mg)		
No digitonin	1.00 ± 0.03	1.00 ± 0.04
Digitonin-soluble fraction	0.34 ± 0.02	0.05 ± 0.03
Particulate fraction	0.55 ± 0.03	0.93 ± 0.05
Lactate dehydrogenase (nmol/min per mg of protein)		
No digitonin	864 ± 39	n.a.
Digitonin-soluble fraction	831 ± 67	n.a.
Particulate fraction	87 ± 11	n.a.
Glutamate dehydrogenase (nmol/min per mg of protein)		
No digitonin	143 ± 8	652 ± 16
Digitonin-soluble fraction	13 ± 2	25 ± 5
Particulate fraction	150 ± 9	640 ± 20
Acetyl-CoA (pmol/mg of protein)		
No digitonin	32.4 ± 2.9	50.6 ± 5.0
Digitonin-particulate fraction	9.1 ± 0.7	46.1 ± 4.6
CoA (pmol/mg of protein)		
No digitonin	497 ± 66	616 ± 15
Digitonin-particulate fraction	171 ± 11	534 ± 16

Table 2. Pyruvate oxidation and [^{14}C]ACh synthesis in rat brain synaptosomes incubated with different effectors of acetyl-moiety metabolism

Synaptosomes isolated on a Ficoll/mannitol density gradient [8] were incubated and handled as described in the Materials and methods section. The rate of pyruvate oxidation was calculated by subtraction of the rate of lactate formation from overall rate of pyruvate consumption. Results are means ± S.E.M. for seven experiments done in duplicate: * $P < 0.05$ with respect to control, † $P < 0.05$ with respect to appropriate control without verapamil, by paired t test [25].

Additions	Pyruvate oxidation (nmol/min per mg of protein)	[^{14}C]ACh synthesis (pmol/min per mg of protein)
None	6.6 ± 0.5	9.3 ± 1.3
3-Bromopyruvate (0.25 mM)	2.2 ± 0.2*	3.1 ± 1.3*
Ca^{2+} (0.1 mM)	3.8 ± 0.5*	10.3 ± 1.3
Ca^{2+} (1.0 mM)	4.0 ± 0.3*	10.7 ± 1.0
Verapamil (0.5 mM)	6.4 ± 0.8	6.2 ± 1.0*
Ca^{2+} (0.1 mM) + verapamil (0.5 mM)	4.9 ± 1.1*	6.8 ± 1.5*†

RESULTS

Effect of digitonin on subcellular fractions

Incubation of synaptosomes with digitonin at a concentration of 0.35 mg/ml released of 38% of protein, 70% of acetyl-CoA and 91% of lactate dehydrogenase activity to the soluble fraction. Simultaneously 62% of protein, 30% of acetyl-CoA and 92% of glutamate dehydrogenase activity remained in the particulate fraction (Table 1). On the other hand, whole brain mitochondria incubated with digitonin for 60 s lost about 9 and 13% of their acetyl-CoA and CoA respectively (Table 1).

Acetyl-CoA content and ACh synthesis in synaptosomes

In freshly prepared synaptosomes levels of acetyl-CoA and CoA were 32.4 and 497 pmol/mg of protein respectively. Suspension of synaptosomes in the medium, followed by their instantaneous centrifugation, did not affect the nucleotide content. Incubation of synaptosomes at 37 °C in media containing no exogenous Ca^{2+} caused gradual loss of about 20% of their acetyl-CoA and CoA contents within 30 min (results not shown).

3-Bromopyruvate (0.25 mM) resulted in 64 and 67% inhibition of pyruvate oxidation and ACh synthesis respectively (Table 2). The inhibitor also caused 100 and 84% loss of acetyl-CoA from particulate (mitochondrial) and synaptoplasmic subfractions respectively (Table 3). In the presence of 0.1 mM-Ca^{2+} a 42% decrease in pyruvate oxidation was accompanied by a slight activation of ACh synthesis. No significant change in acetyl-CoA level in whole synaptosomes was detected. However, the acetyl-CoA content in particulate subfraction was substantially decreased (32%) (Table 3). An increase in Ca^{2+} concentration to 1 mM brought about no further changes in rates of ACh synthesis and pyruvate oxidation (Table 2), but it decreased acetyl-CoA in whole synaptosomes by 29% and that in the particulate subfraction by 74% (Table 3). On the other hand,

Table 3. Acetyl-CoA content in cytoplasmic and particulate fractions of rat brain synaptosomes incubated with different effectors of its metabolism

Synaptosomes were incubated and handled as described in the Materials and methods section. Content of acetyl-CoA in synaptoplasmic fraction was calculated as the difference between acetyl-CoA levels in whole synaptosomes and particulate subfraction. Results are means ± S.E.M. for seven experiments done in duplicate: * $P < 0.05$ with respect to control, † $P < 0.05$ with respect to appropriate control without verapamil, by paired t test [25].

Additions	Acetyl-CoA content (pmol/mg of fraction protein)		
	Whole synaptosomes	Particulate fraction	Soluble fraction
None	24.9±2.9	12.3±2.5	46.8±5.6
3-Bromopyruvate (0.25 mM)	2.7±0.7*	0	7.4±0.6*
Ca^{2+} (0.1 mM)	22.6±3.9	8.4±0.9*	47.7±10.7
Ca^{2+} (1.0 mM)	17.7±4.6*	3.0±2.0*	43.8±9.0
Verapamil (0.5 mM)	22.3±3.4	11.4±2.2	41.3±8.5*
Ca^{2+} (0.1 mM) + verapamil (0.5 mM)	17.1±2.5†	5.2±0.3*†	38.1±6.6*†

Ca^{2+} had no apparent effect on acetyl-CoA content in synaptoplasm.

Verapamil is a known inhibitor of Ca^{2+} influx into synaptoplasm both from the extracellular space and from mitochondria [14,15]. Its addition either to the basic medium or to it containing Ca^{2+} caused 33% inhibition of ACh synthesis, but no changes in oxidation of pyruvate (Table 2). In the absence of Ca^{2+}, verapamil resulted in only a slight decrease in acetyl-CoA in both synaptosomal subfractions. In the presence of 0.1 mM-Ca^{2+} it brought about 38 and 20% decreases in acetyl-CoA in the particulate fraction and synaptoplasmic compartment respectively (Table 3). Verapamil did not change [^{14}C]ACh content remaining in the synaptosomal pellet at the end of incubation, which was equal to 25±5 pmol/mg of protein, under all experimental conditions (result not shown).

DISCUSSION

The high concentration of KCl used in this work assured continuous ACh release from synaptosomes and thereby created non-equilibrium conditions for the choline acetyltransferase reaction [16,17]. In addition 1 mM-choline, present in the medium, was sufficient to saturate its high- and low-affinity transport systems and the enzyme itself [5,18]. In consequence, in this experimental model the supply of acetyl-CoA to synaptoplasm could be the only rate-limiting factor for ACh synthesis.

The concentration of digitonin used here was sufficient for solubilization of synaptosomal plasma membranes, since it caused the release of over 90% of lactate dehydrogenase to the soluble fraction. Simultaneously digitonin did not impair integrity of the mitochondrial membrane, as over 90% of glutamate dehydrogenase and acetyl-CoA remained inside the particulate (mitochondrial) fraction (Table 1). Thanks to this, reliable measurements of the acetyl-CoA content in synaptosomal compartments could be performed (Table 3).

The experiment with 3-bromopyruvate provides direct evidence that ACh synthesis is influenced by the level of acetyl-CoA in synaptoplasm, which depends on the rate of its generation by pyruvate dehydrogenase in intrasynaptosomal mitochondria (Tables 2 and 3) [4,6,19]. Apparent inhibition of pyruvate oxidation by synaptosomes in the presence of Ca^{2+} might result from the decrease by Ca^{2+} of the activation state of the pyruvate dehydrogenase complex and from inhibition of pyruvate transport through the mitochondrial membrane (Table 2) [20–22]. On the other hand, these data are in conflict with a view, based on experiments with pyruvate dehydrogenase inhibitors, that the rate of ACh synthesis is closely dependent on the rate of pyruvate oxidation [4,19]. Namely, they show that the rate of ACh synthesis by synaptosomes in the presence of Ca^{2+} is more or less activated in spite of significant inhibition of pyruvate utilization (Table 2) [4,7,21,23]. This apparent discrepancy is explained in this work by the finding that Ca^{2+}-evoked loss of acetyl-CoA was confined only to intrasynaptosomal mitochondria, whereas the nucleotide content in synaptoplasm remained unchanged (Table 3). This phenomenon could be due to the increase by Ca^{2+} of a selective permeability of the mitochondrial membranes to acetyl-CoA [24], resulting in its direct efflux to synaptoplasm (Table 3). Thanks to this the concentration of acetyl-CoA in synaptoplasm would be, to some degree, independent of the rate of its synthesis inside intraterminal mitochondria.

The inhibitory effect of verapamil on ACh synthesis can be explained by the inhibition of Ca^{2+} influx to synaptosomes leading to decreased permeability of the mitochondrial membranes to acetyl-CoA and its lowered level in synaptoplasm (Tables 2 and 3) [14,15,24]. On the other hand, this inhibition seems not to be due to suppression of ACh release by verapamil, as the same amounts of radiolabelled ACh were retained in the synaptosomal pellet both in the presence and in the absence of the drug.

These data indicate that part of the intramitochondrial acetyl-CoA may be transferred directly to the site of ACh synthesis by a Ca^{2+}-activated mechanism [24] (Table 3). They also suggest that the increase in Ca^{2+} concentration in synaptoplasm of activated cholinergic terminals might automatically adjust the acetyl-CoA level in this compartment to maintain an appropriate rate of ACh synthesis. In such a situation the multiple pathways of

transfer of acetyl units through the mitochondrial membrane [2,6] would not compete, but rather complement each other to meet this demand.

The work was supported in part by Polish Academy of Sciences project 06.02.II.6.4.

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Received 20 March 1989/5 June 1989; accepted 15 June 1989