Roles of Extracellular Ca\(^{++}\) and Calmodulin in Roxatidine-Stimulated Secretion and Synthesis of Mucus by Cultured Rabbit Gastric Mucosal Cells

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ABSTRACT

We found that roxatidine stimulates mucus secretion and synthesis by cultured rabbit gastric mucosal cells. In this study, we examined the roles of the extracellular Ca\(^{++}\) and calmodulin in these effects of roxatidine. Reduction of the extracellular Ca\(^{++}\) concentration decreased the roxatidine-induced increases in mucus secretion and synthesis by gastric mucosal cells. Roxatidine concentration-dependently promoted Ca\(^{++}\) influx and caused an increase in intracellular Ca\(^{++}\). After the addition of roxatidine, the increases in the secretion and synthesis reflected those in Ca\(^{++}\) influx and intracellular Ca\(^{++}\) concentration and then disappeared as Ca\(^{++}\) influx and intracellular Ca\(^{++}\) concentration returned to the control level. The roxatidine-stimulated Ca\(^{++}\) influx and intracellular Ca\(^{++}\) mobilization were abolished by reduction of the extracellular Ca\(^{++}\) concentration. Nifedipine and diltiazem inhibited both the effects of roxatidine, but even at 10 \(\mu\)M, the inhibition was partial. Furthermore, W-7 (a calmodulin antagonist) completely abolished the effects of roxatidine on mucus secretion and synthesis without causing a reduction of the stimulated Ca\(^{++}\) influx. Taken together, these results suggest that roxatidine promoted Ca\(^{++}\) influx through both voltage-sensitive Ca\(^{++}\) channels and other Ca\(^{++}\) entry gates and the subsequent intracellular Ca\(^{++}\) mobilization, leading to potentiation of mucus secretion and synthesis by rabbit gastric mucosal cells. In addition, Ca\(^{++}\) activated calmodulin may play a pivotal role in these stimulatory effects of roxatidine.

Materials and Methods

Preparation of gastric mucosal cells. Gastric mucosal cells were prepared from rabbit stomachs according to the method of Watanabe et al. (1994). Briefly, male Japanese White rabbits (Nihon S.L.C., Shizuoka, Japan), weighing 2.5–3.5 kg, were anesthetized with Nembutal (50 mg/kg, i.v.; Abbott, North Chicago, IL). After a stomach had been excised, the surface of theoxytic mucosa was removed with a razor blade and minced immediately. The minced tissue was incubated in Hank’s balanced salt solution containing 0.07% collagenase (Wako Chemicals, Osaka, Japan) for 15 min at 37°C, and then washed with Ca\(^{++}\), Mg\(^{++}\)-free Hank’s solution containing 1 mM EDTA and 1 mg/ml bovine serum albumin. These procedures were repeated twice. The mucosal cells were obtained by filtration through metal meshes (diameters, 300 \(\mu\)m and 100 \(\mu\)m). The viability of the isolated cells was more than 85%, as determined by the dye exclusion test (Phillips, 1973).

Cell culture. Coon’s modified Ham’s F12 medium (GIBCO BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 units/ml streptomycin and 0.25 \(\mu\)g/ml amphotericin B, and culture plates and dishes coated with collagen type
I (Sigma Chemicals, St. Louis, MO) were used. The medium contained 2 mM CaCl₂. Gastric mucosal cells (2 × 10⁵ cells/1 ml) were inoculated onto 12-well plates (Corning & Costar, Corning, NY). For measurement of intracellular Ca²⁺ concentration, cells were inoculated onto glass-bottomed 35 mm-dishes (Mat Tek, Ashland, MA). The cultures were maintained at 37°C under 5% CO₂ in air, the medium being changed every day. The cells reached confluence 2–3 days later. Most of the cultured cells were morphologically epithelial-like, and 80–90% of them were confirmed to be mucus-producing ones by periodic acid-Schiff staining, as described previously (Takahashi and Okabe, 1995; Takahashi et al., 1995; Takahashi and Okabe, 1996a).

**Determination of mucus secretion and synthesis.** The amounts of secreted and synthesized mucus were determined according to the methods of Terano et al. (1982) and Keates and Hanson (1990) with a slight modification. Gastric mucosal cells grown to confluence were washed with PBS and then incubated with 1 ml of the medium containing [³H] glucosamine (18.5 kBq, 1800 GBq/mmol; New England Nuclear, Boston, MA) in the presence of the indicated drugs or vehicle at 37°C. Unless otherwise stated, the Ca²⁺ concentration in the medium was 2 mM. At appropriate times, the medium was recovered and centrifuged at 3000 × g for 3 min, and then an aliquot (0.8 ml) of the resulting supernatant was mixed with 0.2 ml of 50% trichloroacetic acid. The mixture was held on ice for 5 min and then centrifuged at 10,000 × g for 5 min at 4°C. After the resulting pellet was solubilized with 0.25 ml of 5% Triton X-100, an aliquot (0.2 ml) was subjected to Sepharose CL-4B column (4 ml) chromatography. The radioactivity in the void fractions was measured as the amount of mucus secreted by the cells into the medium. For estimation of mucus synthesis in the cells, the amount of [³H] glucosamine incorporated into the cells was determined. The remaining cells were washed twice with PBS and then solubilized with 0.25 ml of 5% Triton X-100. As described above, when an aliquot (0.2 ml) was loaded onto the same column, the radioactivity in the void fractions was measured. About 80% of the precipitated labeled materials in the medium and cells were identified as mucus glycoprotein on gel filtration chromatography.

**Determination of Ca²⁺ influx into gastric mucosal cells.** Ca²⁺ influx was evaluated as ⁴⁵Ca⁺⁺ uptake by gastric mucosal cells. ⁴⁵Ca⁺⁺ uptake was determined according to the method of Tanaka et al. (1990) with a slight modification. After mucosal cells grown to confluence had been washed with PBS, they were incubated in 0.5 ml of the medium containing 2 mM ⁴⁵CaCl₂ (22.2 kBq, >370 GBq/mmoll; New England Nuclear) at 37°C. At appropriate times, the cells were washed with PBS and then solubilized with 0.2 ml of 0.3N NaOH. Then the radioactivity in the lysate was measured.

**Determination of intracellular Ca²⁺ concentration.** Intracellular Ca²⁺ concentration was measured in the cells loaded with Fura-2. Gastric mucosal cells were incubated with 2 μM Fura-2AM (Dojindo Laboratories, Kumamoto, Japan) for 1 hr at 37°C. The cells were washed with medium and then held for 30 min. After being washed with medium, the cells were incubated with 10 μM roxatidine or vehicle for 4 hr. Relative fluorescence intensity was monitored at an excitation wavelength of 340 nm/380 nm and an emission wavelength of 510 nm, with a digital fluorescence analyzer (Attension; Carl Zeiss, Oberkochen, Germany). Intracellular Ca²⁺ concentration was calculated from the fluorescence intensity using an application software (Atto Graph).

**Determination of cell viability.** Cell viability was determined by the mitochondrial function and membrane permeability assays, as described previously (Takahashi and Okabe, 1996b). Mitochondrial function was assessed by the colorimetric method involving MTT (Sigma Chemicals). After mucosal cells had been incubated with 10 μM roxatidine or vehicle for 4 hr, 0.2 ml of 5 mg/ml MTT solution was added. Two hours later, the MTT was extracted with 3 ml of isopropanol containing 0.04 N HCl, and the color change of the extract was measured at 595 nm. The membrane permeability was assessed by the dye exclusion method. Cells were washed after incubation with 10 μM roxatidine or vehicle, and then 0.2 ml of 0.1% trypan blue solution was added. Three minutes later, the numbers of stained and nonstained cells were determined in four randomly chosen fields in each well under a microscope (CK2; Olympus, Tokyo, Japan; ×100). Cell viability was determined as Nonstained cells/ (Stained cells + Nonstained cells).

**Drugs.** Roxatidine (Teikoku Hormone Mfg. Co., Tokyo, Japan), nifedipine (Sigma Chemicals), diltiazem (Tanabe Seiyaku Co., Osaka, Japan) and W-7 (Seikagaku Corp., Tokyo, Japan) were dissolved in dimethyl sulfoxide. For each assay, dimethyl sulfoxide was diluted to a final concentration of less than 0.8% in the medium. All other chemicals were of reagent grade.

**Statistical analysis.** Data are presented as means ± S.E. Statistical differences were evaluated using Student's t-test or Dunnett's multiple comparison test, and a P value <.05 was regarded as significant.

**Results**

**Effect of extracellular Ca²⁺ concentration on roxatidine-induced increases in mucus secretion and synthesis.** We examined the effects of extracellular Ca²⁺ concentration on the roxatidine-induced increases in mucus secretion and synthesis by gastric mucosal cells (fig. 1). At 2 mM extracellular Ca²⁺, exposure to 10 μM roxatidine for 4 hr induced significant increases in mucus secretion and synthesis. However, reduction of the extracellular Ca²⁺ concentration caused decreases in the roxatidine-stimulated secretion and synthesis in a concentration-dependent manner, and reduction to 0.002 mM completely abolished these stimulatory effects of roxatidine. Basal secretion and synthesis also decreased with reduction of extracellular Ca²⁺ concentration, but the cells apparently secreted and synthesized mucus even at 0.002 mM extracellular Ca²⁺. At 0.02 mM extracellular Ca²⁺, the basal secretion and synthesis were not different from the corresponding secretion and synthesis in the presence of 2 mM extracellular Ca²⁺. In contrast, the roxatidine-stimulated secretion and synthesis were significantly reduced compared with those at 2 mM extracellular Ca²⁺. As determined by both MTT and dye exclusion methods, cell viability was similar among cells incubated at 2 mM and 0.002 mM extracellular Ca²⁺ in the presence and absence of roxatidine for 4 hr.

**Stimulatory effect of roxatidine on Ca²⁺ influx into gastric mucosal cells.** We examined whether roxatidine promotes Ca²⁺ influx into gastric mucosal cells (fig. 2). Mucosal cells incorporated extracellular Ca²⁺ without any external stimulus for 2 hr. Roxatidine caused a concentration-dependent increase in Ca²⁺ influx, and significant effects were observed at 1 and 10 μM, the increases being 14.2% and 21.8%, respectively.

The time courses of the effects of 10 μM roxatidine are shown in figure 3. Ca²⁺ influx was time-dependently enhanced until 2 hr after the addition of roxatidine and then gradually decreased to the control level during the following 6 hr. Under the same conditions, the addition of 5 mM EGTA to the medium caused complete reduction of Ca²⁺ influx at 1 hr (10.5 ± 1.9% of the control, n = 8). In contrast, mucus secretion and synthesis were not affected by treatment with roxatidine for 1 hr but thereafter were time-dependently stimulated until 4 hr after the addition. Similar to the Ca²⁺ influx, the increases in mucus secretion and synthesis also decreased to control levels during the following 8 hr. Significant increases in Ca²⁺ influx appeared at 2 and 4 hr, whereas those in mucus secretion and synthesis were observed at 4 and 8 hr. Thus the effects of roxatidine on mucus secretion and synthesis are expressed after an increase in Ca²⁺ influx, and reducing the increase in Ca²⁺ influx might lead to disappearance of the stimulatory effects of roxatidine.

We further examined intracellular Ca²⁺ concentration in response to 10 μM roxatidine (fig. 4). The concentration of intracellular Ca²⁺ in the control cells was constant around 100 nM for 4 hr. When 1 μM ionomycin was added to the control cells at 4 hr, the concentration was markedly elevated within 1 min, and thereafter the high
level persisted for at least 10 min (479.5 ± 30.2% compared with the basal value, \(n = 8\)). In the case of roxatidine treatment, slow and moderate increase in intracellular \(\text{Ca}^{++}\) was found. Roxatidine induced an increase in intracellular \(\text{Ca}^{++}\) from 0.75 hr to 3 hr after the addition. The significant increases were observed at 0.75 to 2.5 hr, the maximal increase being 37.6 ± 6.4% at 1.75 hr compared with the control. However, when extracellular \(\text{Ca}^{++}\) concentration was reduced to 0.002 mM, the roxatidine-induced increase in intracellular \(\text{Ca}^{++}\) at 2 hr was completely abolished (control, 24.5 ± 3.9 nM; 10 \(\mu\)M roxatidine, 24.4 ± 4.4 nM, \(n = 8\)).

**Effects of nifedipine and diltiazem on roxatidine-induced increases in \(\text{Ca}^{++}\) influx, mucus secretion and synthesis.** To determine whether voltage-sensitive \(\text{Ca}^{++}\) channels are involved in the roxatidine action on \(\text{Ca}^{++}\) mobilization, we examined the effects of two \(\text{Ca}^{++}\) channel blockers, nifedipine and diltiazem, on the stimulated \(\text{Ca}^{++}\) influx (fig. 5A). Basal \(\text{Ca}^{++}\) influx for 2 hr was not affected by nifedipine or diltiazem, even at 10 \(\mu\)M. The roxatidine (10 \(\mu\)M)-induced increase in \(\text{Ca}^{++}\) influx was slight (around 12%) but was significant even in the presence of nifedipine and diltiazem, as compared with that in the corresponding cells without roxatidine. However, nifedipine and diltiazem significantly reduced the roxatidine-stimulated \(\text{Ca}^{++}\) influx by 60.8% and 66.3%, respectively. Similarly, the increase (10–15%) in intracellular \(\text{Ca}^{++}\) induced by 10 \(\mu\)M roxatidine at any time-point was also observed, but it was reduced by nifedipine and diltiazem. As shown in figure 5B, the inhibition at 2 hr by 10 \(\mu\)M nifedipine was 52.4% and by 10 \(\mu\)M diltiazem was 56.0%. Because \(\text{Ca}^{++}\) channel blockers were found partially to inhibit the roxatidine-induced increases in \(\text{Ca}^{++}\) influx and intracellular \(\text{Ca}^{++}\), we examined the effects of \(\text{Ca}^{++}\) channel blockers on mucus secretion and synthesis (fig. 6). Neither nifedipine nor diltiazem at 10 \(\mu\)M inhibited basal secretion or synthesis of mucus, whereas such \(\text{Ca}^{++}\) channel blockers significantly reduced the roxatidine (10 \(\mu\)M)-stimulated secretion and synthesis. However, much like inhibition of the roxatidine-stimulated \(\text{Ca}^{++}\) mobilization, their inhibitory effects were not complete even at 10 \(\mu\)M, and the increases in mucus secretion and synthesis caused by roxatidine remained.

**Effects of W-7 on roxatidine-induced increases in \(\text{Ca}^{++}\) influx, mucus secretion and synthesis.** To evaluate further the roles of intracellular \(\text{Ca}^{++}\) in mucus secretion and synthesis in response to roxatidine, we investigated the relation between \(\text{Ca}^{++}\)
and calmodulin. The significant increase in Ca\(^{++}\) influx caused by 10 \(\mu M\) roxatidine was similarly observed even in the presence of 3 \(\mu M\) W-7 (a calmodulin antagonist). However, W-7 concentration-dependently inhibited the increases in both mucus secretion and synthesis caused by 10 \(\mu M\) roxatidine (fig. 7). At 3 \(\mu M\), W-7 completely abolished the stimulatory effects of roxatidine on the secretion and synthesis of mucus.

**Discussion**

We found that the stimulatory effects of roxatidine on mucus secretion and synthesis by gastric mucosal cells are dependent on the presence of extracellular Ca\(^{++}\). This suggests that Ca\(^{++}\) influx plays an important role in the actions of roxatidine. The important role of the extracellular Ca\(^{++}\) is also evidenced by the following results. First, roxatidine concentration-dependently promoted Ca\(^{++}\) influx into cells, and the concentration for the effect on Ca\(^{++}\) influx was consistent with those for mucus secretion and synthesis (Takahashi and Okabe, 1995). In addition, roxatidine caused an increase in intracellular Ca\(^{++}\), but reduction of extracellular Ca\(^{++}\) concentration abolished this response to roxatidine. Second, roxatidine increased Ca\(^{++}\) influx and intracellular Ca\(^{++}\) concentration, followed by enhancement of mucus secretion and synthesis. The reductions of the increases in Ca\(^{++}\) influx and intracellular Ca\(^{++}\) might lead to disappearance of the stimulatory effects of roxatidine on mucus secretion. Third, Ca\(^{++}\) channel blockers inhibited the roxatidine-stimulated Ca\(^{++}\) influx, intracellular Ca\(^{++}\) mobilization and mucus secretion and synthesis. Taken together, these results indicate that the increased influx of Ca\(^{++}\) caused by roxatidine might contribute to the increases in mucus secretion and synthesis by gastric mucosal cells. Certainly, reduction of the extracellular Ca\(^{++}\) concentration may decrease the viability of cells, resulting in the loss of a cell response to roxatidine, because reduction in extracellular Ca\(^{++}\) was accompanied by decreases in basal secretion and synthesis of mucus. We confirmed that complete depletion of extracellular Ca\(^{++}\) for 4 hr by EGTA induces cell damage. However, the above possibility is unlikely, because the cell viability was not affected by reduction of the extracellular Ca\(^{++}\) concentration or by treatment with roxatidine. In addition, reduction of the extracellular Ca\(^{++}\) concentration to 0.002 mM significantly inhibited the roxatidine-stimulated secretion and synthesis of mucus, although the reduction slightly affected basal secretion and synthesis.

Previous studies revealed that extracellular Ca\(^{++}\)-dependent and -independent pathways of gastric mucus secretion exist (Seidler and Sewing, 1989; Micots et al., 1993; Hata et al., 1994). In the case of roxatidine, it is apparent that the drug potentiates extracellular Ca\(^{++}\)-dependent secretion of mucus. It is of note that the inhibition by nifedipine and diltiazem of the roxatidine-induced increases in Ca\(^{++}\) influx, intracellular Ca\(^{++}\) and mucus secretion and synthesis was not complete even at 10 \(\mu M\), although such channel blockers have been widely used for complete blocking of voltage-sensitive Ca\(^{++}\) channels at around 1 \(\mu M\) in cell culture. Furthermore, reduction of extracellular Ca\(^{++}\) concentration abolished the stimulatory effect of roxatidine on intracellular Ca\(^{++}\) mobilization. Our results also indicate that roxatidine induces the increase in Ca\(^{++}\) influx through both voltage-sensitive Ca\(^{++}\) channels and other Ca\(^{++}\) entry gates, result-
ing in elevation of intracellular Ca\textsuperscript{2+} concentration. As reported by Tepperman et al. (1991), rabbit gastric mucosal cells may possess voltage-sensitive Ca\textsuperscript{2+} channels. However, it is unlikely that voltage-sensitive Ca\textsuperscript{2+} channels are involved in the basal secretion and synthesis of mucus, because nifedipine and diltiazem did not affect basal Ca\textsuperscript{2+} influx, intracellular Ca\textsuperscript{2+} concentration or mucus secretion and synthesis. Tepperman et al. (1991) also stated that under physiological conditions, it is uncertain whether voltage-sensitive Ca\textsuperscript{2+} channels contribute to the regulation of gastric mucosal cell functions. Considering that the extracellular Ca\textsuperscript{2+} concentration influenced basal mucus secretion and synthesis, it seems that other Ca\textsuperscript{2+} gates play crucial roles in basal secretion as well as in the roxatidine-stimulated secretion. In fact, Ca\textsuperscript{2+} influx into gastric mucosal cells constitutively occurred in the presence and absence of Ca\textsuperscript{2+} channel blockers. Such Ca\textsuperscript{2+} entry gates remain unidentified, so further investigation is needed.

Given the finding that W-7 potently inhibited the roxatidine-induced increases in mucus secretion and synthesis, calmodulin might play a pivotal role in the actions of roxatidine. It is well established that calmodulin is activated by Ca\textsuperscript{2+}-binding, and Ca\textsuperscript{2+}/calmodulin subsequently exert numerous biological effects in cells. Accordingly, calmodulin might mediate the action of the Ca\textsuperscript{2+} influx induced by roxatidine in gastric mucosal cells. In addition, calmodulin may be involved in basal secretion and synthesis of mucus, because basal secretion and synthesis were significantly reduced by W-7 at high concentrations.

It has been reported that sustained Ca\textsuperscript{2+} overloading of gastric mucosal cells causes cell injury (Tepperman et al., 1991; Tepperman and Soper, 1993). In the case of roxatidine treatment, it is apparent that the roxatidine-induced increase in the intracellular Ca\textsuperscript{2+} does not induce cell injury, because cell viability was not affected by treatment with roxatidine. In fact, roxatidine caused a slow increase in intracellular Ca\textsuperscript{2+} for about 2 hr, but the degree of the increase was lower (about 20–30 nM), which indicates that roxatidine does not induce Ca\textsuperscript{2+} overloading. In contrast, when Ca\textsuperscript{2+} overloading was induced by treatment of cells with 1 μM ionomycin, Ca\textsuperscript{2+} influx and intracellular Ca\textsuperscript{2+} mobilization were markedly enhanced to about 5-fold of the basal level.
and were sustained thereafter. In gastric mucosal cells, the basal concentration of intracellular Ca\(^{2+}\) was higher (about 100 nM) than those in other cells. Tepperman and Soper (1993) obtained a similar result. These results suggest that gastric mucosal cells may intrinsically possess resistance to toxicity because of slightly higher intracellular Ca\(^{2+}\) concentration. Moreover, the elevated Ca\(^{2+}\) influx, intracellular Ca\(^{2+}\) concentration and mucus secretion and synthesis induced by roxatidine returned to control levels, which suggests some regulation of responses to roxatidine. However, although the elevated intracellular Ca\(^{2+}\) suggests some regulation of responses to roxatidine. However, reduced by roxatidine returned to control levels, which sug-

**References**


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