Volume 243, number 2, 153–155 FEB 06712 January 1989

Polarographic observation of substrate-level phosphorylation and its stimulation by acetylcholine

M.N. Kondrashova and N.M. Doliba*

Institute of Biological Physics, USSR Academy of Sciences, 142292 Pushchino, Moscow Region and **Chair of Physiology, Lvov State University, Shcherbakova 4, 290005 Lvov, USSR*

Received 21 November 1988

Substrate-level phosphorylation was observed under the conditions optimal for this process and opposite to those for oxidative phosphorylation. Polarographic registration of Ca^{2+} stimulated α-ketoglutarate oxidation and self-inhibition of uncoupled α-ketoglutarate (KG) oxidation was used. Acetylcholine (ACh) administration stimulated KG oxidation and substrate-level phosphorylation in isolated mitochondria. These effects are stronger in tissues with a higher level of endogenous acetylcholine, such as guinea pig liver vs rat liver and pancreas vs liver. The specific stimulation of KG oxidation by ACh is related to a decrease of succinate oxidation and is contrary to the specific stimulating effect of adrenaline on succinate oxidation. Therefore the existence of reciprocal hormone-substrate-nucleotide systems is suggested. The described set of conditions optimal for substrate-level phosphorylation observation by polarographic registration of respiration is as convenient as the ADP test for the investigation of oxidative phosphorylation.

Substrate-level phosphorylation; Mitochondria; Acetylcholine

1. INTRODUCTION

We showed earlier that acetylcholine (ACh) stimulates α-ketoglutarate (KG) oxidation and inhibits succinate oxidation [1]. Due to the reciprocal effects on the oxidation of these substrates stimulation is better revealed under elimination of succinate input into total respiration when exogenous KG is used. Malonate addition and 40–60 min storage of mitochondria in ice were used for this purpose. Specific stimulation by ACh of KG oxidation coupled with inhibition of succinate oxidation suggested ACh stimulation of substrate-level phosphorylation (SP). In this work we investigate the effect of ACh on SP by polarographic measurements of respiration under the conditions favourable for this process and abolishing the competing oxidative phosphorylation (OP).

Correspondence address: M.N. Kondrashova, Institute of Biological Physics, USSR Academy of Sciences, 142292 Pushchino, Moscow Region, USSR

2. MATERIALS AND METHODS

Fed male Wistar rats (weighing 200–220 g) and guinea pigs (weighing 500–700 g) were used. Liver and pancreas mitochondria were prepared using the method of native mitochondrial isolation [2,3]. The damage of mitochondrial is prevented due to exemption from washing, preparation of more concentrated suspensions and to some other factors. Obtained mitochondria are present in the suspension in the form of aggregates as corresponds to their native state in the intact cell [4].

Animals were decapitated. Liver or pancreas were quickly excised, chilled and weighed. The isolation medium of liver mitochondria was composed of 300 mM sucrose, 10 mM Tris (pH 7.4); the isolation medium of pancreas mitochondria contained 250 mM sucrose, 20 mM Tris, 0.5 mM EDTA, 0.25 mg/ml of soybean trypsin inhibitor, 150 mg/ml of bovine serum albumin. The unwashed mitochondrial pellet was gently rehomogenized in a homogenization medium without EDTA and albumin. The liver mitochondrial concentrations under storage and under incubation were 70–80 mg protein/ml and 4–5 mg protein/ml, respectively; the pancreas mitochondrial concentrations under storage and incubation were 30–40 mg protein/ml and 2.0–2.5 mg protein/ml, respectively. Protein was measured with Folin phenol reagent [5]. Liver mitochondria were incubated at 26°C in medium containing 150 mM sucrose, 50 mM KCl, 1 mM KH_2PO_4, 3 mM Tris (pH 7.4); pancreas mitochondria were incubated at 28°C in the medium containing 250 mM sucrose, 5 mM KH_2PO_4, 40 mM KCl,

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

10 mM Tris, 0.5% serum bovine albumin (pH 7.4). Respiration was recorded polarographically [6]. SP was registered by self-inhibition of uncoupled KG oxidation [7,8]. It is of importance that the rate of uncoupled respiration should be moderate, 1/3 to 1/2 of the maximal value. Tonicity of the incubation medium was increased, i.e. 300 mM sucrose, 5 mM Tris, 50 mM KCl, 3 mM KH_2PO_4 (pH 7.4). 50 μg/100 g of ACh was administered intraperitoneally, 15 min before decapitation. An equal volume of 0.9% NaCl was injected into control animals.

3. RESULTS

Kinetic predominance of endogenous succinate and succinate generated during incubation decreases KG oxidation and SP. As mentioned in section 1, succinate influence was prevented by malonate addition and by storage of mitochondria in ice. OP as such competes with SP, which may be abolished by using Ca^{2+} instead of ADP or using uncoupler for stimulating respiration. Ca^{2+} addition favours SP as Ca^{2+} abolishes ADP phosphorylation and because the physiological balance between adenine and guanine nucleotides is not altered [9].

The data on the ACh effect on ADP- and Ca^{2+}-stimulated respiration of rat liver and guinea pig liver and pancreas mitochondria are given in table 1. As seen from the table, the stimulating effect of ACh on KG oxidation both in the liver and pancreas mitochondria is considerably higher when respiration is stimulated by Ca^{2+} than by ADP. The results also show that ACh stimulation is greater in guinea pig liver mitochondria than in those of rat and in pancreas mitochondria than in liver. This may be explained by the higher level of endogenous ACh in guinea pig as compared with rat and in pancreas as compared with liver.

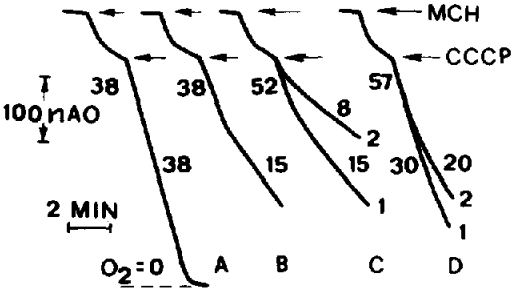

Fig.1. Polarographic registration of self-inhibition of uncoupled respiration of guinea pig and rat liver mitochondria. A,B,C, guinea pig, D, rat liver. Substrates (1.2 mM in all cases): A, pyruvate + malate; B, pyruvate + malate + KG; C,D, KG. 1, control; 2, acetylcholine. ClCCP, 2.5×10^{-7} M. Concentration of mitochondrial protein: 2.8 mg/ml.

In further experiments we compare the effect of ACh in different tissues. SP was observed in the presence of ClCCP as self-inhibition of uncoupled KG oxidation. This reaction was described by Olson and according to his data it is due to dihydrolipoyl dehydrogenase inhibition by the GTP formed in SP. Our experiments showed that self-inhibition is not always reproducible. According to a later work of Olson, oligomycin is necessary to observe self-inhibition. We found that 0.5 mM $MgCl_2$ (not 2–3 mM) may also restore self-inhibition. However, a small increase of incubation medium tonicity with sucrose (not with KCl) was found to be more effective in providing well reproducible self-inhibition [10]. We measured SP using the last modification as described in section 2.

The effects of ACh administration on self-inhibition of KG oxidation in guinea pig and rat

Table 1

Effect of acetylcholine (ACh) administration on ADP or Ca^{2+}-stimulated oxidation of α-ketoglutarate by mitochondria of different organs (in ngatom O/min per mg protein)

Stimulation by	ACh	Rat liver	Stimulation (%)	Guinea pig liver	Stimulation (%)	Guinea pig pancreas	Stimulation (%)
ADP	–	66.44 ± 3.65	36	30.33 ± 2.70	46	33.0 ± 4.16	86
	+	90.40 ± 4.39*		44.20 ± 4.10*		60.52 ± 6.06*	
Ca^{2+}	–	72.46 ± 6.83	61	21.0 ± 1.80	75	20.69 ± 0.71	167
	+	116.08 ± 10.98*		36.75 ± 3.91*		55.46 ± 5.29*	

Results are values ± SE for 8–12 experiments. Statistical significance was calculated by using Student's t-test; * $p < 0.05$

liver mitochondria are given in fig.1. As seen from the figure, self-inhibition of respiration is absent when pyruvate and malate are oxidized. However, it appears when KG is added to pyruvate and malate, but is more apparent with KG alone and is further enhanced by ACh administration: the inhibition develops more rapidly and is more complete. As with Ca^{2+} stimulated respiration, the effect of ACh is stronger in guinea pig mitochondria than in those of rat. We used fed animals. Starvation may abolish self-inhibition in guinea pig preparations due to activation of phospho*enol*-pyruvate carboxykinase mostly located in mitochondria.

4. DISCUSSION

The described data on Ca^{2+} stimulated and uncoupled oxidation of KG may be explained as a result of the stimulating effect of ACh on SP. Maybe this effect contributes to the increase of GTP and cGMP level by ACh. On the other hand, succinate-supported OP is diminished by ACh. Hence the effects of ACh on KG-supported SP and on succinate-supported OP are as opposite as the conditions optimal for SP and OP. The reciprocal relationship of these hormones and substrates is also manifested in the stimulating effect of adrenaline on succinate oxidation, ATP and cAMP synthesis coupled with a decrease of the cGMP level [11–13]. All the data considered support the view that the two investigated substrates are involved in two reciprocal hormone-substrate-nucleotide systems:

Acetylcholine–α-ketoglutarate–GTP–cGMP

Adrenaline–succinate–ATP–cAMP (1)

Non-simultaneous signals of ACh and adrenaline in an organism should also be related to a subsequent and no-simultaneous activation of oxidation of KG and succinate coupled with SP or OP, respectively. This coincides with our data showing that optimal manifestation of SP or OP in isolated mitochondria is also possible not simultaneously but subsequently in separate samples.

The described set of conditions favourable for SP observation by polarographic registration of respiration can compensate the discrimination of SP in current experiments on OP. The conditions run as follows: (i) providing pure KG oxidation by eliminating succinate input; (ii) excluding disturbance of SP by ADP addition; (iii) preserving SP by increased tonicity; (iv) last but not least, using ACh administration or tissues with a high ACh content.

REFERENCES

[1] Shostakovskaya, I.V., Doliba, N.M., Gordy, S.K., Babsky, A.M. and Kondrashova, M.N. (1986) Ukr. Biokhim. Zh. (USSR) 58, 54–61.
[2] Kondrashova, M.N. and Grigorenko, E.V. (1985) Zh. Obshch. Biol. (USSR) 46, 516–526.
[3] Kondrashova, M.N. and Grigorenko, E.V. (1985) EBEC III Short Reports, p.704, ISCU Press, Cambridge.
[4] Kondrashova, M.N., Grigorenko, E.V., Temnov, A.V. et al. (1987) Biofizika 32, 313–323.
[5] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[6] Chance, B. and Williams, G.R. (1956) Adv. Enzymol. 17, 65–134.
[7] Olson, M.S. and Allgyer, T.T. (1972) Biochim. Biophys. Acta 267, 232–248.
[8] Olson, M.S. and Allgyer, T.T. (1973) J. Biol. Chem. 248, 1582–1597.
[9] Tokumitsu, J. and Ui, M. (1973) J. Biochem. 74, 551–560.
[10] Doliba, N.M., Maevsky, E.J. and Kondrashova, M.N. (1986) Molecular Mechanisms and Regulation of Energy Metabolism, p.124, ONTI NCBI Pushchino.
[11] Shostakovskaya, I.V. and Babsky, A.M. (1984) Ukr. Biokhim. Zh. (USSR) 56, 57–62.
[12] Babsky, A.M., Kondrashova, M.N. and Shostakovskaya, I.V. (1985) Fiziol. Zh. (USSR) 31, 301–306.
[13] Andreev, A.A., Vulfius, C.A., Budantsev, A.Yu., Kondrashova, M.N. and Grishina, E.V. (1986) Cell. Mol. Neurobiol. 6, 407–420.