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The Antiulcer Effect of Verapamil in Relation to Gastric Calcium Levels in Stressed Rats

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KOO, M W L , C H CHO AND C W OGLE *The antiulcer effect of verapamil in relation to gastric calcium levels in stressed rats* PHARMACOL BIOCHEM BEHAV **34**(1) 73–76, 1989 —The antiulcer effect of verapamil, and its relationship to stomach calcium levels, were examined in rats restrained at 4°C (stress) Stress for 2 hr significantly increased muscle calcium and induced mucosal ulceration in the gastric glandular segment, calcium concentrations in the glandular mucosa and serum were unaffected Verapamil or calcium gluconate given 30 min before stress prevented the rise in gastric muscle calcium, and attenuated ulcer severity Bis(β-aminoethylether)-NNN'N'-tetra-acetic acid (EGTA) pretreatment, however, further elevated stomach muscle calcium and markedly worsened lesion formation These findings suggest that increased stomach muscle calcium could be a causal factor in stress-induced gastric glandular ulceration

Stress ulceration Calcium Verapamil EGTA Calcium gluconate

CALCIUM is involved in many physiological processes Increases in free calcium concentration within excitable cells can stimulate muscle contraction (3,8), synthesis and secretion of transmitters, and hormone release (14,15), as well as enzyme activity and membrane permeability (6) Thus, it is conceivable that such changes could be involved in stress-evoked ulceration (2,23) Indeed, the calcium channel blockers, verapamil and nifedipine, protect against gastric ulcers produced by cold-restraint stress in rats (9,16). This communication reports the results of a study on the effects of stress, and of calcium channel blockade of verapamil, on gastric tissue and serum calcium levels

METHOD

Animals

Female Sprague-Dawley rats (170–200 g) were supplied by the Laboratory Animal Unit, University of Hong Kong The animals were housed in an air-conditioned room with constant temperature (22 ± 1°C) and relative humidity (65–70%) Rats were starved for 48 hr before use but had free access to sucrose (BDH) 8% w/v in NaCl (BDH) 0 2% w/v This drinking solution was removed 1 hr before experimentation. The animals were either left in the room where they were normally housed (nonstressed controls) or placed in individual close-fitting tubular wire mesh cages and exposed to 4°C (cold-restraint stress) for 30 min or 2 hr, after which they were killed by a sharp blow on the head and rapidly cutting their throats

Drug Treatment and Measurement of Gastric Mucosal Lesions

Verapamil hydrochloride (Knoll) and calcium gluconate (E Merck) were prepared in normal saline (0 9% NaCl, w/v), whereas ethyleneglycol bis(β-aminoethylether)-NNN'N'-tetra-acetic acid (EGTA) (Sigma) was dissolved in phosphate buffer. These agents were injected intraperitoneally (IP) 30 min before exposure to cold-restraint stress After the rats were killed at the end of each experiment, their stomachs were removed and opened along the greater curvature The mucosa of the glandular segment was then examined, using an illuminated magnifier (3 ×) Lesions were measured (mm) along their greatest lengths, in the case of petechiae, five such lesions were considered the equivalent of a 1-mm ulcer (7)

Measurement of Gastric Tissue and Serum Calcium

The stomach was rinsed thrice with deionized water after the lesions were measured, and the mucosa of the glandular segment of the stomach scraped off with a glass slide. The glandular mucosa and muscle layer were then weighed after being placed into separate crucibles, the samples were finally ashed in an oven (Naber, Model N11) at 600°C for 24 hr. Following 24 hr of ashing, all the crucibles were taken our and their contents dissolved in 2 ml of a solution of 2 M HNO_3 (BDH) in $LaCl_3$

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TABLE 1

EFFECTS OF VERAPAMIL (GIVEN IP 30 MIN BEFOREHAND) ON STRESS-INDUCED CHANGES IN GASTRIC GLANDULAR TISSUE AND SERUM CALCIUM LEVELS AND GASTRIC GLANDULAR ULCERS IN RATS

Pretreatment Group	Dose	Calcium Content: Muscle (μmole/g)	Calcium Content: Mucosal (μmole/g)	Calcium Content: Serum (μmole/ml)	Glandular Ulcer Index (mm)
A No Stress (unrestrained at 22°C for 2 hr)					
Saline	2 ml/kg	3 2 ± 0 1	13 6 ± 0 4	2 35 ± 0 03	0 02 ± 0 01
Verapamil	1 mg/kg	3 4 ± 0 3	14 2 ± 0 5	2 20 ± 0 05	0 02 ± 0 01
Verapamil	2 mg/kg	3 3 ± 0 2	13 4 ± 0 4	2 23 ± 0 04	0 01 ± 0 01
Verapamil	4 mg/kg	3 6 ± 0 3	12 9 ± 0 4	2 38 ± 0 03	0 03 ± 0 01
Verapamil	8 mg/kg	3 3 ± 0 1	13 3 ± 0 5	2 27 ± 0 02	0 02 ± 0 01
B Stress (restrained at 4°C for 2 hr)					
Saline	2 ml/kg	3 9 ± 0 2†	13 5 ± 0 3	2 26 ± 0 04	6 28 ± 0 95‡
Verapamil	1 mg/kg	3 4 ± 0 3	13 6 ± 0 8	2 30 ± 0 06	4 71 ± 0 63‡
Verapamil	2 mg/kg	3 5 ± 0 3	12 1 ± 0 6	2 28 ± 0 05	3 26 ± 1 05‡§
Verapamil	4 mg/kg	3 3 ± 0 2§	13 8 ± 0 4	2 25 ± 0 08	2 85 ± 0 71‡¶
Verapamil	8 mg/kg	3 1 ± 0 2¶	12 4 ± 0 5	2 33 ± 0 04	2 12 ± 0 86*¶

Values are the means ± S E M of 20 rats in each group
*$p<0$ 02, †$p<0$ 01, ‡$p<0$ 001 when compared with the corresponding control in A
§$p<0$ 05, ¶$p<0$ 01 when compared with the saline-pretreated group in B

(BDH) 0 7% w/v Two empty crucibles were also exposed to 600°C for 24 hr so that the net calcium content of the tissues could be calculated after deducting the averaged residual calcium levels in both receptacles Arterial blood was collected into a centrifuge tube and allowed to clot for 10 min at room temperature (22°C), it was then spun at 4,500 rpm for 15 min The clear serum was pipetted into another tube and subjected to the same centrifugation speed for another 10 min An aliqout of the supernatant was then diluted to 50% with deionized water, 0 2 ml of this dilution was added to 1 8 ml of a solution of 2 M HNO_3 in $LaCl_3$ The amount of calcium present in the tissue and serum was determined, using an atomic absorption spectrophotometer (Perkin Elmer, model 103) with its wavelength set at 495 nm Calcium levels in the samples were calculated from a standard curve

Statistical Analysis

The data were analyzed for statistical significance by means of the two-tailed Student's *t*-test (1) Verapamil effects on calcium levels and glandular ulcer formation were further analyzed by the one-way ANOVA (1)

RESULTS

Effects of Verapamil Treatment on Gastric Tissue and Serum Calcium in Cold-Restrained Rats

Verapamil pretreatment did not have any effect on the serum calcium of nonstressed rats, similarly, no difference was found in the gastric glandular mucosal and muscle layers (Table 1), hemorrhagic gastric lesions were not observed in these control animals Stress for 2 hr elevated the gastric muscle calcium level in saline-pretreated rats, this was accompanied by hemorrhagic gastric glandular ulceration The calcium content of the gastric mucosa and serum was, however, not significantly affected by cold-restraint stress Verapamil pretreatment dose-dependently and significantly lessened stress-induced gastric glandular ulceration when analyzed by Student's *t*-test and by ANOVA ($F=3$ 77, $p<0$ 01) The drug also significantly prevented the elevation of muscle calcium levels when given in the higher dose range (Table 1)

Effects of Calcium Gluconate of EGTA Treatment on Gastric Tissue and Serum Calcium in Cold-Restrained Rats

Under nonstress conditions, neither saline, phosphate buffer nor EGTA pretreatment, given 30 min beforehand, influenced calcium levels in gastric glandular tissue and serum throughout the whole 2-hr experimental period (Tables 2 and 3) Calcium gluconate induced a transient rise of calcium in the glandular muscle layer and serum 1 hr after its IP injection, however, no ulceration was observed (Table 2) Restraint at 4°C for 30 min did not induce any change in the gastric tissue and serum calcium in both vehicle- and EGTA-treated animals This period of stress did not produce glandular mucosal ulceration in either group Calcium gluconate pretreatment also increased the calcium levels in the glandular muscle and serum in 30-min stress conditions, as in the nonstressed group, no ulceration was observed (Table 2) The calcium levels in the gastric tissue and serum of all 2-hr non-stressed rats were comparable to those at 30 min (except for muscle and serum calcium following calcium gluconate) after injection of the drugs, no hemorrhagic lesions were observed in these animals (Table 3) Cold-restraint stress for 2 hr significantly increased the gastric glandular mucosal ulcer index When compared with the controls left at room temperature (22°C), there was significant elevation of the glandular muscle calcium levels in the vehicle- and EGTA-treated animals, but not in those given calcium gluconate (Table 3) Calcium gluconate, however, increased serum calcium The ulcer index of EGTA-treated animals was found to be significantly greater when compared to that of the vehicle-treated group Calcium gluconate administration tended to prevent the stress-induced elevation of gastric glandular muscle calcium and to lessen ulcer formation significantly (Table 3)

DISCUSSION

In this study, 2-hr cold-restraint stress significantly increased

TABLE 2

EFFECTS OF CALCIUM GLUCONATE OR EGTA (GIVEN IP 30 MIN BEFOREHAND) ON STRESS-INDUCED CHANGES IN GASTRIC GLANDULAR TISSUE AND SERUM CALCIUM LEVELS AND GASTRIC GLANDULAR ULCERS IN RATS

Pretreatment Group	Dose	Calcium Content Muscle (μmole/g)	Mucosa (μmole/g)	Serum (μmole/ml)	Glandular Ulcer Index (mm)
A No Stress (unrestrained at 22°C for 0 5 hr)					
Saline	2 ml/kg	4 35 ± 0 51	12 7 ± 1 0	2 26 ± 0 03	0 01 ± 0 01
Phosphate buffer	2 ml/kg	4 70 ± 0 54	12 4 ± 1 4	2 32 ± 0 07	0 03 ± 0 02
Calcium gluconate	100 mg/kg	5 82 ± 0 42*	13 3 ± 1 0	2 77 ± 0 04†	0 02 ± 0 01
EGTA	20 mg/kg	4 67 ± 0 73	12 6 ± 1 5	2 34 ± 0 03	0 02 ± 0 01
B Stress (restrained at 4°C for 0 5 hr)					
Saline	2 ml/kg	4 51 ± 0 68	13 6 ± 1 3	2 31 ± 0 09	0 02 ± 0 01
Phosphate buffer	2 ml/kg	4 93 ± 0 62	13 2 ± 1 1	2 25 ± 0 08	0 01 ± 0 01
Calcium gluconate	100 mg/kg	6 43 ± 0 50*	12 5 ± 0 9	2 84 ± 0 09†	0 03 ± 0 01
EGTA	20 mg/kg	3 54 ± 0 50	13 8 ± 1 4	2 46 ± 0 06	0 04 ± 0 02

Values are the means ± S E M of 8 rats in each group
*$p<0$ 05, †$p<0$ 001 when compared with the corresponding vehicle-pretreated group
EGTA = ethyleneglycol bis(β-aminoethylether)-NNN'N'-tetra-acetic acid

the stomach glandular muscle calcium content, whereas no change occurred in the mucosa and serum Gastric glandular mucosal ulceration was also observed in these stressed animals Thus, an increase in muscle calcium during stress may be related to ulcer formation It is difficult to be certain whether this calcium elevation is the cause, or the result, of the ulceration An increase in intracellular calcium has been shown to facilitate muscle contractility in stress-induced ulceration (24,25), this effect is thought to be due to vagal stimulation which occurs in stress (4,11) Acetylcholine release from the vagal nerve fibre depolarizes in smooth muscle membranes and opens the potential-operated slow channels, allowing an influx of calcium (21) Stress also increases the release of catecholamines (18) which activate receptor-operated slow channels to allow more calcium influx (20,22) It has further been found that the activity of the enzyme Ca^{++}-ATPase, which is partly responsible for the extrusion of cellular calcium, is depressed by low temperatures (7), thus, when animals are subjected to cold-restraint stress, the fall in body temperature may depress this calcium extrusion process The elevation of calcium levels in the gastric glandular muscle layer could, therefore, be due to these factors collectively producing an increase in gastric wall contraction during stress.

TABLE 3

EFFECTS OF CALCIUM GLUCONATE OR EGTA (GIVEN IP 30 MIN BEFOREHAND) ON STRESS-INDUCED CHANGES AND IN GASTRIC GLANDULAR TISSUE AND SERUM CALCIUM LEVELS AND GASTRIC GLANDULAR ULCERS IN RATS

Pretreatment Group	Dose	Calcium Content Muscle (μmole/g)	Mucosa (μmole/g)	Serum (μmole/ml)	Glandular Ulcer Index (mm)
A No Stress (unrestrained at 22°C for 2 hr)					
Saline	2 ml/kg	3 32 ± 0 24	12 8 ± 0 9	2 28 ± 0 05	0 11 ± 0 05
Phosphate buffer	2 ml/kg	3 38 ± 0 42	13 4 ± 0 7	2 15 ± 0 04	0 04 ± 0 02
Calcium gluconate	100 mg/kg	3 69 ± 0 38	13 1 ± 0 7	2 26 ± 0 08	0 09 ± 0 06
EGTA	20 mg/kg	3 12 ± 0 34	14 4 ± 1 0	2 21 ± 0 06	0 08 ± 0 03
B Stress (restrained at 4°C for 2 hr)					
Saline	2 ml/kg	4 36 ± 0 28*	12 3 ± 0 9	2 25 ± 0 08	7 43 ± 1 52§
Phosphate buffer	2 ml/kg	4 62 ± 0 43*	12 3 ± 0 8	2 20 ± 0 05	8 24 ± 1 86§
Calcium gluconate	100 mg/kg	3 91 ± 0 32	13 7 ± 1 2	2 43 ± 0 09*	3 17 ± 1 10†¶
EGTA	20 mg/kg	5 01 ± 0 47‡	13 6 ± 1 1	2 38 ± 0 08	13 86 ± 1 73§¶

Values are the means ± S E M of 8 rats in each group
*$p<0$ 05, †$p<0$ 02, ‡$p<0$ 01, §$p<0$ 001 when compared with the corresponding control in A
¶$p<0$ 05 when compared with the corresponding vehicle-pretreated group in B
EGTA = ethyleneglycol bis(β-aminoethylether)-NNN'N'N-tetra-acetic acid

Verapamil, a calcium antagonist (10), was found to prevent stress-induced elevation of muscle calcium levels, as well as gastric ulceration in the glandular segment (Table 1) This supports the idea that increased calcium levels in the muscle wall could be an important causative factor in stress ulcer formation Verapamil treatment, however, did not significantly influence muscle layer calcium levels under nonstress conditions, this may be explained by the ability of verapamil to block preferentially calcium channels only when they are in the activated state (13,23) Specific binding sites for calcium blockers have been demonstrated in rat stomach tissue (12) Thus, one of the antiulcer effects of verapamil could be due to it being bound to these receptors, this would prevent over-loading of calcium in the gastric tissue and inhibit gastric contraction and acid secretion (9, 16, 17) However, the importance of acid secretion in stress-evoked ulceration is still debatable Complete neutralization of the luminal acid does not prevent glandular formation (19), furthermore, nifedipine, which is less potent than verapamil in reducing gastric acid secretion, is more effective in attenuating stress-induced mucosal lesions (9) Thus, it is unlikely that gastric acid reduction by verapamil could play a major role in the antiulcer action of this calcium channel blocker

Calcium gluconate injection significantly increased the gastric muscle and serum calcium levels 1 hr after its administration, both under nonstress and stress (30-min duration) conditions (Table 2) However, this elevation of calcium was not accompanied by ulceration in the gastric glandular mucosa Thus, when stressing the animals for only 30 min, the increased stomach tissue and serum calcium levels during this period were unable to produce ulceration However, it is possible that excess calcium alone is unable to initiate ulcer formation during this early period of stress As the calcium content in the glandular mucosa remained unchanged even in the presence of ulcer formation, this finding suggests that the gastric mucosal calcium level may have no direct relationship to the mechanism of stress-evoked lesions The observation that EGTA administration further increased muscle calcium levels and aggravated ulceration in 2-hr stress experiments (Table 3) indeed points to a direct relationship between an elevated glandular muscle calcium content and ulcer severity In the same experiment, calcium gluconate injection did prevent the stress-induced rise in muscle calcium levels when compared with the saline-pretreated controls, and this was associated with less lesion formation Such an observation also suggests that gastric muscle calcium may be closely related to ulcer aggravation during the later phase of 2-hr cold-restraint stress

It is concluded that the manipulation of gastric tissue and serum calcium levels by calcium gluconate administration does not induce gastric glandular ulceration in nonstressed animals Verapamil or calcium gluconate protects against, whereas EGTA worsens, ulcer formation in rats stressed for 2 hr The effects of verapamil, calcium gluconate and EGTA pretreatment on stress-induced ulceration could be mediated through their interaction with membrane-bound calcium and free calcium influx into the glandular muscle cells, this in turn influences cell membrane stability and muscle contraction, as well as the integrity of the gastric glandular mucosa

REFERENCES

1 Armitage, P Statistical method in medical research Oxford Blackwell Scientific Publications, 1971
2 Brodie, D A Ulceration of the stomach produced by restraint in rats Gastroenterology 43 107–109, 1962
3 Chapman, R A Control of cardiac contractility at the cellular level Am J Physiol 245 H535–H552, 1983
4 Cho, C H , Ogle, C W Acute gastric ulcer formation in response to electrical vagal stimulation in rats Eur J Pharmacol 35 215–219, 1976
5 Cho, C H , Ogle, C W Does increased gastric mucus play a role in the ulcer-protecting effects of zinc sulphate? Experientia 34 90–91, 1978
6 Cohen, P The role of protein phosphorylation in neural and hormonal control of cellular activity Nature 296 613–620, 1982
7 Droogmans, G , Casteel, R Temperature-dependence of ^{45}Ca fluxes and contraction in vascular smooth muscle cells of rabbit ear artery Pflugers Arch 391 183–189, 1981
8 Fabiato, A , Fabiato, F Calcium and cardiac excitation-contraction coupling Annu Rev Physiol 331 599–635, 1979
9 Glavin, G B Verapamil and nifedipine effects on gastric acid secretion and ulcer formation in rats J Pharm Pharmacol 40 514–515, 1988
10 Godfraind, T Calcium entry and calcium entry blockade In Godfraind, T , Vanhoutte, P M , Govoni, S , Paoletti, R , eds Calcium entry blockers and tissue protection New York Raven Press, 1985 1–19
11 Goldman, H , Rosoff, C B Pathogenesis of acute gastric stress ulcers Am J Pathol 52 227–243, 1968
12 Janis, R A , Rampe, D , Su, C M , Triggle, D J Calcium channel ligand-induced antagonism and activation In Godfraind, T , Vanhoutte, P M , Govoni, S , Paoletti, R , eds Calcium entry blockers and tissue protection New York Raven Press, 1985 21–30
13 Katz, A M What are calcium channels and how do drugs act on them? J Cardiovasc Med 8 435–450, 1983
14 Katz, B , Miledi, R The timing of calcium action during neuromuscular transmission J Physiol 189 535–544, 1967
15 Katz, B , Miledi, R Further study of the role of calcium in synaptic transmission J Physiol 207 789–801, 1970
16 Koo, M W L , Cho, C H , Ogle, C W Effects of cold-restraint stress on gastric ulceration and motility in rats Pharmacol Biochem Behav 25 775–779, 1986
17 Koo, M W L , Ogle, C W , Cho, C H The effect of cold-restraint stress on gastric emptying in rats Pharmacol Biochem Behav 23 969–972, 1985
18 Leduc, J Catecholamine production and release in exposure and acclimation to cold Acta Physiol Scand 53(Suppl 183) 5–101, 1961
19 Ogle, C W , Cho, C H , Dai, S Intragastric $NaHCO_3$ perfusion and vagal-induced ulcer formation in the rat stomach Eur J Pharmacol 37 197–201, 1976
20 Pappano, A J Calcium-dependent action potentials produced by catecholamines in guinea pig atrial muscle fibres depolarized by potassium Circ Res 27 379–390, 1970
21 Rubin, R P The role of Ca^{++} in the release of neurotransmitter substances and hormones Pharmacol Rev 22 389–428, 1970
22 Shinebourne, E A , Hess, M L , White, R J , Hamer, J The effect of noradrenaline on the calcium uptake of the sarcoplasmic reticulum Cardiovasc Res 3 113–117, 1969
23 Van Zwieten, P A Calcium antagonists—recent developments Prog Pharmacol 5 1–94, 1982
24 Watanabe, K Some pharmacological factors involved in formation and prevention of stress ulceration in rats Chem Pharm Bull 14 101–107, 1966
25 Yano, S , Akahane, M , Harada, M Role of gastric motility in development of stress-induced gastric lesions of rats Jpn J Pharmacol 28 607–615, 1978