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THE EFFECT OF BONGKREKIC ACID ON THE Ca^{2+}-STIMULATED OXIDATION IN RAT-LIVER MITOCHONDRIA AND ITS RELATION TO THE EFFLUX OF INTRAMITOCHONDRIAL ADENINE NUCLEOTIDES

T. A. OUT, A. KEMP, JR. AND J. H. M. SOUVERIJN

Laboratory of Biochemistry, B.C.P. Jansen Institute, University of Amsterdam, Amsterdam (The Netherlands)*

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SUMMARY

1. Bongkrekic acid inhibits the uncoupling of succinate oxidation induced by addition of Ca^{2+} and P_i.

2. It also inhibits the efflux of intramitochondrial adenine nucleotides induced by this treatment.

3. It is concluded that the inhibitory action of bongkrekic acid on the adenine nucleotide translocator is favoured by the presence of endogenous adenine nucleotides.

INTRODUCTION

Bongkrekic acid has been shown to be an inhibitor of the adenine nucleotide translocator[1–5]. In this paper its effect on the Ca^{2+}-stimulated oxidation of succinate is described.

When low amounts of Ca^{2+} are added to mitochondria in the presence of oxidizable substrate and phosphate there is a rapid increase in the rate of oxygen uptake, which is either temporary or permanent, dependent on the concentration of Ca^{2+} and of phosphate used[6,7]. For reasons that are not yet fully understood, ATP, ADP and oligomycin promote a return, after the initial stimulation of the oxygen uptake, to the original resting rate[6,8,9], whereas the presence of atractyloside favours somewhat the maintenance of the rapid respiration[10]. This rapid respiration is uncoupled.

ERNSTER[11] suggested that the loss of phosphorylating capacity induced by Ca^{2+} is caused by a loss of intramitochondrial ATP, a suggestion that receives experimental support from MEISNER AND KLINGENBERG's[12] demonstration that Ca^{2+} and phosphate bring about an efflux of mitochondrial adenine nucleotides. The relation between Ca^{2+}-stimulated oxidation and the efflux of adenine nucleotides have been further studied in this paper.

* Postal address: Plantage Muidergracht 12, Amsterdam, The Netherlands.

METHODS

Rat-liver mitochondria were prepared according the method of Hogeboom as described by MYERS AND SLATER[13].

Oxygen uptake was measured polarographically using a Clark-type electrode.

The efflux of intramitochondrial adenine nucleotides was calculated by following the loss of radioactivity from mitochondria prelabelled with [8-^{14}C]ADP by incubation for 30 min at 0°, followed by twice washing with 0.25 M sucrose.

The mitochondria were separated from the reaction medium by millipore filtration and washed with 0.15 M NaCl. The filters were dried and the radioactivity was measured in a system with toluene, ethanol, 2,5-diphenyloxazole and 1,4-bis-(5-phenyloxazolyl-2)-benzene in a liquid scintillation counter.

RESULTS

In Fig. 1 the effects of bongkrekic acid and atractyloside on the Ca^{2+}-stimulated oxygen uptake are compared. It can be seen that neither have any effect on the initial stimulation of oxygen uptake, and that they have a different effect on the second rapid phase of oxygen uptake, that sets in shortly after cessation of the initial stimulation. Atractyloside promotes[10] whereas bongkrekic acid in very low concentrations inhibits this reaction. The rate of oxygen uptake in the second rapid phase depends on both the amount of Ca^{2+} added (Fig. 2) and on the concentration of phosphate used

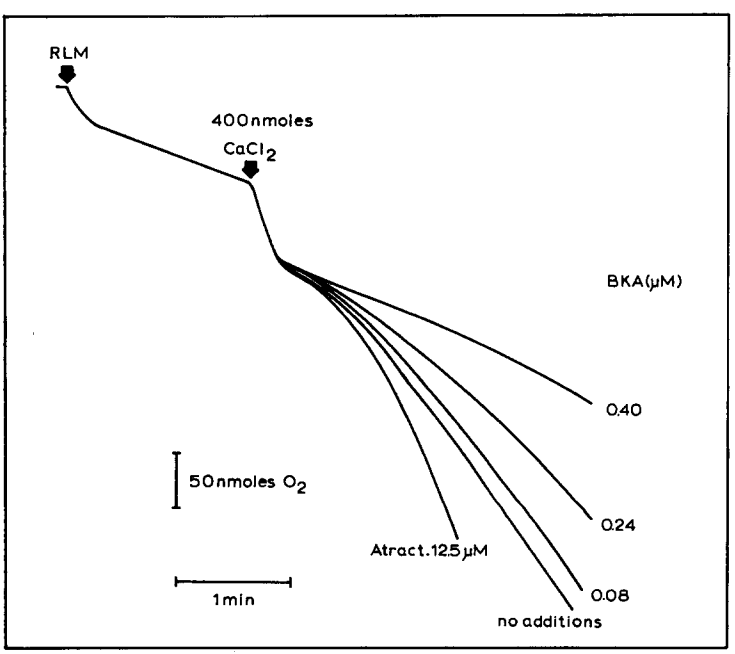

Fig. 1. The effect of bongkrekic acid and of atractyloside on Ca^{2+}-stimulated succinate oxidation. Rat-liver mitochondria (RLM) (2.0 mg protein) were incubated in a medium containing 50 mM triethanolamine–HCl buffer (pH 6.8), 55 mM sucrose, 15 mM KCl, 12 mM potassium succinate, 0.5 mM potassium phosphate, rotenone (0.15 μg/ml protein) and atractyloside (Atract.) or bongkrekic acid (BKA) as indicated. Reaction volume, 2.0 ml. Temp., 25°.

(Fig. 3). The Ca^{2+}/O ratio (calculated in exactly the same way as an ADP/O ratio) was 2.9–3.1; it was independent of these variables and was not affected by bongkrekic acid or atractyloside. In these experiments the mitochondria were incubated with the inhibitor for 1.5 min before the Ca^{2+} was added. In other experiments it was found

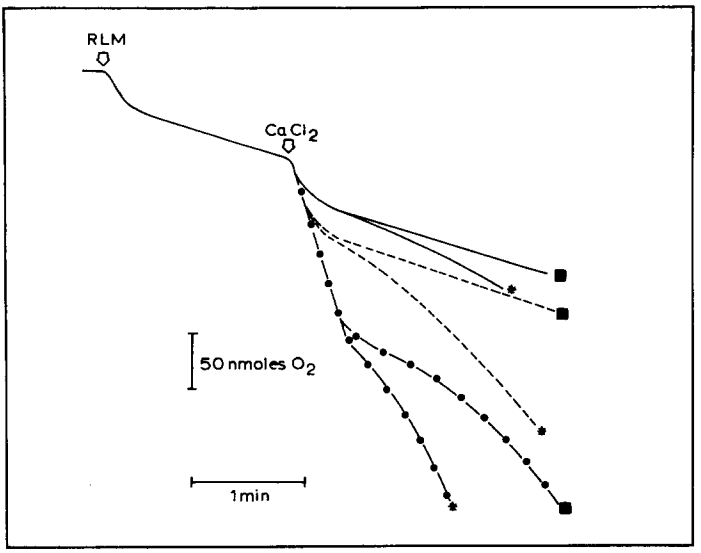

Fig. 2. The effect of varying the concentration of Ca^{2+} on the inhibition by bongkrekic acid of Ca^{2+}-stimulated succinate oxidation. Conditions as in Fig. 1. ———, 200 nmoles $CaCl_2$ added; – – – –, 400 nmoles $CaCl_2$ added; ●—●, 1000 nmoles $CaCl_2$ added; *, no bongkrekic acid; ■, 0.80 μM bongkrekic acid.

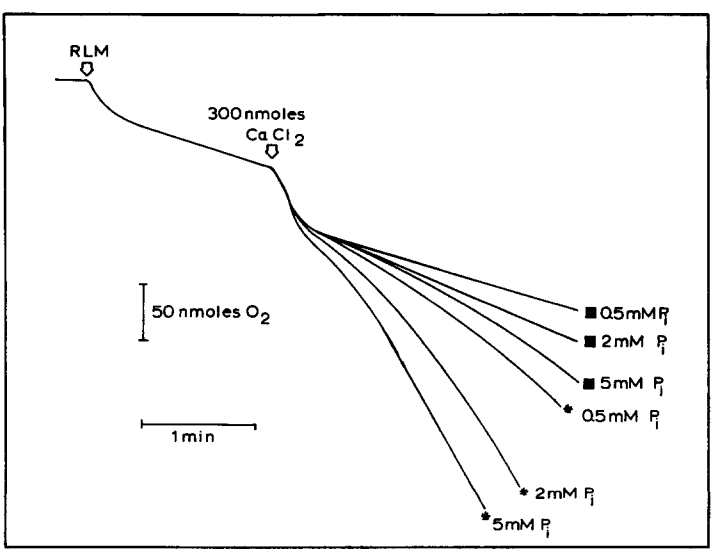

Fig. 3. The effect of varying the concentration of P_i on the inhibition by bongkrekic acid of Ca^{2+}-stimulated succinate oxidation. Conditions as in Fig. 1. P_i concentration as indicated. *, no bongkrekic acid; ■, 0.80 μM bongkrekic acid.

that the time dependency of the inhibitory action of bongkrekic acid is the same as that previously reported for the adenine nucleotide translocator[4,5].

The amount of endogenous adenine nucleotides in the mitochondria were measured under the same conditions as used in the experiments described in Figs. 1–3. In agreement with earlier reports[12,14] low concentrations of phosphate added to an isotonic medium brought about an efflux of adenine nucleotides; calcium increased the rate of this P_i-induced efflux, which bongkrekic acid inhibited (Fig. 4). Atractyloside increased the rate of efflux (*cf.* ref. 12).

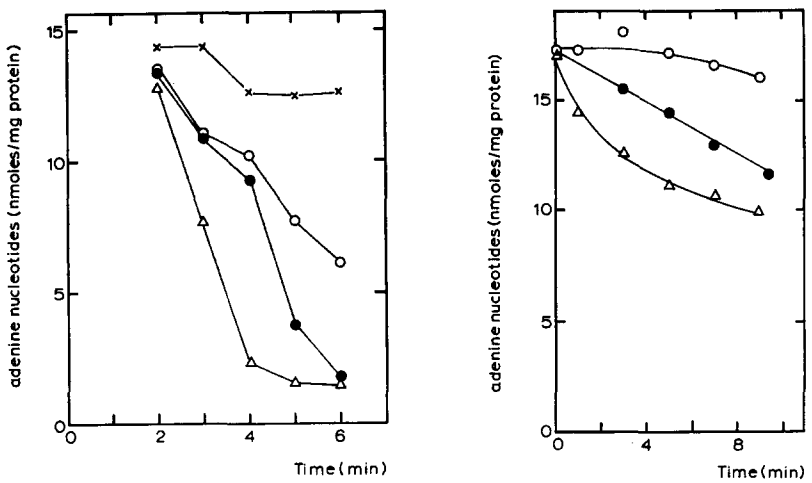

Fig. 4. The effect of bongkrekic acid on the efflux of adenine nucleotides induced by Ca^{2+} and P_i. Prelabelled rat-liver mitochondria (2.7 mg protein) were incubated under continuous stirring in a medium containing 50 mM triethanolamine–HCl buffer (pH 6.8), 55 mM sucrose, 15 mM KCl, 12 mM potassium succinate, 0.5 mM potassium phosphate and rotenone (0.13 μg/mg protein). $CaCl_2$ was added at 1.5 min. Reaction volume, 1.5 ml. Temp., 25°. ○—○, 500 nmoles $CaCl_2$; ●—●, 800 nmoles $CaCl_2$; ×—×, 800 nmoles $CaCl_2$, 1.04 μM bongkrekic acid. △—△, 800 nmoles $CaCl_2$, 16.5 μM atractyloside.

Fig. 5. The effect of bongkrekic acid on the efflux of adenine nucleotides. Prelabelled rat-liver mitochondria (3.15 mg protein) were incubated under continuous stirring in a medium containing 50 mM triethanolamine–HCl buffer (pH 6.8), 55 mM sucrose, 15 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 12 mM potassium succinate and rotenone (0.11 μg/mg protein). Final volume, 1.5 ml. Temp., 25°. ○—○, no additions; △—△, 5 mM P_i; ●—●, 5 mM P_i + 15.6 μM bongkrekic acid.

As reported earlier[4] phosphate delays inhibition by bongkrekic acid of ADP-stimulated oxidation, whereas ATP or ADP increase the rate of the inhibition. In agreement with the findings of KEMP[15] and VIGNAIS AND DUEE[16] an efflux of adenine nucleotides was found in the presence of substrate, Mg^{2+}, P_i and EDTA (see Fig. 5). Bongkrekic acid inhibits this efflux.

DISCUSSION

It has been suggested that the second rapid phase of oxygen uptake after adding Ca^{2+} to mitochondria in the presence of substrate and of phosphate is due to an interaction of phosphate with a high-energy intermediate containing Ca^{2+} (refs. 6, 17). The experiments reported here seem to indicate that this apparent uncoupling is due to the efflux of adenine nucleotides out of the mitochondria.

(i) ATP (or ADP), oligomycin[7] and bongkrekic acid inhibit this uncoupling. ATP, ADP and oligomycin[11] as well as bongkrekic acid (this paper) inhibit the efflux of adenine nucleotides.

(ii) When acetate is used as the permeant anion no second rapid phase of oxygen uptake is found after addition of Ca^{2+} (refs. 18, 19). Similarly, no efflux is found when phosphate is replaced by acetate[14].

(iii) Atractyloside induces a stimulation of both the efflux and the second rapid phase of oxygen uptake.

The experiments reported in this paper suggest also an explanation for the role of ATP or ADP in stabilizing Ca^{2+} accumulation in the presence of high concentrations of P_i; the added nucleotides prevent an efflux of endogenous adenine nucleotides. The question of whether the partly atractyloside-sensitive uptake of adenine nucleotides accompanying Ca^{2+} uptake, as reported by ROSSI *et al.*[17], is related to the phenomenon described in this paper, needs further investigation.

Another process that is affected by atractyloside and bongkrekic acid in opposite ways is the phosphate-induced large-amplitude swelling. This is stimulated by atractyloside[20] and prevented by bongkrekic acid (unpublished observations). Furthermore, oligomycin and ADP have been demonstrated to inhibit this swelling[20]. This supports the suggestion of MEISNER AND KLINGENBERG[12] that it is caused by a loss of intramitochondrial adenine nucleotides.

It is clear that bongkrekic acid inhibits two processes, the exchange[1–3] and the efflux of adenine nucleotides. The latter is not inhibited by atractyloside[12,15] except in very high concentrations[15]. In disagreement with our results, HENDERSON *et al.*[5] reported that bongkrekic acid does not inhibit the efflux of adenine nucleotides, but it is doubtful whether a specific efflux of adenine nucleotides occurs in the presence of EDTA and in the absence of phosphate and Mg^{2+}, the incubation conditions used by these authors.

MEISNER AND KLINGENBERG[12] and KEMP[15] have proposed an efflux of adenine nucleotides mediated by the adenine nucleotide exchanging translocator balanced by a phosphate entry. The different effects of atractyloside and of bongkrekic acid on the efflux might be explained by assuming that in intact mitochondria atractyloside acts on the outside and bongkrekic acid acts both on the inside and the outside of the mitochondrial inner membrane. However, the adenine nucleotide translocator is thought to involve a 1:1 exchange of adenine nucleotides, and the rate of translocation exceeds greatly the rate of efflux. If the efflux of adenine nucleotides is mediated by another mitochondrial system this system and the adenine nucleotide translocator must have very related properties.

The finding that the efflux of adenine nucleotides causes a delay in the inhibitory action of bongkrekic acid may indicate that the binding of bongkrekic acid to adenine nucleotides[21] on the inside of the inner membrane plays an important role in the inhibitory action.

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