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ACTIVATION OF CALCIUM UPTAKE IN RAT LIVER MITOCHONDRIA BY AMINOGLUCOSIDE ANTIBIOTICS

HANS KRÖNER

Institut für Physiologische Chemie I, Universität Düsseldorf, D-4000 Düsseldorf, Federal Republic of Germany

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Abstract—Ca uptake in rat liver mitochondria is accelerated by various aminoglucoside antibiotics and, to a lesser degree, by triethylenetetramine and by protamine. With 1 mM Mg^{2+} and at a concentration of Ca^{2+} = 2 μM maximal (about six-fold) activation is achieved with 20 μM neomycin; at higher concentrations of the antibiotic the velocity of Ca uptake decreases. Activation to the same degree by gentamicin, kanamycin or streptomycin requires higher concentrations of the antibiotics. The reason for the acceleration of Ca uptake by neomycin is an allosteric alteration of the kinetics corresponding to that previously observed in the presence of spermine. The conformity with effects of spermine and possible inferences of that conformity are discussed.

In previous investigations new characteristics of Ca uptake in rat liver mitochondria were described [1, 2] in relation to a hitherto unknown change in the kinetics of the Ca uniporter. The change of the sigmoidal kinetics, usually observed in liver mitochondria, to hyperbolic ones can be produced by calcium itself as well as by spermine [2, 3] and has great implications. Some of the puzzling lack of clarity referred to by Igbavboa and Pfeiffer [4] can be explained by this phenomenon: varying kinetic constants, e.g. Hill coefficients, according to the methods employed become obvious. The Ca^{2+} concentration gradient across the inner mitochondrial membrane should be determined by the kinetics of the Ca uniporter and not by the membrane potential. This further implies an independent regulation of mitochondrial and cytoplasmic Ca^{2+} concentration.

Because the changed kinetics mean an increase in the affinity for calcium, and hence a considerable acceleration of Ca uptake in the range of low Ca concentrations, their significance in the physiological regulation of liver cell calcium has been postulated [2]. This could be demonstrated in an *in vitro* system, simulating conditions prevailing *in vivo* during the action of α-adrenergic agonists or vasoactive peptides on liver and during the early phase of carbon tetrachloride intoxication [5]. In any case, calcium released from the endoplasmic reticulum is completely adequate to activate the mitochondrial Ca uniporter. The consequence of this activation is a decrease of the extramitochondrial Ca^{2+} concentration, observed *in vitro*. *In vivo* this decrease should be compensated by a passive entry of calcium into the cell via its membrane.

In this connection an observation of Altin and Bygrave [6] seems interesting. They find that the Ca^{2+} influx which is normally associated with the administration of vasopressin in the perfused liver and which is stimulated by co-administration of glucagon is inhibited by neomycin. If that influx is not separately regulated as suggested [6] but is a passive entry of Ca^{2+} consequent on the activation of Ca uptake in mitochondria [5], neomycin should influence mitochondrial Ca uptake. A further hint at the aminoglucoside antibiotics is found in the observations of Palade [7]. In a quite different system he finds a remarkable conformity of aminoglucoside antibiotics with ruthenium red and spermine. All these substances are polyamines and all are capable of blocking Ca^{2+} releases mediated by a number of agents that activate a Ca^{2+}-induced Ca^{2+} release channel in triadic and terminal cisterna sarcoplasmic reticulum subfractions. It should be stressed that relatively low doses of the aminoglucosid antibiotics are effective in that experimental system.

The inhibitory effect of ruthenium red on the mitochondrial Ca uniporter is well known and effects of spermine on this transporter were recently described [3, 8, 9], so this investigation sought an influence of aminoglucoside antibiotics and some related drugs on the mitochondrial Ca transport. Because a like conformity with the spermine effect was found one may infer structural similarities in the different Ca transport systems.

MATERIAL AND METHODS

Neomycin sulfate was from Byk Gulden (Konstanz, F.R.G.) protamine sulfate from Merck (Darmstadt, F.R.G.), gallamin triethyliodide from Aldrich Chemical Co. (Steinheim, F.R.G.), streptomycin sulfate, gentamicin sulfate, kanamycin sulfate and triethylenetetramine from Serva (Heidelberg, F.R.G.).

Liver mitochondria from female Wistar rats were prepared as previously described [1]. They were incubated (1 mg protein/mL unless stated otherwise) at 30° and pH 7.0 in a medium containing 75 mM KCl, 110 mM sucrose, 10 mM TES,* 5 mM potasium acetate, 2 mM succinate, 1 mM magnesium chloride and 1 μM rotenone. Incubations with concentrations

* Abbreviations: DMNTA, 2,2-dimethylnitrilotriacetate; EGTA, [1,2 ethanediylbis-(oxy-2,1-ethanediylnitrilo)] tetraacetic acid; TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl] amino} ethanesulfonic acid.

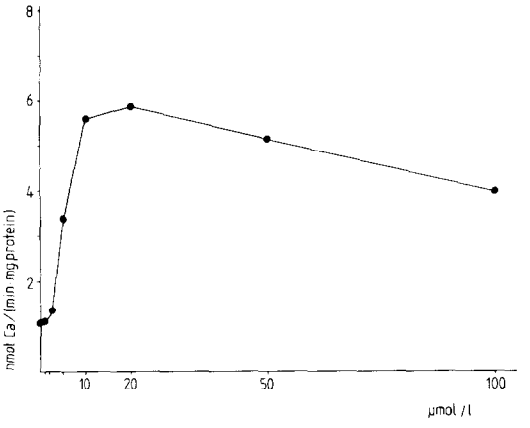

Fig. 1. Dependence of the initial velocity of Ca uptake in rat liver mitochondria on the concentration of neomycin. The experiments were done as described in Materials and Methods with 1 mg mitochondrial protein/mL and a Ca^{2+} concentration of 2 μM. Given are the means of four independent experiments, standard deviations are below 15% of the means.

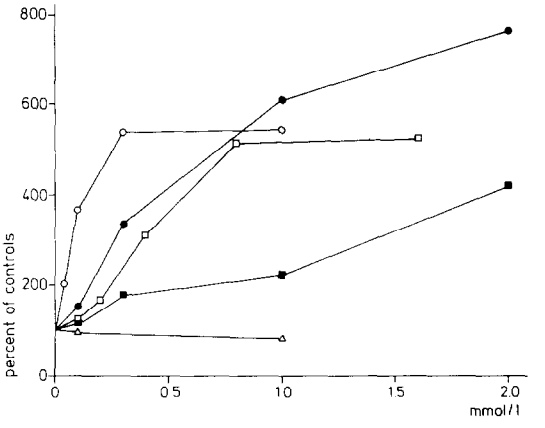

Fig. 2. Dependence of the initial velocity of Ca uptake in rat liver mitochondria on the concentration of gentamicin (○), kanamycin (●), streptomycin (□), triethylenetetramine (■), gallamin (△). Given are the means of at least three independent experiments in per cent of the control value: 1.00 ± 0.14 nmol/min/mg protein (N = 6).

of mitochondrial protein below 1 mg/mL were done with addition of albumin, 16 μmol/L. Ca uptake was started by addition of DMNTA to 5 mM final concentration, Ca-45 to 0.25 μCi/mL and $CaCl_2$ to 31 μmol/L resulting in 2 μM Ca^{2+} ions, unless stated otherwise. Mitochondria were separated by filtration through cellulose nitrate filters (Schleicher u. Schüll, poresize 0.45 μm) and washed twice with a medium of 75 mM KCl, 110 mM sucrose, 10 mM TES and 1 mM EGTA.

The dried filters were counted with 2,5-diphenyloxazole in toluene (6 g/L) in a Beckman LS 1801 liquid scintillation counter. The concentration of calcium in all assays as well as the mitochondrial calcium content were determined by atom absorption spectrometry [10]. The mitochondrial protein was determined with the Biuret reagent [11] using bovine serum albumin as standard. Concerning DMNTA and Ca buffering see Ref. 1.

RESULTS

The initial velocity of Ca uptake in the control experiments of Fig. 1 at 2 μM Ca^{2+} was 1.06 ± 0.16 nmol Ca/mg protein/min and remained nearly constant for several minutes. When the same experiment was performed after addition of neomycin sulfate to the incubation medium, the initial velocity of Ca uptake increased with increasing neomycin concentrations up to 20 μM. At higher drug concentrations the initial velocity of Ca uptake again decreased (Fig. 1). Additionally, in such experiments e.g. with 1 mM neomycin, the velocity of Ca uptake rapidly decreased with time and after a few minutes Ca uptake into mitochondria ceased altogether. This obviously inhibitory effect of higher neomycin doses was not investigated in detail.

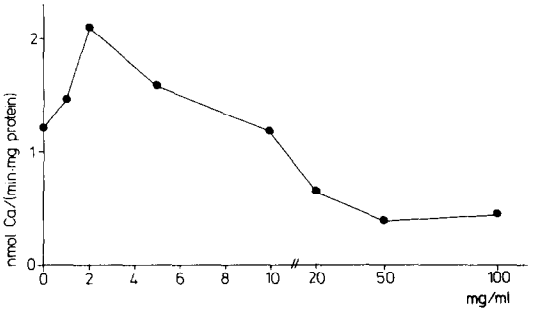

Fig. 3. Dependence of the initial velocity of Ca uptake in rat liver mitochondria on the concentration of protamine sulfate. Given are the means of at least four independent experiments. Control value: 1.21 ± 0.23 nmol/min/mg protein (N = 7).

In Fig. 2 effects of other aminoglucoside antibiotics as kanamycin, gentamicin and streptomycin on calcium uptake are shown. At appropriate concentrations of the drugs the initial velocity increases to almost the same extent as with neomycin but higher concentrations in the range of mmol/L are necessary to obtain maximal effects. Ca uptake in mitochondria is also accelerated by triethylenetetramine but this aliphatic polyamine is clearly less effective compared to the antibiotics or spermine [3]. The non depolarizing muscle relaxant gallamin has no activating effect at all but a slightly inhibitory one on mitochondrial Ca uptake. Finally, the effect of protamine resembles that of neomycin (Fig. 3); there is some activation at low and a distinct inhibition at higher protamin concentrations.

The activation of the Ca uniporter described above were measured at a substrate concentration of 2 μM Ca^{2+}, which is far below the concentration of saturation with substrate. Because under equivalent conditions the activation of the Ca uniporter by Ca^{2+}

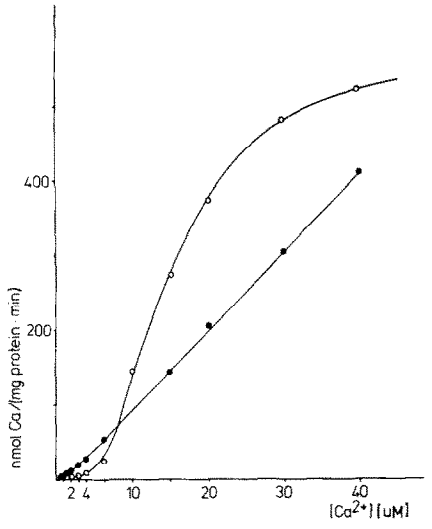

Fig. 4. Dependence of the initial velocity of Ca uptake upon Ca^{2+} concentration in the presence of 20 μM neomycin (●) and without neomycin (○). Mitochondria were incubated (0.2 mg mitochondrial protein/mL) in a medium containing 16 μM albumin. Given are the means of three independent experiments.

and by spermine proved to be a consequence of an allosteric transition from sigmoidal to hyperbolic kinetics [2, 3], the kinetics of Ca uptake in the presence of 20 μM neomycin was investigated. The kinetics with this most activating neomycin concentration is compared to normal kinetics as previously described [2, 3] (Fig. 4).

In principle the effect of neomycin on the kinetics corresponds to that of spermine and to the activation by calcium. The Hill coefficient, normally in the range of 3.0, declines to 1.41 in the presence of neomycin. The corresponding coefficients for activation by calcium and spermine are 1.33 and 1.30 respectively. Yet there is a difference between the kinetics with neomycin and the other ones: the slope in the v/s plot is not as steep as previously observed. This implies an additional 42% inhibition of Ca uptake by neomycin up to Ca^{2+} concentrations of 20 μM, compared to the kinetics with spermine [3].

DISCUSSION

A direct influence of aminoglucoside antibiotics especially of neomycin on mitochondrial Ca transport has not been described previously. There are, however, some effects of neomycin found in investigations with isolated liver cells or with perfused livers which should be addressed as they relate to mitochondria. The observations of Altin and Bygrave [6] concerning the perfused rat liver, already quoted above, should be considered in more detail. The authors have described a Ca^{2+} efflux consequent on the liberation of calcium from the endoplasmic reticulum by phenylephrine and vasopressin via inositoltrisphosphate. Pre-perfusion of the liver with 10 mM neomycin results in an increased efflux of Ca^{2+}. The following Ca^{2+} influx which is greater than would be necessary to replenish the calcium stores of the endoplasmic reticulum and which is stimulated by the co-administration of glucagon, is inhibited by neomycin. I postulate an inhibition of the mitochondrial Ca uniporter by neomycin applied at the extremely high concentration of 10 mM. Mitochondria cannot take up all the calcium liberated from the endoplasmic reticulum, the Ca uniporter cannot be activated by that calcium and therefore, only a small Ca^{2+} influx in order to replenish the stores of the endoplasmic reticulum takes place. The two or more separate mechanisms existing for regulation of Ca^{2+} influx in the liver, which were postulated by Altin and Bygrave [6] would be superfluous.

Bosch *et al.* [12] have described an increase of cytoplasmic calcium, induced by epidermal growth factor and also by vasopressin in isolated liver cells, which is abolished by 2 mM neomycin. They postulate an inhibition of inositoltrisphosphate formation by neomycin, though they could not detect an increase of inositoltrisphosphate induced by epidermal growth factor. In consequence of the lower concentration of neomycin (2 mM) and the shorter time of application (1/2 min) here the mitochondrial Ca uniporter should be activated. This could explain the results quite simply.

While preparing this manuscript I became aquainted with the investigation of Hughes *et al.* [13] where the effects of neomycin in isolated hepatocytes are analysed in more detail. Because the authors found no effect of neomycin on glucagon stimulated phosphorylase activity, the observed alterations of glycogen phosphorylase should apply to changes of intracellular Ca^{2+}. All these changes can be conditioned by an allosteric activation of the mitochondrial Ca uniporter, with no need to assume a further effect of neomycin at the cell membrane to inhibit Ca inflow. In Fig. 2 [13] a faster decrease of the phosphorylase activity due to neomycin is shown in hepatocytes stimulated with vasopressin and also in normal ones. The increase in phosphorylase activity consequent to the addition of calcium to hepatocytes incubated in Ca free medium is lower in the presence of neomycin. This is shown in vasopressin-stimulated cells (Fig. 3) as well as in normal ones (Fig. 5). Particularly the observation of Hughes *et al.* [13] that the neomycin effect can be abolished by increasing extracellular Ca concentration supports the assumption of passive entry of Ca^{2+} into the cell in consequence of activated mitochondrial Ca uptake.

On principle the interpretation of neomycin effects in such a complex biological system as the liver or the liver cell, is problematic. The task can be facilitated by reducing the doses. For the interaction of neomycin with the formation of inositol-trisphosphate in intact liver cells, concentrations of 10 mM neomycin [13] and in permeabilized cells about 1 mM [14] are necessary. The effective doses found in this investigation contrasts favourably to those values, although one has to consider a concentration gradient of neomycin at the cell membrane.

Though results with the high doses of neomycin probably will be of no physiological relevance and

though neomycin in high doses seems to be an unsuitable tool to study the role of inositol phospholipids in intracellular signalling [14], experiments in simpler systems, which are easier to survey may help to understand the medically relevant secondary effects of neomycin and the other aminoglucoside antibiotics. In such experiments the relationship between neomycin and spermine is striking. That applies to the inhibition of the ornithine decarboxylase [15] as well as to the inhibition of backward swimming of paramecium [16].

In this respect the results from this investigation particularly should be compared to those from Palade [7]. Here an influence on Ca transport into mitochondria which is stimulated by Ca^{2+} [2, 5] is shown, there the influence on Ca release from the sarcoplasmic reticulum which is induced by Ca^{2+} is described. While there the release is mediated by numerous chemical substances, here the uptake is mediated via the membrane potential. There all substances, especially neomycin spermine and ruthenium red, act inhibitorily, here only ruthenium red inhibits the Ca transport while neomycin in low concentrations and spermine have an activating effect. The results presented by Palade [7] do suggest that ruthenium red, spermine and the aminoglucosid antibiotics interact directly with the Ca^{2+} channel and I cannot imagine that this does not apply to the mitochondrial Ca transporter. On account of the conformity, one may speculate structural similarities in the several Ca transport systems.

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