Mucosal gastric injury was induced in vivo by oral administration of 75% ethanol to rats that were intraperitoneally pretreated with saline (controls), 4 mg/kg of verapamil, 0.4 mg/kg of nifedipine or 160 mg/kg of MgCl₂. The glandular stomachs were used for macroscopic and histologic evaluation of mucosal lesions. The gastroprotective effects of substances tested against ethanol-induced mucosal damage in vitro were examined: 1) in rats intraperitoneally pretreated with calcium antagonists and 2) after adding of 10⁻⁴ M verapamil, 10⁻⁶ M nifedipine or 10⁻² M MgCl₂ directly to minced gastric mucosa of untreated rats. This effect was measured by DNA synthesis.

Gross and histologic evaluation showed that rats pretreated with nifedipine or MgCl₂ had significantly decreased ethanol-induced gastric injury compared to controls, whereas those pretreated with verapamil had significantly increased injury. On the contrary, all verapamil, nifedipine and MgCl₂ treatments, administrated intraperitoneally or by exposure to an incubation mixture were equally effective in reducing gastric mucosal damage following to ethanol treatment in vitro.

We conclude that the differential effects of verapamil and nifedipine on ethanol-damaged gastric mucosa in vivo, but not in vitro, suggests the existence of different actions of these calcium antagonists more on systemic rather than local protective mechanisms, such as gastric mucosal blood flow, mucosal barrier, or cell renewal.

Key words: gastroprotection; verapamil; nifedipine; MgCl₂; ethanol.

INTRODUCTION

Calcium channel blockers inhibit calcium entry into the cell and may affect the function of many organs, among them the gastric mucosa. Although calcium channel blockers can affect gastric mucosal damage, basic mechanisms of their cytoprotective action are still poorly understood. In fact, even opposite effects of these agents on experimental gastric injury have been described [1—12]. Whereas nifedipine exhibited apparent gastroprotective
effects, both protecting and enhancing effects on induced gastric lesions were observed during verapamil treatment. The mechanism of this difference remains unknown.

The aim of this study was to compare effects of calcium antagonists (verapamil, nifedipine, and magnesium ions) on gastric mucosal damage induced by ethanol in vivo and in vitro in rats.

**METHODS**

Male Wistar rats (180—230 g) fasted overnight with unlimited access to drinking water before experiments. Each control and experimental group consisted of 2—3 rats and the results of at least four repeated experiments were pooled.

**Mucosal gastric lesions induced in vivo.**

Animals randomly divided into groups were intraperitoneally pretreated with 0.9% NaCl, 4 mg/kg of verapamil, 0.4 mg/kg of nifedipine, or 160 mg/kg of MgCl₂. Thirty minutes after pretreatment, 2 ml of 75% ethanol was administered into the stomach, and 60 min. later the rats were killed by decapitation. Then, stomachs were excised, opened along the greater curvature, rinsed in 0.9% NaCl, and pinned to a cardboard. Then the mucosa was photographed. An enlarged color picture of the glandular stomach was used for analysis of macroscopic mucosal changes. The area of macroscopic necrosis was measured with the planimeter and was expressed as a percentage of total glandular area.

After the picture for the gross evaluation of mucosal lesions had been taken, stomachs were rolled up, fixed in buffered formaldehyde, routinely processed, and embedded in paraffin. Tissue sections comprised, therefore, of the entire width of the glandular stomach wall. Three paraffin sections of each stomach, cut in different distances from the pylorus, were mounted on glass slides and stained with hematoxylin and eosin. Slides were coded, and the damage of the mucosa was scored on a scale of 1—3: grade — superficial mucosal necrosis; grade 2 — half-thickness mucosal necrosis; grade 3 — full-thickness mucosal necrosis. The histological damage index was expressed for each slide as a single value calculated from both the length and number of mucosal lesions multiplied by their scored mucosal depth of injury. Results were expressed for each stomach as a mean of three slides damage index.

**Mucosal gastric damage induced in vitro.**

In these experiments, gastroprotective effects of the substances tested were examined in two separate series of experiments (A and B).

In the A series, rats were pretreated with intraperitoneal injections of 0.9% NaCl, verapamil (4 mg/kg), nifedipine (0.4 mg/kg) or MgCl₂ (160 mg/kg). Thirty minutes later, stomachs were removed, washed in 0.9% NaCl, and numerous biopsy samples, comprising the full thickness of the mucosal layer (each about 15—20 mg wet weight) were taken. Tissue material was immediately minced and transferred to Eppendorf tubes containing 1 ml of Minimal Essential Medium with 2 μCi/ml of ³H-thymidine. Half of these tubes served as controls. Ethanol to 25% of final concentration was added (as described previously by Domschke et al [13]) to the remaining tubes.
In the B series, equal amounts of minced gastric mucosal material of untreated rats were transferred to Eppendorf tubes. The tubes were divided into four sets. The first set of tubes served as control. Verapamil, nifedipine, or MgCl₂ to a final concentration of 10⁻⁴M, 10⁻⁶M, or 10⁻²M, respectively, was added to the second set of tubes. In the third and fourth sets of tubes, ethanol to equal 25% of the final concentration was added alone or with the substances tested.

The tubes were incubated with shaking in 5/95% CO₂/O₂ gas mixture at 37°C for 60 min. Following incubation the reaction was stopped with 0.4 N perchloric acid, tubes were centrifuged (12,000 g, 4°C, 10 min.), and resulting pellets were washed twice with ice cold 0.2 N perchloric acid. Then, pellets were kept in 0.3 N KOH for 60 min at 37°C with mixing. After centrifugation, supernatants were placed on ice for 10 min. and then centrifuged. Resulting pellets containing DNA were solubilized in 10% perchloric acid by heating to 70°C for 20 min. After a final centrifugation, incorporation of ³H-thymidine into DNA was determined by radioactivity counts and the estimation of DNA concentration in the supernatant. The DNA content of the samples was measured by the fluorometric method [14]. The rate of DNA synthesis was calculated as DPM per microgram of DNA. All measurements were performed in triplicate.

Statistics.

The results were statistically evaluated by the nonparametric Mann-Whitney test.

**RESULTS**

Preliminary studies showed that the protective effects of verapamil, nifedipine and MgCl₂ on ethanol-damaged rat gastric mucosa in vitro were dose dependent, and maximal effects were achieved with intraperitoneal injections of 4 mg/kg, 0.4 mg/kg, and 160 mg/kg, respectively. The same concentrations of substances studied were used for the evaluation of their effects on gastric mucosa injury in vivo. When calcium antagonists were added directly to minced gastric mucosa of untreated rats, the protective effects were also dose dependent being the maximal at 10⁻⁴M verapamil, 10⁻⁶M nifedipine or 10⁻²M MgCl₂.

No macroscopic or histologic evidence of gastric mucosal changes was noted in rats treated with verapamil, nifedipine or MgCl₂. Stomachs exposed to 75% ethanol administered orally revealed obvious evidence of severe hemorrhagic necrotic lesions of the mucosa. As shown in Fig. 1, gross evaluation of stomach from rats treated with 4 mg/kg of verapamil followed by oral ethanol administration showed significant increases in the area of mucosal damage compared to control rats (p < 0.01). On the contrary, rats pretreated with 0.4 mg/kg of nifedipine or 160 mg/kg of MgCl₂ had significantly decreased macroscopic necrosis after ethanol instillation compared to controls (p < 0.01).

The semiquantitative data for microscopic necrosis calculated as the histological damage index showed results similar to those of gross evaluation. Ethanol-injured gastric mucosa of rats pretreated with verapamil showed
Fig. 1. Effect of pretreatment with verapamil (4 mg/kg), nifedipine (0.4 mg/kg) or MgCl₂ (160 mg/kg) on ethanol-induced rat gastric mucosal lesions evaluated grossly. Bars represent medians of mucosal lesion area (percent of glandular mucosa showing gross damage at the end of experiment) of 20 rats from each studied group. Asterisks indicate significant increase or decrease (p<0.01) in lesion area induced by oral administration of 75% ethanol in rats pretreated with calcium antagonists compared to controls.

significantly increased intensity in the depth and extensiveness of gastric mucosal lesions (p<0.05), whereas nifedipine and MgCl₂ significantly reduced the histological necrotic damage index (p<0.01), as compared to control rats. (Fig. 2).

Under experimental conditions, verapamil, nifedipine, and MgCl₂ by themselves administered intraperitoneally (the A series) or directly to the incubation tubes (the B series) did not influence the rate of DNA synthesis estimated by thymidine incorporation (data not shown).

Twenty-five percent ethanol significantly decreased the ³H-thymidine uptake by gastric mucosa cells in vitro. Verapamil, nifedipine, and MgCl₂
Fig. 2. Effect of pretreatment with verapamil (4 mg/kg), nifedipine (0.4 mg/kg) or MgCl₂ (160 mg/kg) on histologically evaluated ethanol-induced rat gastric mucosal lesions. Bars represent medians of the histological damage index of 20 stomachs from each studied group. Asterisks indicate significant increase (p<0.05) or decrease (p<0.01) in microscopic ethanol-induced necrosis in rats pretreated with calcium antagonists compared to controls.

significantly reduced this ethanol-induced decrease in the synthetic activity of DNA, both in the A (Fig. 3) and B series (Fig. 4) of experiments (p<0.01).

DISCUSSION

Because of ethanol lipid solubility, its rapid diffusion into the gastric mucosa after oral administration causes severe necrotized mucosal injury. Exact mechanisms of the necrotizing effect of ethanol have not been elucidated. It is suggested [15, 16] that the ethanol-induced gastric lesions are connected
with release of vasoactive mediators from injured superficial mucosal cells that results in vascular contractions. Since inappropriate influx of calcium ions due to opening of calcium channels by ethanol is considered as one of the mechanisms that can enhance ethanol-induced mucosal disruption [17, 18], it is reasonable to assume that intracellular calcium depletion of gastric mucosal cells by calcium antagonists acts gastroprotectively. The protection of gastric mucosa against alcohol injury may also result from blocking the generation of
vascular contractions (as smooth muscle contraction is calcium-dependent), by inhibition of the release of vasoactive products, or via both processes.

Verapamil prevents pyloric-ligation-, aspirin-, and stress-induced gastric lesions in rats [4, 7—12] but exacerbates ethanol-induced gastric damage [5, 7]. Another commonly used calcium channel blocker, nifedipine, attenuates gastric mucosal injury induced by ethanol [1, 2]. Results of this study confirmed that verapamil worsens mucosal damage whereas nifedipine and MgCl₂ prevent ethanol-induced gastric lesions in vivo. On the other hand, all of the calcium antagonists used (verapamil, nifedipine, and MgCl₂) were equally

![Graph](image)

Fig. 4. \[^{3}H\]thymidine incorporation into DNA of rat gastric mucosa in response to 25% ethanol, nifedipine (10⁻⁶M) plus 25% ethanol, verapamil (10⁻⁴) plus 25% ethanol, or MgCl₂ (10⁻²M) plus 25% ethanol. Both calcium antagonists and ethanol were added directly to the incubation mixture. Values were expressed as DPM/µg DNA, and are illustrated as the percentage of decrease in the value of thymidine incorporation into DNA of undamaged by ethanol gastric mucosal cells relative to control. Bars represent medians of 30 observations. Asterisks indicate a significant increase (p<0.01) in gastric mucosal DNA synthesis in rats treated with calcium antagonists in response to 25% ethanol, compared to DNA synthesis of control rat gastric mucosa in response to ethanol.
effective in reducing gastric mucosal damage in response to ethanol treatment in vitro, as assessed by thymidine incorporation. This protective effect found in in vitro experiments was independent of the route of administration of calcium antagonists. The differential effects of verapamil and nifedipine on ethanol-damaged gastric mucosa in vivo, but not in vitro, may indicate the existence of different methods of action for these calcium antagonists in systemic, but not in local protective mechanisms. In fact, many factors, such as increases in the mucosal blood flow, decreases in acid secretion, and increases in endogenous protective prostaglandin or sulfhydryl production, were assumed to participate with the calcium antagonists in protecting the gastric mucosal against damage. The worsening effects of verapamil on ethanol-induced mucosal injury could result from the exacerbation of some undefined ulcerogenic stimuli, or from influencing systems that are present in the stomach in situ which protect gastric mucosa, such as gastric mucosal blood flow, gastric mucosal barrier, or mucosal cell renewal. It can only be speculated that the differential effects of calcium antagonists on gastric mucosa indicate the existence of different classes of calcium channels in gastric mucosal cells. However, the basic mechanisms of cytoprotection by calcium antagonists are still poorly understood.

Since gastroprotection is the property of various agents that protect gastric mucosa from damage caused by necrotizing substances [19], two essential elements are required in the assessment of this phenomenon — experimental cell damage and a method to evaluate the protective action of the substances tested.

Gastric damage induced in vivo can be evaluated by means of macroscopic or histologic measurements. In the in vitro induced cell injury, its severity can be measured by several methods such as release of lysosomal enzymes, RNA, DNA or 51chromium or inhibition of DNA synthesis rate by damaged cells. According to the results of this work, it seems that in studies on gastroprotection, in vitro experiments cannot fully replace experiments performed in vivo. Complete evaluation of the protective effect of substances tested still requires measurements of gastric mucosal injury in situ since both local and systemic factors may be involved in gastroprotection.

REFERENCES


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