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EFFECT OF GLYCYRRHETINIC ACID ON THE CYCLIC NUCLEOTIDE SYSTEM OF THE RAT STOMACH

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Abstract—Glycyrrhetinic acid (carbenoxolone) inhibited cyclic nucleotide phosphodiesterase (PDE) activity in a number of tissues from rat, guinea pig and dog. In the fundic mucosa, it was more active when cyclic AMP rather than cyclic GMP was used as substrate in the PDE assays. Glycyrrhetinic acid also raised the intracellular levels of cyclic AMP in rat pyloric and fundic mucosae *in vivo* with little effect on cyclic GMP levels. This effect, at least in the rat, was apparently mediated via PDE-inhibition, since the drug did not affect adenylyl cyclase activity from either mucosa. Studies with theophylline and papaverine, in addition to glycyrrhetinic acid, suggested that the effects of PDE inhibitors on gastric acid secretion could be related to their relative activities on cyclic AMP-PDE's or cyclic GMP-PDE's, owing to the possibly opposite effects mediated by the two cyclic nucleotides on acid secretion by the stomach.

LICORICE extracts have long been used in the treatment of peptic ulcers, since the days of ancient China.[1] More recently, the value of glycyrrhetinic acid, one of the main constituents of licorice extracts, in the treatment of peptic ulcers has been widely acclaimed.[2–5] The disodium salt of glycyrrhetinic acid hemisuccinate (carbenoxolone sodium) is marketed in Europe* and is claimed to be of value in the treatment of gastric and possibly duodenal ulcers.[6–11]

The mechanisms by which glycyrrhetinic acid produces its desirable effects in man are largely unknown. It was reported to: (a) inhibit acid secretion in man[4,12] and rat,[13,14] (b) reduce peptic activity in man[4,15] and (c) increase mucus secretion, in addition to a number of other effects.[5,16–18] Nothing is known about the intracellular mechanisms involved in these effects.

Since the cyclic nucleotides, cyclic adenosine-3′,5′-monophosphate (cyclic AMP) and cyclic guanosine-3′,5′-monophosphate (cyclic GMP), may be involved in the control of gastric acid secretion,[19–22] it was interesting to examine the effects of glycyrrhetinic acid on the cyclic nucleotide system in the rat stomach. Studies *in vitro* on the effects of glycyrrhetinic acid on the enzymes catalyzing the formation of the two cyclic nucleotides, adenylyl cyclase (AC) and guanylyl cyclase (GC), and the enzymes catalyzing their degradation were conducted. In addition, the effects of glycyrrhetinic acid on the levels of the cyclic nucleotides *in vivo* were also determined.

* Biogastrone, Berk.

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MATERIALS AND METHODS

All animals used in the present experiments were fasted overnight before sacrifice.

Enzyme studies in vitro. The animals were sacrificed by decapitation* and the tissues quickly excised. The entire length of the aorta from the heart to the bifurcation was used. The fundic and pyloric mucosae were separated manually from the smooth muscle layers before use. After removal of adherent connective tissue, the tissues were homogenized in ice-cold 0·25 M sucrose to yield a 20% homogenate based on the wet weight of the tissue. The whole homogenate was kept at 4° for a maximum of 2 hr until used in the enzyme assays. The whole homogenates were used as such, without further purification, as the enzyme source for phosphodiesterase (PDE), AC and GC assays. Protein was determined by the method of Lowry *et al.*[23]

PDE assays. PDE activity was assayed according to Thompson and Appleman[24] using either cyclic AMP or cyclic GMP as substrate (0·8 or 1 and 2×10^{-6} M cyclic AMP; 1 and 2×10^{-6} M cyclic GMP). The use of low substrate concentrations would be expected to reflect the activity of the low K_m form of PDE that was reported to exist in the fundic and pyloric mucosae[25,26] and other tissues as well[27] (for review, see Ref. 28). This is probably the more important form biologically due to the low levels of either cyclic AMP or cyclic GMP encountered *in vivo*. Two substrate (above) and four to six drug concentrations were used for the determination of the inhibitor constant (K_i) values by the method of Dixon and Webb.[29] In the cases of duplicate experiments, the variation in the K_i values never exceeded 20 per cent of the value shown in the tables.

AC or GC assays. The incubation mixture for AC contained 0·5 μmole ATP-G-^{3}H, 1 μmole theophylline, 32·5 μg pyruvate kinase, 10 μmoles phosphoenolpyruvate, 20 μmoles Tris buffer at pH 7·4, 7·5 μmoles $MgSO_4$ and 250 μg cyclic AMP in a total volume of 0·5 ml. For GC assays, the incubation mixture was similar except that 0·5 μmole GTP-8-^{3}H was substituted for the labeled ATP, 6 μmoles Mn_2Cl_2 and 4 μmoles $CaCl_2$ for the $MgSO_4$ and cold cyclic GMP for the cold cyclic AMP. The incubation for both AC and GC was continued for 10 min at 37°. The reaction was linear up to 20 min. At the end of the incubation, the reaction was stopped by placing the tubes in a boiling water bath for 5 min. The cyclic nucleotides formed were purified by chromatography on Dowex-1-formate columns[30] and radioactivity was determined in the respective cyclic AMP or cyclic GMP fraction. Appropriate standards have shown that this method is superior to other published methods for the assay of labeled cyclic AMP or cyclic GMP formed during AC or GC assays, respectively. Recovery of cyclic AMP was 85–95 per cent and that of cyclic GMP 65–80 per cent. The results were corrected for losses during chromatography via the use of appropriate standards.

Studies in vivo. The method developed in this laboratory and based on the procedure reported by Schmidt *et al.*[31] was used. Briefly, glycyrrhetinic acid was given orally at a dose of 300 mg/kg and the animals were sacrificed 45 min later by microwave irradiation for 30 sec. The tissues were then removed for the determination of their cyclic nucleotide contents by the method of Murad and Gilman[32] after separation of cyclic AMP from cyclic GMP on Dowex-formate columns. No AC, GC or PDE activities could be demonstrated in either pyloric or fundic mucosa at the end

* The dog used in this study was sacrificed by carbon dioxide asphyxiation.

of the 30-sec microwave irradiation. The identity of the cyclic nucleotides determined was checked by treating aliquots of the samples with PDE. No cyclic nucleotides could be detected in the PDE-treated aliquots.

Glycyrrhetinic acid was dissolved in water and neutralized with sodium hydroxide to pH 7·0 immediately before use.

Agents employed. Cyclic AMP, ATP, GTP, phosphoenolpyruvate, pyruvate kinase and dibutyryl cyclic AMP were obtained from Nutritional Biochemicals Corp. (Cleveland, Ohio); cyclic GMP, from Boehringer Mannheim Corp. (New York); and ATP-G-^{3}H, GTP-8-^{3}H, cyclic GMP-G-^{3}H and cyclic AMP-G-^{3}H, from New England Nuclear (Boston, Mass.).

RESULTS

Studies in vitro. As can be seen in Table 1, glycyrrhetinic acid strongly inhibited the low K_m-PDE (measured at low substrate concentrations) from most of the tissues examined, whether cyclic AMP or cyclic GMP was used as substrate. The inhibition was of a magnitude similar to that produced by papaverine (see Table 2). Since papaverine is one of the most potent inhibitors of the enzyme known,[28] glycyrrhetinic acid can thus be classified as a potent PDE inhibitor. Like papaverine, glycyrrhetinic acid also inhibited the enzyme non-competitively (Fig. 1).

TABLE 1. INHIBITOR CONSTANTS (K_i VALUES $\times 10^{-5}$ M) OF GLYCYRRHETINIC ACID ON PDE FROM DIFFERENT TISSUES OF THE RAT, GUINEA PIG AND DOG

	Rat			Guinea pig			Dog		
	Substrate			Substrate			Substrate		
Tissue	Cyclic AMP	Cyclic GMP	G/A*	Cyclic AMP	Cyclic GMP	G/A*	Cyclic AMP	Cyclic GMP	G/A*
Aorta	6·0	2·5	0·4	3·0	1·9	0·6	1·7	9·5	5·5
Brain	2·0	5·2	2·6	3·0	2·0	0·6	6·0	78·8	13·1
Gall bladder	NT†	NT	NT	NT	NT	NT	3·5	2·9	0·8
Heart	10·0	9·0	0·9	3·0	2·0	0·6	Inactive	4·7	NT
Kidney	4·5	1·4	0·3	3·0	2·6	0·8	6·5	2·2	0·3
Liver	5·0	4·4	0·8	12·0	7·5	0·6	7·0	6·2	0·8
Ling	6·0	5·8	0·9	10·8	4·6	0·4	4·7	1·9	0·4
Mucosa, fundic	4·0	10·0	2·5	1·1	3·0	2·8	7·0	13·0	1·9
Mucosa, pyloric	2·3	2·8	1·2	2·2	3·4	1·5	1·5	1·6	1·0

* G/A = [K_i (cyclic GMP) M/K_i (cyclic AMP) M]; numbers greater than 1 indicate greater selectivity for cyclic AMP-PDE.

† NT = not tested.

Glycyrrhetinic acid also appeared to possess some selectivity for the cyclic AMP-PDE from the fundic mucosa of each of the three species studied, exhibiting a relatively smaller K_i value than most other tissues (Table 1). However, the differences in K_i values between the different tissues were small.

More interesting is the selectivity of glycyrrhetinic acid for cyclic AMP-PDE vs cyclic GMP-PDE in the fundic mucosa. G/A ratios higher than 1 (Table 1), indicating that lower K_i values were obtained when cyclic AMP rather than cyclic GMP was used as substrate, were consistently observed with preparations from the fundic mucosa of the three species examined. This is apparently also true in the case of rat

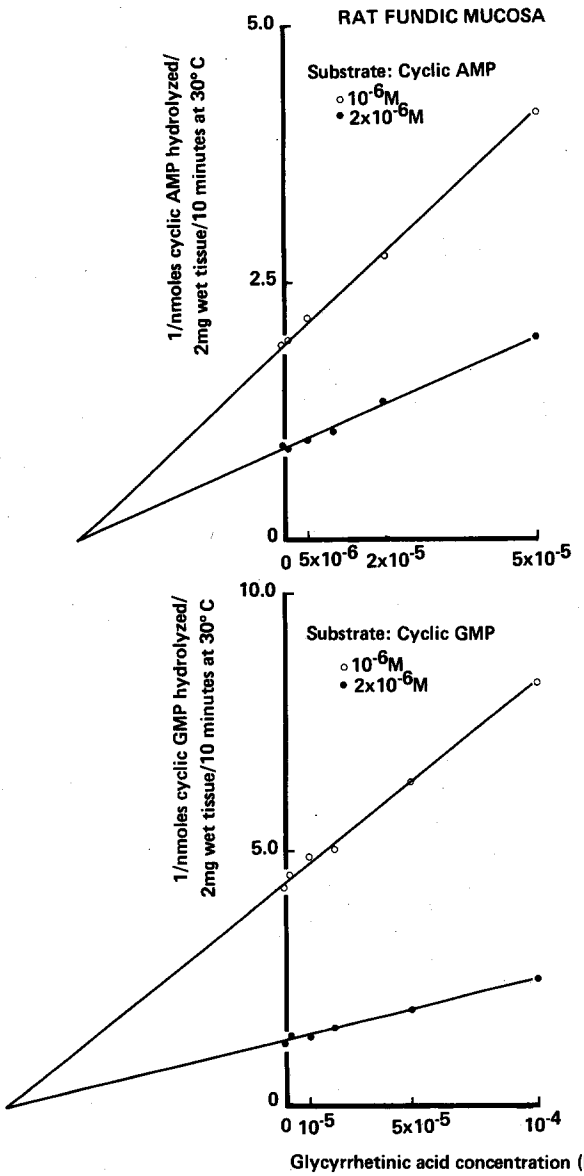

FIG. 1. Dixon plots for the effects of glycyrrhetinic acid on rat fundic mucosal PDE activity. The K_i values obtained were 4×10^{-5} M and 10^{-4} M when cyclic AMP and cyclic GMP were used as substrates respectively.

and dog brains and the dog aorta. It seems, therefore, that glycyrrhetinic acid exhibits PDE selectivity in that it is more active on the cyclic AMP-PDE's than on the cyclic GMP-PDE's, particularly in the fundic mucosa.

The use of two other known PDE inhibitors, theophylline and papaverine, demonstrated that selectivity for either cyclic AMP-PDE or cyclic GMP-PDE could be different with the different inhibitors of the enzyme (Table 2). Glycyrrhetinic acid

TABLE 2. SELECTIVITY OF THEOPHYLLINE, PAPAVERINE HCl AND GLYCYRRHETINIC ACID TOWARD LOW K_m CYCLIC AMP OR CYCLIC GMP PHOSPHODIESTERASES FROM THE FUNDIC MUCOSA OF THREE ANIMAL SPECIES

	Inhibitor constants (K_i)Δ* ($M \times 10^{-5}$)								
	Dog			Rat			Rabbit		
	Substrate			Substrate			Substrate		
Inhibitors	Cyclic AMP	Cyclic GMP	G/A†	Cyclic AMP	Cyclic GMP	G/A†	Cyclic AMP	Cyclic GMP	G/A†
Theophylline	50	26	0·5	38	17	0·5	44	17	0·4
Papaverine	2	2·6	1·3	2·5	2·8	1·1	2·5	3	1·2
Glycyrrhetinic acid	7	13	1·9	4·0	10	2·5	7·5	15	2·0

* Determined by the method of Dixon at two substrate (0·8 and 1×10^{-6} M cyclic AMP and 1 and 2×10^{-6} M cyclic GMP) and six drug concentrations bracketing the K_i value.

† G/A = [K_i (cyclic GMP as substrate) M/K_i (cyclic AMP as substrate) M]; numbers greater than 1 indicate greater selectivity for cyclic GMP-PDE.

showed greater selectivity for cyclic AMP-PDE in the fundic mucosa of the three species examined than did either theophylline or papaverine. Theophylline appeared to be the least selective for cyclic AMP-PDE. Papaverine, although the strongest PDE inhibitor, showed almost no selectivity for either cyclic AMP-PDE or cyclic GMP-PDE.

Studies with AC and GC activities of the fundic and pyloric mucosae from the rat indicated that glycyrrhetinic acid does not affect the activity of either enzyme (Table 3). Although the enzyme activities increased with time and were sensitive to classical stimulants (NaF for AC and acetylcholine for GC), glycyrrhetinic acid was without significant effect on either AC or GC from either tissue. Similar results were also obtained with the enzymes from the rabbit fundic and pyloric mucosae.

Studies in vivo. The effects of oral administration of glycyrrhetinic acid in a dose of 300 mg/kg (10 per cent of the oral LD_{50} of glycyrrhetinic acid in rats[33]) on the cyclic nucleotide levels in pyloric and fundic mucosae of rats are shown in Table 4. It is

TABLE 3. EFFECT OF GLYCYRRHETINIC ACID ON ADENYLYL CYCLASE AND GUANYLYL CYCLASE ACTIVITIES FROM RAT PYLORIC AND FUNDIC MUCOSAE*

Treatment	Adenylyl cyclase activity†		Guanylyl cyclase activity‡	
	Fundic mucosa	Pyloric mucosa	Fundic mucosa	Pyloric mucosa
Control, 5-min incubation	2·0 ± 0·2	3·0 ± 0·4	1·1 ± 0·2	1·3 ± 0·3
Control, 10-min incubation	3·3 ± 0·6	4·6 ± 0·7	1·8 ± 0·3	2·2 ± 0·4
Control, 20-min incubation	6·7 ± 0·8	8·3 ± 1·0	3·3 ± 0·5	4·3 ± 0·5
Glycyrrhetinic acid, 5×10^{-4} M	3·5 ± 0·6	4·9 ± 0·7	1·7 ± 0·3	2·1 ± 0·5
Glycyrrhetinic acid, 10^{-4} M	3·3 ± 0·6	4·3 ± 0·6	1·8 ± 0·2	2·2 ± 0·3
Glycyrrhetinic acid, 5×10^{-5} M	3·4 ± 0·7	4·7 ± 0·6	1·9 ± 0·4	2·3 ± 0·4
Sodium fluoride, 8 mM	4·5 ± 0·7§	6·2 ± 0·9§	NA	NA
Acetylcholine chloride, 10^{-4} M	NA‖	NA	3·3 ± 0·4§	3·6 ± 0·4§

* Average of three to twelve experiments ± S.E.

† Nmoles cyclic AMP formed/10 mg wet tissue (1·1 mg protein)/10 min at 37°.

‡ Nmoles cyclic GMP formed/10 mg wet tissue (1·1 mg protein)/10 min at 37°.

§ Difference from control (10-min incubation) statistically significant, $P < 0·05$.

‖ NA = not applicable.

TABLE 4. EFFECT OF ORAL ADMINISTRATION OF GLYCYRRHETINIC ACID ON INTRACELLULAR LEVELS OF CYCLIC AMP AND CYCLIC GMP IN PYLORIC AND FUNDIC MUCOSAE OF INTACT FEMALE RATS*

Tissue	Cyclic AMP level†		Cyclic GMP level†	
	Control	Glycyrrhetinic acid	Control	Glycyrrhetinic acid
Mucosa, fundic	0·91 ± 0·41 (6)	3·80 ± 1·31 (4)‡	0·048 ± 0·006 (6)	0·069 ± 0·012 (4)
Mucosa, pyloric	0·25 ± 0·02 (5)	0·83 ± 0·23 (5)‡	0·042 ± 0·005 (5)	0·059 ± 0·008 (5)

* Glycyrrhetinic acid was used as an aqueous suspension at a dose of 300 mg/kg. Animals were sacrificed 45 min after injection.

† Data reported as nmoles/g of tissue (wet wt); mean ± S.E. The number in parentheses = number of animals used.

‡ Different from control; $P < 0·05$.

evident that glycyrrhetinic acid significantly elevated cyclic AMP levels in both the pyloric and fundic mucosae (4-fold in the fundic and 3-fold in the pyloric). On the other hand, although there was an apparent rise in the cyclic GMP contents in both tissues, the difference from control was not statistically significant.

DISCUSSION

The spectrum of activity of glycyrrhetinic acid in inhibiting PDE activity in a variety of tissues from three species was surprising. This appears to be the first time a compound of this structural type has been reported to inhibit PDE activity.[28] The relative selectivity of glycyrrhetinic acid toward the enzyme from the fundic mucosa, its greater selectivity for cyclic AMP-PDE than for cyclic GMP-PDE, and its greater effect on the accumulation of intracellular cyclic AMP than of cyclic GMP in that same tissue *in vivo* are extremely interesting and may underlie its beneficial effects on the stomach.[4–18] Glycyrrhetinic acid appears to act exclusively via PDE inhibition, since it is devoid of activity on AC in the tissues examined. The relative selectivity of the drug for cyclic AMP-PDE rather than for cyclic GMP-PDE probably accounts for the lack of any significant increases in cyclic GMP levels in either the pyloric or fundic mucosae *in vivo* (Table 3), since the drug is far less active on cyclic GMP-PDE. Separate cyclic AMP- and cyclic GMP-PDE's appear to exist in a number of tissues.[28,34] Other factors may also be invoked to explain the lack of cyclic GMP accumulation in response to glycyrrhetinic acid. Among these factors are the low activity of GC in the fundic and pyloric mucosae, the expected low nucleotide accumulation in the face of PDE inhibition, and the possible stimulatory effects of the accumulated cyclic AMP on cyclic GMP hydrolysis. Cyclic AMP in certain concentrations can stimulate cyclic GMP-PDE.[35]

The selectivity of glycyrrhetinic acid for cyclic AMP-PDE may be extremely important, since the two nucleotides may mediate opposing effects on acid secretion by the stomach.[19,20] Glycyrrhetinic acid may indeed alter the balance of the two naturally occurring cyclic nucleotides in favor of the more inhibitory cyclic AMP and against the possibly stimulatory cyclic GMP.[20,21] This was re-emphasized by the results obtained with theophylline and papaverine. Theophylline stimulates acid secretion in some systems and potentiates it in others[19] and has the highest selectivity for cyclic GMP-PDE and thus is expected to preferentially raise intracellular cyclic GMP levels. Papaverine, on the other hand, shows little selectivity for either cyclic AMP or cyclic GMP-PDE's and would not be expected to appreciably change the

intracellular ratio of the two cyclic nucleotides and is devoid of any activity on acid secretion[36–38] despite its potency as a PDE inhibitor.[39–42] It would seem, therefore, that PDE inhibition *per se* may not be the important factor in determining the possible effects of PDE inhibitors on gastric acid secretion but rather their possible selectivity toward either cyclic AMP-PDE or cyclic GMP-PDE.

The effects of glycyrrhetinic acid on the cyclic nucleotide system of the stomach may also provide an explanation for its inhibitory effect on the release of gastrin.[4–8,12–14] Gastrin is produced by the pyloric mucosa in response to acetylcholine[26,43] which is known to lower the intracellular levels of the cyclic nucleotide in other systems.[44] α-Adrenergic stimulation generally lowers intracellular cyclic AMP levels[44] and also appears to stimulate gastrin release.[45] Thus, the rise of cyclic AMP levels in the pyloric mucosa effected by glycyrrhetinic acid would be consonant with its antagonism of gastrin release.

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