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THE ROLE OF HEXOKINASE AS A POSSIBLE MODULATOR OF Ca^{2+} MOVEMENTS IN ISOLATED RAT BRAIN MITOCHONDRIA

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SUMMARY. The present study shows that in brain mitochondria the active calcium uptake and the sodium-dependent calcium efflux are modulated by the porin-bound enzyme hexokinase. The release of the enzyme, promoted by glucose-6-phosphate (G-6-P), under conditions which do not affect mitochondrial functions, is accompanied by a decrease of the rates of fluxes of the cation. This phenomenon is discussed and correlated with the formation of microcompartments between the inner and outer mitochondrial membranes, where the hexokinase-porin complex may constitute a regulating gate system for calcium.

The problem of mitochondrial Ca^{2+} transport has been investigated intensively in the past twenty years in mitochondria isolated from different tissues, although some molecular aspects have still to be clarified (for a review see 1). A small acidic protein, named calvectin (2), reversibly associated to the outer surface of the inner membrane (3), has been recognized as the main component of the Ca^{2+} transport system (4). It was isolated from liver and heart mitochondria (2,5) and extensively characterized. The results indicate that it is involved in both the inward and outward movements of the ion, with the exception of the Na^{+}-dependent Ca^{2+} efflux (6).

Studies of Ca^{2+} movements in brain mitochondria, on the contrary, are rather fragmentary (7-16) and concern mainly clinical aspects (17-19). It is well established, on the other hand, that brain mitochondria differ from others in the fact that they possess the en-

ABBREVIATIONS: G-6-P: glucose-6-phosphate; arsenazo III: 2-2'-(1,8-dihydroxy-3,6-disulfo-2,7-naphthalene-bis-azo) bis-benzenarsonic acid

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zyme hexokinase (E.C. 2.7.1.1). About 80% of the enzyme firmly but reversibly bound to the porin protein is situated at the external surface of the outer membrane (for a review see 20,21). According to Brdiczka et al. (21), the hexokinase-porin complex is in close association with points of contacts of the inner with the outer mitochondrial membrane. Microcompartments are therefore created by these junctions and the hexokinase-porin complex may regulate the molecular traffic from cytosol to the mitochondrial matrix. Two findings are relevant in this context: a) Ca^{2+} plays an important role in contact formation and b) Ca^{2+} in uM concentrations in liver mitochondria stimulates hexokinase binding to these junctions (Brdiczka, personal communication). The aim of this work was to assess whether hexokinase controls the Ca^{2+} movements in brain mitochondria.

MATERIALS AND METHODS

Non-synaptosomal brain mitochondria were routinely prepared from fed male albino rats according to Rehncrona et al.(22). The method of Clark and Nicklas (23) was also occasionally employed. Liver mitochondria were prepared according to Schnaitman and Greenawalt (24). Anticalvectin antibodies purified by affinity chromatography were obtained by using an Affi-Gel 10 immobilized pure liver calvectin as already described (25). Calcium movements were followed spectrophotometrically at 665-685 nm (dual wavelength spectrophotometer) using purified (26) arsenazo III as suggested by Fletcher et al. (28). The amount of hexokinase bound to mitochondria has been determined both in the pellet and in the supernatant obtained by rapid centrifugation of the content of the cuvette where calcium spectrophotometric monitoring was followed; the assay was carried out according to Chou and Wilson (29). Membrane potential was measured by means of safranin (30). Proteins were determined by the biuret method (31). All media were routinely prepared by using distilled, deionized and filtered water produced by a Milli-Q system (Millipore).

RESULTS AND DISCUSSION

Rat brain mitochondria were prepared according to the procedure described by Rehncrona et al. (22), since they show better biochemical properties than those prepared without nagarse according to Clark and Nicklas (23). Since it was not known whether brain mitochondria contain calvectin in the Ca^{2+} transport system, they were

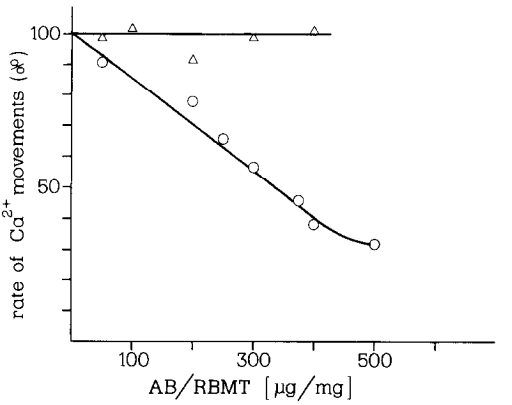

Figure 1. Effect of anticalvectin antibodies on the Ca^{2+} uptake and Na^{+}-induced Ca^{2+} efflux in isolated rat brain mitochondria. Experimental conditions: dual wavelength spectrophotometer operating at 665-685 nm. The assay solution (3ml) contained 1.5mg mitochondrial proteins (RBMT) in 0.1M mannitol, 25mM Tris-HCl, 50mM KCl (pH 7.4), 3mM K^{+} phosphate (pH 7.4), 15 µM rotenone, 5mM glutamate and 5mM malate, 0.1mM purified arsenazo III. 30ngions Ca^{2+} were added in each sample. 0.2 µM purified ruthenium red (RR) and 15mM Na^{+} were added to elicit the Ca^{2+} efflux. Treatment with antibodies (AB) was carried out at 0°C for 30 min in 200 µl final vulume. Samples were then transferred in the measuring cuvette, brought to a volume of 3ml and further processed. Controls were also incubated under the same conditions without antibodies. (○): Ca^{2+} uptake (100% corresponds to 225ngions $Ca^{2+}.min^{-1}.mg^{-1}$ protein); (△): Na^{+}-induced Ca^{2+} efflux (100% corresponds to 16 ngions $Ca^{2+}.min^{-1}.mg^{-1}$ protein).

incubated with increasing amounts of anticalvectin antibodies, and Ca^{2+} movements were followed thereafter. Figure 1 shows the rates of Ca^{2+} uptake and Na^{+}-induced Ca^{2+} release as a function of different antibodies/mitochondrial protein ratios. It appears that only the uniport system is inhibited as previously observed in liver (25) and heart (5) mitochondria. The Na^{+}-dependent Ca^{2+} efflux remains unaffected as already known for heart mitochondria (6). These findings allow us to conclude that: a) calvectin plays a pivotal role in active Ca^{2+} uptake also in brain mitochondria and b) the Na^{+}-dependent efflux of the ion proceeds through a pathway which is independent from calvectin.

The first approach to investigate the modulation of Ca^{2+} fluxes in brain mitochondria by hexokinase consisted in the partial removal of this enzyme from the outer membrane porin. It is well known that, among various substances tested, ATP and G-6-P release hexokinase

from the outer membrane binding sites (32,33). G-6-P is the most widely used, and in the range of 10^{-4}-10^{-3}M, 90% of the bound hexokinase could be removed from mitochondria. The conditions, however, employed for this removal are too drastic to retain the integrity of mitochondria. It was therefore compulsory for our purpose to find a compromise between maintenance of mitochondrial intactness, structure and function, and partial release of hexokinase from them. The conditions employed consist of a short incubation time (60 s) at room temperature in the presence of 2mM G-6-P in the cuvette used for the spectrophotometric determination of Ca^{2+} fluxes. This treatment results in a 30% release of hexokinase.

Figure 2 illustrates the spectrophotometric traces of Ca^{2+} uptake and Na^{+}-dependent Ca^{2+} efflux both in the absence and in the presence of G-6-P. Rates of both Ca^{2+} influx and release are inhibited by the addition of phosphorylated glucose which reduces also the extent of Ca^{2+} uptake.

As shown in Figure 3, the same response is observed when the G-6-P is added after Ca^{2+} accumulation by mitochondria. The impairment of the ion movements, therefore, is independent of the quantity of Ca^{2+} inside the mitochondria.

From a quantitative point of view the release of about 30% of hexokinase from G-6-P-treated mitochondria causes 60% decrease of the active cation influx and 50% of Na^{+}-induced extrusion respectively.

Data so far collected clearly indicate that not only the electrophoretic energy-driven Ca^{2+} uniport (calvectin-dependent) is modulated by the release of bound hexokinase, but also the passive antiport. The two pathways are independent, and their sensitivity toward the same modulator points to a generalized mechanism of the porin-hexokinase complex. This system could possibly regulate movements of other ions and/or metabolites.

Experimental conditions employed to obtain these data were carefully examined more in detail in order to exclude possible artifacts. By following the method of Åkerman and Vikström (30), it was possible to demonstrate that the membrane potential is not modified

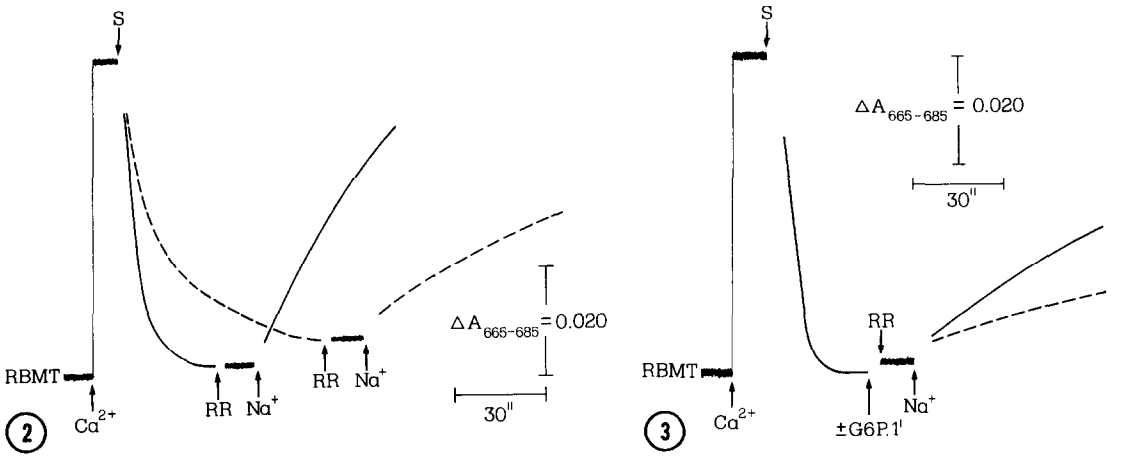

Figure 2. Effect of G-6-P (added before the Ca^{2+} uptake) on the Ca^{2+} uptake and Na^{+}-induced Ca^{2+} efflux in isolated rat brain mitochondria. Experimental conditions as in fig.1. Mitochondria (RBMT) were incubated for 1min at 22°C in the absence (——) and in the presence (----) of 2 mM G-6-P in the assay cuvette before the addition of Ca^{2+} and substrates.

Figure 3. Effect of G-6-P (added after the Ca^{2+} accumulation) on the Na^{+}-induced Ca^{2+} efflux in isolated rat brain mitochondria. Experimental conditions as in fig.1. Mitochondria (RBMT) were incubated at 22°C for 1min in the absence (——) and in the presence (----) of 2mM G-6-P in the assay cuvette after the complete accumulation of Ca^{2+}.

by G-6-P. The possibility that a change in membrane potential is responsible of the impairment of Ca^{2+} uptake by G-6-P is thus excluded. By using liver instead of brain mitochondria, both the extent and rates of the Ca^{2+} uptake were the same in controls and in G-6-P-treated mitochondria (data not shown). These results point to a specific effect of G-6-P in brain mitochondria and fit well with the idea that the removal of hexokinase affects Ca^{2+} movements and not other mitochondrial properties. Another piece of evidence in favour of a relationship between hexokinase and Ca^{2+} movements is provided by the fact that glucose, instead of G-6-P, is completely inert. Although some G-6-P may be formed by hexokinase during the experiment, the concentration is clearly too low to produce significant release of the enzyme: furthermore ATP produced by the energized mitochondria is consumed in the active transport of Ca^{2+}. 3 mM phosphate was routinely present in all the experiments: this is the concentration which elicits the maximum calcium uptake.

In conclusion results presented in this paper suggest a new role for the hexokinase-porin complex: that of modulating Ca^{2+} movements in brain mitochondria. The fact that a partial removal of hexokinase affect the fluxes of the cation is probably due to a rearrangement of the membrane domain, where the complex is located. Indeed not only Ca^{2+} uptake, calvectin-dependent, is affected, but also the antiporter system. The fact that porin is situated on the outer mitochondrial membrane (20,21,34) and calvectin on the external face of the inner one (3), indicates that the transmission of a signal (namely the hexokinase release) from the former to the latter must occur. These results provide indirect evidence that porin is close to the structure that links the two mitochondrial membranes and that the hexokinase-porin system is capable of controlling Ca^{2+} transport in brain mitochondria. Recently De Pinto et al. (35), by identifying the 35 KDa DCCD-binding protein with porin in heart mitochondria, postulate also an important role for this protein in regulating the metabolism of the cell and the mitochondria.

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