Interaction of Cu^+ with Mitochondria

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The uptake of Cu^+ by rat liver mitochondria is rapid and extensive. Respiration is stimulated by 10 μM Cu^+ then inhibited and the inhibition could not be relieved with uncoupling agents. Collapse of the membrane potential is induced by 5–10 μM Cu^+. These effects are partially inhibited by radical scavengers indicating the involvement of radical production in these events. Reduction of the GSH content and production of peroxidation products by higher amounts of Cu^+ was also demonstrated. Swelling of non-respiring rat liver and heart mitochondria in sodium or lithium acetate was used to study effects of Cu^+ on the Na^+/H^+ exchanger. Swelling is stimulated by 5–100 μM Cu^+. In the presence of a radical scavenger the swelling is reduced. In sodium nitrate media diltiazem-sensitive stimulated swelling is observed. Amiloride was found to inhibit Cu^+-induced efflux of Ca^{2+}. At high concentrations of Cu^+, a general increase in permeability was the dominant feature.

The interaction of cuprous ions, Cu^+, with biological systems has received little attention. Cupric ions, Cu^{2+}, are known to increase K^+ and Mg^{2+} permeabilities and to stimulate swelling with binding of large amounts of Cu^{2+}, especially in non-respiring mitochondria.[1] It may be expected that Cu^+, under certain conditions, might compete with Na^+ for transport owing to the almost identical crystal radii (Cu^+, 0.96 nm; Na^+, 0.97 nm)[2] and charge, analogously to K^+ and Tl^+,[3]. Indeed, Cu^+ has been found to inhibit Na^+/H^+ exchange in frog-skin epithelium.[4] Interaction with Ca^{2+} transport may also be possible since the ionic radius of Ca^{2+}, 0.99 nm, also is very close.[2] In view of the similarities in ionic radii, we therefore investigated whether Na^+ or Ca^{2+} transport in mitochondria was affected by Cu^+. However, transition metals such as Cu and Fe[5] are known to produce reactive oxygen radicals and to cause a general increase in the permeability of the inner mitochondrial membrane.[6] We found that Cu^+ is taken up by the mitochondria and strongly interacts with them, causing a general increase in permeability and, in respiring mitochondria, collapse of the membrane potential. This made it difficult to demonstrate effects on specific cation-transport systems. However, amiloride and diltiazem inhibited some effects of Cu^+, possibly due to interaction of Cu^+ with the mitochondrial Ca^{2+}/Na^+ antiporter, which is sensitive to these drugs.[7,8]

* Amiloride: 3,5-diamino-*N*-(aminoiminomethyl)-6-chloropyrazine-2-carboxamide; BHT, 2,6-di-*tert*-butylphenol: diltiazem, 3-acetoxy-5-[2-(dimethylamino)ethyl]-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzodiazepam-4(5*H*)-one; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. Hepes, 4-(2-hydroxyethyl)-1-piperizinylethanesulfonic acid.

Experimental

Liver and heart mitochondria from male Sprague–Dawley rats were prepared by standard methods and their protein content estimated as described elsewhere.[9] Swelling was monitored by measuring the absorbance at 520 nm with the Shimadzu UV-3000 photometer (Japan). Respiration was measured polarographically with a Clark-type electrode. Experiments were carried out at room temperature (22–23 °C) in media of composition described in the figure legends. Copper was added as $CuSO_4$ and was reduced to Cu^+ with 1 mM hydrazine, added before the Cu^{2+}. Binding and uptake of Cu^+ by mitochondria were measured by sedimenting the mitochondria for 1 min in an Eppendorf microcentrifuge and estimating the concentration remaining in the supernatant by using disodium 4,4′-dicarboxy-2,2′-biquinoline (Sigma Chemical Co, St. Louis, MO) as a chromogen.[10] A stock solution of 0.1 M chromogen in 0.2 M sodium carbonate was diluted by a factor of 100 before use, and 100–400 μl of supernatant were added to a final volume of 2.5 ml, and the absorbance measured at 560 nm. Na^+/Ca^{2+} antiporter activity was measured as Na^+-stimulated release of accumulated ^{45}Ca (Radiochemical Centre, Amersham, UK) in respiring mitochondria after inhibition of Ca^{2+} uptake with ruthenium red (BDH, Poole, UK), measuring the radioactivity of mitochondria retained on 0.45 μm poresize filters. Diltiazem* was obtained from Sigma Chemical Co, St. Louis, MO. Mitochondrial membrane potential was measured by means of the safranine technique.[11] Lipid peroxidation products were measured by the thiobarbituric acid assay[12] by addition of 1 ml of the mitochondrial suspension to 2 ml of a freshly prepared solution containing 15 % (w/v) trichloroacetic acid, 0.375 % (w/v) thiobarbituric acid and 0.25 M HCl, in-

cubating for 15 min in a boiling water bath and measuring the absorbance at 535 nm. Reduced glutathione (GSH) was measured[13] by extracting the mitochondria with 5 % (w/v) trichloroacetic acid containing 5 mM EDTA. To 1 ml of the extract was added 2 ml 0.5 M potassium phosphate buffer pH 7.0 and 0.2 ml 2.78 mM 5,5′-dithiobis(2-nitrobenzoic acid).

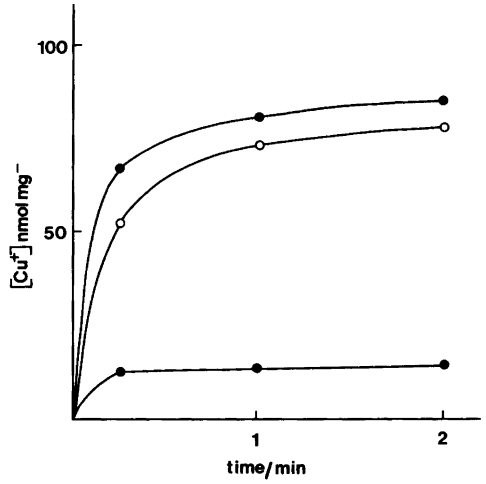

Fig. 1. Binding and uptake of Cu^+ by mitochondria. Mitochondria were rapidly sedimented and their Cu^+ content measured as described in detail in the Materials and methods section. Rat-liver mitochondria, 1.0 mg protein per ml, were added to a medium containing 100 mM sodium acetate, 1 mM hydrazine, 6 μM rotenone, and in the trace with open circles, 2 mM succinate. In the two top traces, 100 μM Cu^+ was added, in the bottom trace 25 μM.

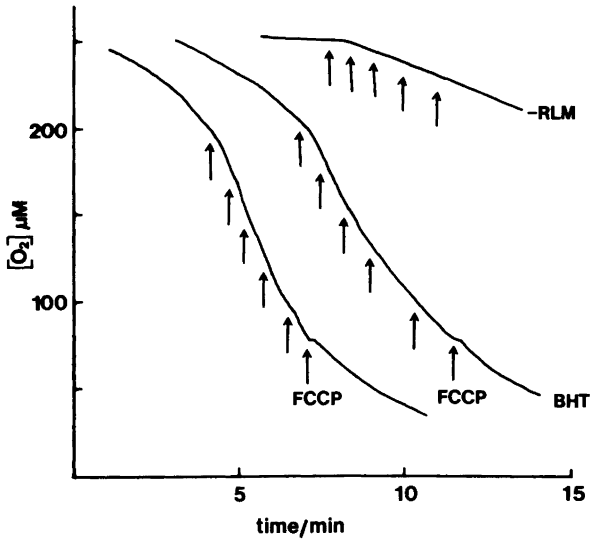

Fig. 2. Effect of Cu^+ on mitochondrial respiration. The medium contained 50 mM sodium acetate, 10 mM Hepes-buffer, pH 7.2, 2 mM succinate, 1 mM hydrazine and 6 μM rotenone. In the uppermost trace mitochondria were omitted in order to determine the rate of autoxidation of Cu^+; in the middle trace, 50 μM BHT was present. Arrows indicate additions of 10 μM aliquots of copper sulfate and finally 100 mM FCCP was added.

Results and discussion

Uptake of Cu^+. In acetate media, cation uptake, including Na^+, occurs mainly by exchange (antiport) against H^+ supplied in the matrix by dissocation of the acetic acid that diffuses from the external medium.[14] In nitrate-containing media uptake occurs mainly by uniport,[14] i.e. the cation moves without direct coupling to the movement of other ions. In addition there may be apparent uptake due to surface binding of Cu^+ to –SH groups and other binding sites.

The uptake of Cu^+ was extensive and rapid, Fig. 1. In acetate-containing media the uptake was more extensive in non-respiring than in respiring mitochondria. In this regard Cu^+ behaves as Cu^{2+}.[1] In nitrate-based media the uptake was slightly less (not shown).

Respiration and uncoupling. Respiration of rat-liver mitochondria on succinate was stimulated by addition of 10 μM Cu^+, Fig. 2, while further additions progressively caused inhibition, that was not relieved by addition of an uncoupling agent, FCCP. This indicates a general inhibition of respiration. In the presence of 50 μM BHT, a radical scavenger, the stimulation and subsequent inhibition of respiration by Cu^+ was similar, though the respiratory rates

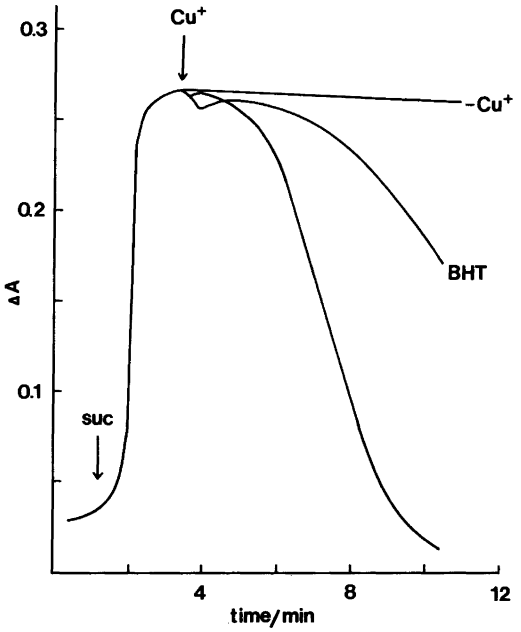

Fig. 3. Effect of Cu^+ on the mitochondrial membrane potential. The rat-heart mitochondria, 1 mg protein per ml, were suspended in a medium containing 210 mM sucrose, 40 mM sodium acetate, 1 mM hydrazine, 20 μM safranine and 6 μM rotenone. The membrane potential changes were followed at the wavelength couple 554–524 nm with an upward deflection showing an increase in the potential. Succinate, 2 mM, was added to initiate respiration and increase the membrane potential. The uppermost trace is a control without Cu^+; in the other traces, 5 μM Cu^+ was added; in the middle trace, 50 μM BHT was also present.

appeared to be lower. These data indicate that the mitochondria become uncoupled at 10 μM Cu^+ followed by inhibition of respiration. There was some consumption of oxygen even in the absence of mitochondria, which indicates autoxidation of Cu^+ under these conditions.

The effect of Cu^+ on the membrane potential was studied with safranine as a probe. Addition of succinate to mitochondria induced the formation of a membrane potential as indicated by the upward deflection of the trace in Fig. 3. In a control experiment the membrane potential formed was stable. Addition of 5 μM Cu^+ caused a gradual loss of the membrane potential. Thus, Cu^+ either acts as an uncoupling agent or is transported by an electrogenic uniport mechanism, consuming the membrane potential. In the presence of BHT the collapse of the membrane potential was clearly retarded. The collapse of the membrane potential is therefore likely to result from uncoupling due to the formation of oxygen radicals in the autoxidation of Cu^+.[5] Ruthenium red had no effect on the collapse of the membrane potential (not shown) which indicates that Cu^+ is not transported by the Ca^{2+} uniporter. Nor did cyclosporin A have any effect (not shown), either because opening of an unspecific, cyclosporin-A-sensitive pore[15,16] is not involved or that the drug is not able to prevent opening under these conditions.

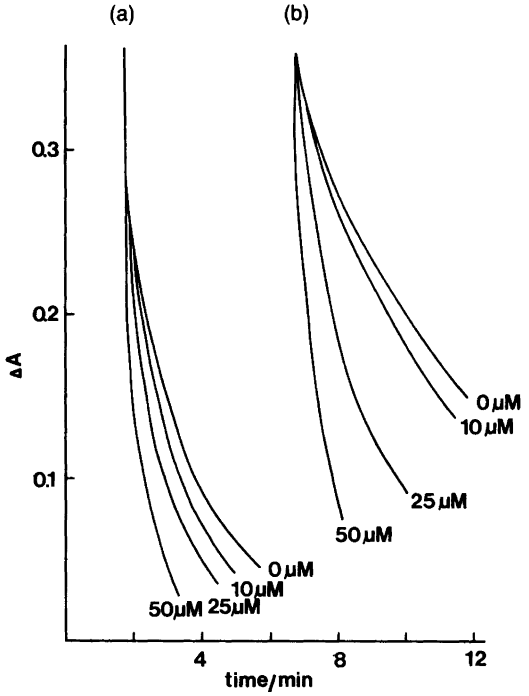

Fig. 4. Effects of Cu^+ on the swelling of rat liver mitochondria in sodium- or lithium-acetate-containing media. Swelling was monitored by the decrease in adsorbance at 520 nm. In Panel A, rat liver mitochondria, 1.0 mg protein per ml, were suspended in a medium containing 100 mM sodium acetate, pH 7.2, 1 mM hydrazine and 5 μM rotenone. In Panel B, sodium acetate was replaced by lithium acetate. The concentration of Cu^+ was varied between 0 and 50 μM as indicated.

Na^+/H^+ antiport. Non-respiring liver mitochondria underwent swelling in sodium-acetate-containing media [Fig. 4(a)] as previously shown for heart mitochondria.[14] In the presence of 10 μM or more Cu^+, the rate of swelling was stimulated. Diltiazem or amiloride did not influence the swelling (not shown). Hydrazine alone had no effect upon the swelling. In lithium acetate the findings were qualitatively similar, but the swelling was more sluggish than in sodium acetate, Fig. 4(b).

The finding that neither diltiazem or amiloride interfered with the Cu^+-induced swelling shows that these agents do not inhibit mitochondrial Na^+/H^+ antiport under these conditions. They have been reported to inhibit the Na^+/Ca^{2+} antiporter,[7,8,17] and also Na^+/H^+ antiport[18,19] and K^+/H^+ antiport,[20] when the matrix space is acidified.

Ca^{2+}/Na^+ antiport. The Ca^{2+}/Na^+ antiporter binds two cations simultaneously.[21] It is able to catalyze the Ca^{2+}/Ca^{2+} exchange[22] and it seems likely that it catalyzes the Na^+/Na^+ exchange as well. It was therefore of interest to study the interaction of Cu^+ with the Ca^{2+}/Na^+ antiporter in more detail using known inhibitors of this mechanism.

There is a fairly high Ca^{2+}/Na^+ antiporter activity in heart mitochondria[22,23] though some activity is also present in liver mitochondria.[24,25] The activity can be seen in non-respiring mitochondria as an amiloride- or diltiazem-sensitive stimulated swelling by Ca^{2+} in $NaNO_3$-containing media. In these media, Cu^+ may also penetrate the membrane electrogenically in response to the nitrate-induced

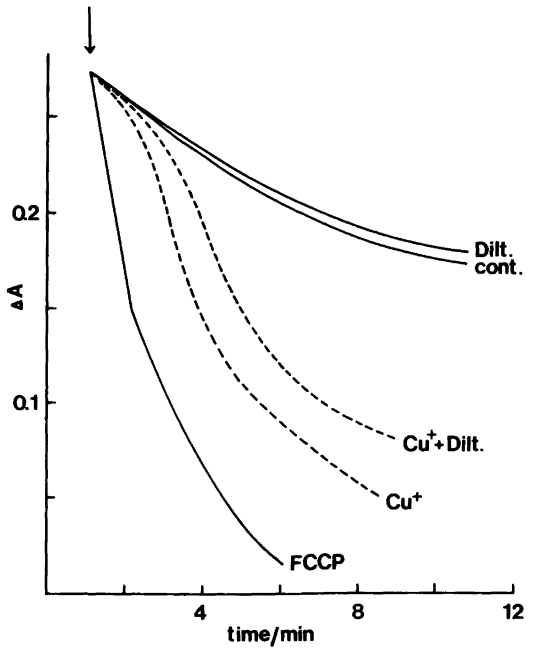

Fig. 5. Swelling of mitochondria in sodium-nitrate-containing media. The experimental conditions were as in Fig. 4 but for the replacement of acetate by sodium nitrate and the use of heart mitochondria. In the control experiment no further additions were made. Where indicated, 10 μM Cu^+, 500 nM FCCP and 200 μM diltiazem were present.

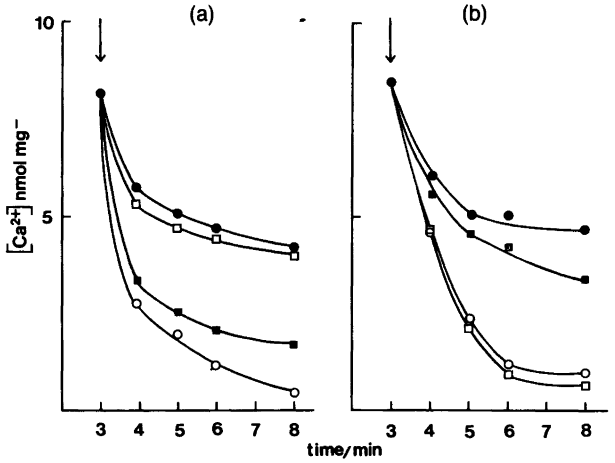

Fig. 6. Effects of Cu^+ on Ca^{2+} efflux. Rat-heart mitochondria, 1.0 mg protein per ml, were suspended in a medium containing 210 mM sucrose, 20 mM potassium sulfate, 10 mM Hepes, pH 7.2, 2 mM succinate, 1 mM hydrazine, 25 μM BHT and 6 μM rotenone. The mitochondria were allowed to accumulate an added amount of 10 μM Ca^{2+}, then 2 μM ruthenium red was added at the arrow together with 10 mM Na^+, Panel A, or, in Panel B, 20 μM Cu^+ (preceded by 1 mM hydrazine). Filled circles, controls without Na^+ or Cu^+; open circles, Na^+ or Cu^+, 0.2 mM diltiazem present.

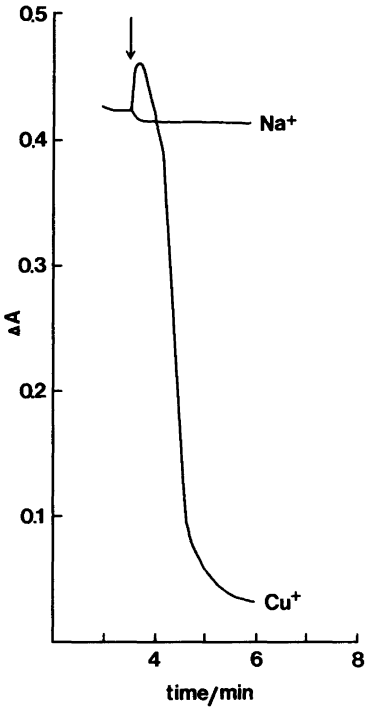

Fig. 7. Induction of a general increase in permeability by Cu^+. The medium contained 210 μM sucrose, 20 mM sodium acetate and 10 mM Hepes, pH 7.2. In the control trace, sodium acetate was added at the arrow. In the other trace, it was present from the beginning, and 100 μM Cu^+ was added.

diffusion potential, negative on the matrix side.[14] Fig. 5 shows that 5 μM Cu^+ was able clearly to stimulate swelling of rat-liver mitochondria as did addition of FCCP, presumably because in its presence, H^+ becomes available for Na^+/H^+ antiport. The same may be true for stimulation by Cu^+. However, diltiazem somewhat delayed, i.e. inhibited the Cu^+-induced swelling, which indicates that Cu^+ may be transported on the Ca^{2+}/Na^+ antiporter. At higher concentrations of Cu^+, the effect of diltiazem diappeared, presumably because the deleterious effects of Cu^+ became dominant.

Fig. 6 summarizes experiments on rat-heart mitochondria. Respiring mitochondria were allowed to accumulate Ca^{2+}, then ruthenium red was added to block the uniporter and efflux of Ca^{2+} followed. The Na^+-dependent Ca^{2+} efflux, due to the exchange activity, is seen as the difference between the traces with open and filled circles, respectively, in Fig. 6(a). Diltiazem caused a substantial inhibition of efflux, while that of amiloride was weaker. In the control traces, filled circles, the Na^+-independent efflux of Ca^{2+} was also rather extensive in the presence of sulfate (chloride would bind Cu^+). It is of interest that Cu^+ mimicked the effect of Na^+, Fig. 6(b). The Cu^+-induced efflux was, however, not inhibited by diltiazem under these conditions, Fig. 6(b) (open squares) while amiloride potently inhibited the efflux (filled squares). The difference in potency of inhibition between diltiazem and amiloride when Ca^{2+} efflux is induced by Na^+ and Cu^+, respectively, is not readily apparent. The Cu^+-induced efflux could be due to the collapse of the membrane potential and efflux of Ca^{2+} by reversal of the uniporter, in which case it would not be inhibited by either diltiazem or amiloride. However, there was clear inhibition by amiloride. In these experiments we used a minimum concentration of Cu^+ (5 μM) in the presence of 25 μM BHT as a protection against oxygen radical-induced damage. At higher concentrations of Cu^+ this would become so massive that a general increase in permeability would mask any effects upon specific transport systems.

General increase in permeability induced by Cu^+. The swelling of rat-liver mitochondria was studied in a sucrose-based medium in the presence of a small amount, 20 mM, of sodium acetate. The uptake of sodium acetate at this concentration caused, at most, small volume changes, Fig. 7, trace 1. Addition of a substantial amount of Cu^+, 100 μM, trace 2, caused extensive swelling. This must be due to the induction of a general increase in permeability so that the main solute, sucrose, was also able to penetrate the inner membrane.

Oxidative stress induced by Cu^+. It is generally known that copper ions cause increased production of oxygen radicals. Under our conditions Cu^+ was rapidly autoxidized and the presence of hydrazine was necessary to keep Cu^+ in the reduced state. These conditions can be expected to result in the profuse production of oxygen radicals and severe oxidative stress in mitochondria resulting in increased perme-

ability and swelling.[26,27] It is therefore not surprising that, in the thiobarbituric acid test, the absorbance during swelling induced by 100 μM Cu^+ increased from a negligible value of 0.04 to 0.13 showing considerable formation of peroxidation products. Cu^+, in addition to stimulating the production of oxygen radicals, might reduce the protective capability of mitochondria by binding to SH-groups, including that of glutathione, GSH. In the samples studied, the control mitochondria contained 10.3 mmol GSH per mg protein; with 100 μM Cu^+ the amount was reduced to 5.6 GSH per mg protein. These data support the interpretation that oxidative stress is involved in inducing a general increase in permeability, which may be due to opening of a pore.[6,15,16,26,27]

Conclusions

(1) Cu^+ is rapidly taken up or bound by mitochondria. (2) Cu^+ at 5–10 μM causes stimulation of respiration and loss of the membrane potential. At higher concentrations respiration becomes inhibited. (3) Cu^+ at 5–100 μM stimulates swelling of non-respiring mitochondria in acetate- or nitrate-containing media in the presence of both Na^+ and Li^+. (4) Cu^+ may be transported by the Na^+/Ca^{2+} antiporter since Cu^+-stimulated release of Ca^{2+} was sensitive to amiloride, an inhibitor of this activity. (5) Cu^+ induced a general increase in permeability, at least at higher concentrations. (6) Cu^+ caused oxidative stress in mitochondria which may be the cause of the general increase in permeability.

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