Irsogladine Inhibits Ionomycin-Induced Decrease in Intercellular Communication in Cultured Rabbit Gastric Epithelial Cells

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ABSTRACT—Effects of irsogladine on ionomycin-induced decrease in intercellular communication and increase in intracellular concentration of Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) were investigated in cultured rabbit gastric epithelial cells. Ionomycin (10\(^{-7}\)–10\(^{-6}\) M) transiently and concentration-dependently inhibited intercellular communication concomitantly with the elevation of [Ca\(^{2+}\)]\(_i\), in the presence and absence of extracellular Ca\(^{2+}\). Irsogladine (10\(^{-5}\) M), which has been shown to facilitate intercellular communication, suppressed the ionomycin-induced elevation of [Ca\(^{2+}\)]\(_i\) and decrease in intercellular communication. The suppression of the ionomycin effects by irsogladine was independent of extracellular Ca\(^{2+}\). TMB-8 [8-(diethylamino)octyl-3,4,5-trimethoxy-benzoate hydrochloride] (10\(^{-6}\) M) also suppressed the ionomycin-induced elevation of [Ca\(^{2+}\)]\(_i\) and decrease in intercellular communication. These results indicate that the ionomycin-induced decrease in intercellular communication may be due to Ca\(^{2+}\) mobilization from intracellular stores. Inhibitory effects of irsogladine and TMB-8 on the ionomycin-induced decrease in intercellular communication may be produced by suppressing Ca\(^{2+}\) mobilization.

Keywords: Ionomycin, Irsogladine, TMB-8, Intracellular calcium ion concentration, Gap-junctional intercellular communication

Gap junctions permit the passage of small molecular cytoplasmic constituents up to 1200 Da in weight, including ions, sugars and small peptides (1–3). Gap-junctional intercellular communication provides a rapid propagation of electric signals between excitable cells (4). In most nonexcitable cells, intercellular communication participates in regulating various cellular functions such as proliferation (5) and differentiation (6). In gastric mucosa, intercellular communication is thought to play an important role in the secretion of mucus and maintenance of the defensive function and homeostasis (7).

The functions of gap junctions have been shown to be modulated by second messengers. In cultured rabbit gastric epithelial cells, the intercellular communication is enhanced by cyclic AMP (8). On the other hand, an increase in intracellular concentration of Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) has been shown to close gap junction channels in several cell systems (9–11). Changes in the morphology of gap junctions induced by the elevation of [Ca\(^{2+}\)]\(_i\) have been demonstrated by electron microscopy and X-ray diffraction in the isolated liver (12). It has been reported that a Ca\(^{2+}\) ionophore, such as ionomycin or A23187, also inhibited intercellular communication by Ca\(^{2+}\) mobilization in salivary gland cells (13) and lacrimal gland cells (14).

Irsogladine, an antiulcer drug, enhances intercellular communication (8, 15). In the present study, the effects of irsogladine on the ionomycin-induced decrease in intercellular communication and elevation of [Ca\(^{2+}\)] were investigated in the presence or absence of extracellular Ca\(^{2+}\) in rabbit gastric epithelial cells.

MATERIALS AND METHODS

Measurement of intercellular communication

Intercellular communication in rabbit gastric epithelial cells was measured by the method described previously (15). Briefly, rabbit gastric epithelial cells were isolated from Japanese white rabbit fetuses by the method of Matuoka et al. (16) and subcultured at a split ratio of 1:2 until the population doubling level of 7. After confluence in a 35-mm dish (Corning™, New York, NY, USA), the culture medium was changed from Dulbecco’s Modified Eagle’s Medium (Nissui Pharm, Tokyo) containing 20% fetal bovine serum (Gibco, Grand Island, NY, USA), 100 µg/ml of Kanamycin (Meiji Seika, Tokyo) and 100 unit/ml of penicillin G potassium salt.
(Meiji Seika) to Hank’s balanced salt solution containing 1.35 mM calcium (Nissui Pharm). Drugs were added to the Hank’s balanced salt solution. Glass capillaries (diameter of the aperture: 0.1 μm, GD-1; Narishige, Tokyo) were prepared with a vertical pipette puller (PB-7, Narishige). The glass capillary was filled with a 10% solution of Lucifer Yellow CH (Sigma, St. Louis, MO, USA) in 0.33 M LiCl. The fluorescent solution (0.5 pl) was injected into a cell under nitrogen gas pressure (20 psi) applied for 0.05 sec by a pneumatic picopump (PV-800; World Precision Instruments, New Haven, CT, USA). The cell-to-cell diffusion of the fluorescence tracer was recorded by a video system (HCC-3600; Flovel, Tokyo) under fluorescence microscopy (IMT-2; Olympus, Tokyo); this was played back for analysis. The number of cells containing fluorescent Lucifer Yellow increases in a time-dependent manner until 5 min (8). The number of fluorescent cells within 3 min after the microinjection served as an index of intercellular communication.

Measurement of $[\text{Ca}^{2+}]_i$

For monitoring $[\text{Ca}^{2+}]_i$, $5 \times 10^{-6} \text{M} \ 1-(2-(5''-\text{carboxy}-ozaozol-2''-yl)-6-\text{aminobenso-furan-5''-oxy})-2-(2''-\text{amino}-5''\text{-methylphenoxo})\text{-ethane N,N,N',N'-tetraacetic acid pentaacetoxymethyl ester (fura-2 AM; Wako, Osaka), a Ca}^{2+} \text{ probe (17), was loaded into rabbit gastric epithelial cells cultured in Dulbecco’s Modified Eagle’s medium containing 20% fetal calf serum on a coverslip attached to a four-well petri dish (Hereraeus Flexiperm-Disc; W.C. Heraeus GmbH, Hanau, Germany) at 37°C. Thirty minutes later, the medium was changed to Hank’s balanced salt solution. Then the four-well dish was placed on the thermostat stage of a fluorescent microscope (IMT-2, Olympus) maintained at 37°C, and drugs were added to the Hank’s balanced salt solution. The cell-associated fura-2 fluorescence was analyzed with a fluorescent microscope equipped with a dual wavelength excitation device (OSP-3, Olympus). The excitation wavelengths of 340 and 380 nm provided with a 75-w Xe lamp were selected by computer-controlled movement of narrow path filters. Cytosolic fluorescence intensities through a 510 nm filter at 340 and 380 nm excitation were measured with a photomultiplier at a 0.1-sec interval. The fluorescence of endogenous substance was measured every time and subtracted as background from the fluorescence of fura-2. The level of $[\text{Ca}^{2+}]_i$, was calculated from the ratio of the fluorescence intensities obtained by excitations at 340 and 380 nm. The $[\text{Ca}^{2+}]_i$, was calibrated by measuring the ratio of 340/380 nm fluorescence intensities of $10^{-6} \text{M}$ fura-2 solution in free $\text{Ca}^{2+}$ concentration (46–8600 nM)-controlled medium. These controlled media were prepared from a solution consisting of 50 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid-KOH (pH 7.4), 100 mM KCl, 1 mM MgCl$_2$, 2 mM $O,O'$-bis (2-aminoethyl)ethylenglycol-$N,N,N',N''$-tetraacetic acid (EGTA) and various concentrations of CaCl$_2$ at pH 7.4 (18).

Drugs and solutions

Irsogladine (Nippon Shinyaku Co., Ltd., Kyoto), TMB-8 [8-(diethylamino)octyl-3,4,5-trimethoxy-benzoate hydrochloride] (Funakoshi, Tokyo) and ionomycin (Calbiochem, La Jolla, CA, USA) were dissolved in dimethylsulfoxide and diluted with distilled water (the final concentration of dimethylsulfoxide was 0.01% in experimental media).

Statistical analyses

The significance of the differences between the means was determined by the Dunnett’s method at P<0.05.

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Fig. 1. Effects of ionomycin on $[\text{Ca}^{2+}]_i$, (A) and dye transfer (B) between rabbit gastric epithelial cells in 1.35 mM Ca$^{2+}$-containing medium. ○: control, ●: $10^{-7}$ M ionomycin, □: $3 \times 10^{-7}$ M ionomycin, ■: $10^{-6}$ M ionomycin. A: Each value represents the mean and S.E. of 5 different experiments. *P<0.05, **P<0.01, compared with the controls at the corresponding time. B: Lucifer Yellow was microinjected 1, 6 and 12 min after administration of ionomycin. Each value represents the mean and S.E. of 9 different experiments. *P<0.05, **P<0.01, compared with the controls at the corresponding time.
RESULTS

Effects of ionomycin on \([\text{Ca}^{2+}]_i\) and intercellular communication

Figure 1 shows the effects of ionomycin on \([\text{Ca}^{2+}]_i\) and dye transfer in 1.35 mM \(\text{Ca}^{2+}\)-containing medium. The basal \([\text{Ca}^{2+}]_i\) was ca 150 nM (Fig. 1A). Ionomycin (10\(^{-7}\)–10\(^{-6}\) M) concentration-dependently produced a transient increase in \([\text{Ca}^{2+}]_i\), which reached its maximum within 15–30 sec and returned near to the basal level within 3 min. The maximum levels of \([\text{Ca}^{2+}]_i\) were 317±57, 680±202 and 1380±241 nM by 10\(^{-7}\), 3 \times 10\(^{-7}\) and 10\(^{-6}\) M ionomycin, respectively. Intercellular communication was decreased transiently within 1 min after the application of 10\(^{-7}\)–10\(^{-6}\) M ionomycin in a concentration-dependent manner and then returned to the control level 6 min later (Fig. 1B).

The dependence on the extracellular \(\text{Ca}^{2+}\) of ionomycin’s ability to increase \([\text{Ca}^{2+}]_i\) and decrease intercellular communication was examined in \(\text{Ca}^{2+}\)-free medium containing 2 mM EGTA. Ionomycin (10\(^{-7}\)–10\(^{-6}\) M) transiently increased \([\text{Ca}^{2+}]_i\) in a concentration-dependent manner even in the \(\text{Ca}^{2+}\)-free medium (Fig. 2A). However, the maximal level of \([\text{Ca}^{2+}]_i\) stimulated by ionomycin in \(\text{Ca}^{2+}\)-free medium was lower than that in 1.35 mM \(\text{Ca}^{2+}\)-containing medium. The maximum levels of \([\text{Ca}^{2+}]_i\) in \(\text{Ca}^{2+}\)-free medium were 190±12, 369±19 and 419±94 nM by 10\(^{-7}\), 3 \times 10\(^{-7}\) and 10\(^{-6}\) M ionomycin, respectively. Ionomycin also transiently decreased intercellular communication in the \(\text{Ca}^{2+}\)-free medium (Fig. 2B). The intercellular communication responses to ionomycin were similar in the medium containing 1.35 mM \(\text{Ca}^{2+}\) and in the \(\text{Ca}^{2+}\)-free medium.

Fig. 2. Effects of ionomycin on \([\text{Ca}^{2+}]_i\) (A) and dye transfer (B) in \(\text{Ca}^{2+}\)-free medium. ○: control, ●: 10\(^{-7}\) M ionomycin, □: 3 \times 10\(^{-7}\) M ionomycin, ■: 10\(^{-6}\) M ionomycin. A: Each value represents the mean and S.E. of 5 different experiments. *P<0.05, **P<0.01, compared with the controls at the corresponding time. B: Lucifer Yellow was microinjected 1, 6 and 12 min after administration of ionomycin. Each value represents the mean and S.E. of 9 different experiments. *P<0.05, **P<0.01, compared with the controls at the corresponding time.

Fig. 3. Effects of 10\(^{-5}\) M irsogladine on the increase in \([\text{Ca}^{2+}]_i\) induced by 10\(^{-6}\) M ionomycin in 1.35 mM \(\text{Ca}^{2+}\)-containing medium (EGTA(−) (A) and in \(\text{Ca}^{2+}\)-free medium (EGTA(+) (B). ○: 10\(^{-6}\) M ionomycin, ●: 10\(^{-6}\) M ionomycin+10\(^{-5}\) M irsogladine. Irsogladine was added 1 min before administration of ionomycin. Each value represents the mean and S.E. of 5 different experiments. *P<0.05, **P<0.01, compared with 10\(^{-6}\) M ionomycin at the corresponding time.
Effects of irsogladine and TMB-8 on the changes in [Ca\(^{2+}\)], and intercellular communication induced by ionomycin

The effect of irsogladine on the ionomycin-induced changes in [Ca\(^{2+}\)] was studied in 1.35 mM Ca\(^{2+}\)-containing medium and Ca\(^{2+}\)-free medium (Fig. 3). Irsogladine (10\(^{-5}\) M) had no effect on the fluorescent signal of fura-2 (data not shown) and basal [Ca\(^{2+}\)], for 1 min. When 10\(^{-5}\) M irsogladine was added 1 min before stimulation by ionomycin, it inhibited the increase in [Ca\(^{2+}\)] induced by ionomycin in 1.35 mM Ca\(^{2+}\)-containing medium and in Ca\(^{2+}\)-free medium.

The effect of irsogladine on the ionomycin-induced decrease in intercellular communication was investigated both in 1.35 mM Ca\(^{2+}\)-containing medium and in Ca\(^{2+}\)-free medium. Treatment of the cells for 1 min with 10\(^{-5}\) M irsogladine significantly increased intercellular communication (Fig. 4A). When the cells were treated with irsogladine (10\(^{-7}\)–10\(^{-5}\) M) 1 min before the addition of 10\(^{-6}\) M ionomycin, irsogladine concentration-dependently inhibited the decrease in intercellular communication induced by 10\(^{-6}\) M ionomycin independently of extracellular Ca\(^{2+}\) (Fig. 5).

The effects of TMB-8, a putative intracellular Ca\(^{2+}\) antagonist, on the ionomycin-induced changes in [Ca\(^{2+}\)], and intercellular communication were studied in 1.35 mM Ca\(^{2+}\)-containing medium. Treatment of cells with TMB-8 alone for 10 min did not change the fura-2 fluorescent signals (data not shown) and [Ca\(^{2+}\)], under resting conditions (Fig. 6). However, TMB-8 inhibited the increase in [Ca\(^{2+}\)] induced by ionomycin when it was added 10 min before stimulation by ionomycin. Treatment with 10\(^{-6}\) M TMB-8 alone for 6 and 12 min significantly increased in-
tercellular communication by about 40% more than the control level (Fig. 4B). TMB-8 applied 12 min before 10^{-6} M ionomycin treatment partially inhibited the ionomycin-induced decrease in intercellular communication in a concentration-dependent manner (Fig. 7).

**DISCUSSION**

The blockage of intercellular communication was caused concomitantly with a transient elevation of \([\text{Ca}^{2+}]_i\) induced by ionomycin. When \([\text{Ca}^{2+}]_i\) had returned to the resting level, intercellular communication also returned to the control level. These results indicate that the increase in \([\text{Ca}^{2+}]_i\) induced by ionomycin closes gap junctional channels.

Ionomycin (4 \times 10^{-6} M) has been thought to increase \([\text{Ca}^{2+}]_i\), by causing an influx of \(\text{Ca}^{2+}\) across the plasma membrane (19). However, ionomycin (10^{-7} M) has been shown to increase \([\text{Ca}^{2+}]_i\), transiently in the absence of extracellular \(\text{Ca}^{2+}\) in Ehrlich ascites-tumor cells (20). This fact indicates that ionomycin mobilizes \(\text{Ca}^{2+}\) from intracellular stores. It has been reported that at low concentrations less than 10^{-7} M, A23187 mobilizes \(\text{Ca}^{2+}\) selectively from intracellular stores and that at high concentrations more than 10^{-6} M, A23187 causes \(\text{Ca}^{2+}\) influx (21). Ionomycin is considered to act in the same manner as A23187.

In the present study, ionomycin transiently increased \([\text{Ca}^{2+}]_i\), even in the absence of extracellular \(\text{Ca}^{2+}\), but the maximal level of \([\text{Ca}^{2+}]_i\) was lower than that in the presence of extracellular \(\text{Ca}^{2+}\). These results indicate that the elevation of \([\text{Ca}^{2+}]_i\) induced by ionomycin in the presence of extracellular \(\text{Ca}^{2+}\) may be due mainly to \(\text{Ca}^{2+}\) influx from the extracellular space in addition to \(\text{Ca}^{2+}\) mobilization from intracellular stores. However, the extent of ionomycin’s block of intercellular communication was similar in the presence and absence of extracellular \(\text{Ca}^{2+}\). Therefore, it is suggested that cytosolic \(\text{Ca}^{2+}\) mobilization from intracellular stores was dominantly involved in the ionomycin-induced decrease in intercellular communication.

Compartmentalization of \(\text{Ca}^{2+}\) has been demonstrated in decreasing intercellular communication (13). That is, microinjection of \(\text{Ca}^{2+}\) only into the region near gap junctions has been shown to inhibit intercellular communication, but not injection into regions distal to gap junctions. Gap junctions are numerous in the lateral membrane at the level of the nucleus in gastric surface mucous cells (7). The influx of \(\text{Ca}^{2+}\) may occur in the basolateral membrane, considering the in vivo orientation of gastric epithelial cells. The marked elevation of \([\text{Ca}^{2+}]_i\) to 300–1400 nM induced by ionomycin in the presence of extracellular \(\text{Ca}^{2+}\), however, caused a similar decrease in intercellular communication to a slight elevation of \([\text{Ca}^{2+}]_i\) in the \(\text{Ca}^{2+}\)-free medium. These results indicate that \(\text{Ca}^{2+}\) influxed from the extracellular space through the basolateral membrane may not play a role in the inhibition of intercellular communication.

Irsogladine, which has been shown to facilitate intercellular communication (8, 15), inhibited both the decrease in intercellular communication and elevation of \([\text{Ca}^{2+}]_i\) induced by ionomycin in both the presence and absence of extracellular \(\text{Ca}^{2+}\). These results indicate that irsogladine blocks the ionomycin-induced mobilization of
Ca\(^{2+}\) from intracellular stores and then inhibits the ionomycin-induced closure of gap junctional channels. TMB-8 has been reported to inhibit Ca\(^{2+}\) mobilization from the intracellular stores, but it does not inhibit Ca\(^{2+}\) influx (22). However, it should be noted that TMB-8 may also act on protein kinase C and plasma membrane Ca\(^{2+}\) influx (23). In this experiment, TMB-8 also inhibited both the elevation of [Ca\(^{2+}\)], and the decrease in intercellular communication induced by ionomycin. These results suggest that the inhibitory effect of TMB-8 on ionomycin-induced closure of gap junctional channels may be derived from the same way as the effect of irsogladine.

Gap-junctional intercellular communication between surface mucous cells is thought to play an important role in maintenance of the defensive capacity against the noxious effect of digestive secretions, food and drugs (7). Irsogladine facilitating intercellular communication under the resting condition (8, 15) suppressed the ionomycin-induced decrease in intercellular communication by inhibiting mobilization of Ca\(^{2+}\). These effects of irsogladine on gap junctional intercellular communication may participate in the protective mechanism against various ulcero-genic stimuli (24).

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