Intercellular Communication in Cultured Rabbit Gastric Epithelial Cells

Fusao Ueda, Takashi Kyoj, Kiyotaka Mimura, Kiyoshi Kimura and Masao Yamamoto

Research Laboratories, Nippon Shinyaku Co. Ltd., Kyoto 601, Japan

Department of Anatomy, Hiroshima University School of Medicine, Hiroshima 734, Japan

Received May 31, 1991 Accepted July 17, 1991

ABSTRACT—The effects of drugs related to cyclic AMP and a tumor promoter, phorbol ester, on intercellular communications via gap junctions were investigated by the Lucifer Yellow-transfer method in cultured rabbit gastric epithelial cells. Cells were in contact with each drug for 4 hr before the microinjection of the dye into a cell. Dye transfer capacity was significantly increased by dibutyryl cyclic AMP (10⁻³ M), theophylline (10⁻³ M), 3-isobutyl-1-methylxanthine (10⁻⁴ M), forskolin (10⁻⁶ M) and irsogladine (10⁻⁴ M); and it was inhibited by 12-O-tetradecanoyl-phorbol-13-acetate (100 ng/ml). These results suggest that the intercellular communication between cultured rabbit gastric epithelial cells is upregulated by cyclic AMP.

It has been reported that gap junctions are more numerous in surface mucous cells than in gland cells, including mucous neck and chief cells of the gastric mucosa in mice and rats (1), and that the capacity of intercellular communication (IC) via gap junctions is found in normal gastric epithelial cells, but not in cancerous cells (2). The role of IC is considered to be mainly the maintenance of tissue homeostasis and the regulation of signal transmissions (reviewed in refs. 3–5). The tissue homeostatic role of IC is the buffering of various channel-permeable molecules and the equalization of chemical substances and electrical potentials among individual cells in a cellular community. One of the most important roles of IC in regulating signal transmissions is genetic regulation, which is required for normal proliferation and differentiation. The IC via gap junctions is regulated mainly by intracellular cyclic AMP, H⁺ and Ca²⁺ (6–9) and by growth factors (10). Cyclic AMP acting as a stimulatory second messenger generally upregulates IC (6, 11, 12), except in retinal horizontal cells (13–15). Our aim was to elucidate whether IC in cultured rabbit gastric epithelial cells is upregulated by intracellular cyclic AMP as well as other cultured cell systems. Since the antiulcer drug irsogladine maleate (16) has been demonstrated to increase cyclic AMP in the rat gastric mucosa (17), we also investigated the effect of irsogladine on IC in cultured gastric epithelial cells.

MATERIALS AND METHODS

Cultured cells

Cultured rabbit gastric epithelial cells were prepared by the method of Matuoka et al. (18). Briefly, the fetuses of JW/NIBS white rabbits were removed on day 24 of gestation, and the stomachs were isolated, everted, and digested by 0.2% Pronase E (Kaken Chemi-
The dispersed epithelial cells were centrifuged, washed, seeded in plastic dishes, and subcultured in a split ratio of 2:1 in Dulbecco's Modified Eagle Medium (Nissui Pharm) containing 20% fetal bovine serum (Gibco), 80 μg/ml Kanamycin (Meiji Seika), 100 units/ml of penicillin G potassium salt (Meiji Seika) and 50 units/ml of Dispase I (Godo Shusei) in a CO₂ incubator (5% CO₂ in humid air set at 37°C). At population doubling level 3, the cells were frozen at -80°C and stocked. Before the experiments, the frozen cells were thawed and seeded in a medium containing 10% serum without Dispase I. The cells after confluence were used in the following experiments.

Histochemical analysis
The cultured cells were fixed with methanol:acetic acid (3:1). Populations of mucous, mucous neck, parietal and chief cells in the culture were examined microscopically after Azan-staining by the method of Matuoka et al. (18).

Electron microscopic examination
The cultured cells were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.4) for 1 hr at room temperature, dehydrated with graded alcohol solutions, and embedded in Epon 812 resin. Thin sections were doubly stained with uranyl acetate and lead citrate and then examined under a Hitachi H-500 electron microscope.

Dye transfer methods
Under a phase-contrast microscope, 0.5 pl of 10% Lucifer Yellow CH (Sigma) dissolved in 0.33 M LiCl was microinjected into a cell with a pneumatic picopump (PV-800, World Precision Instruments) assembled in an injectoscope system (Olympus IMT-2). After microinjection, the extent of dye transfer was photographed under fluorescence microscopy. The capacity of IC between the cells was assessed by counting dye-transferred cells on the fluorescence photograph. The effect of each drug on the capacity of IC was evaluated by the changes in the numbers of dye-transferred cells 5 min after the microinjection. Drugs were added to the culture medium 4 hr before the microinjection of dye in consideration of the pretreatment periods previously reported in the experiments using dibutyryl cyclic AMP (6, 19) and 12-O-tetradecanoylphorbol-13-acetate (TPA) (12, 19–21) in several kinds of cultured cells. The effects of forskolin and irsogladine on IC were investigated in the presence of a subthreshold concentration of a phosphodiesterase inhibitor, 10⁻⁴ M theophylline.

Drugs
Irsogladine was synthesized in the Research Laboratories of Nippon Shinyaku Co., Ltd. Theophylline was purchased from Nacalai Tesque; dibutyryl cyclic AMP and forskolin, from Sigma; 3-isobutyl-1-methylxanthine (IBMX) and TPA, from Funakoshi.

Statistical analysis
Statistical analyses were performed by SAS (SAS Institute Inc.). The significance of the differences between the means was verified by the Dunnett method.

RESULTS
Cultured rabbit gastric epithelial cells were classified into the following 4 types by the size and shape of the cells and nuclei and the histochemical characteristics of the surface mucous, mucous neck, parietal and chief cells (Fig. 1).

With an electron microscopy, the cells were characterized morphologically by their flat shape and the narrow space between the apical cell membrane and the nucleus. The existence of gap junctions between cultured cells was morphologically confirmed (Fig. 2).

Lucifer Yellow injected into a cell was transferred to neighboring cells (Fig. 3). There were no changes in the capacity of IC among the 4 different types of cultured cells. The
number of Lucifer Yellow-transferred cells increased progressively until 5 min after the microinjection, and no increases in dye-transferred cells were observed 10 or 15 min after the microinjection (Fig. 4). Therefore, drug effects on the capacity of IC were evaluated by the changes in the number of dye-transferred cells 5 min after the microinjection. Theophylline ($10^{-3}$ M) and IBMX ($10^{-4}$ M) significantly enhanced the capacity of IC be-

**Fig. 1.** Heterogeneity of cultured rabbit gastric epithelial cells. Cells at population doubling level 4 in confluent cultures were stained with azan. According to Matuoka et al. (ref. 18), these cells were surface mucous (a), parietal (b), mucous neck (c) and chief (d) cells. bar = 10 $\mu$m.
tween the cells (Fig. 5). These increases were 60.7% and 73.1%, respectively. Forskolin (10^{-6} M) in the presence of theophylline (10^{-4} M) enhanced the IC by 65.8%. Dibutyryl cyclic AMP also increased the capacity of IC dose-dependently (Fig. 6); the increase was 56.8% at 10^{-3} M. In contrast, TPA (100 ng/ml) decreased the capacity of IC by 70.7% (Fig. 6). Irsogladine in the presence of theophylline (10^{-4} M) enhanced the capacity

Fig. 2. Gap junction between cultured rabbit gastric epithelial cells. The arrow shows a gap junction. bar = 0.5 μm.

Fig. 3. Pattern of dye transfer in cultured rabbit gastric epithelial cells. A: A phase contrast micrograph. A cell (star) was microinjected with Lucifer Yellow CH. B: Five minutes after the microinjection, the dye was transferred from a donor cell into surrounding cells (fluorescence micrograph of the dish shown in A). bar = 100 μm.
of IC dose-dependently; the increase was 45.7% at $10^{-4}$ M (Fig. 7).

**DISCUSSION**

Intracellular cyclic AMP has been reported to increase gap junctional membrane permeability by cyclic AMP-dependent protein phosphorylation of a gap junction protein (22, 23). For instance, electrical coupling in voltage-clamped cell pairs of rat hepatocytes is considered to be regulated through the activity of a cyclic AMP-dependent protein kinase that phosphorylates specific sites of gap junction proteins.
protein (24). In addition, cyclic AMP has been suggested to increase gap junctional membrane permeability depending on the increased formation of gap junction plaques (6, 25). Synthesis of gap junction protein precursors, assembly and insertion of these precursors into the plasma membrane may require a longer period than the phosphorylation of gap junction protein. Recently, it has been reported that the long-term effect of intracellular cyclic AMP on gap junctional membrane permeability between rat hepatocytes may involve an increase in the stability of gap junction mRNA, thereby increasing the synthesis of gap junction protein (26). In the present study, IC between cultured rabbit gastric epithelial cells was enhanced by phosphodiesterase inhibitors, theophylline and IBMX; a non-specific adenylate cyclase activator, forskolin; and a cell membrane permeable cyclic AMP analog, dibutyryl cyclic AMP. These results indicate that gap junctional IC in cultured gastric epithelial cells can be enhanced by increase in the intracellular concentration of cyclic AMP as well as in the other cultured cells. Mechanisms for the increase of IC capacity induced by cyclic AMP-related drugs may involve several actions of cyclic AMP including phosphorylation and synthesis of gap junction protein.

Irsogladine maleate, an antiulcer drug, has been reported to increase cyclic AMP in rat gastric mucous cells and cultured rabbit gastric epithelial cells (17). Therefore, IC capacity enhanced by irsogladine is considered to be caused by the increase in cyclic AMP content produced by irsogladine. This effect of irsogladine on IC might lead to an augmentation of the gap junctional transfer of nutrients, metabolites and other effector molecules for maintaining basal tissue homeostasis. We propose that the increase in IC capacity caused by irsogladine may be one of the defensive mechanisms against gastric epithelial cell damage caused by various kinds of necrotizing agents (27).

In the present study, TPA remarkably inhibited the capacity of IC in rabbit gastric epithelial cells. It is well-known that active tumor promoting phorbol esters such as TPA inhibit IC between cultured cells (28, 29). The onset of TPA-induced inhibition of dye transfer is rapid, and its inhibition continues for at least several hours in several kinds of cultured cells (12, 19–21). The ability of phorbol esters to inhibit IC is considered to be associated with activation of protein kinase C (21, 28, 30). The TPA-induced inhibition of IC may be derived from direct phosphorylation of gap junction protein, because purified protein kinase C phosphorylates the 27-kDa rat liver gap junction protein (31).

It seems to be apparent that the function of gap junctions may be modulated in a variety of ways from channel gating to synthesis of gap junction proteins. In the present study,
drugs were added to the culture medium 4 hr before microinjection of dye in order to investigate the drug effects on several steps which modulate gap junctional membrane permeability. Changing of the incubation time of cells with drugs will make the feasible mechanisms for regulation of IC clear.

In conclusion, the capacity of IC between cultured rabbit gastric epithelial cells is upregulated by cyclic AMP. The enhancement of IC induced by irsogladine may be associated with increased cyclic AMP content.

REFERENCES

1 Kataoka, K., Maesako, J., Yamamoto, M. and Toyota, T.: Intercellular junctions in the gastric mucosa of mice and rats. Proc. Xth Int. Cong. on Electron Microscopy, 2877 (1986)
2 Kanno, Y. and Matsui, Y.: Cellular uncoupling in cancerous stomach epithelium. Nature 218, 775–776 (1968)
3 Loewenstein, W.R.: Junctional intercellular communication: The cell-to-cell membrane channel. Physiol. Rev. 61, 829–913 (1981)
4 Spray, D.C. and Bennett, M.V.L.: Physiology and pharmacology of gap junctions. Annu. Rev. Physiol. 47, 281–303 (1985)
5 Sheridan, J.D. and Atkinson, M.M.: Physiological roles of permeable junctions: some possibilities. Annu. Rev. Physiol. 47, 337–353 (1985)
6 Flagg-Newton, J.L., Dahl, G. and Loewenstein, W.R.: Cell junction and cyclic AMP. I. Upregulation of junctional membrane particles by administration of cyclic nucleotide or phosphodiesterase inhibitor. J. Membr. Biol. 63, 105–121 (1981)
7 Spray, D.C., Stern, J.H., Harris, A.L. and Bennett, M.V.L.: Gap junctional conductance: comparison of sensitivities to H and Ca ions. Proc. Natl. Acad. Sci. U.S.A. 79, 441–445 (1982)
8 Bassnett, S. and Duncan, G.: The influence of pH on membrane conductance and intercellular resistance in the rat lens. J. Physiol. (Lond.) 398, 507–521 (1988)
9 Pacchicci, C.: Increase in gap junction resistance with acidification in crayfish septate axons is closely related to changes in intracellular calcium but not hydrogen ion concentration. J. Membr. Biol. 113, 75–92 (1990)
10 Maldonado, P.E., Rose, B. and Loewenstein, W.R.: Growth factors modulate junctional cell-to-cell communication. J. Membr. Biol. 106, 203–210 (1988)
11 Kessler, J.A., Spray, D.C., Saez, J.C. and Bennett, M.V.L.: Determination of synaptic phenotype: insulin and cAMP independently initiate development of electronic coupling between cultured sympathetic neurons. Proc. Natl. Acad. Sci. U.S.A. 81, 6235–6239 (1984)
12 Kanno, Y., Enomoto, T., Shiba, Y. and Yamasaki, H.: Protective effect of cAMP on tumour promoter-mediated inhibition of cell-cell communication. Exp. Cell Res. 152, 31–37 (1984)
13 Piccolino, M., Neyton, J. and Gerschenfeld, H.M.: Decrease of gap junction permeability induced by dopamine and cyclic adenosine 3′,5′-monophosphate in horizontal cells of turtle retina. J. Neurosci. 4, 2477–2488 (1982)
14 Lacaster, E.M. and Dowling, J.E.: Dopamine decreases conductance of the electrical junctions between cultured retinal horizontal cells. Proc. Natl. Acad. Sci. U.S.A. 82, 3025–3029 (1985)
15 Teranishi, T., Negishi, K. and Kato, S.: Dopamine modulates S-potential amplitude and dye-coupling between external horizontal cells in carp retina. Nature 301, 243–246 (1983)
16 Ueda, F., Aratani, S., Mimura, K., Kimura, K., Nomura, A. and Enomoto, H.: Effect of 2,4-diamino-6-(2,5-dichlorophenyl)-s-triazine maleate (MN-1695) on gastric ulcers and gastric secretion in experimental animals. Arzneimittelforschung 34, 474–477 (1984)
17 Ueda, F., Watanabe, M., Hirata, Y., Ideguchi, K., Kyoi, T., Yano, T., Kimura, K. and Enomoto, H.: Mechanism for the antiulcer action of 2,4-diamino-6-(2,5-dichlorophenyl)-s-triazine maleate (MN-1695). Pharmacometrics 33, 157–163 (1987)
18 Matsuka, K., Tanaka, M., Mitsui, Y. and Murota, S.: Cultured rabbit gastric epithelial cells producing prostaglandin I_2. Gastroenterology 84, 498–505 (1983)
19 Enomoto, T., Martel, N., Kanno, Y. and Yamasaki, H.: Inhibition of cell communication between Balb/c 3T3 cells by tumor promoters and protection by cAMP. J. Cell. Physiol. 121, 323–333 (1984)
20 Enomoto, T., Sasaki, Y., Shiba, Y., Kanno, Y. and Yamasaki, H.: Tumor promoters cause a rapid and reversible inhibition of the formation and maintenance of electrical cell coupling in culture. Proc. Natl. Acad. Sci. U.S.A. 78, 5628–5632 (1981)
21 Zeilmaker, M.J. and Yamasaki, H.: Inhibition of junctional intercellular communication as a possible short-term test to detect tumor-promoting agents: results with nine chemicals tested by dye
transfer assay in Chinese hamster V79 cells. Cancer Res. 46, 6180–6186 (1986)
22 Azarnia, R. and Russell, T.R.: Cyclic AMP effects on cell-to-cell junctional membrane permeability during adipocyte differentiation of 3T3-L1 fibroblast. J. Cell Biol. 100, 265–269 (1985)
23 Saez, J.C., Nairn, A.C., Czernik, A.J., Spray, D.C., Hertzberg, E., Greengard, P. and Bennett, M.V.L.: Phosphorylation of connexin 32, a hepatocyte gap-junction protein, by cAMP-dependent protein kinase, protein kinase C and Ca2+/calmodulin-dependent protein kinase II. Eur. J. Biochem. 192, 263–273 (1990)
24 Saez, J.C., Spray, D.C., Nairn, A.C., Hertzberg, E., Greengard, P. and Bennett, M.V.L.: cAMP increases junctional conductance and stimulates phosphorylation of the 27kDa principal gap junction polypeptide. Proc. Natl. Acad. Sci. U.S.A. 83, 2473–2477 (1986)
25 Azarnia, R., Dahl, G. and Loewenstein, W.R.: Cell junction and cyclic AMP: III. Promotion of junctional membrane permeability and junctional membrane particles in a junction-deficient cell type. J. Membr. Biol. 63, 133–146 (1981)
26 Saez, J.C., Gregory, W.A., Watanabe, T., Dermietzel, R., Hertzberg, E.L., Reid, L., Bennett, M.V.L. and Spray, D.C.: cAMP delays disappearance of gap junctions between pairs of rat hepatocytes in primary culture. Am. J. Physiol. 257, C1–C11 (1989)
27 Ueda, F., Aratani, S., Mimura, K., Kimura, K., Nomura, A. and Enomoto, H.: Effect of 2,4-diamino-6-(2,5-dichlorophenyl)-s-triazine maleate (MN-1695) on gastric mucosal damage induced by various necrotizing agents in rats. Arzneimittelforschung 34, 478–484 (1984)
28 Fitzgerald, D.J., Knowles, S.E., Ballard, F.J. and Murry, A.W.: Rapid and reversible inhibition of junctional communication by tumor promoters in a mouse cell line. Cancer Res. 43, 3614–3618 (1983)
29 Enomoto, T. and Yamasaki H.: Phorbol ester-mediated inhibition of intercellular communication in Balb/c 3T3 cells: relationships to enhancement of cell transformation. Cancer Res. 45, 2681–2688 (1985)
30 Enomoto, T. and Yamasaki, H.: Rapid inhibition of intercellular communication between Balb/c 3T3 cells by diacylglycerol, a possible endogenous functional analogue of phorbol ester. Cancer Res. 45, 3706–3710 (1985)
31 Takeda, A., Hashimoto, E., Yamamura, H. and Shimazu, T.: Phosphorylation of liver gap junction protein by protein kinase C. FEBS Lett. 210, 169–172 (1987)