COMPARATIVE EFFECTS OF IONOPHORES GRISORIXIN, ALBORIXIN AND TWO DERIVATIVES ON K^+ GLUTAMATE EFFLUX IN RAT LIVER MITOCHONDRIA

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(Received for publication February 26, 1979)

The alkali cations discrimination on a liquid membrane electrodes system, was determined for the carboxylic ionophores grisorixin, alborixin and two derivatives, dihydrogrisorixin and hexahydroalborixin. The two antibiotics exhibited a great preference for K^+. Dihydrogrisorixin again showed the selectivity curve of a carboxylic ionophore, but with a discrimination power lowered compared with grisorixin. Hexahydroalborixin had lost all the complexing properties of the natural molecule. The selectivity scales measured for cations, were directly correlated with the K^+ and glutamate effluxes measured in rat liver mitochondria. The chemical modifications of the natural structures of grisorixin and alborixin resulted in a drastic reduction of their ionophoric properties. The loss of K^+-glutamate might occur in two steps, the efflux of K^+ catalysed by the ionophores then causing a loss of negative charges in the form of glutamate.

Carboxylic ionophores of the nigericin group are a large and growing class of antibiotics[1], the biological properties of which have been recently reviewed[2]. They form neutral lipophilic complexes with IA or IIA cations which give them the ability to transport these ones through biological membranes, in such a way they induce an electroneutral exchange of cations for H^+. Grisorixin and alborixin are two members of this group isolated by us[3~4], which complex preferentially K^+.

These compounds are useful tools for studying bio-energetic processes which implicate cations and or/H^+ in their mechanism. Our interest is focused on the transport systems in mitochondria, especially phosphate and glutamate ones. We have shown that grisorixin is a powerful inhibitor of phosphate and glutamate entry in rat liver mitochondria[5]. There are two systems of glutamate transport, a glutamate-aspartate[6~8] exchange and a glutamate-H^+ co-transport which latter is sensitive to N-ethylmaleimide[9~13]. It has also been shown that glutamate-K^+ co-transport[14] and, more recently glutamate-Ca^{++} co-transport[15] is possible. Results are presented here concerning the effects of grisorixin, alborixin and two derivatives on the N-ethylmaleimide sensitive efflux of potassium glutamate with the same biological system. By using a similar way of investigation as ŌTAKE and collaborators[16] we discuss the structure-activity relationships which can be established between the complexing properties of these molecules and their mode of action across the internal membrane of mitochondria.

Materials and Methods

Chemicals

Grisorixin and alborixin were the stock samples in our laboratory. The action of sodium boro-

hydride upon these two molecules, in ethanolic solution, opened hemiacetalic cycles by a method described for monensin and nigericin.[17] As noted elsewhere, in relation to the dihydro-derivatives of A-204[18], the stereospecificity of the opening was not certain. The compounds were purified by thin layer chromatography on silica gel and their structure was confirmed by mass spectrometry.

Measurements of the cationic selectivity

The measurements on liquid membrane electrodes used the following galvanic cell: Ag, AgCl/ 0.01 M KCl in water//0.03 M ionophore in decan-1 O1//0.01 M MCl in water//decan-1 O1//0.01 M KCl in water/Ag, AgCl (M^+ = alkali cation). The electrodes used for our measurements were Orion liquid membrane electrode bodies (series 92) with RAHP millipore membranes. The liquid ion exchanger was a 0.03 M solution of the ionophore in 0.2 ml decan-1 O1. The reference electrode was of the same type with decan-1 O1 alone. This method has already been used for this type of ionophore[19,20].

Mitochondria

Rat liver mitochondria were isolated according to JOHNSON and LARDY[21] in 0.25 M sucrose, 2 mM Tris-HCl*, pH 7.4. The protein concentration was determined by the biuret method[22].

Measurements of swelling, K^+ and glutamate translocation in mitochondria

Mitochondrial swelling was followed by recording the absorbance change of the suspension at 546 nm using an Eppendorf photometer according to CHAPELL and CROFTS[23].

The potassium content of mitochondria was determined by atomic absorption with a Perkin Elmer spectrophotometer 420.

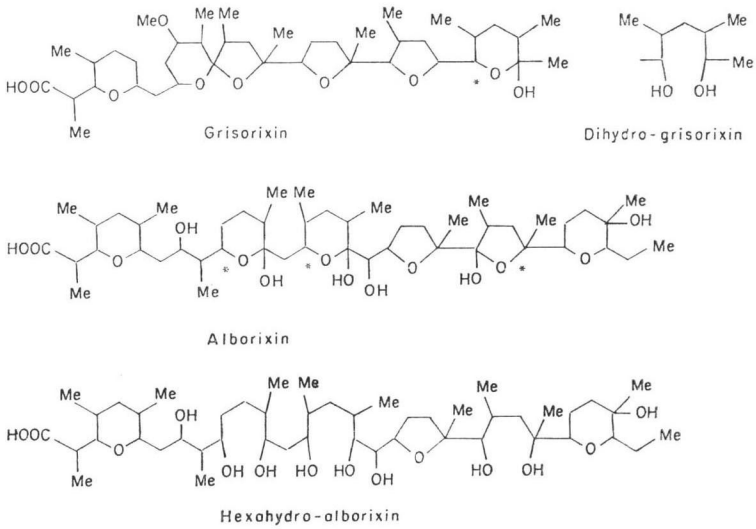
Glutamate movements were followed with (^{3}H)-glutamate. The technique used was derived from that of MEYER and VIGNAIS[10] and recently described in details[5].

Results and Discussion

1. Physicochemical Properties of the Molecules

Fig. 1 shows the structures of the compounds studied. The action of sodium borohydride resulted in the opening of the hemiacetalic cycles, giving rise to dihydro-derivatives. There was thus a little change in the primary structure. Nevertheless this report shows that the ionophorous properties are considerably altered.

Fig. 1. Chemical structures of the molecules under investigation.
* Indicates hemiacetal functions affected by the sodium borohydride reaction.



The e.m.f. measured from the galvanic cell described are plotted on Fig. 2. These values are indicatives of ionophores selectivities for alkali cations of different size.

For grisorixin, we have shown previously[20] that there was a satisfactory agreement between the selectivity scale obtained by

* Abbreviations: Tris, Tris (hydroxymethyl) aminomethane; HEPES, N-2-hydroxy-ethyl-piperazine-N'2 ethanesulfonic acid.

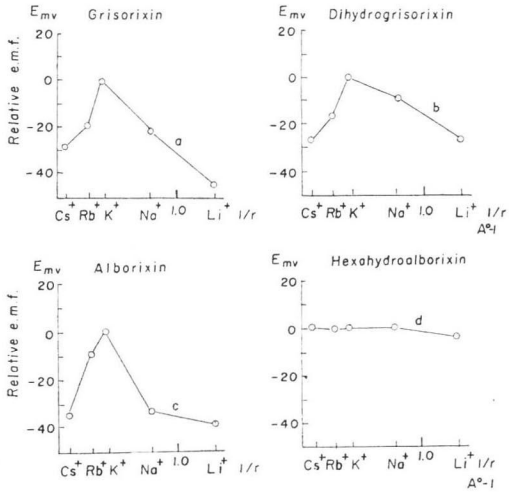
this method compared with the thermodynamic association constants of the grisorixin salts in methanol. In both cases we observed the order $K^+ > Rb^+ > Na^+ > Cs^+ > Li^+$, which means that this molecule is preferentially a potassium carrier.

Alborixin showed a similar cation binding sequence, although the C_{36} carbon backbone was larger than in grisorixin (C_{30})[1]. It seems likely that in solution as in the cristalline state[4] three intramolecular hydrogen bonds would increase the rigidity of the complexing cavity, increasing slightly the K^+/Na^+ selectivity measured.

The opening of three hemiacetal functions in alborixin, although creating three new hydroxyl groups, resulted in a hexahydro-derivative which had lost all the complexing properties of the natural molecule. This can be explained by the considerable loss of entropy in the open chain molecule compared with alborixin, where the six heterocycles are maintained in a well defined conformation, which is close to that one observed in the cristalline state.

Fig. 2. Selectivities for alkali metal cations.

Grisorixin (*a*), dihydrogrisorixin (*b*), alborixin (*c*) and hexahydroalborixin (*d*) from liquid membrane electrode measurements. E.m.f. versus the size of the cation (r=M.F.C. Ladd ionic radius). Owing to the symmetry of the galvanic cell described in methods, we have a direct representation of the affinities of cations, relatively to potassium for the ionophore under investigation. The e.m.f. are mean values for three runs; they are standardized from the zero value choosen for K^+. The e.m.f. between K^+ and the less associated cations is indicative of the discrimination power of the ionophore.



The dihydro-derivative of grisorixin, corresponding to the opening of the terminal hemiacetal again showed the selectivity curve of a carboxylic ionophore, but the difference between K^+ and the other cations was lowered compared to grisorixin. This can be attributed to the decreasing in the discrimination power of the ligand which depends on the thermodynamic association constants between cations and the ligand[20].

2. K^+-Glutamate Efflux in Rat Liver Mitochondria

The experiment of Fig. 3 shows the effects of the ionophores and their derivatives on K^+ efflux (Fig. 3A) and on volume changes of mitochondria; the latter was measured by the changes in absorbance (Fig. 3B). The experiment was carried out as follows: The swelling of mitochondria occurred upon the loading with K^+-glutamate after addition of valinomycin. Then, 1 minute 30 seconds after the addition of valinomycin, the contraction of mitochondrial volume in the presence of each of the ionophores and their derivatives was followed. The addition of grisorixin and of a alborixin induced a loss of about 170 natoms K^+/mg protein/30 sec (Fig. 3A, assays *c* and *d*). Dihydrogrisorixin caused a loss of about 45 natoms K^+/mg protein/30 sec (assay *e*). Hexahydroalborixin stabilized the K^+ concentration of the matrix at about 180 natoms K^+/mg protein (assay *f*). Although dihydrogrisorixin appeared to have more effect on K^+ efflux than hexahydroalborixin (this experiment was repeated three times and always gave the same results), the differences observed in their activities were too slight to be significant. Fig. 3B shows that grisorixin and alborixin induced a strong contraction of mitochondria (Fig. 3B, assays *c* and *d*). In contrast, the derivatives of these ionophores did not noticeably alter the

Fig. 3. Effects of ionophores and their derivatives on K^+ efflux, and on volume changes in rat liver mitochondria.

The incubation medium was composed of 200 mM sucrose, 20 mM Hepes-KOH buffer pH 7, 5 mM $MgCl_2$, 5 mM glutamate-Tris.

Mitochondria (2 mg protein) suspended in 1 ml of medium were incubated with valinomycin (50 ng/mg prot.). After 1 min. 30 sec. antibiotics (50 ng/mg prot.) were added as indicated and as follows: *c*) grisorixin, *d*) alborixin, *e*) dihydrogrisorixin, *f*) hexahydroalborixin, *a*) neither valinomycin nor carboxylic antibiotic were added, *b*) only valinomycin was added.

A: Reaction was stopped within 30 seconds at different times by rapid centrifugation and the potassium content of the pellet was determined in natoms per mg protein.

B: Swelling was monitored in the same conditions.

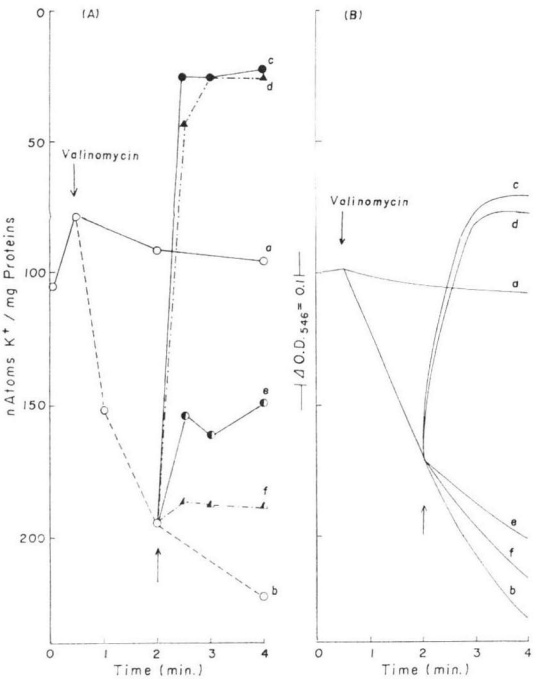
Experiments A and B were done on the same mitochondrial preparation.

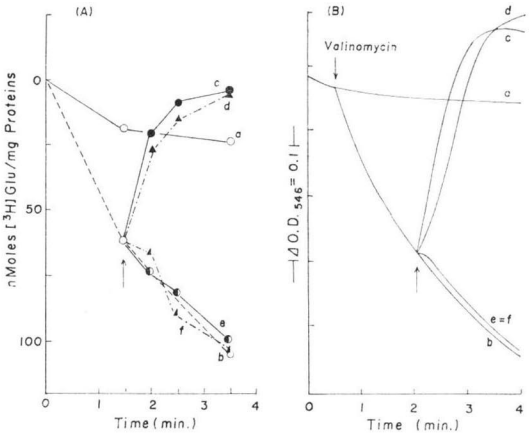
Fig. 4. Effects of ionophores and their derivatives on (^{3}H)-glutamate efflux and volume changes in rat liver mitochondria.

A: The incubation medium was the same as in Fig. 3 without glutamate. Mitochondria (2 mg protein) were preincubated in this medium (1 ml) with valinomycin (50 ng/mg prot.). Glutamate transport was initiated by adding (^{3}H)-glutamate (5 mM final concentration) and stopped within 30 sec. by rapid centrifugation at different times. The (^{3}H)-glutamate content of the pellet was determined in nmoles per mg prot. For more details about radioactivity measurements see Materials and Methods. As in Fig. 3 antibiotics were added 1 min. 30 sec. after the zero time of incorporation and as follows: *c*) grisorixin, *d*) alborixin, *e*) dihydrogrisorixin, *f*) hexahydroalborixin, *a*) neither valinomycin nor antibiotic, *b*) valinomycin only.

B: Swelling experimental conditions were the same as described in Fig. 3.

Experiments A and B were investigated on the same mitochondrial preparation.





absorbance measured in the presence of valinomycin alone (assays *e*, *f* and *b*).

Fig. 4A shows the effects of the ionophores on (^{3}H)-glutamate movements, measured in the same conditions as for K^+. Valinomycin addition, in the presence of K^+-glutamate, induced penetration of about 60 nmoles/mg protein/1 min. 30 sec. The addition of grisorixin or alborixin (assays *c* and *d*) resulted in an equivalent loss of (^{3}H)-glutamate. The derivatives were without effect on glutamate movement and the swelling-contraction cycle of the mitochondria (Fig. 4B).

In all these experiments, there was always a good agreement between the variations of absorbance and K^+- or (^{3}H)-glutamate movements. From these results, the following conclusions can be drawn:

(a) The chemical modification of the skeletons of grisorixin and alborixin by opening the hemi-

acetalic groups drastically reduces the ionophoric properties tested in rat liver mitochondria.

(b) Grisorixin and alborixin induce a much more rapid loss of K^+ than of glutamate. The quantity of K^+ lost is much greater than the quantity of glutamate, 2 minutes after the addition of the ionophores. There is no stoechiometry between glutamate and K^+ transport. In our experimental conditions the Glu/K^+ ratio is close to 0.5, either measuring K^+-glutamate influx induced by valinomycin or K^+-glutamate efflux induced by grisorixin or alborixin. HARRIS[24] found 0.22 for the same ratio using the former method. Our data show that the rate of K^+ efflux is much higher than that of glutamate efflux. It may be suggested that the loss of K^+-glutamate occurs in two steps: the efflux of K^+ catalysed by the ionophores consequently causes a loss of negative charges in the form of glutamate.

(c) The decreasing order of affinity for K^+ of dihydrogrisorixin and hexahydroalborixin (Fig. 3) correlates with the effect of these ionophores on K^+ efflux in rat liver mitochondria.

PRESSMAN stressed the importance of the turnover number of nigericin in mitochondria during H^+/K^+ exchange[25]. It seems likely that, in our case, the modification of natural structures results in a considerable reduction of this turnover number.

For the molecules under investigation, comparison of the thermodynamic values (ΔH, ΔS, ΔG, Kf) for the reaction of complexation in homogeneous solution should be of great interest and experiments are in progress.

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