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MAGNESIUM–PHENETHYLBIGUANIDE COMPETITION IN Mg^{2+}-DEPLETED HEPATIC MITOCHONDRIA

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Abstract—Guinea pig liver mitochondria 40–70 per cent depleted of magnesium are three times more sensitive to phenethylbiguanide inhibition of state 3 respiration than are comparable controls. Similar results are obtained whether the depletion is accomplished by the divalent cation ionophore A23187 or by incubation with inorganic phosphate. In contrast to controls, inhibition in Mg-depleted mitochondria is competitively reversed by Mg^{2+}. These phenomena are observed with NAD^+-linked substrates, stimulated by either ADP or Ca^{2+}, but not with succinate. The Mg-depleted mitochondria can be restored to the normal degree of phenethylbiguanide sensitivity by incubation with high levels of Mg^{2+} followed by washing. As in controls, phenethylbiguanide inhibition in Mg-repleted mitochondria is not reversed by Mg^{2+}. These results suggest a dual action of Mg^{2+}: (1) a structural role of bound magnesium which regulates the activity of cation transporters, and (2) competition between Mg^{2+} in solution and guanidinium derivatives for a cation carrier.

One approach to investigating the mode of action of hypoglycemic drugs related to guanidine has been the study of the mechanism of their uptake into mitochondria. Pressman and Park [1] demonstrated competition between guanidine and Mg^{2+} uptake into rat liver mitochondria. Subsequently, Davidoff [2] showed that Mg^{2+} also prevented phenethylbiguanide uptake into guinea pig heart mitochondria. Exploring this relationship further, we have been unable to demonstrate Mg^{2+}-biguanide competition in intact guinea pig liver mitochondria. However, as we show in the present report, Mg^{2+} depletion of these mitochondria, either by exposure to the divalent cation ionophore A23187 or by contact with P_i, enhances the inhibitory potency of phenethylbiguanide several-fold. Furthermore, the presence of Mg^{2+} in the medium during exposure of Mg^{2+}-depleted mitochondria to phenethylbiguanide competitively protects against respiratory inhibition by the biguanide. Once Mg^{2+} has been restored to the mitochondria, however, they again revert to their original state of insensitivity to biguanide inhibition and the competitive effect of Mg^{2+} on biguanide inhibition is lost.

MATERIALS AND METHODS

Guinea pig liver mitochondria were prepared as described previously [3]; only the initial homogenization buffer contained 0.5 mM EDTA. All sugar solutions were deionized prior to use. O_2 uptake was measured polarigraphically using a Clark platinum electrode in a Gilson oxygraph. For measurement of Mg^{2+} content, mitochondria were suspended at a concentration of 12 mg protein/ml in medium consisting of either (a) 0.28 M sucrose, 0.010 M *N*-2-hydroxyethylpiperazine-*N*'-2-ethane sulfonic acid (HEPES), pH 7.4, 0.005 M glutamate, and 0.5% bovine serum albumin, for ionophore experiments; or (b) 0.1 M sucrose, 0.075 M KCl, 0.015 M HEPES, pH 7.4, and 0.5% fatty acid-free bovine serum albumin, for P_i experiments. The mitochondria were then washed once in sucrose–HEPES and finally resuspended in distilled water. Mg^{2+} content was assayed by atomic absorption using a Perkin–Elmer spectrophotometer, model 290B.

RESULTS

Mg^{2+} content of control vs ionophore or P_i-treated mitochondria

Control mitochondria contained 26.3 ± 0.7 nmoles Mg^{2+}/mg of protein (mean ± S.E.M., $N = 5$); after exposure to 0.2 nmole A23187/mg of protein for 10 min at 30°, followed by the washing procedure, the Mg^{2+} content dropped to 7.6 ± 0.9 nmoles/mg of protein ($N = 2$); treatment with 2.5 mM P_i produced a smaller drop in Mg^{2+} content, to 16.1 ± 1.0 nmoles/mg of protein ($N = 3$). These changes are comparable to those obtained by Reed and Lardy [4] with ionophore, and Höser and Dargel [5] with P_i.

After the first wash, mitochondria were also re-exposed to 6mM Mg^{2+} for 5 min at 30°, then washed again and analyzed. After such re-exposure to Mg^{2+}, the Mg^{2+} content of non-depleted, control mitochondria increased to 38.3 nmoles/mg of protein, while that of P_i-treated mitochondria rose to 33.2 nmoles/mg.

Phenethylbiguanide inhibition of control and ionophore-treated mitochondria

Substrate specificity. Respiratory control was related to the degree of Mg^{2+} depletion. Thus, control was well preserved in mitochondria exposed to 0.1 nmole A23187/mg of protein (Table 1) while at 0.2 nmole ionophore/mg of protein an approximately 2-fold increase in state 2 rate was observed. With the smaller degree of Mg^{2+} depletion obtained with P_i (Table 2), respiratory control was also

Table 1. Phenethylbiguanide (PEBG) inhibition of respiration: effects of prior exposure to A23187 and presence of PEBG or Mg^{2+} in assay*

	Oxygen uptake rate									
	Glutamate-malate					Succinate				
	Control		[A23187]			Control		[A23187]		
PEBG	0	+	0	+	+	0	+	0	+	+
Mg^{2+}, 1.5 mM	0	0	0	0	+	0	0	0	0	+
State	(10^{-9} atoms O/min/mg protein)									
2	8	8	8	10	10	17	15	17	17	17
3	28	22	27	18	23	42	28	38	25	25
4	10	10	10	12	12	18	17	20	17	17
Δ3–4	18	12	17	7	12	23	12	18	8	8
Per cent inhibition		36		60	30		50		54	54
3_{Ca}	30	23	32	20	27					
4_{Ca}	8	8	10	10	10					
Δ3–4	32	15	22	10	17					
Per cent inhibition		31		54	23					

* Mitochondria were prepared by incubation in sucrose–HEPES–glutamate BSA medium either without or with 0.1 nmole A23187/mg of protein, as described in text under "Mg^{2+} content" and finally washed twice and suspended in sucrose–HEPES at 0°. Control mitochondria were put through the same procedure, only omitting the ionophore. Mitochondria were then preincubated without or with PEBG (0.15 or 0.45 mM in glutamate or succinate experiments respectively) and/or Mg^{2+}, as indicated, for 5 min at 30° prior to the O_2 uptake assay. Medium for O_2 uptake assay contained 120 mM KCl, 20 mM HEPES, pH 7.2, and either 5 mM glutamate–5 mM malate–0.5 mM malonate or 10 mM succinate–2 μM rotenone. O_2 uptake was measured at 30° in a 1.5 ml of oxygraph cell with 7 mg protein. After the state 2 rate was established, respiration was stimulated by adding 167 μM ADP + 167 μM P_i; 333 μM $CaCl_2$ was added where indicated. Respiratory state was as defined by Ref. 6.

unaffected. After pretreatment with either level of ionophore, the maximal ADP + P_i- or Ca^{2+}-stimulated respiratory rate was usually slightly lower than in untreated mitochondria.

Respiration with NAD^+-linked substrate (glutamate-malate) was consistently more sensitive to phenethylbiguanide inhibition in ionophore-treated mitochondria than in untreated controls (Table 1 and Fig. 1A), whether the respiratory stimulus was ADP + P_i or Ca^{2+}. Thus, in mitochondria depleted of Mg^{2+} by exposure to 0.2 nmole ionophore/mg of protein, 50 per cent inhibition of glutamate-malate oxidation occurred at 101 ± 13 μM phenethylbiguanide (N = 5), while controls required 300 ± 29 μM biguanide (N = 3; see also Ref. 7) for the same degree of inhibition. As noted below, with

Table 2. Phenethylbiguanide (PEBG) inhibition of respiration: effects of prior exposure to P_i or Mg^{2+}, and presence of PEBG or Mg^{2+} in assay*

Additions during assay pre-incubation	Oxygen uptake rate															
	Control				Exposed to P_i				Control, exposed to Mg^{2+}				Exposed to P_i followed by Mg^{2+}			
Mg^{2+}, 6 mM	0	+	0	+	0	+	0	+	0	+	0	+	0	+	0	+
PEBG, 0.15 mM	0	0	+	+	0	0	+	+	0	0	+	+	0	0	+	+
State	(10^{-9} atoms O/min/mg protein)															
2	7	7	7	7	8	7	8	7	7	7	7	7	7	7	7	7
3_{Ca}	40	32	28	23	50	38	27	37	37	28	27	22	42	35	30	27
$\Delta 3_{Ca-2}$	33	25	22	17	42	32	18	30	30	22	20	15	35	28	23	20
Per cent inhibition			35	33			56	5			33	31			33	29

* Mitochondria were depleted of Mg^{2+} by incubation in sucrose–KCl–HEPES–albumin medium containing 2.5 mM P_i for 7 min at 30°, then washed as described in Materials and Methods; control mitochondria were exposed to the same procedure, omitting the P_i. For Mg^{2+} repletion, portions of control or treated mitochondria were incubated in sucrose–HEPES with 6 mM $MgCl_2$ for 5 min at 30°, then washed in sucrose–HEPES. Control, Mg^{2+}-depleted and Mg^{2+}-repleted mitochondria were then pre-incubated in oxygen uptake assay medium without or with Mg^{2+} and/or PEBG where indicated, as described in Table 1. They were then transferred to the oxygraph cell where O_2 uptake rates were measured with 333 μM Ca^{2+} as respiratory stimulus.

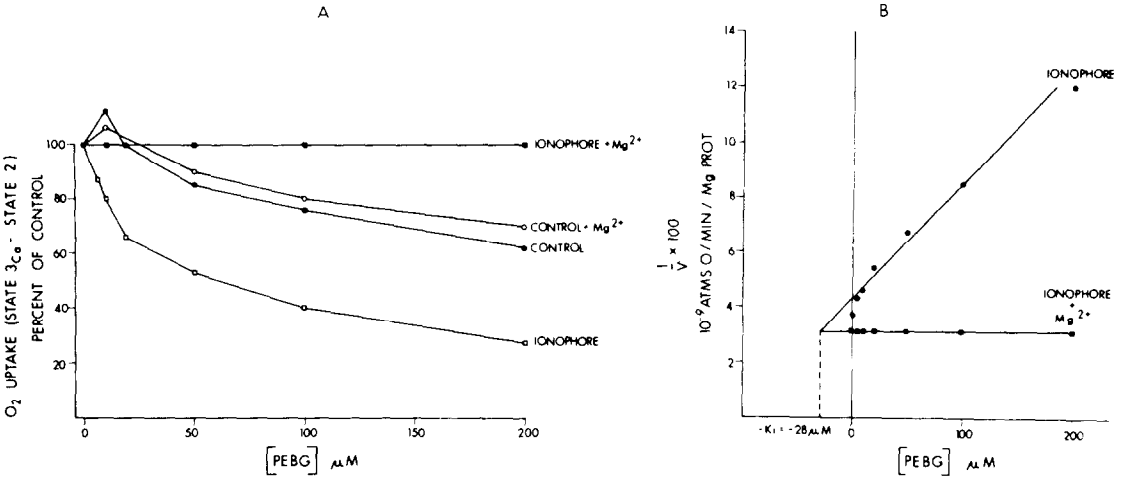

Fig. 1. Sensitivity of guinea pig liver mitochondria to phenethylbiguanide inhibition: effect of prior Mg^{2+} depletion by A23187 and presence of Mg^{2+} during subsequent exposure to phenethylbiguanide. (A) mitochondria were exposed to A23187 and washed as in Table 1 except that 0.2 nmole ionophore/mg of protein was used. After a 5-min preincubation with phenethylbiguanide and/or 5 mM Mg^{2+}, O_2 uptake rates were measured with the oxygen electrode using 333 μM Ca^{2+} as the respiratory stimulus. (B) data from the single experiment of panel A replotted according to Dixon [9]. Line drawn by method of least squares.

ionophore-treated mitochondria the K_i for phenethylbiguanide calculated from the Dixon plot was even lower (cf. Fig. 1B).

Guanidine, (+)-α-phenethylguanidine and octylguanidine all gave results qualitatively similar to those with phenethylbiguanide. It should be noted that Ca^{2+} stimulation of respiration responded in the same way to ionophore treatment of mitochondria whether the Ca^{2+} was added independently or after a cycle of ADP + P_i-stimulated respiration (Table 1).

The concentration of phenethylbiguanide required to inhibit succinate oxidation was approximately 3-fold greater than that required for comparable inhibition of glutamate-malate (Table 1; also compare Refs. 3 and 7). Mg^{2+} depletion was entirely without effect on the susceptibility of succinate oxidation to biguanide inhibition (Table 1).

It should be noted at this point that, after their exposure to ionophore, the mitochondria used in experiments such as those in Table 1 and Fig. 1 were washed in buffer containing no albumin. The effects of the ionophore, therefore, theoretically could have been due to the presence of residual ionophore tightly bound to the mitochondrial membrane. Subsequent experiments were performed using an albumin-containing wash, a procedure which has been shown to remove all membrane-bound ionophore [8]. These two types of experiment gave identical results (cf. Fig. 2), indicating that with either wash procedure the effects of ionophore exposure and subsequent Mg^{2+} addition (see section below) were due to Mg^{2+} depletion and repletion *per se*, and consequently independent of the presence of bound ionophore.

Effects of exogenous Mg^{2+}. Addition of Mg^{2+} to non-depleted mitochondria oxidizing glutamate-malate did not detectably alter respiratory control or antagonize phenethylbiguanide inhibition (Figs. 1 and 2). In contrast, Mg^{2+} added to mitochondria which were previously treated with 0.2 nmole ionophore/mg of protein reduced the elevated state 2 rate, restoring respiratory control toward normal,

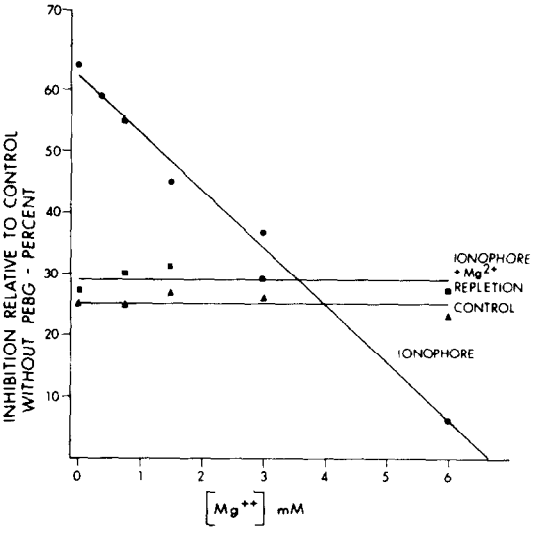

Fig. 2. Quantitative study of the effects of Mg^{2+} on intact, Mg^{2+}-depleted and Mg^{2+}-repleted mitochondria: phenethylbiguanide inhibition. Mitochondria were depleted of Mg^{2+} by exposure to ionophore as in Fig. 1, and then washed twice, once with sucrose–HEPES buffer containing 0.5% bovine serum albumin, and once with sucrose–HEPES alone. Mg^{2+} was restored to depleted mitochondria by incubating them in sucrose–HEPES containing 6 mM Mg^{2+} for 5 min at 30° and finally washing with sucrose–HEPES alone. Final pre-incubation was then carried out in oxygraph medium containing 0.15 mM phenethylbiguanide plus $MgCl_2$ in increasing concentrations as indicated for 5 min at 30° prior to O_2 uptake assay using 333 μM Ca^{2+} as respiratory stimulus. Results are expressed relative to individual control samples for each condition containing no phenethylbiguanide.

a further indication that the slight loss of respiratory control in mitochondria exposed to ionophore was a function of Mg^{2+} depletion *per se*. When Mg^{2+} was added to ionophore-treated mitochondria during their final pre-incubation with phenethylbiguanide, immediately preceding the O_2 uptake assay, the presence of Mg^{2+} appeared to protect strongly against respiratory inhibition (Fig. 1). The degree of protection was a function of Mg^{2+} concentration (Fig. 2): at Mg^{2+} concentrations of 5 mM or greater, ionophore-treated mitochondria were completely protected against inhibition by phenethylbiguanide at concentrations up to 200 μM (Figs. 1 and 2). This protective effect should be contrasted with the total lack of Mg^{2+} effect on intact mitochondria (see above), and on Mg^{2+}-repleted mitochondria, as described below.

When ionophore-treated mitochondria were pre-incubated with increasing concentrations of phenethylbiguanide in the absence and the presence of 5 mM Mg^{2+}, the data shown in Fig. 1A were obtained. Replotting these data according to the method of Dixon [9] indicated competition between Mg^{2+} and phenethylbiguanide (Fig. 1B). From six such experiments, the K_i for phenethylbiguanide was calculated to be 19.0 ± 4.3 μM in ionophore-treated mitochondria.

In contrast to the effects observed with mitochondria oxidizing NAD^+-linked substrates, addition of Mg^{2+} along with phenethylbiguanide to ionophore-treated mitochondria oxidizing succinate provided no detectable protection against biguanide inhibition (Table 1).

When washed, ionophore-treated mitochondria were incubated with 6 mM $MgCl_2$, then again washed and studied for sensitivity to phenethylbiguanide inhibition without and with Mg^{2+} in the oxygen uptake assay medium, the biguanide sensitivity was identical to that of intact, non-ionophore-treated mitochondria (Fig. 2). As with intact mitochondria, Mg^{2+} did not protect these depleted–repleted mitochondria against phenethylbiguanide inhibition.

Studies in P_i-treated mitochondria

To provide independent confirmation that Mg^{2+} depletion was responsible for the ionophore effects described above, we depleted mitochondria of Mg^{2+} by exposure to P_i, as described by Höser and Dargel [5]. As shown in Table 2, even the relatively lesser degree of Mg^{2+} depletion produced under these conditions (see above) led to a significant increase in sensitivity to biguanide inhibition. Introduction of Mg^{2+} into the medium during biguanide exposure markedly antagonized inhibition. As was the case with ionophore-treated mitochondria, repletion of Mg^{2+} in P_i-treated mitochondria, followed by washing, restored the same biguanide and Mg^{2+} insensitivity as untreated mitochondria.

DISCUSSION

It has not been clearly established whether biguanides inhibit mitochondrial respiration by binding non-specifically to the external surface of phospholipid bilayers in mitochondrial membranes by simple electrostatic interaction [10] or through more selective binding to mitochondrial proteins within a membrane barrier [2, 11]. The present studies indicate that both mechanisms may occur, operating at different sites. Thus, at high concentrations (~0.5 mM phenethylbiguanide) biguanides inhibit at coupling site II by a process which is unaffected by Mg^{2+} depletion and repletion (Table 1). This site II inhibition is, therefore, distinguishable from the more complex inhibition at coupling site I, since the latter occurs at lower concentrations and is strongly affected by Mg^{2+} removal and replacement.

With regard to inhibition at coupling site I, we [2] and others [12] have shown previously that uptake of biguanide by an energy-dependent process is required for inhibition at low biguanide concentrations. It is important to note that, in this instance, the uptake of biguanide into the mitochondrion may occur at a locus and through a mechanism quite distinct from the site and mechanism by which biguanide subsequently binds to inhibit respiration. Based on the recognition that biguanide inhibition at coupling site I may be a two-step process, we may generate two distinct models to explain our present observations of an increase in inhibitory potency of phenethylbiguanide created by Mg^{2+} depletion and its reversal by Mg^{2+} repletion.

If we first make the assumption that Mg^{2+} affects biguanide inhibition at site I through alterations in biguanide uptake, the marked increase in sensitivity to inhibition at this site after exposure to ionophore or P_i would then indicate that Mg^{2+} depletion increases the access of biguanide to the inhibitory locus within this site. Such an increase in biguanide permeability after Mg^{2+} depletion in turn implies that, in the intact mitochondrion, biguanide access to the inhibitory locus is restricted by a barrier, probably the inner membrane, which normally must have very low permeability to biguanide in its cationic form.

In intact mitochondria, therefore, we conclude that biguanide is probably entering by some mechanism other than via a cation carrier. Although the exact mechanism by which biguanides traverse the membrane barrier has not been identified, the data from at least two laboratories suggest that the major pathway of entry into intact mitochondria or bacterial chromatophores may be via non-ionic diffusion [2, 13]. Such a mechanism is consistent with the complete lack of effect of added Mg^{2+} on site I biguanide inhibition presently observed in intact hepatic mitochondria (Tables 1 and 2, Fig. 2).

One of the most striking observations in the present studies is the totally different effect of Mg^{2+} added to depleted mitochondria concurrently with biguanide compared with the effect of Mg^{2+} repletion prior to biguanide exposure. Thus, in intact or Mg^{2+}-repleted mitochondria, further Mg^{2+} added just prior to the O_2-uptake assay has no detectable effect on respiration or biguanide inhibition, while with depleted mitochondria, Mg^{2+} added only during the exposure to phenethylbiguanide competitively antagonizes biguanide inhibition, and at high Mg^{2+} biguanide ratios, protection is total. These observations indicate that Mg^{2+} exerts effects on biguanide inhibition by two completely different mechanisms:

the Mg^{2+} present in intact mitochondria or reintroduced by repletion of depleted mitochondria is probably bound to structural sites. In contrast, the competitive protection of Mg^{2+}-depleted mitochondria by Mg^{2+} present in the medium indicates that it is Mg^{2+} in solution which is the active agent. From these observations, we may draw three further important conclusions: (1) in Mg^{2+}-depleted mitochondria, biguanide appears to enter via a mechanism different from that in intact mitochondria; (2) this new biguanide uptake mechanism, "unmasked" by Mg^{2+} depletion, is probably a cation carrier; and (3) this unmasked biguanide carrier also transports Mg^{2+}. As an aside, it should be noted that a Mg^{2+} carrier which is unmasked by Mg^{2+} depletion and hence, suppressed by Mg^{2+} restoration, may well be of considerable physiological importance.

At least two mitochondrial cation carrier mechanisms activated by Mg^{2+} depletion have been described elsewhere, either of which could serve as the proposed biguanide carrier. First, Duszyński and Wojtczak [8] have recently shown that Mg^{2+} depletion with divalent cation ionophore reversibly activates a K^+/H^+ antiporter across the inner mitochondrial membrane. However, this carrier is not likely to be the one involved in biguanide transport for the following reasons: (a) our experiments which revealed increased sensitivity to biguanide inhibition in Mg^{2+}-depleted mitochondria were conducted in a high K^+ medium, a condition which should minimize biguanide entry via a carrier with K^+ specificity, and (b) previous work from this laboratory has shown that, although K^+ interferes with biguanide uptake into P_i-treated mitochondria, the interference is non-competitive [2]. Second, EDTA removes a relatively small fraction of tightly bound Mg^{2+} from the mitochondrial membrane in a respiration-dependent process which is completely dependent on rapid inward movement of Na^+: K^+, Tris or choline cannot substitute for Na^+ [14]. Since Mg^{2+} depletion by this procedure does not increase the passive Na^+ permeability of the mitochondrial membrane, the inward movement of Na^+ appears to be dependent on a Na^+ carrier, activated by Mg^{2+} depletion. Independent evidence that Na^+ and guanidinium compounds can compete for a single site in mitochondria has been obtained from studies of the interaction of octylguanidine and Na^+ on the mitochondrial response to dinitrophenol [15]; Na^+ also interferes with phenethylbiguanide uptake into heart mitochondria [2].

Of the two carriers unmasked by Mg^{2+} depletion, the carrier with Na^+ specificity thus appears more likely than does the K^+/H^+ antiporter to be responsible for biguanide uptake in depleted mitochondria.

If, in contrast, we assume that the effects of prior Mg^{2+} depletion on sensitivity to biguanide inhibition occur at an inhibitory binding site within the membrane, as distinct from action on a biguanide carrier then the greater sensitivity to biguanide inhibition after Mg^{2+} depletion would most likely represent a simple loss of the matrix pool of Mg^{2+} [16]. Mg^{2+} in this matrix pool would otherwise compete with biguanide for inhibitory loci within the matrix itself or on the matrix surface of the inner membrane. Since exogenous Mg^{2+} does not affect biguanide inhibition in intact mitochondria (Figs 1 and 2), the effects of Mg^{2+} depletion are difficult to explain in terms of the binding-site model, particularly in view of the effectiveness of Mg^{2+} in antagonizing biguanide in Mg^{2+}-depleted mitochondria.

In summary, the present data support the existence of an inhibitory locus for mono- and biguanides at coupling site I within a membrane barrier which is normally impermeable to the cationic form of these compounds. Depletion of mitochondrial Mg^{2+} may activate a cation carrier mechanism, perhaps the same as the Na^+ carrier described by Settlemire *et al.* [14], which conveys the guanidinium compounds across the membrane; Mg^{2+} appears to compete for this carrier. Mg^{2+} depletion also increases the sensitivity to inhibition by triethyltin [17] and diphenylene-iodonium [18] at coupling site I. Whether these two organic cations share uptake and inhibitory loci with guanidinium compounds remains to be determined.

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