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Factors Affecting the Inhibition of Adenine Nucleotide Translocase by Bongkrekic Acid*

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ABSTRACT: The translocation of adenine nucleotides and their structural analogs across the membranes of rat liver mitochondria is inhibited by bongkrekic acid; the extent of inhibition achieved depends on the ratio of bongkrekic acid concentration to mitochondrial protein concentration, the temperature, and period of time for which the mitochondria are exposed to the inhibitor. By contrast, inactivation of the translocase enzyme by atractyloside is relatively independent of these parameters. The greater potency of bongkrekic acid, when compared to atractyloside on a concentration basis, is probably due to the relatively weak antagonism by adenosine diphosphate, whereas adenine nucleotides competitively diminish inhibition by atractyloside.

Welling *et al.* (1960) first reported that the antibiotic bongkrekic acid is an inhibitor of oxidative phosphorylation in rat heart mitochondria. Subsequent experiments with mitochondria from rat liver have shown that bongkrekic acid inactivates an adenine nucleotide translocase and that this effect is probably responsible for the inhibition of ADP phosphorylation, ATPase, and other adenine nucleotide requiring reactions (Henderson and Lardy, 1970a,b). This conclusion is confirmed in this communication by further observations on the exchange of adenine nucleotides across the mitochondrial membrane, a direct assay of the adenine nucleotide translocase activity (Klingenberg and Pfaff, 1968).

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TABLE I: Exchange of Labeled Internal ADP with Added Adenine Nucleotides or Their Analogs.[a]

Nucleotide	Exchange Act. (%) Control	+Bongkrekic Acid
ADP	62	5
ATP	46	8
AMP	10	1
Deoxy-ADP	25	4
Deoxy-ATP	13	5
Deoxy-AMP	6	2
Adenosine 5′-methylene diphosphonate (AOPCP)	21	2
5′-Adenylylmethylene diphosphonate (AOPOPCP)	13	5
ADP-ribose	3	2

[a] Mitochondria (4–5 mg of protein) containing ^{14}C-labeled ADP were incubated at 0° for 10 min in 0.30 ml of KCl medium containing either 200 nmoles of bongkrekic acid or an equal amount of the antibiotic solvent. The indicated nucleotide (250 nmoles) was then added, and the exchange activity occurring in the subsequent 10-min period was measured.

An inconsistency in our previous observations was the finding that very different concentrations of bongkrekic acid were necessary for inhibition of phosphorylation of ADP (less than 1 nmole/mg of protein), dephosphorylation of ATP (approximately 15 nmoles/mg), and ADP or ATP uptake into mitochondria (approximately 60 nmoles/mg) (Henderson and Lardy, 1970a,b). Only in the phosphorylation assays were the mitochondria incubated with the antibiotic before initiation of the assay, and also the temperature for measurement of ADP or ATP uptake was 23° as opposed to 30° for the phosphorylation and dephosphorylation assays. The discrepancy is resolved here by the observation that a prior incubation of mitochondria with the inhibitor is necessary to allow time for the bongkrekic acid to equilibrate with the susceptible site, and the time required is related to the temperature. In addition, the effectiveness of bongkrekic acid is shown to be strongly dependent on protein concentration.

This dependence on time, temperature, and protein concentration differentiates bongkrekic acid from the other inhibitor of the adenine nucleotide translocase, atractyloside (see reviews by Bruni, 1966, and Heldt, 1969). A preliminary kinetic analysis is presented to demonstrate that inhibition of oxidative phosphorylation by bongkrekic acid is partly diminished by increasing ADP concentrations, but the biphasic nature of the response differs markedly from the linearity obtained with atractyloside (Bruni *et al.*, 1965; Vignais *et al.*, 1966). The second phase of bongkrekic acid inhibition is apparently noncompetitive; this seems to be a factor determining that bongkrekic acid is a more efficient inhibitor than atractyloside.

Experimental Section

Adenine nucleotide translocase activity was measured by the "back-exchange" technique of Pfaff and Klingenberg (1968). Samples of mitochondria previously labeled with [^{14}C]ADP or -ATP were incubated for 10 min in 80 mM KCl, 50 mM Tris-Cl, 20 mM sucrose, and 1 mM EDTA (pH 7.3) (KCl medium) in the presence of an inhibitor or an equal volume of the solvent for the inhibitor, and then unlabeled adenine nucleotide or analog was rapidly mixed in. The total volume of each sample (contained in 400-μl Microfuge tubes) varied between 0.37 and 0.39 ml; the volume was kept constant in any single experiment. After exactly 2 min (10 min in some experiments), the mitochondria were sedimented by centrifugation on the Beckman Microfuge, and the radioactivity which had appeared in the supernatant solution was measured. The efflux independent of added nucleotide, or "leakage" (Pfaff and Klingenberg, 1968), was determined in control samples. Leakage was decreased only 0–5% by concentrations of bongkrekic acid which completely inhibited nucleotide-dependent efflux, or "exchange," and this variability was allowed for in the calculation of "per cent exchange activity" (Pfaff and Klingenberg, 1968). The values of exchange activity obtained do not represent a quantitative kinetic treatment (see Pfaff *et al.*, 1969; Duée and Vignais, 1969) since the rate of exchange was not necessarily linear during the interval of measurement, but the fixed time interval measurements gave the advantage of rapidity in comparing exchange activities with different inhibitors, and widely varying conditions of temperature, time, and concentrations of substrate, inhibitor, or protein.

Respiration of coupled mitochondria was assayed by measuring oxygen uptake exactly as described in Henderson and Lardy (1970a). Oxidative phosphorylation was assayed by the disappearance of P_i as a result of conversion into ATP and then incorporation into glucose-6-P; samples of mitochondria in 2-ml volumes containing 135 mM sucrose, 12.5 mM KCl, 10 mM triethanolamine-Cl, 4 mM $MgCl_2$, 12.5 mM glutamate, 50 mM glucose, and 6 mg of yeast hexokinase were shaken in erlenmeyer flasks maintained at 30° on a Dubnoff metabolic incubator. Also present were ADP and bongkrekic acid in amounts to be indicated in the figures. After a period of 10 min, 40 μmoles of Tris-P_i was added to each flask to initiate oxidative phosphorylation, and the P_i remaining after a further 10 min was measured after terminating the reaction with 5% trichloroacetic acid and centrifuging to remove protein.

Results

Specificity of the Inhibition with Respect to Different Adenine Nucleotides and Their Structural Analogs. Pfaff and Klingenberg (1968) and Duée and Vignais (1968, 1969) have determined the specificity of the adenine nucleotide translocase by measuring release of ^{14}C-labeled ADP from mitochondria in response to different added nucleotides. In excellent agreement with their results, Table I shows that ADP-ribose was inactive, deoxy-AMP and AMP were least effective, ADP was more effective than ATP, and each ADP analog was better than the corresponding ATP derivative. Bongkrekic acid prevented the exchange with all the effective nucleotides

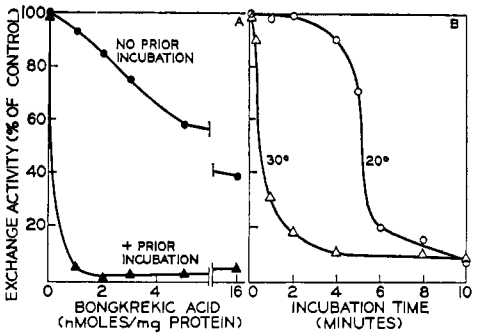

FIGURE 1: Influence of incubation period on inhibition of ADP–ADP exchange by bongkrekic acid. (A) Exchange activity was measured at the end of a 10-min exposure of 5.4 mg of mitochondrial protein to 300 nmoles of ADP and the amounts of bongkrekic acid indicated. In the lower curve the antibiotic was added to the mitochondria 10 min before the ADP; in the upper curve the mitochondria were added to the already mixed antibiotic and ADP; temperature, 23°. (B) Mitochondrial protein (2.7 mg) was exposed to 2.0 nmoles of bongkrekic acid for the indicated time before addition of 300 nmoles of ADP, and the exchange activity in the subsequent 2 min was measured. In both parts A and B corrections were applied for leakage of adenine nucleotide.

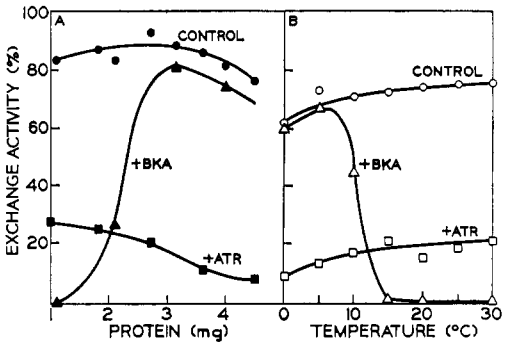

FIGURE 2: Depression of bongkrekic acid inhibition by increased protein concentrations or decreased temperatures. (A) The indicated amounts of mitochondrial protein were incubated for 10 min at 24° with 1.1 nmoles of bongkrekic acid (BKA) or 200 nmoles of atractyloside (ATR), and in controls; 300 nmoles of ADP was then added to all except the leakage controls, and the exchange activity was measured over a 2-min period. (B) Conditions similar to part A except that 20 nmoles of bongkrekic acid and 150 nmoles of atractyloside were incubated with 5.4 mg of mitochondrial protein at each temperature.

(Table I) and in this way resembles atractyloside (Duée and Vignais, 1968, 1969).

Dependence of Bongkrekic Acid Inhibition on Incubation Period. Figure 1A depicts dose–response curves for the inhibition of ADP–ADP exchange by bongkrekic acid. When the mitochondria containing ^{14}C-labeled ADP were incubated with the antibiotic for 10 min before addition of unlabeled ADP, 1 nmole of bongkrekic acid/mg of protein completely prevented nucleotide exchange, whereas 16 nmoles/mg was necessary to achieve only 60% inhibition when the mitochondria were exposed to ADP and bongkrekic acid simultaneously. It should be noted that in the absence of inhibitor the ADP–ADP exchange is 70–80% completed 2 min after addition of the ADP. The effect of varying the period of exposure to the inhibitor is shown in Figure 1B; at 20° a lag period of about 3 min was observed before an inhibition became apparent, and a nearly maximal extent was achieved within 10 min. Raising the temperature accelerated the appearance of inhibition (Figure 1B).

These results conform to the lag period observed also for bongkrekic acid inhibition of mitochondrial substrate level phosphorylation (Henderson and Lardy, 1970a), oxidative phosphorylation, and ATPase activities (unpublished observations). They also resolve the discrepancy between the 50–60 nmoles of bongkrekic acid/mg of protein necessary to prevent uptake of ADP or ATP into mitochondria, and the inhibition of oxidative phosphorylation by less than 1 nmole/mg, since the uptake assay was performed without prior incubation with inhibitor, and the oxidative phosphorylation assay employed a 10-min incubation period (Henderson and Lardy, 1970a). Similar low concentrations of bongkrekic acid (0.9 nmole/mg) completely inhibited the ATPase reaction when the inhibitor was incubated with the mitochondria before addition of ATP, in contrast to the 10–15-fold greater quantity previously reported to be necessary in assays omitting the preliminary incubation period (Henderson and Lardy, 1970a,b).

In a number of ADP–ADP-, ATP–ATP-, and ADP–ATP-exchange studies at 23°, 0.7–1.1 nmoles of bongkrekic acid/mg of protein was sufficient for complete inhibition with a 10-min incubation before nucleotide addition, whereas 50–70 nmoles/mg was required without the prior incubation period. By contrast, the extent of inhibition by a fixed concentration of atractyloside in identical exchange experiments was not augmented by prior incubation with the mitochondria, and atractyloside inhibition of adenine nucleotide exchange, oxidative phosphorylation, and ATPase reactions appeared to be instantaneous (see also Heldt, 1969).

Dependence on Mitochondrial Protein Concentration and Temperature. Early we observed a considerable variation in the concentration of bongkrekic acid required to inhibit different preparations of rat liver mitochondria. This variability disappeared when the amount of antibiotic was related to the concentration of mitochondrial protein. Thus, in the ADP–ADP-exchange reaction, 1.1 nmoles of bongkrekic acid (2.8 μM) caused complete inhibition in a system containing 1 mg of protein, but was ineffective with 3 or more mg of protein (Figure 2A). Consequently, it is more useful to express dose–response curves in moles inhibitor per milligram protein than moles per liter (*e.g.*, Figure 1A, also Henderson and Lardy, 1970a,b). Under the same conditions the extent of inhibition exhibited by a fixed concentration of atractyloside was relatively unaffected by changing the protein concentration (Figure 2A).

It was previously noted that bongkrekic acid is a less effective inhibitor of adenine nucleotide uptake into mitochondria at low temperatures (Henderson and Lardy, 1970a). For example, Figure 2B shows that 3.7 nmoles of bongkrekic acid/mg of protein inhibited ADP–ADP exchange at 15° and above, but was completely ineffective below 10°, despite the use of a 10-min incubation period. Other experiments have revealed that the inactivity is due to both an extension of the necessary incubation period (Figure 1B) and a requirement for higher bongkrekic acid concentration (Table I). The inhibition by atractyloside was independent of the temperature variable (Figure 2B).

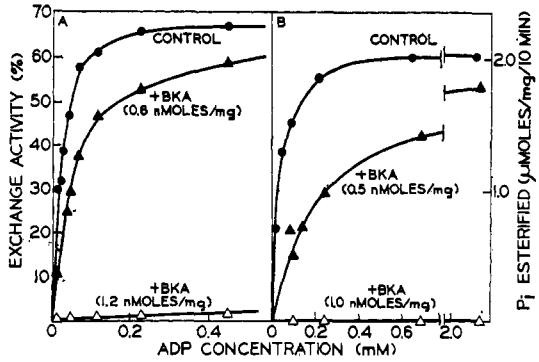

FIGURE 3: Effect of changing ADP concentration on the inhibition of ADP–ADP exchange and oxidative phosphorylation by bongkrekic acid. (A) After a 10-min preincubation of 3.5 mg of protein with 2.1 nmoles of bongkrekic acid, ADP was added to the indicated concentration and the exchange occurring in 2 min measured; temperature 24°. (B) Protein (9.5 mg) was incubated with bongkrekic acid and ADP as indicated for 10 min, P_i was added, and the esterification occurring in the next 10 min was measured; temperature 30°.

Ability of ADP to Overcome Partially the Inhibition by Bongkrekic Acid. In Figure 3A it can be seen that a concentration of bongkrekic acid (0.6 nmole/mg) causing about 50% inactivation of ADP–ADP exchange at 20 μM ADP produced only 10% inactivation at 450 μM ADP. Similarly, in measurements of the rate of oxidative phosphorylation (Figure 3B), a progressive increase in ADP concentration markedly reduced the potency of the inhibitor. However, in both experiments a twofold increase in bongkrekic acid concentration increased the extent of inhibition beyond the point where it could be diminished by the same increase in ADP concentration.

This partially competitive nature of the ADP antagonism is more clearly illustrated in Figure 4, which contains plots of reciprocal rates of oxidative phosphorylation measured at fixed concentrations of ADP and variable concentrations of bongkrekic acid. It should be mentioned that the mitochondria were incubated with the antibiotic for 10 min at 30° before initiation of the reaction by addition of ADP, and that the subsequent rates of oxygen uptake were linear for at least 25 min. It may be seen that, at each ADP concentration, an increase in bongkrekic acid concentration at first caused a reduction in velocity that indicated a reasonably linear relationship between reciprocal velocity and inhibitor concentration, as required by a Michaelis–Menten-type equation (Dixon, 1953); also, the slopes of the lines were inversely proportional to the concentration of ADP, consistent with a competitive type of interaction. However, at each ADP concentration, the slope of each line started to increase at a critical bongkrekic acid concentration. The new slopes are verified by additional data points in the reciprocal velocity range of 150–500, which are not shown. This may reflect an allosteric phenomenon, as suggested before (Henderson and Lardy, 1970a), but alternative explanations will be considered in the discussion section. Meanwhile the apparent K_i value of 1.3 μM (Figure 4) should be regarded with caution.

At the higher ADP concentrations, a second anomaly became apparent in the form of a small, but reproducible activation of the system as the bongkrekic acid concentration was

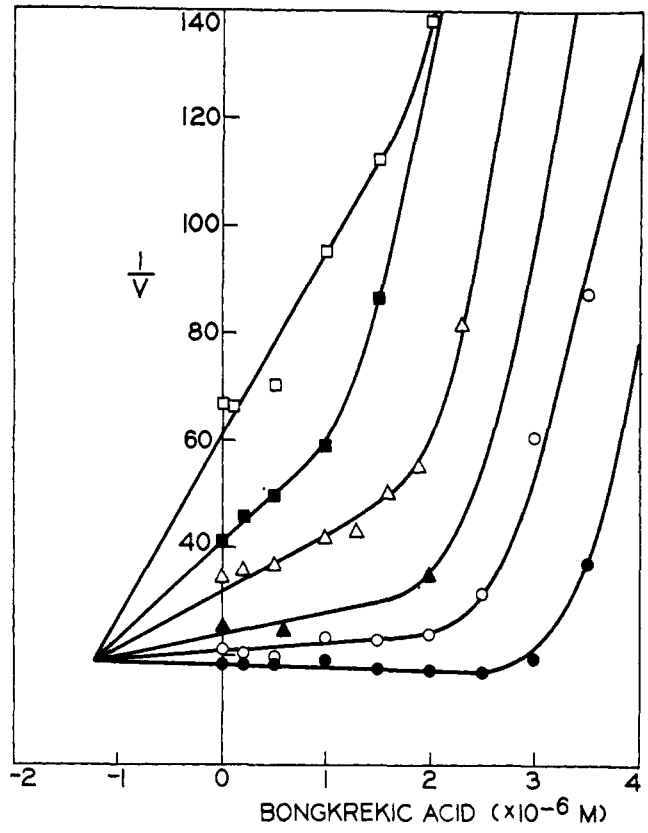

FIGURE 4: Dixon plot of inhibition of coupled respiration by bongkrekic acid. (□) 0.023 mM ADP, (■) 0.043 mM ADP, (△) 0.090 mM ADP, (▲) 1.89 mM ADP, (○) 4.10 mM ADP, and (●) 9.00 mM ADP. The assay measured oxygen uptake at 30° (method in Henderson and Lardy, 1970a, Figure 8) expressed as μatoms of O_2 (min x mg of protein)$^{-1}$ and corrected for the rate observed without added ADP

increased. This is most clearly seen in the tendency of the line at 10 μM ADP to slope *downward*, exceeding the expected limiting case where ADP completely overcomes the inhibition by bongkrekic acid and the line is horizontal.

Discussion

Bongkrekic acid had very little effect on the nonspecific "leakage" of ADP or ATP from mitochondria at concentrations of the antibiotic (0.7–1.1 nmoles/mg of protein) that totally inhibited the efflux dependent on added ADP, ATP, or their structural analogs. This exchange activity has been elegantly assigned to an adenine nucleotide translocase (reviewed by Klingenberg and Pfaff, 1968; Klingenberg, 1970), so these experiments confirm that bongkrekic acid, like atractyloside, is a specific inhibitor of this enzyme. After a preliminary communication of our results (Lardy, 1969), they have been confirmed by Kemp (1970) and Klingenberg *et al.* (1970a,b). The inactivation of mitochondrial oxidative phosphorylation, ATPase and other reactions requiring adenine nucleotide is clearly a consequence of the translocase inhibition, since given amounts of bongkrekic acid inhibit all these activities equally, provided that a sufficient prior incubation with the antibiotic is carried out.

The dependence of bongkrekic acid inhibition on time of

incubation, temperature, and protein concentration—observed also by Klingenberg and coworkers (1970a)—differentiates it from atractyloside (Figure 2; *cf.* Heldt, 1969). The temperature and time relationship may indicate an energy requirement for combination of the first inhibitor with the translocase, so that, with its independence of these parameters, atractyloside could be regarded as the more "efficient" inhibitor. Yet our results (Henderson and Lardy, 1970a,b) indicate that, when compared on a concentration basis, bongkrekic acid is usually more potent provided that a sufficient time interval is allowed for the temperature at which the experiment is conducted. The best quantitative basis for comparison of inhibitors is estimation of their respective K_i values, but it is difficult for bongkrekic acid since the apparent K_i is a function of protein concentration (unpublished observations). This indicates that the dissociation constant of the enzyme–inhibitor complex is well below the concentration of enzyme used in the experimental system, so that the inhibitor is virtually titrating out the enzyme molecules. However, if rat liver mitochondria contain 0.18 μmole of translocase/g of protein (Klingenberg *et al.*, 1970a), the enzyme concentration would be 0.72–0.95 μM in these experiments, whereas about 1.5 μM bongkrekic acid was required for 50% inhibition at the lowest ADP concentrations and the apparent K_i was 1.3 μM. Klingenberg *et al.* also noted that the amount of bongkrekic acid required for inhibition was in excess of the expected number of translocase sites. These points are being investigated further.

Another important factor to be considered is that the major fraction of the inhibition by bongkrekic acid is noncompetitive with respect to adenine nucleotides (*e.g.*, Figure 4), whereas inhibition by atractyloside is totally competitive (Bruni, 1966; Vignais, 1969; Weidemann *et al.*, 1969, 1970; but see Winkler and Lehninger, 1968). Thus, at low nucleotide concentrations, atractyloside approaches the potency of bongkrekic acid (Henderson and Lardy, 1970a). This difference accords with the report of Weidemann *et al.* (1970) that atractyloside *decreases* the affinity of the adenine nucleotide translocase for ADP, whereas bongkrekic acid *increases* the affinity of the enzyme for ADP. Nevertheless, at concentrations of bongkrekic acid causing low fractional inhibition, a competitive effect of ADP became apparent, and this has been confirmed in other unpublished experiments. It may be that the translocase enzyme itself is interacting in a competitive fashion with ADP and low concentrations of bongkrekic acid, as supported by the experiment of Figure 3A, but a number of other explanations are possible for the competitive phase of inhibition of oxidative phosphorylation. For example, the translocase may not be rate limiting under the assay conditions, but an impairment of its activity could still alter the balance of internal ATP and ADP, causing a competitive inhibition of the rate-limiting phosphorylation reactions, since ADP and ATP appear to interact competitively with these enzymes (Mitchell and Moyle, 1970). A kinetic study of these possibilities is presently in progress.

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