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EFFECTS OF GRAMINE ON ENERGY METABOLISM OF RAT AND BOVINE MITOCHONDRIA

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Abstract—The indole alkaloid gramine is found in several plant families. Its effects on mammalian mitochondria and submitochondrial particles were studied. Low concentrations of gramine slightly stimulated basal electron transport, totally inhibited the Ca^{2+}-induced respiratory control and partially abolished the enhancement of 8-anilino-1-naphthalene sulfonate fluorescence induced by proton translocation coupled to ATP hydrolysis or to succinate oxidation. At higher concentrations gramine inhibited specifically the electron transport at the level of Complex I of the respiratory chain. The I_{50} values (2–6 mM) were dependent on the presence of uncouplers. Higher concentrations of the alkaloid also inhibited coupled succinate oxidation and ATP hydrolysis (I_{50} = 10 mM). Possible explanations for these effects are discussed.

It has been postulated that alkaloids, products of secondary plant metabolism, can act as allelochemical agents playing an important role in the interaction between different plant species or in the mechanism of defense against plant pathogens or predators [1, 2]. The biochemical basis of this action is, with few exceptions, not well known. One of the enzymic systems possibly affected by allelochemical agents is cellular energy metabolism [3].

Several indole alkaloids isolated from fodder plants belonging to Gramineae, Leguminoseae and other plant families [4] have been shown to produce acute toxicity or death in ruminants [5, 6]. This toxic action has been postulated to be due to the known physiological effects of tryptamine-related compounds [7].

In this paper we examine the effects of 3-(*N*,*N*-dimethyl-aminomethyl)-indole (gramine) on rat liver mitochondria and bovine heart submitochondrial particles and discuss possible molecular modes of its action.

MATERIALS AND METHODS

Rat liver [8] and heavy bovine heart [9] mitochondria were prepared as described. Submitochondrial particles (Mg^{2+}-ATP particles) were prepared from heavy bovine heart mitochondria as described [10].

Electron transport. Electron transport in RLM† (1.5 to 2.5 mg protein) was measured following oxygen consumption with a Clark electrode connected to a Gilson oxygraph in a reaction medium containing 250 mM sucrose, 30 mM KCl, 6 mM $MgCl_2$, 1 mM EDTA, 10 mM KH_2PO_4 and 25 mM Tris–HCl, pH 7.4. In submitochondrial particles (SMP), electron transport was measured as above in a medium containing 180 mM sucrose and 25 mM Tris–HCl, pH 7.5.

ATPase activity in SMP. SMP (60 μg protein) were added to a reaction medium (1 ml) containing 180 mM sucrose, 6 mM $MgCl_2$, 2 μM FCCP and 50 mM Tris–HCl, pH 7.5. After 5 min of preincubation, the reaction was started by adding ATP (4 mM final concentration). The reaction was stopped 5 min later with 0.05 ml of 100% trichloroacetic acid. After centrifugation (10 min, 3000 rpm) aliquots were withdrawn from the supernatant fraction and analyzed for inorganic phosphate according to Sumner [11].

Oxidative phosphorylation in SMP. The reaction was measured in a medium containing (final concentrations) 180 mM sucrose, 1 mM $MgCl_2$, 10 mM succinate, 0.5 mM EDTA, 3 μM rotenone, 50 mM glucose, 2 mM ATP, 5 I.U. of yeast hexokinase (EC 2.7.1.1) and 50 mM Tris–HCl, pH 7.4. Particles (0.38 mg protein) were added to this medium and incubated for 5 min. ATP synthesis was started by adding 5 μmoles KH_2PO_4, 2×10^6 cpm of carrier free $^{32}P_i$ and 3 μmoles $MgCl_2$. The final volume was 1 ml. The preincubation and the reaction were carried out under aerobiosis obtained with a gyratory water bath shaker. After 5 min the reaction was stopped and inorganic phosphate was quantitatively precipitated by the procedure of Sugino and Miyoshi [12] as modified by Roveri *et al.* [13]. The tubes were centrifuged for 10 min at 3000 rpm. Aliquots were analyzed for [^{32}P]glucose-6-phosphate by Cerenkov counting in a Beckman 8100 liquid scintillation counter.

Energy-linked 8-anilino 1-naphthalene sulfonate fluorescence enhancement. The measurements were carried out in a Perkin Elmer 650–40 fluorescence spectrophotometer. 8-Anilino-1-naphthalene sul-

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† Abbreviations: RLM, rat liver mitochondria; SMP, submitochondrial particles; DNP, 2,4-dinitrophenol; FCCP (carbonylcyanide *p*-trifluoromethoxyphenyl hydrazone); and ANS (8-anilino-1-naphthalene sulfonate).

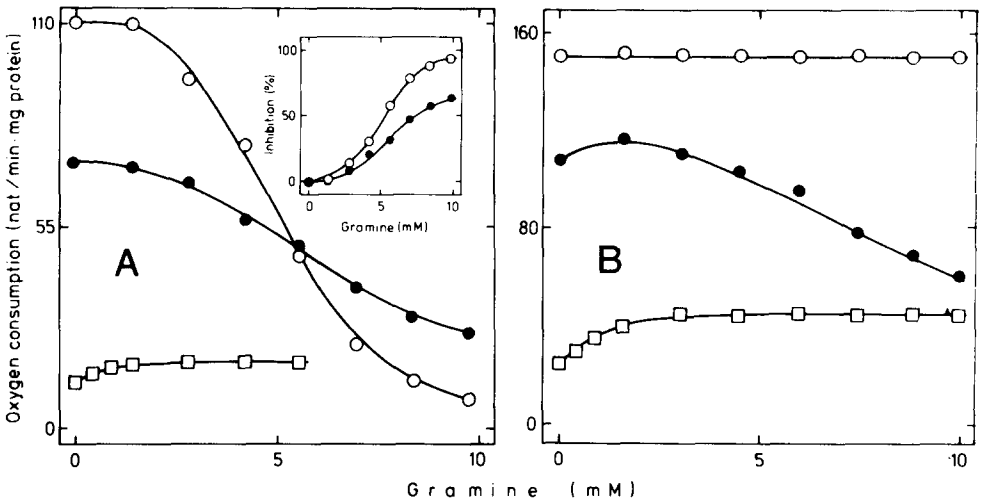

Fig. 1. Effect of gramine on basal, active and uncoupled electron transport in RLM. Basal electron transports (□) were obtained with malate/glutamate (10 mM each) (A) or 5 mM succinate (B). Active states were obtained by addition of 1.5 mM ADP (●) and uncoupled states by addition of 60 μM DNP (○). Other conditions are described in Materials and Methods.

fonate (ANS) fluorescence was excited at 380 nm and measured at 480 nm essentially as described by Ferguson *et al.* [14].

Protein determinations were carried out using a modified biuret procedure [15].

Gramine (obtained from Sigma Chemical Co.) was recrystallized once from acetone before use. Further recrystallizations did not change its behavior as a single compound as judged by u.v. spectra and by thin-layer chromatograms using different elution solvent mixtures. Gramine was dissolved in dimethyl sulfoxide prior to addition to RLM or SMP suspensions. Controls with the solvents (less than 2%) were performed for all the biochemical reactions studied. Measurements were carried out at 25°.

RESULTS

Oxidation of malate/glutamate by RLM was inhibited by gramine in both coupled (active) and uncoupled conditions (Fig. 1A). On the other hand, the oxidation of succinate in uncoupled conditions was not affected at all by gramine up to 10 mM (Fig. 1B). These results indicate that gramine inhibited the entry of malate/glutamate to the mitochondrion, the corresponding NAD-linked dehydrogenases, or

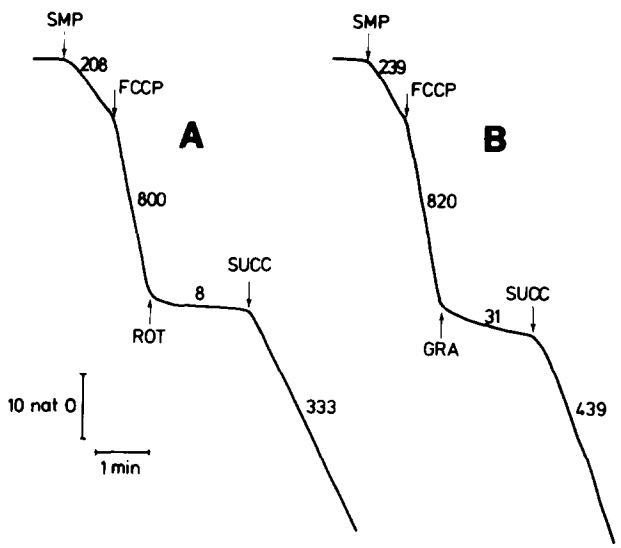

Fig. 2. Comparison of the effects of gramine and rotenone on electron transport in SMP from bovine heart. Basal electron transport was obtained by addition of SMP (0.5 mg protein) to the reaction medium containing 1 mM NADH. The uncoupled state was obtained by addition of 2.5 μM FCCP. Other conditions were: ROT 3.5 μM rotenone; GRA 9 mM gramine; and SUCC 10 mM succinate. Numerals on the slopes represent oxygen consumption in nat oxygen/min/mg protein.

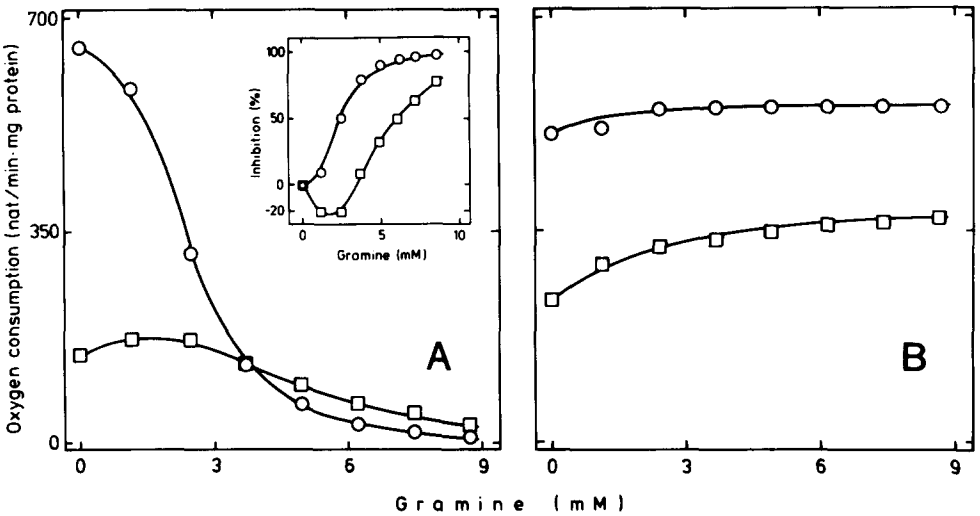

Fig. 3. Effect of gramine on electron transport in SMP. Electron transport in SMP (0.5 mg protein) was measured following oxygen consumption as described in Materials and Methods. Substrates were 1 mM NADH (A) or 10 mM succinate (B). In both cases, electron transport was determined in the basal state (□) or in the uncoupled state (○) obtained by addition of 2.5 μM FCCP.

the electron transport in Complex I of the respiratory chain. Figure 2 shows that the effect of gramine on electron transport in SMP was qualitatively similar to that produced by rotenone, a known inhibitor of Complex I (NADH-ubiquinone segment) of the respiratory chain. In addition, gramine inhibited electron transport in SMP only when NADH was used as substrate, without affecting the electron transport from succinate to oxygen (Fig. 3). These results strongly suggest that gramine inhibits electron transport specifically at the level of Complex I or NADH-ubiquinone oxidoreductase in the respiratory chain. The effect of gramine on the oxygen uptake rate was dependent on the presence of uncouplers. In RLM (see Fig. 1A, inset), the I_{50} values were 5 mM (in the presence of uncoupler) and 7 mM (in the absence of uncoupler). In SMP (Fig. 3A, inset) the differences in I_{50} values were even larger: 2 and 6 mM in the presence and in the absence of uncoupler respectively.

Several interesting effects were observed at gramine concentrations lower than those that inhibited the electron transport. Figures 1 and 3 show that gramine stimulated the electron transport rate in basal conditions in RLM and SMP. When malate/glutamate or NADH was used as substrate in RLM

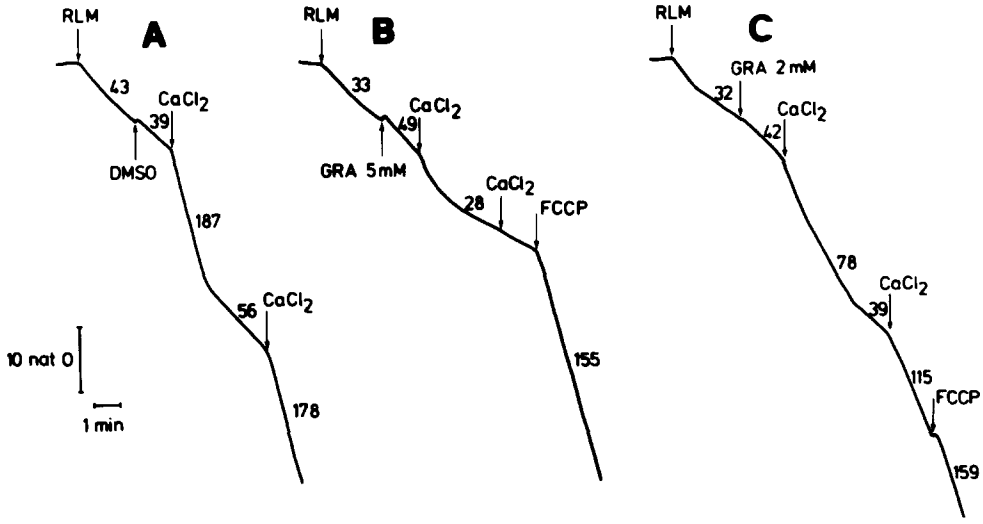

Fig. 4. Effect of gramine on the stimulation and uncoupling of mitochondrial respiration by Ca^{2+}. Oxygen uptake by RLM (1 mg protein) was determined in a medium containing 80 mM NaCl, 20 mM succinate, 2.5 mM P_i, 9 μM rotenone and 10 mM Tris–HCl, pH 7.4. Additions were: $CaCl_2$ (500 nmoles in each addition); DMSO, 7 μl; GRA, final concentrations as stated; and FCCP, 2.5 μM. Numerals on the slopes represent oxygen consumption in nat oxygen/min/mg protein.

and SMP, respectively, the stimulatory effect was overcome by the inhibitory effect produced at higher concentrations of gramine on Complex I (see Figs. 1A and 3A). When succinate was used as substrate, the stimulatory effect was seen more clearly since succinate oxidation is insensitive to gramine (Figs. 1B and 3B).

Ca^{2+} mitochondrial metabolism was also affected by low gramine concentrations. Addition of small amounts of Ca^{2+} to mitochondria that are oxidizing succinate in basal conditions induces a respiratory control which accompanies the energy-dependent accumulation of Ca^{2+} inside the mitochondria [16]. When Ca^{2+} was added at higher amounts, a large stimulation of the respiration was observed which did not return to the basal level (Fig. 4A). Gramine (5 mM) almost totally inhibited the respiratory control by low Ca^{2+} and completely suppressed the uncoupling effect by high Ca^{2+} amounts (Fig. 4B). At low gramine concentrations (2 mM), Ca^{2+} still induced a respiratory control which was similar in amplitude to that obtained in the absence of gramine, but the rate of Ca^{2+}-stimulated respiration was 60% inhibited by the alkaloid. A similar effect was produced by gramine on the Ca^{2+} uncoupled rate of oxygen uptake (Fig. 4C).

According to Mitchell's hypothesis, a protonmotive force is generated across the inner mitochondrial membrane due to proton translocation coupled to ATP hydrolysis or to electron transport. This leads to an "energization" of the membrane which can be followed by the changes of 8-anilino-1-naphthalene sulfonate (ANS) fluorescence. ANS fluorescence increases upon binding to submitochondrial particles. The energization of the inner mitochondrial membrane induces a further enhancement of ANS fluorescence. This energization can be produced by succinate oxidation, by ATP hydrolysis, or by a potassium diffusion potential (in the presence of valinomycin) [14]. Figure 5 shows the ANS fluorescence enhancement induced by the addition of ATP-Mg^{2+} to SMP under coupled conditions. The enhancement was reversed completely when a classical uncoupler such as FCCP was added. Gramine, when added after the maximal enhancement had been achieved, decreased the ANS fluorescence to a level that was intermediate between those corresponding to the energized and de-energized states. The addition of FCCP after gramine decreased the fluorescence to the basal de-energized level (Fig. 5B). The maximal effect of gramine was obtained at about 0.35 mM alkaloid. The ANS response was inhibited only up to 60% (see Fig. 5, inset). When gramine was added prior to ATP-Mg^{2+} (Fig. 5C), not only was the amplitude of the fluorescence enhancement diminished but its time course was also changed drastically. In the absence of gramine, the ANS fluorescence increased rather slowly whereas in its presence the increase was significantly faster. Similar results were obtained when SMP were energized by coupled succinate oxidation (data not shown).

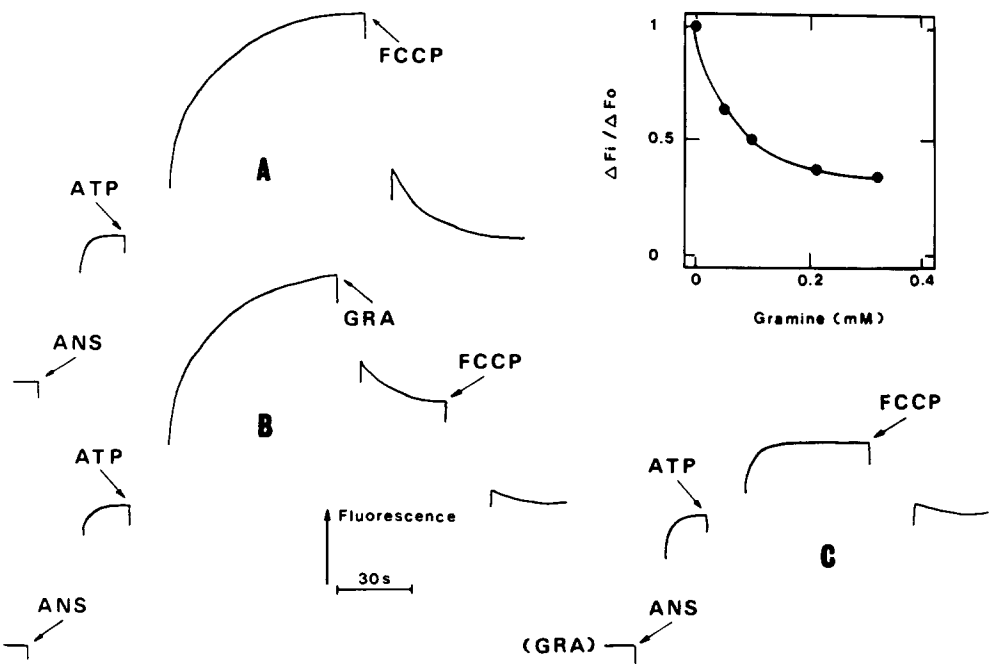

Fig. 5. Effect of gramine on ANS response. ANS fluorescence was measured as described under Materials and Methods. SMP (0.5 mg protein) were added to a reaction medium (1.5 ml) containing 180 mM sucrose and 25 mM Tris–HCl (pH 7.5). Additions were: ANS 5 μM; ATP (ATP-Mg^{2+}) 2 mM; FCCP, 2.5 μM; and GRA, 0.35 mM. In trace (C) gramine was present from the beginning. In the inset is shown the titration of the effect of gramine. On the ordinate is expressed the relative ANS fluorescence enhancement calculated as the ratio between the enhancement in the presence of gramine (ΔF_i) and the enhancement in the absence of the alkaloid (ΔF_o).

DISCUSSION

Gramine totally inhibited the oxygen uptake rate by SMP when the substrate was NADH. Since succinate oxidation is insensitive to gramine, the effect of the alkaloid is exerted at the level of Complex I of the respiratory chain. The potency of this effect depended on the presence of uncouplers. Not only was the relative inhibition produced by gramine increased by the addition of uncouplers (see Figs. 1A and 3A, insets) but also the absolute value of the oxygen uptake rate was diminished markedly by the combination of the alkaloid with the uncoupler. At low gramine concentrations DNP increased the rate of oxygen uptake without reaching the value obtained in the absence of gramine (Fig. 6, A and B). At higher concentrations of gramine (Fig. 6A), the addition of DNP inhibited the electron transport even more, clearly suggesting that the uncoupler potentiates the action of the alkaloid. Gramine is a tertiary amine and it is protonated at the pH values at which the experiments were carried out. The partition coefficient between an organic phase containing toluene and *n*-butanol (7:3) and an aqueous phase containing 25 mM Tris–HCl (pH 7.5) was equal to 0.6. This partition coefficient increased to 1.5 when DNP was added in a 1:1 molar ratio with respect to gramine. Thus, the uncoupler promotes the partition of the gramine into the organic phase. Green and Vande Zande [18] have reported previously that uncouplers are capable of transporting lysine into an organic phase. Therefore, it can be postulated that uncouplers potentiate the effect of gramine by increasing its effective concentration in the membrane in which the electron transport component affected by gramine is embedded. An alternative explanation for such potentiation is the possibility that the affinity of the electron transport component for gramine depends on the energy state of the membrane, which is altered by the addition of uncouplers.

At low concentrations, gramine slightly stimulated basal electron transport (see Figs. 1 and 3). This effect differed from that produced by a classical uncoupler in that (i) the stimulation in all cases was much smaller than that produced by protonophoric uncouplers such as DNP and FCCP and (ii) the ADP/O ratio was not affected by gramine concentrations which produced maximal stimulation of basal respiration (Fig. 6C).

Low gramine concentrations (2–5 mM) almost totally inhibited the Ca^{2+}-induced respiratory control in RLM oxidizing succinate. This phenomenon cannot be explained by an effect on the electron transport chain, since the uncoupled electron transport was not altered by gramine (Fig. 5). The possibility that gramine can act as an uncoupler is also excluded, since the amplitude of the Ca^{2+}-induced respiratory control was not affected by alkaloid concentrations that inhibited the Ca^{2+}-stimulated oxygen consumption by 60%. It can also be excluded that the effect of gramine on Ca^{2+} metabolism could be produced by Ca^{2+} complexation since it was not possible to detect any decrease in the free ionic concentration

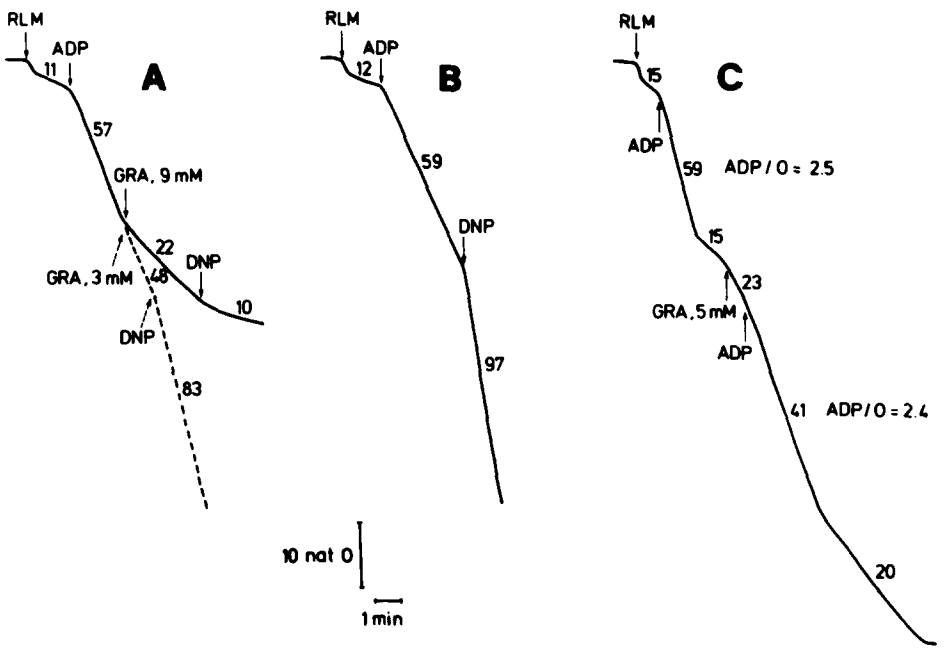

Fig. 6. Effects of gramine on the different metabolic states of RLM. Basal electron transport was obtained by addition of mitochondria to the reaction medium containing malate/glutamate (10 mM each) in the absence of phosphate acceptor. Coupled electron transport was obtained by addition of ADP and uncoupled electron transport by addition of DNP. Additions are as follows: ADP, 1.5 mM in A and B (final concentrations) or 400 nmoles in C (each addition); DNP, 60 μM; and GRA, final concentrations as stated. The final volume was 1.7 ml. Numerals on the slopes represent oxygen consumption in nat oxygen/min/mg protein. ADP/O ratios were calculated from the figure according to Chance and Williams [17]. Other conditions are described in Materials and Methods.

of Ca^{2+} by addition of gramine. The mitochondrial Ca^{2+} uptake is driven by the protonmotive force generated by the electron transport and is accompanied by H^+ efflux. The effect of gramine can be due to a direct effect on the Ca^{2+} transport system or to a secondary effect on the availability of H^+ inside the mitochondria. It has been pointed out above that gramine can partition into an organic phase at pH 7.5. Others have suggested that gramine should cross several permeability barriers very fast in order to exert its toxic action [5]. Moreover, the partition of gramine is favored by alkaline pH values, at which the alkaloid is deprotonated. Therefore, it is reasonable to suppose that the unprotonated form is the one that will be able to cross the membrane. Taking into account that the pH inside the mitochondria is more alkaline, the entry of gramine could result in an increased buffer capacity and concomitantly in a decrease in the availability of H^+ to be exchanged by Ca^{2+}.

The most striking effect of gramine is that produced on the ANS response. According to Ferguson *et al.* [14], the enhancement of ANS fluorescence driven by ATP hydrolysis or succinate oxidation is due to changes in the binding properties of the membrane induced by its energization rather than to direct monitoring of the electrical potential difference across the inner mitochondrial membrane. Nevertheless, ANS fluorescence can be used as an indicator of "energization" of the membrane. The addition of gramine reduced the extent of the ANS response to energization when the particles were energized by ATP-Mg^{2+} (Fig. 5) or by succinate. The maximal effect was obtained with 0.35 mM gramine which inhibited the ANS response by 60%. Gramine did not change either the quantum yield of ANS fluorescence or its emission spectra (data not shown) so a direct interaction between gramine and ANS can be reasonably ruled out. On the other hand, ANS fluorescence enhancement driven by a potassium diffusion potential induced by addition of KCl in the presence of valinomycin was not affected by gramine (now shown). According to Ferguson *et al.* [14], the potassium diffusion potential-driven ANS response can be due to monitoring membrane potential. Therefore, it should be possible to conclude that gramine does not affect the electrical component of the protonmotive force. But its effect on ANS response to energization by ATP-Mg^{2+} or succinate clearly indicates that it interferes with some other component related in some way with the protonmotive force. If gramine could cross, as it has been stated above, the membrane in its deprotonated form, it would tend to distribute according to the pH difference across the membrane and to collapse the Δ pH component of the protonmotive force (Δp). Since in mitochondria the major component of the Δp is the electrical potential difference ($\Delta\Psi$), in the absence of a permeable anion gramine could be able to collapse Δ pH by changing it in $\Delta\Psi$ without uncoupling oxidative phosphorylation. This rationale could explain the slight stimulus of basal respiration and the effect on ANS fluorescence enhancement. A prediction of such a hypothesis should be that, in chloroplasts, in the presence of chloride (a permeant anion for the thylakoid membrane), gramine should behave as a true uncoupler as NH_3 and other amines do [19]. A classical uncoupling effect on chloroplasts was observed by Andreo *et al.* [20].

Gramine also exerts other actions on mitochondrial energy metabolism. The coupled electron transport with succinate as substrate (see Fig. 1B) and the ATP hydrolysis by RLM and SMP (data not shown) were inhibited by high concentrations of gramine (I_{50} = 10 mM). These effects indicate that gramine can also behave as an energy transfer inhibitor, but this action is produced at such high alkaloid concentrations that its specificity and physiological relevance are doubtful.

In conclusion, gramine exerts several actions on mitochondrial energy metabolism. The most relevant of such effects are those produced on mitochondria Ca^{2+} metabolism and on the ANS response to energization. This last effect was observed at concentrations lower than 0.35 mM. In addition, the interference of the alkaloid with the mitochondrial metabolism of Ca^{2+} could result in effects on the regulation of cellular metabolism mediated by Ca^{2+} levels which have been postulated to be under mitochondrial control [21, 22]. The effect of the alkaloid on the energization of the inner mitochondrial membrane together with its effect on Ca^{2+} metabolism must be taken into account as an alternative mechanism to its toxic action.

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