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PHENETHYLBIGUANIDE EFFECTS ON MITOCHONDRIAL Ca^{2+} AND Mg^{2+} CONTENT

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Abstract—Phenethylbiguanide (PEBG) in low doses (12 mg/kg) lowered blood sugar in fasted and in glucagon-pretreated fed guinea pigs but not in fed controls. The Ca^{2+} content of hepatic mitochondria subsequently isolated from biguanide-treated animals was reduced significantly, the loss occurring predominantly in the loosely bound external fraction. Mitochondrial matrix Mg^{2+} content increased significantly after PEBG administration, except in glucagon-treated animals, and the Mg^{2+}/Ca^{2+} ratio increased after PEBG under all metabolic conditions studied. Except in mitochondria from fasted animals, PEBG added *in vitro* did not reduce mitochondrial Ca^{2+} content, nor did it increase Mg^{2+} content. Insulin pretreatment in doses which reduced blood sugar to the same extent as PEBG had no effect on mitochondrial Ca^{2+} or Mg^{2+} content. We conclude that PEBG administered *in vivo* produces consistent and significant effects on hepatic mitochondrial divalent metal ion content through a mechanism which is more complex than competitive binding. These metal ion shifts may be involved in the hypoglycemic activity of the drug, probably by limiting the gluconeogenic rate of the liver under a "glucagon-predominant" signal. Insulin appears to inhibit gluconeogenesis by a different mechanism.

Phenethylbiguanide (PEBG) added *in vitro* at concentrations which are expected after therapeutic doses, i.e. 1–30 μM, enhances the rate of energized Ca^{2+} uptake into isolated hepatic mitochondria [1]. This finding has suggested that biguanides might inhibit hepatic gluconeogenesis through alterations in hepatic mitochondrial divalent cation distribution. To explore this possibility further, we have measured Ca^{2+} and Mg^{2+} in liver mitochondria isolated from fed, fasted or glucagon-treated guinea pigs after administration of biguanide in low doses, using isolation conditions which enabled us to distinguish the loosely from the tightly bound cations. Biguanides produced a consistent decrease in loosely bound Ca^{2+} and an increase in matrix Mg^{2+}.

Insulin perfusion of the intact liver decreases Ca^{2+} uptake rate into mitochondria isolated after the perfusion, while glucagon perfusion gives rise to the opposite effect [2]. Furthermore, insulin added to intact adipocytes alters the distribution of Ca^{2+} among the cellular organelles and within the mitochondria isolated from treated cells [3], supporting the general hypothesis that Ca^{2+} may play a key regulatory role in cellular metabolism. Therefore, we compared the effects of physiological doses of insulin with biguanide after injection into intact animals. In contrast to biguanide, insulin produced no detectable alteration in total hepatic mitochondrial divalent cation content. The effects of insulin on the dynamics of Ca^{2+} movement in mitochondria thus do not appear to be reflected in changes in total cation content, at least in the intact animal. Furthermore, biguanides and insulin probably affect hepatic metabolism by different mechanisms.

METHODS

In vivo *studies*

Guinea pigs weighing approximately 400 g were either fed *ab lib.* up to 3 hr before death or fasted for 45–50 hr. Animals receiving PEBG were injected intraperitoneally 3 hr prior to death with 12 mg/kg body wt (low dose) or 30 mg/kg body wt (high dose). Animals receiving glucagon were injected with 20 μg subcutaneously 1 hr before death; animals receiving both glucagon and insulin were first injected with glucagon (20 μg) at 2 hr and then with insulin (1 unit) subcutaneously at 1 hr before death.

After appropriate pretreatment of the animal, the liver was removed and cut into three approximately equal parts. The three parts were homogenized in either sucrose–Hepes, sucrose–Hepes–1 mM EDTA, or sucrose–Hepes–5 μM ruthenium red.* These same mixtures were used throughout the mitochondrial isolations [4]. The washed mitochondrial suspensions were then assayed for protein content [5] and 10 mg of mitochondrial protein were added to 10 ml of deionized water. The protein was precipitated with 0.1 ml of 70% perchloric acid, centrifuged off at 10,000 *g* for 3 min, and the supernatant fraction assayed for Ca^{2+} and Mg^{2+}, using a Perkin–Elmer spectrophotometer, model 290B.

In vitro *studies*

Guinea pig liver mitochondria were prepared as

* Hepes = 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

described previously [4]. All sugar solutions were deionized prior to use. Mitochondria (1 mg protein/ml) were incubated with shaking in 105 mM KCl, 10 mM sucrose, 10 mM Hepes, containing as substrate 20 mM succinate, plus 2 μM rotenone, pH 7.4, at 30° in 3 ml total volume. After 7 min, 5 μM ruthenium red, 1 mM EDTA or H_2O was added to various tubes of mitochondrial suspension. The tubes were centrifuged for 5 min at 10,000 *g*, and the pellets were washed in incubation mix containing ruthenium red, EDTA or no additions, as appropriate, and recentrifuged. The pellets were then suspended in 3 ml of deionized water, 50 μl of 70% perchloric acid were added, and the tubes were recentrifuged at 10,000 *g* for 3 min. The supernatant fraction was assayed as above for Mg^{2+} and Ca^{2+}.

Intramitochondrial distribution of Mg^{2+}

Guinea pig liver mitochondria were isolated according to our usual procedure in the absence of EDTA. The washed mitochondria were suspended in about 70 ml of 20 mM phosphate buffer, pH 7.4, containing 0.02% fatty acid-free bovine serum albumin. After 20 min at 0°, the suspension was centrifuged at 35,000 *g* for 20 min. The supernatant fluid was carefully removed and saved as the intermembrane space. The pellet was suspended in 60 ml of ice-cold deionized water and centrifuged at 48,000 *g* for 30 min. The supernatant fluid was saved as the matrix and the pellet was saved as the membrane fraction. The fractions were assayed by atomic absorption for Mg^{2+} content, which was expressed as nmoles/mg of total mitochondrial protein [6]. Using standard assays, the fractions were checked for two marker enzymes, adenylate kinase and malic dehydrogenase [7]. According to this assay there was only 5–8 per cent cross-contamination between matrix and intermembrane space fractions.

Blood glucose assay

For the *in vivo* studies, blood glucose was determined on samples obtained from the neck vessels immediately after the animal was decapitated. Glucose was determined on whole blood using Worthington Glucostat reagents.

Statistical methods

The significance of the results was calculated using a two-tailed *t*-test for comparison of means.

RESULTS

Blood sugar responses

Blood glucose levels measured at the time of sacrifice are given in Table 1. The lower dose of PEBG reduced blood glucose significantly in fasted animals and in animals treated with glucagon, when compared with appropriate controls. In contrast, this dose did not lower blood sugar in fed animals. The high dose of PEBG actually raised the blood glucose in fed animals when assayed 3 hr after the drug was given. A similar hyperglycemic response has been observed with phenethylguanidine in rabbits [8].

Glucagon alone raised blood sugar, and this was depressed to hypoglycemic levels by both PEBG and insulin.

Analysis of mitochondrial Ca^{2+} *distribution*

Calcium in cellular organelles exists in both ionized pools and complexed or stable pools [9]. Since EDTA lowers the measured Ca^{2+} content of mitochondria, as well as of other organelles [3], it is presumed that the residual Ca^{2+} detected after isolation of mitochondria in the presence of EDTA represents tightly complexed Ca^{2+} in internal and external sites.

Ruthenium red at a concentration of 5 μM blocks all Ca^{2+} movement across membranes. The Ca^{2+} content of adipocyte mitochondria is much greater when isolated in the presence of ruthenium red. Therefore, it appears that during isolation substantial Ca^{2+} loss takes place from mitochondria of adipocytes which is prevented by ruthenium red blockade [3].

Table 1. Effect of PEBG or insulin pretreatment on blood glucose of fasted or fed guinea pigs with or without glucagon*

	Blood glucose (mg/dl)				
	Pretreatment				
		PEBG			
Nutritional/ hormonal state	None	12 mg/kg	30 mg/kg	Insulin	P†
Fed	131 ± 5 (5)	125 ± 5 (3)	163 ± 5 (2)		NS‡
					<0.01
Fasted	100 ± 6§ (4)	68 ± 20 (4)			<0.05
Fed + glucagon	188 ± 5‖ (7)	60 ± 19 (5)			<0.001
				65 ± 32 (4)	<0.005

* Blood glucose values are means ± SEM. Numbers in parentheses are numbers of animals in each group.

† P for PEBG or insulin pretreatment vs none.

‡ NS = not significant.

§ $P < 0.005$, for comparison with fed controls.

‖ $P < 0.001$, for comparison with fed controls.

Table 2. Ca^{2+} pools of mitochondria from guinea pig liver*

Nutritional/hormonal state	Isolation condition	Ca^{2+} content (nmoles/mg protein)				
		Pretreatment				
		None	PEBG	Insulin	ΔCa^{2+}†	P†
Fed	No additions	27.8 ± 0.3	17.3 ± 1.3		−10.5	<0.001
	+ EDTA	10.5 ± 0.7	8.3 ± 0.8		−2.2	NS‡
	+ Ruthenium red	16.7 ± 1.0	14.1 ± 1.3		−2.6	NS
Fasted	No additions	29.4 ± 2.9	19.4 ± 1.1		−10.0	<0.025
	+ EDTA	8.1 ± 0.4	7.0 ± 0.7		−1.1	NS
	+ Ruthenium red	18.0 ± 1.3	15.7 ± 1.5		−2.3	NS
Fed + glucagon	No additions	25.8 ± 0.9	17.4 ± 1.2		−8.4	<0.001
	+ EDTA	12.6 ± 1.5	7.4 ± 0.8		−5.2	<0.025
	+ Ruthenium red	21.5 ± 1.0	12.5 ± 1.1		−9.0	<0.001
Fed + glucagon	No additions	25.8 ± 0.9		25.9 ± 1.2	+0.1	NS
	+ EDTA	12.6 ± 1.5		11.1 ± 0.7	−1.5	NS
	+ Ruthenium red	21.5 ± 1.0		23.7 ± 2.1	+2.2	NS

* Number of animals in each treatment group is given in Table 1. Ca^{2+} values are means ± SEM.
† ΔCa^{2+} and P are for comparison of PEBG or insulin pretreatment vs none.
‡ NS = not significant.

When we isolated mitochondria from the livers of fed guinea pigs in the presence of EDTA, the Ca^{2+} content was reduced by over 60 per cent (Table 2). The residual pool of tightly complexed Ca^{2+} in hepatic mitochondria (11 nmoles/mg of protein) is quite comparable to that found in adipocyte mitochondria (14 nmoles/mg of protein [3]). In contrast to the Ca^{2+} increase observed with ruthenium red in adipocyte mitochondria, however, this reagent reduced the Ca^{2+} content of hepatic mitochondria by about one-third (Table 2). This discrepancy probably is due to basic differences between adipocyte and hepatic mitochondria in Ca^{2+} binding and permeability. In the case of adipocyte mitochondria there are apparently few loose external Ca^{2+} binding sites; ruthenium red thus acts primarily to prevent Ca^{2+} from leaking out during preparation. Guinea pig liver mitochondria process a large number of loose external binding sites [4]; it therefore appears that, even though ruthenium red may prevent some leakage of internal Ca^{2+} from guinea pig liver mitochondria, it primarily displaces native Ca^{2+} from these external sites. This finding suggests further that the total spontaneous Ca^{2+} leak from hepatic mitochondria during isolation is very much less than from adipocyte mitochondria, a conclusion supported by the higher total Ca^{2+} content of hepatic mitochondria than of adipocyte mitochondria isolated without ruthenium red (Table 2 and Ref. 3: 28 vs 21 nmoles Ca^{2+}/mg of protein). The Ca^{2+} found in hepatic mitochondria isolated with ruthenium red therefore probably represents ionized internal Ca^{2+} plus all tightly complexed Ca^{2+} at inner and outer sites. These relationships are diagrammed in Fig. 1.

Effects of starvation, glucagon, PEBG and insulin on the Ca^{2+} pools of guinea pig liver mitochondria

Fasting did not alter the Ca^{2+} pools of hepatic mitochondria significantly (Table 2); in contrast, glucagon pretreatment did appear to increase the EDTA- and ruthenium red-stable pools substantially, although only with ruthenium red was the difference statistically significant (Table 2: 16.7 ± 1.0 nmoles Ca^{2+}/mg of protein for fed vs 21.5 ± 1.0 for fed and glucagon, $P < 0.01$). When PEBG was administered at a dose of 12 mg/kg, total mitochondrial Ca^{2+} which was measured without additions to the isolation medium was clearly diminished (about 35 per cent) in fed animals, even though, as noted above, blood sugar was not decreased at the time of death. The pools of Ca^{2+} which were stable to EDTA or ruthenium red were not altered significantly by PEBG treatment; hence, the drug-induced decrease in total Ca^{2+} appeared to be limited almost entirely to the loosely complexed external Ca^{2+} fraction. Furthermore, since PEBG, when administered *in vivo*, reduced Ca^{2+} content almost as much as ruthenium red, which was added during the isolation procedure (Table 2: 10.5 nmoles Ca^{2+}/mg of protein for PEBG vs 9.1 for ruthenium red), PEBG treatment appears to affect virtually all of that loosely complexed fraction.

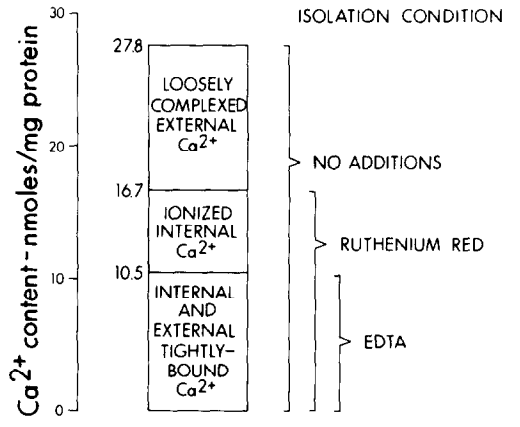

Fig. 1. Calcium content of mitochondria isolated from livers of fed guinea pigs under various conditions. For details of experimental procedure, see Methods.

In two experiments, we administered a higher dose of PEBG (30 mg/kg) to fed animals. The effects on mitochondrial Ca^{2+} were similar to the low-dose experiments, except that all Ca^{2+} pools were reduced 15–20 per cent further by this dose of the drug (data not shown).

Mitochondria from fasted animals showed an identical reduction in loosely complexed Ca^{2+} after PEBG treatment. When PEBG was administered to glucagon-treated, fed animals, mitochondrial Ca^{2+} decreased significantly under all three isolation conditions. Indeed, with ruthenium red present, the PEBG-induced drop in Ca^{2+} was fully as great as that measured with no additions (Table 2: Δ of 9.0 vs 8.4 nmoles/mg of protein). The final absolute content of all Ca^{2+} pools was virtually identical after PEBG administration whether the animals were fed, fasted or treated with glucagon. The larger decrements seen with PEBG in glucagon-treated animals thus resulted from higher Ca^{2+} levels in the controls rather than from lower final PEBG-induced Ca^{2+} content.

In contrast to the effects of PEBG, insulin pretreatment had no detectable effect on any of the Ca^{2+} pool measurements (Table 2), despite a decrease in blood sugar with insulin which was quite comparable to that with PEBG (Table 1).

Effects of starvation, glucagon, PEBG and insulin on the Mg^{2+} pools of guinea pig liver mitochondria

For direct comparison with the Ca^{2+} data, mitochondria were analyzed for Mg^{2+} content after isolation under the same conditions used for Ca^{2+}. Ruthenium red had no consistent effect on Mg^{2+} content (Table 3), as expected, since ruthenium red binds to Ca^{2+} sites with high selectivity; the Mg^{2+} values with ruthenium red are therefore considered together with those measured with no additions. EDTA reduced mitochondrial Mg^{2+} content moderately (about 15–25 per cent) as found by other workers [6, 10].

Fasting produced small increments, in total and EDTA-stable Mg^{2+} pools, which were not statistically significant. Glucagon gave rise to rather impressive increases in both pools (33–50 per cent, $P < 0.001$ for total Mg^{2+}, EDTA- and ruthenium red-stable pools, compared with fed controls).

PEBG at a dose of 12 mg/kg caused both total and EDTA-stable Mg^{2+} to increase by the same absolute amount in fed and in fasted animals. In contrast, PEBG given after glucagon pretreatment produced a reduction in mitochondrial Mg^{2+} in both pools. The 30 mg/kg dose of PEBG did not alter the mitochondrial Mg^{2+} pool sizes in comparison with fed controls.

Insulin had no effect on Mg^{2+} content or distribution.

The Mg^{2+}/Ca^{2+} ratio

The above data indicated that Ca^{2+} and Mg^{2+} contents of mitochondria changed reciprocally in response to PEBG, at least under some metabolic conditions. In order to analyze the total mitochondrial divalent cation responses to various treatments, we therefore calculated the Mg^{2+}/Ca^{2+} ratios for mitochondria isolated with no additions or with EDTA (Fig. 2).

Using the Mg^{2+} and Ca^{2+} contents measured with no additions during isolation (total divalent cations), there was little change in the Mg^{2+}/Ca^{2+} ratio in fed vs starved controls ($P > 0.4$) and a slight but significant increase in the presence of glucagon ($P < 0.001$). PEBG nearly doubled the ratio in fed and starved animals ($P < 0.001$ in both instances), and increased it by about 30 per cent in glucagon-treated animals ($P < 0.01$). Insulin had no effect on this ratio ($P > 0.2$).

The absolute changes in Mg^{2+}/Ca^{2+} ratio produced by fasting ($P < 0.005$) and glucagon ($P = 0.075$) were augmented when calculated on the basis of EDTA-stable pools of these cations. PEBG also shifted the ratio upward consistently in all three metabolic set-

Table 3. Mg^{2+} pools of mitochondria from guinea pig liver*

Nutritional/hormonal state	Isolation condition	Mg^{2+} content (nmoles/mg protein)				
		Pretreatment				
		None	PEBG (12 mg/kg)	Insulin	ΔMg^{2+}†	P‡
Fed	No additions	33.9 ± 1.1	39.2 ± 1.9		+5.3	<0.05
	+ EDTA	25.1 ± 0.5	31.3 ± 1.8		+6.2	<0.025
	+ Ruthenium red	30.9 ± 0.7	40.1 ± 2.0		+9.2	<0.01
Fasted	No additions	35.1 ± 2.7	44.1 ± 2.7		+9.2	<0.01
	+ EDTA	29.7 ± 1.5	36.5 ± 2.5		+6.8	<0.05
	+ Ruthenium red	35.3 ± 0.8	46.2 ± 2.9		+10.9	<0.025
Fed + glucagon	No additions	43.5 ± 0.9	36.0 ± 2.7		−7.5	<0.05
	+ EDTA	37.8 ± 1.2	28.4 ± 3.1		−9.4	<0.025
	+ Ruthenium red	44.3 ± 1.3	36.7 ± 3.0		−7.6	<0.05
Fed + glucagon	No additions	43.5 ± 0.9		46.9 ± 1.7	+3.4	NS‡
	+ EDTA	37.8 ± 1.2		36.5 ± 2.1	−1.3	NS
	+ Ruthenium red	44.3 ± 1.3		46.4 ± 1.7	+2.1	NS

* Number of animals in each treatment group is given in Table 1. Mg^{2+} values are means ± SEM.
† ΔMg^{2+} and P are for comparison of PEBG or insulin pretreatment vs none.
‡ NS = not significant.

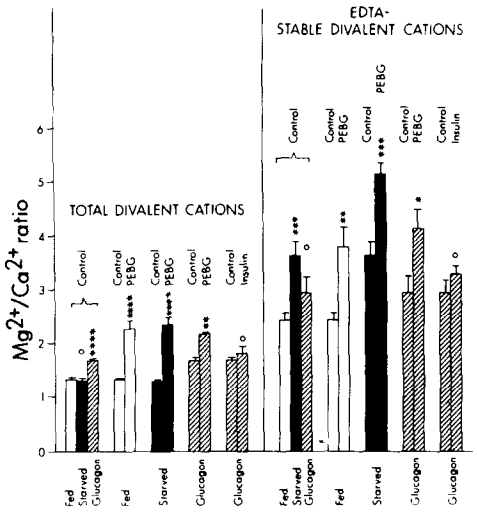

Fig. 2. Mg^{2+}/Ca^{2+} ratio of guinea pig hepatic mitochondria from fed or fasted animals treated with glucagon, PEBG or insulin. P values for experimental vs control in each group: (0) not significant; (*) $P < 0.025$; (**) $P < 0.01$; (***) $P < 0.005$; and (****) $P < 0.001$.

tings ($P < 0.01$, < 0.005 and < 0.025 in fed, fasted and glucagon experiments respectively). Insulin again had no effect on the ratio ($P > 0.4$).

Effects of PEBG added in vitro *on* Ca^{2+} *and* Mg^{2+} *pools*

Calcium. The addition of 10 μM PEBG *in vitro* decreased the total pool of Ca^{2+} only in mitochondria from fasted animals [from 25.0 ± 1.2 to 19.0 ± 1.7 nmoles/mg protein, a reduction of 24 per cent ($P < 0.025$)]. The EDTA-stable pool was unaffected. No significant changes in loose or tightly bound Ca^{2+} content were produced by PEBG in mitochondria from fed or glucagon-treated animals. When animals were starved for 48 hr and then refed 4 hr before the isolation of mitochondria, PBEG no longer reduced the total Ca^{2+} content.

Magnesium. Mg^{2+} content was unaffected by PEBG in mitochondria isolated from animals in any of the various nutritional states, and with or without glucagon. However, the initial incubation medium for *in vitro* experiments did not contain Mg^{2+} and, therefore, we were concerned that its absence would mask an increase in Mg^{2+} content as was seen when PEBG was administered *in vivo*. Hence in several experiments we included 5 mM Mg^{2+} in the incubation medium during *in vitro* exposure to PEBG. There was no stimulation of Mg^{2+} uptake by PEBG under these conditions.

Intramitochondrial distribution of Mg^{2+} *in the presence and absence of injected PEBG*

In order to explore the possibility that the changes in Mg^{2+} content, observed when PEBG was administered *in vivo*, occurred in a single compartment of the mitochondrion, rather than as a diffuse or generalized increase, we applied the fractionation technique of Bogucka and Wojtczak [6] to mitochondria from control and PEBG-treated animals. For these experiments, slightly larger animals were used, with livers averaging 22 g. Inner and outer membranes were not separated since our interest was focused primarily on the two mitochondrial compartments which are known to hold most of the Mg^{2+}, i.e. the matrix and the intermembrane space, rather than the membranes.

Total Mg^{2+} recovery after the fractionation procedure was 94 per cent which compares favourably with a 92–95 per cent recovery of mitochondrial protein and indicates the results were not distorted by technical artifact. The values for Mg^{2+} in the various fractions from control mitochondria are very similar to those reported by Bogucka and Wojtczak [6], for rat liver mitochondria.

The data in Table 4 clearly indicate that essentially all of the increase in Mg^{2+} content of the mitochondria from PEBG-treated animals occurred in the matrix.

DISCUSSION

Phenethylbiguanide affected hepatic mitochondrial Ca^{2+} and Mg^{2+} contents when given *in vivo* to guinea pigs in doses which lowered blood sugar in fasted or glucagon-treated animals, but which were sub-hypoglycemic in fed animals. In fed and fasted animals, PEBG depressed loosely bound Ca^{2+} without affecting tightly bound or ionized internal pools; PEBG increased matrix Mg^{2+} under these conditions. In glucagon-treated animals, PEBG reduced all pools of Ca^{2+} and Mg^{2+} below the control values. Insulin, in contrast, had no detectable effect on mitochondrial Ca^{2+} or Mg^{2+}, even though the dose of insulin used depressed blood sugar to the same extent as the lower dose of PEBG.

The reduction of mitochondrial Ca^{2+} by the lower dose of PEBG could reflect loss or "leak" due to nonspecific or toxic damage. Although more detailed studies are needed, the evidence weighs against such an action. First, total Mg^{2+} increased after PEBG; and second, this Mg^{2+} increase was maintained and was limited to the matrix compartment despite the rather rigorous treatments to which the mitochondria were exposed during the fractionation procedure. One might have expected mitochondria which had been damaged enough by the drug to leak Ca^{2+} to exhibit neither of the observed behaviours with regard to Mg^{2+}. Third, PEBG added *in vitro* in concentrations close to those expected in liver after *in vivo* administration [1] did not affect loosely bound Ca^{2+}, except to a moderate degree under fasted conditions. Again, a nonspecific damaging effect should have been seen equally both *in vivo* and *in vitro*.

The mechanism by which PEBG affects the divalent ion content of mitochondria remains to be established. A simple reduction in bound Ca^{2+} by competitive displacement seems unlikely, since such an effect was not generally observed when PEBG was added *in vitro* in low concentrations. This result was not unexpected, since we have shown previously that the concentrations of PEBG required for half-maximal competitive displacement of Ca^{2+} in both energized and non-energized guinea pig liver mitochondria are in the range of 0.5 to 1.0 mM [4], considerably above those expected after low-dose PEBG administration [11].

Table 4. Effect of PEBG on intramitochondrial Mg^{2+} distribution*

Fraction	Mg^{2+} (nmoles/mg protein)			
	Pretreatment			
	None	PEBG	ΔMg^{2+}†	P†
Whole mitochondria	32.8 ± 0.9	41.3 ± 9	+8.5	<0.005
Intermembrane space	14.5 ± 0.6	13.5 ± 0.2	−1.0	NS‡
Matrix	14.0 ± 0.3	23.1 ± 0.4	+9.0	<0.001
Membranes	2.2 ± 0.2	2.2 ± 0.3	0	NS
Average recovery of Mg^{2+} in fractions: % of total	93.6	93.9		NS

* Mitochondria were isolated from livers of fed guinea pigs without pretreatment or after administration of 12 mg/kg of PEBG. Each group consisted of seven animals.

† ΔMg^{2+} and P are for comparison of PEBG pretreatment vs none.

‡ NS = not significant.

Calcium uptake into mitochondria may be mediated by a glycoprotein carrier contained in the intermembrane space which migrates into the membrane in the presence of Ca^{2+} [12]. Magnesium appears to be required to maintain this glycoprotein bound to the membrane. Since PEBG competes with Mg^{2+} in several systems [13, 14], it is possible that the drug displaces Mg^{2+} from the glycoprotein–membrane complex, thus releasing Ca^{2+}. This model would be consistent with our observation that the fraction of hepatic mitochondrial Ca^{2+} reduced by pretreatment of the animal with PEBG is predominantly the ruthenium red-sensitive component (Table 1), since Ca^{2+} binding to the isolated mitochondrial glycoprotein is also ruthenium red sensitive.

As an alternative explanation for the present results, PEBG might reduce the concentration of cytosolic ionized Ca^{2+}, perhaps by stimulating the Ca^{2+} "pump" at the plasma membrane. Such a reduction in cytosolic Ca^{2+} concentration could secondarily limit both the rate and the final level of Ca^{2+} accumulation in mitochondria.

The PEBG-associated increase in mitochondrial Mg^{2+} content probably represents an increase in nucleotide-associated cation, since most of the Mg^{2+} in the matrix compartment is believed to be complexed to nucleotides [6]. A shift of matrix nucleotide composition from ADP to ATP, without an increase in total nucleotides, could account for a net Mg^{2+} shift into the mitochondrial matrix, since the affinity of ATP for Mg^{2+} is ten times that of ADP [15]. This hypothesis can be tested by direct measurement of the total nucleotide content and composition. PEBG could produce such a shift in the ADP/ATP ratio simply by reducing the rate of nucleotide exchange across the inner mitochondrial membrane. Thus, as coupled respiration proceeded and ATP was generated in the matrix, a relative excess of ATP would accumulate in that compartment.

The effects of PEBG on blood sugar demonstrated in this paper (Table 1) support our hypothesis that, in its usual, low therapeutic concentrations, this drug lowers blood sugar by limiting glucagon-dependent gluconeogenesis [16], rather than by the more nonspecific, toxic mechanism of inhibiting coupled respiration. Thus, fasted animals, whose blood sugar is maintained almost exclusively by gluconeogenesis, were extremely sensitive to these low doses of drug. The same dose of the drug given to fed animals had virtually no effect on blood sugar, which is not surprising since the fed animal maintains blood sugar primarily from gastrointestinal absorption plus varying amounts of hepatic glycogenolysis, rather than from gluconeogenesis. An identical phenomenon has been noted in fed and fasted normal human subjects [17]. Connon [18] has also shown that isolated perfused livers removed from fasted rats are markedly more sensitive to PEBG inhibition of gluconeogenesis than livers from fed controls.

Despite the selectivity of PEBG-induced hypoglycemia for glucagon-predominant states, it is of interest that both the PEBG-associated reduction in mitochondrial Ca^{2+} and the shift in the Mg^{2+}/Ca^{2+} ratio were observed under all three metabolic conditions: feeding, fasting and glucagon pretreatment. Since blood sugar fell only in the latter two situations, we may reasonably conclude that these ion shifts were of no metabolic consequence for carbohydrate metabolism when the liver was under an "insulin predominant" signal, but that these cation shifts became rate limiting when the liver was exposed to a "glucagon predominant" signal.

The exact role of divalent metal ions in the control of hepatic gluconeogenesis has been the subject of much study and speculation. Friedmann and Rasmussen [19] have shown that the earliest detectable metabolic event following exposure of the perfused liver to glucagon is a major release of mitochondrial Ca^{2+}, indicating that mitochondrial Ca^{2+} may well be directly involved in mediating the gluconeogenic response to glucagon. Calcium has also been shown to alter the mitochondrial redox poise of isolated intact hepatocytes [20], probably through a direct interaction with one or more respiratory carriers of the inner mitochondrial membrane. Zahlten *et al.* [21] have demonstrated a striking enhancement of basal gluconeogenic rate by Ca^{2+} in isolated hepatocytes, an effect which they attributed to a direct Ca^{2+}-induced shift in mitochondrial redox poise. These authors have also shown that the incremental response of gluconeogenesis to a glucagon or epi-

nephrine stimulus in these cells depends on the redox poise. The results of these hepatocyte studies, combined with our present data, lead us to suggest that PEBG may limit hepatic gluconeogenic responsiveness to glucagon through a change in the oxidation–reduction state of the cell, which, in turn, may result from a PEBG-induced change in the amount or distribution of mitochondrial Ca^{2+} and Mg^{2+}. The present data also indicate that insulin has no detectable effect on mitochondrial Ca^{2+} or Mg^{2+} content under conditions where changes in these cation levels were clearly and consistently demonstrated with PEBG. We conclude that insulin and PEBG inhibit gluconeogenesis in liver by different mechanisms, at least at the level of mitochondrial involvement.

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