Activation of Ca^{2+}-dependent K^+ Current by Acetylcholine and Histamine in a Human Gastric Epithelial Cell Line

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ABSTRACT The effects of acetylcholine (ACh) and histamine (His) on the membrane potential and current were examined in JR-1 cells, a mucin-producing epithelial cell line derived from human gastric signet ring cell carcinoma. The tight-seal, whole cell clamp technique was used. The resting membrane potential, the input resistance, and the capacitance of the cells were approximately −12 mV, 1.4 GΩ, and 50 pF, respectively. Under the voltage-clamp condition, no voltage-dependent currents were evoked. ACh or His added to the bathing solution hyperpolarized the membrane by activating a time- and voltage-independent K^+ current. The ACh-induced hyperpolarization and K^+ current persisted, while the His response desensitized quickly (< 1 min). These effects of ACh and His were mediated predominantly by m_3-muscarinic and H_1-His receptors, respectively. The K^+ current induced by ACh and His was inhibited by charybdotoxin, suggesting that it is a Ca^{2+}-activated K^+ channel current ($I_{K.Ca}$). The measurement of intracellular Ca^{2+} ($[Ca^{2+}]_i$) using Indo-1 revealed that both agents increased $[Ca^{2+}]_i$ with similar time courses as they increased $I_{K.Ca}$. When EGTA in the pipette solution was increased from 0.15 to 10 mM, the induction of $I_{K.Ca}$ by ACh and His was abolished. Thus, both ACh and His activate $I_{K.Ca}$ by increasing $[Ca^{2+}]_i$ in JR-1 cells. In the Ca^{2+}-free bathing solution (0.15 mM EGTA in the pipette), ACh evoked $I_{K.Ca}$ transiently. Addition of Ca^{2+} (1.8 mM) to the bath immediately restored the sustained $I_{K.Ca}$. These results suggest that the ACh response is due to at least two different mechanisms; i.e., the Ca^{2+} release–related initial transient activation and the Ca^{2+} influx–related sustained activation of $I_{K.Ca}$. Probably because of desensitization, the Ca^{2+} influx–related component of the His response could not be identified. Intracellularly applied inositol 1,4,5-trisphosphate (IP_3), with and without

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inositol 1,3,4,5-tetrakisphosphate (IP_4), mimicked the ACh response. IP_4 alone did not affect the membrane current. Under the steady effect of IP_3 or IP_3 plus IP_4, neither ACh nor His further evoked $I_{K.Ca}$. Intracellular application of heparin or of the monoclonal antibody against the IP_3 receptor, mAb18A10, inhibited the ACh and His responses in a concentration-dependent fashion. Neomycin, a phospholipase C (PLC) inhibitor, also inhibited the agonist-induced response in a concentration-dependent fashion. Although neither pertussis toxin (PTX) nor *N*-ethylmaleimide affected the ACh or His activation of $I_{K.Ca}$, GDPβS attenuated and GTPγS enhanced the agonist response. These results suggest that (*a*) PTX-insensitive G proteins and PLC are involved in the agonist-dependent activation of $I_{K.Ca}$, and (*b*) IP_3 is essentially involved not only in agonist-induced Ca^{2+} release from the intracellular store but also in agonist-mediated Ca^{2+} influx across the cell membrane in JR-1 cells. However, upon reapplication of $[Ca^{2+}]_o$ after a brief exposure of cells to the Ca^{2+}-free bathing solution (during which the Ca^{2+} influx–related component of $I_{K.Ca}$ was abolished), intracellular IP_3 (with and without IP_4) did not cause immediate recovery of the Ca^{2+} influx–related $I_{K.Ca}$, but restored it with a very slow time course (the half-recovery time of ~2–5 min). This slow recovery was markedly accelerated by the application of ACh. This result suggests that ACh may generate an unidentified signal other than IP_3 and IP_4 responsible for Ca^{2+} influx in JR-1 cells.

INTRODUCTION

The gastric epithelium is composed of three types of cells: the parietal, the chief, and the mucin-producing epithelial cells. These cells secrete ions, glycoproteins, enzymes, and mucin in response to various hormones and neurotransmitters, such as histamine (His), acetylcholine (ACh), and gastrin (Ganong, 1991). The secretion of these substances from the epithelium is essential for the physiological functions of the gastrointestinal tract, including digestion and absorption (Ganong, 1991). Although it is established that intracellular second messengers such as cAMP and Ca^{2+} mediate the receptor-dependent secretion in gastric epithelial cells (Soll and Walsh, 1979; Chew and Brown, 1986), the subcellular mechanisms responsible for the stimulation–secretion coupling process in these cells remain largely unknown because of the structural and functional heterogeneity of the epithelium.

Ion channels exist in cell membranes of epithelial cells lining the gastrointestinal tract (Demarest and Loo, 1990). Ueda, Loo, and Sachs (1987) found Ca^{2+}- and cAMP-dependent K^+ channels in the basolateral membrane of the frog acid-secreting cells. In the apical membrane of these cells, Demarest, Loo, and Sachs (1989) identified a cAMP-dependent Cl^- channel. In the basolateral membrane of parietal cells of the rabbit isolated gastric gland, Sakai, Okada, Morii, and Takeguchi (1989) reported an inward-rectifying K^+ channel and a nonselective cation channel which were insensitive to intracellular Ca^{2+} and cAMP. In a human intestinal cell line (Intestine 407), Yada, Oiki, Ueda, and Okada (1989) reported that a Ca^{2+}-activated K^+ channel is stimulated by His, ATP, and the vasointestinal peptide. The second messenger–independent ion channels may determine the resting electrical properties, while the ion channels regulated by second messengers may play an important role in the stimulation–secretion coupling process in these cells. Thus far, no report

is available on the electrical activity and its modulation in response to secretagogues in mucin-producing gastric epithelial cells.

An epithelial cell line derived from signet ring cell carcinoma of the stomach was recently established and named JR-1 (Terano, Nakada, Mutoh, Hiraishi, Ota, Shiina, Shimada, Itoh, Kimura, Shiga, and Sugimoto, 1991). The JR-1 cell has rapid growth potency and secretes mucin. This cell line could be used as a useful model to gain insight into the subcellular mechanisms underlying the agonist-induced cell growth and mucin secretion in the human gastric epithelium. In this study, we examined the effects of ACh and His, two secretagogues of mucin, on the membrane potential and current of JR-1 cells. We report that ACh and His hyperpolarized these cells by activating a Ca^{2+}-activated K^+ channel predominantly via m_3-muscarinic cholinergic and H_1-His receptors. The signaling pathway of these responses was also examined.

MATERIALS AND METHODS

Preparation

JR-1 cells, derived from human gastric signet ring cell carcinoma, were used (Terano et al., 1991). This cell line was originally developed from a scirrhous gastric cancer in a 37-yr-old female. The primary culture of this cell line was obtained from the cerebrospinal fluid of the patient who had brain metastasis. The cells were suspended in the medium described below and incubated on plastic plates (CELLDESK; S.B. Medical Co., Ltd., Tokyo, Japan) in a 24-well tissue culture dish (MULTIWELL; Becton Dickinson Labware, Lincoln Park, NJ) at a density of 10^6 cells/ml. The composition of the medium used for this culture was MEM (GIBCO BRL, Gaithersburg, MD), supplemented with 15 mM sodium bicarbonate, 15 mM HEPES buffer, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml gentamycin, and 10% fetal bovine serum. Passages from 30 to 50 were used in these experiments. Fig. 1 shows JR-1 cells stained with PAS. Cells were spherical (~10 μm in diameter). PAS staining revealed mucin granules in the cytoplasm, and spines, foldings, and caveoles on the surface of the cultured cells.

Solutions and Drugs

The control bathing solution contained (mM): 136.5 NaCl, 0.33 NaH_2PO_4, 5.4 KCl, 1.8 $CaCl_2$, 0.53 $MgCl_2$, 5.5 D-glucose, and 5 HEPES-NaOH buffer (pH 7.4). In the Ca^{2+}-free bathing solution, $CaCl_2$ was omitted and EGTA (0.5 mM) was added to the control bathing solution. When the concentration of K^+ in the bathing solution was increased, Na^+ was replaced with an equimolar amount of K^+. In the whole cell experiments, pipettes were filled with the internal solution containing (mM): 140 KCl, 2 $MgCl_2$, 0.15 EGTA, 3 Na_2ATP, 0.1 GTP (sodium salt), and 5 HEPES-KOH buffer (pH 7.3). In some experiments, the concentration of EGTA in the pipette solution was increased from 0.15 to 10 mM to chelate intracellular Ca^{2+}. Inositol 1,4,5-trisphosphate (IP_3), inositol 1,3,4,5-tetrakisphosphate (IP_4), cAMP, cGMP, diacylglycerol, ACh, and His were purchased from Sigma Chemical Co. (St. Louis, MO). Heparin (sodium salt) obtained from porcine intestinal mucosa was also purchased from Sigma Chemical Co. (H3125, 170 USP units/mg). 4-Diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP, an m_3-muscarinic receptor antagonist), pirenzepine (an m_1 receptor antagonist), 11-[(2-[(diethylamino) methyl]-1-piperidinyl) acetyl]-5,11-dihydro-6*H*-pyrido[2,3-*b*][1,4] benzodiapepine-6-one (AF-DX 117, an m_2-receptor antagonist), chlorphenilamine maleate (an H_1 receptor antagonist), and cimetidine (an H_2 receptor antagonist) were from Research Biochemicals Inc. (Natick, MA). The monoclonal antibody to the IP_3 receptor (18A10) was prepared as described

previously (Nakade, Maeda, and Mikoshiba, 1991). All experiments were performed at 35–37°C.

Electrophysiological Measurements and Data Analysis

The GΩ seal patch-clamp technique was used in the whole cell configuration (Hamill, Marty, Neher, Sakmann, and Sigworth, 1981). A heat-polished patch pipette, filled with an artificial internal solution, had a tip resistance of 5–7 MΩ. In the experiments in which IP_3, IP_3 plus IP_4, or IP_4 was applied into the cells through the pipettes, the tip of the patch pipette (~ 1 mm) was filled with the control internal solution, and the rest of the pipette was filled with the internal solution containing these substances. Membrane current and potential were measured through

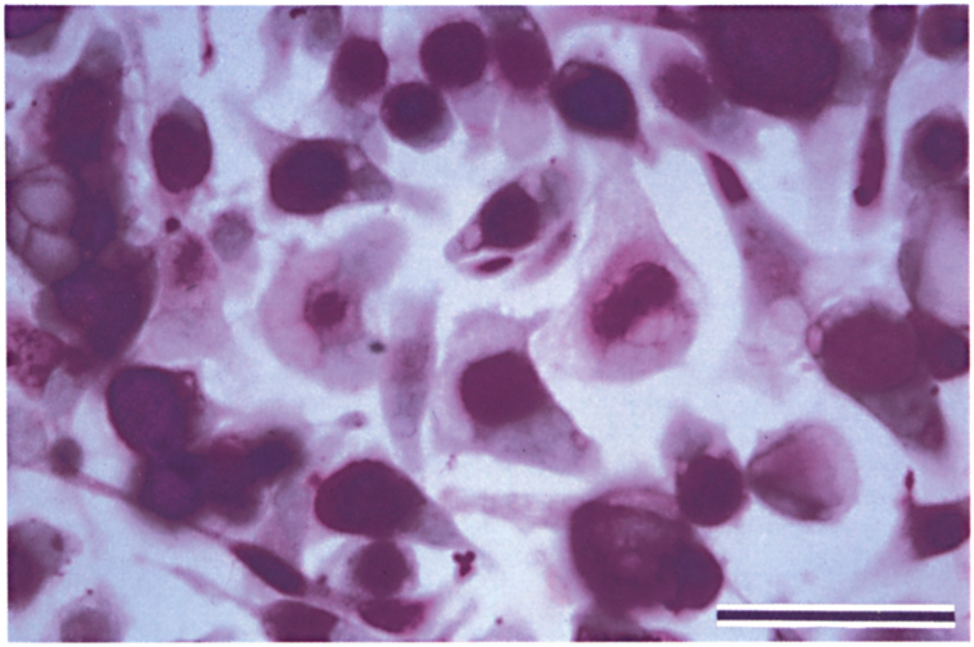

FIGURE 1. PAS staining of cultured JR-1 cells derived from a human gastric signet ring cell cancer. Most of the cells contained PAS-positive granules in the cytoplasm, indicating that JR-1 cells possess mucin granules. The bar is 10 μm.

a patch-clamp amplifier (EPC-7; List Electronics, Darmstadt, FRG) and monitored with a high-gain storage oscilloscope (COS5020-ST; Kikusui Electronic, Tokyo, Japan). Data were stored in a video cassette recorder using the PCM converter system (RP-880; NF Electronic Circuit Design, Tokyo, Japan), reproduced, and low-pass filtered at 1 kHz (at −3 dB) by a filter with Bessel characteristics (48 dB/octave slope attenuation) for analysis and illustration. The data were expressed as mean ± SD. Student's t test was performed whenever appropriate. A value of $P < 0.05$ was considered significant.

Estimation of Intracellular Ca^{2+} Concentration by Microspectrofluorometry

The fluorescent Ca^{2+} probe, indo-1-acetoxymethyl ester (indo-1/AM), was used to measure the cytosolic Ca^{2+} concentration (Grynkiewicz, Poenie, and Tsien, 1985; Peeters, Hlady, Bridge,

and Barry, 1987). Cultured JR-1 cells were exposed to indo-1/AM (10 μM) in 1% dimethylsulfoxide for 15 min and washed with a physiological solution for 30 min at 37°C. For measurement of the indo-1 fluorescence, the cells were placed in a physiological solution containing (mM): 137 NaCl, 3.7 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 5.6 glucose, and 4 HEPES-NaOH (pH 7.35). Cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was estimated as described by Peeters et al. (1987). A high-pressure Hg-arc lamp was used as the excitation source since it provided an intense emission peak at 360 nm. Further selection of this peak was made by narrow bandwidth interference filters. The fluorescent light was collected by the Fluor ×40 objective lens (Nikon, Tokyo, Japan) and divided with a beam splitter to measure the fluorescence at two wavelengths, 410 and 480 nm, using two separate photomultiplier tubes. $[Ca^{2+}]_i$ was calculated from the 410/480-nm fluorescence ratio ($R = F410/F480$) as $[Ca^{2+}]_i = K_d\beta(R - R_{min})/(R_{max} - R)$, where K_d = the dissociation constant of indo-1/AM (250 nM), $\beta = F_{max}480/F_{min}480$, R_{max} (maximum fluorescence ratio) = $F_{max}410/F_{min}480$, and R_{min} (minimum fluorescence ratio) = $F_{min}410/F_{max}480$. To obtain R_{min}, JR-1 cells were loaded with indo-1/AM, washed, and exposed to 140 mM KCl, 4 mM HEPES, and 50 μM digitonin in 0 Ca^{2+} (0.1 mM EGTA) for 2 min at 37°C. After addition of enough Ca^{2+} to increase $[Ca^{2+}]_o$ to 0.1 mM, R_{max} was obtained.

RESULTS

Effects of ACh and His on the Membrane Potential and Currents of JR-1 Cells

JR-1 cells had a resting membrane potential of −11.9 ± 0.7 mV (n = 20) under current-clamp conditions. The total cell capacitance and the input resistance at the resting potential measured under the voltage-clamp condition were 52.9 ± 13.8 pF (n = 20) and 1.41 ± 0.50 GΩ (n = 20), respectively. Fig. 2 *A* shows the effects of ACh and His on the resting membrane potential in JR-1 cells. ACh (1 μM), added to the bathing solution, rapidly hyperpolarized the membrane from −10 to −50 mV (Fig. 2 *A,a*). The ACh-induced hyperpolarization faded slightly during the continuous application of ACh (>5 min). After the washout of ACh, the membrane potential returned to its control level. Histamine (1 μM) added to the bathing solution also hyperpolarized the membrane (Fig. 2 *A,b*). The His-induced hyperpolarization, however, faded within 1 min, despite the continuous presence of His. Within 1–2 min of washout of His from the bath, the cells did not respond to this agonist (see Fig. 2 *B,b*). After ~3–5 min of washout, His regained its capability to fully induce a hyperpolarization (Fig. 2 *A,b*).

In Fig. 2, *B* and *C*, the effects of ACh and His on the holding current of JR-1 cells at −40 mV were examined under voltage-clamp conditions. ACh and His, added to the bathing solution, rapidly induced outward currents (Fig. 2 *B*). The ACh-induced current persisted (Fig. 2 *B,a*), while the His-induced outward current faded within 1 min in the continuous presence of the agonist (Fig. 2 *B,b*), as was the case with the His-induced hyperpolarization. Even after the His-induced current had faded completely, ACh evoked to its full magnitude an outward current (Fig. 2 *B,b*). After washout, the ability of His to induce the outward current gradually recovered (Fig. 2 *B,a*). In Fig. 2 *B,a,* His (50 μM) was further added to the bathing solution during the steady activation of the outward current by ACh. His induced a small additional outward current. The peak value of the outward current during the application of His in addition to ACh amounted to the same value as that of the initial peak induced by 10 μM ACh alone. The magnitude of the current component induced by His (50 μM)

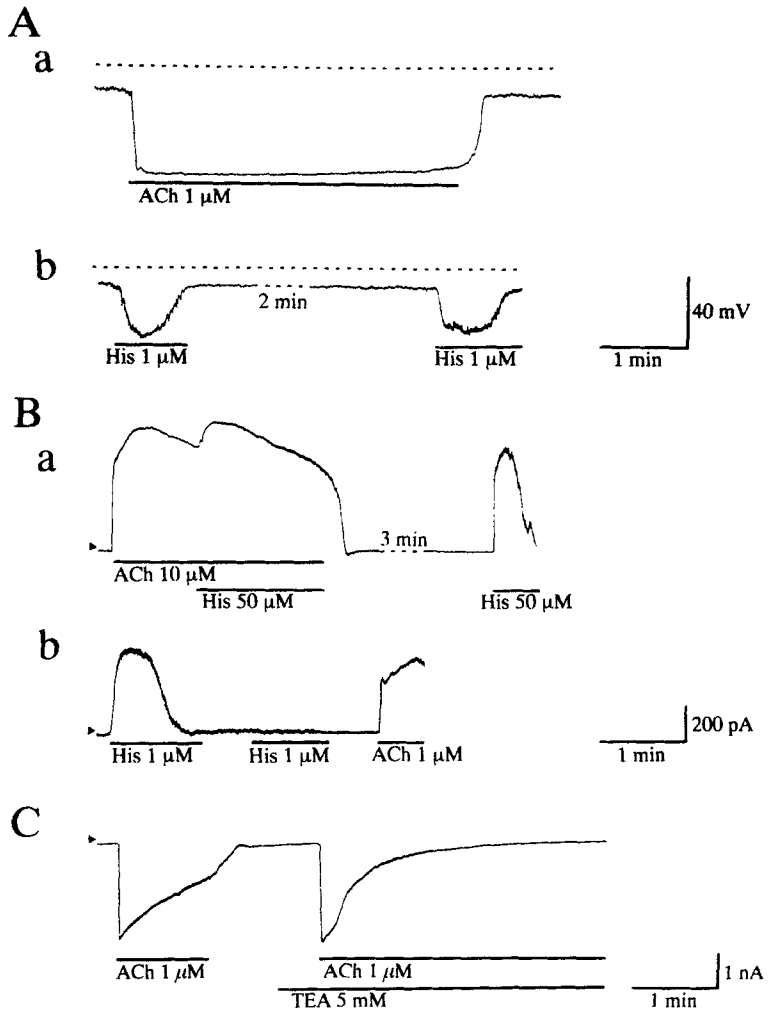

FIGURE 2. Effects of ACh and His on the resting membrane potential (*A*) and the holding current (*B* and *C*) of JR-1 cells. Under the current-clamp condition, ACh (1 μM, *a*) or His (1 μM, *b*) was added to the bathing solution in *A*. The dotted lines indicate the zero potential level. Under the voltage-clamp condition, ACh and His induced an outward current (*B*) or an inward current (*C*). HP = −40 mV. The arrowheads indicate the zero current level. The induction of an inward current was observed in 10 of 120 cells examined. The protocols of perfusing agonists and drugs are indicated by the bars beneath each current trace.

in the presence of ACh was much smaller than that in the absence of ACh. These observations indicate that the induction of the outward currents by ACh and His was not additive, but saturative in JR-1 cells.

ACh and His hyperpolarized the membrane by inducing outward currents in most JR-1 cells as shown in Fig. 2, *A* and *B*. However, in 10 of the 120 cells studied, ACh depolarized the membrane by evoking an inward current. The ACh-induced inward

current (at −40 mV) declined time dependently in the continuous presence of ACh. The current was not affected by tetraethylammonium (TEA, 5 mM), a K^+ channel blocker, and its reversal potential was ~0 mV. The current was not affected by replacing extracellular Na^+ with Tris. Thus, the inward current seemed to flow through a Ca^{2+}-activated Cl^- channel. However, we did not examine its properties in detail because the inward current was only rarely evoked by the agonists in JR-1 cells.

Specific antagonists for muscarinic and His receptors on the ACh- and His-induced outward currents were examined (Fig. 3). Atropine, a nonselective muscarinic

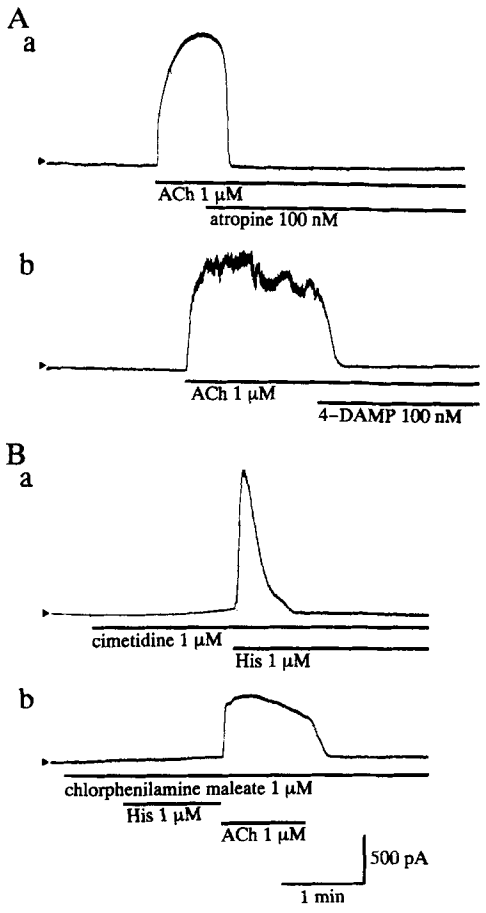

FIGURE 3. Pharmacological characterization of ACh and His induction of an outward current in JR-1 cells. (*A*) The effects of atropine (100 nM, *a*) and 4-DAMP (100 nM, *b*) on the ACh induction of an outward current. (*B*) The effects of cimetidine (1 μM, *a*) and chlorphenilamine maleate (1 μM, *b*) on the His induction of an outward current. The cells were voltage-clamped at 0 mV. The protocols used to perfuse agonists and antagonists are indicated by the bars beneath each current trace.

receptor antagonist, and 4-DAMP, an m_3-muscarinic receptor antagonist (Fisher and Heacock, 1988; Lucchesi, Romano, Scheid, Yamaguchi, and Honeyman, 1989, van Charldorp and van Zwieten, 1989) at 100 nM completely inhibited the ACh-induced outward current (Fig. 3*A*). Pirenzepine (100 nM, an m_1-muscarinic receptor antagonist) and AF-DX116 (100 nM, a m_2-muscarinic antagonist; Hulme, Birdsall, and Buckley, 1990) inhibited the current by ~30 and ~10%, respectively (not shown). The His induction of the outward current was not affected by 1 μM cimetidine, an

H_2-His receptor antagonist, but was abolished by 1 μM chlorphenilamine maleate, an H_1-His receptor antagonist (Fig. 3 *B*). These results suggest that m_3-muscarinic ACh and H_1-His receptors predominantly mediate the ACh and His induction of the outward current in JR-1 cells, respectively.

Voltage-dependent and Pharmacological Properties of ACh- and His-induced Currents in JR-1 Cells

The voltage-dependent properties of the currents induced by ACh and His in JR-1 cells were examined (Fig. 4). Fig. 4 *A* depicts current traces before and during the

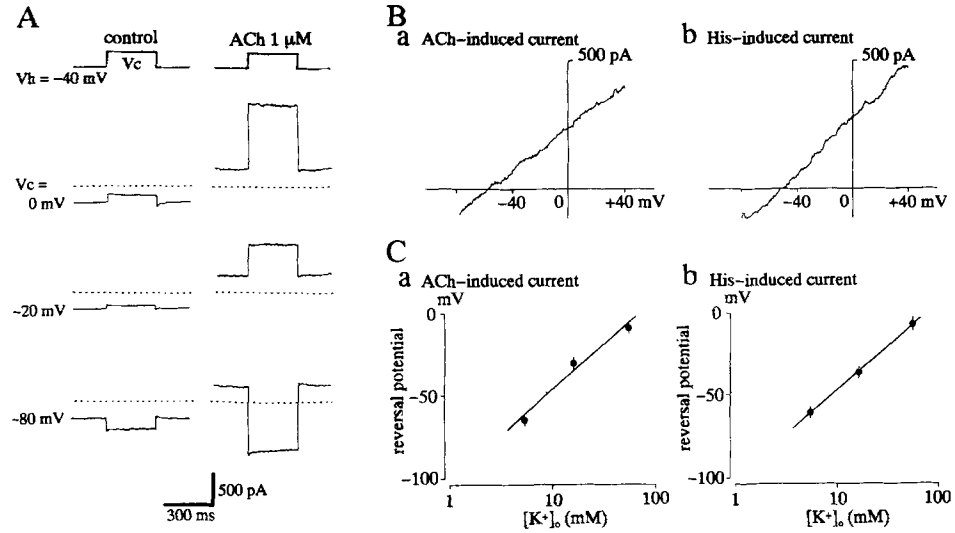

FIGURE 4. The voltage-dependent properties of the ACh- and His-induced currents in JR-1 cells. (*A*) Current traces before and during application of ACh (1 μM) at various potentials are shown. HP = −40 mV. The voltage steps to various potentials (300 ms in duration) were applied every 5 s. Dotted lines indicate the zero current level. (*B*) The current–voltage relations of ACh (*a*)- and His (*b*)-induced currents obtained by voltage-ramp pulses. HP = −40 mV. The voltage-ramp pulse from −80 to +40 mV (at a speed of 50 mV/s) was applied every 5 s. The difference between the control relation and that under application of ACh or His was defined as the ACh (*a*)- or His (*b*)-induced current, respectively. (*C*) The reversal potential of the ACh (*a*)- and His (*b*)-induced currents at various $[K^+]_o$.

application of ACh (1 μM). The cell was held at −40 mV, and command voltage steps (300 ms in duration) to various membrane potentials were applied every 5 s. In the control, no significant voltage-dependent ionic currents were evoked by the command steps. ACh induced outward currents at the holding potential (−40 mV) and during the command steps to −20 and 0 mV. An additional inward current was induced by ACh during a step to −80 mV. The ACh-induced current did not show significant time-dependent relaxation during command pulses. Similar results were obtained in the His-induced current (not shown).

Fig. 4 *B* shows the current–voltage relationship of the ACh- and His-induced currents examined by applying a linear voltage-ramp pulse (from −80 to +40 mV with 50 mV/s alteration) every 5 s to the cell. The current–voltage relationship was almost linear between −80 and +40 mV in both ACh- and His-induced currents, and crossed the zero current level at approximately −65 mV with 5.4 mM of extracellular K^+ ($[K^+]_o$), suggesting that the currents are mainly carried by K^+. The peak current density at 0 mV was 54.2 ± 31.1 μA/cm² (mean ± SD, n = 10) in the ACh response and 52.9 ± 33.7 μA/cm² (n = 10) in the His response, respectively.

The reversal potentials of the ACh- and His-induced currents at various $[K^+]_o$ are plotted in Fig. 4 *C,a* and *b*. As $[K^+]_o$ was increased, the reversal potentials shifted to more depolarized potentials. The reversal potentials of the ACh- and His-induced

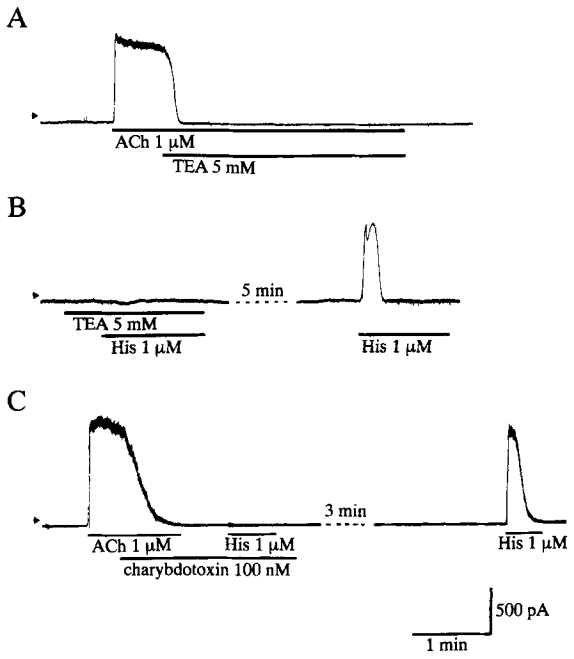

FIGURE 5. Effects of tetraethylammonium chloride (*TEA; A* and *B*) and charybdotoxin (*C*) on the ACh- and His-induced outward currents. The cells were held at 0 mV. The perfusing protocol of various substances is indicated by the bars beneath each current trace.

currents were −65 ± 5 mV (n = 4) and −67 ± 5 mV (n = 4) at 5.4 mM $[K^+]_o$, −38 ± 5 mV (n = 3) and −35 ± 5 mV (n = 3) at 10.8 mM $[K^+]_o$, and −10 ± 3 mV (n = 3) and −9 ± 2 mV (n = 3) at 20 mM $[K^+]_o$, respectively. They changed ~55 mV per 10-fold change of $[K^+]_o$ in both cases. The value is close to the theoretical shift of the K^+ equilibrium potential, which is 60 mV with 150 mM $[K^+]_i$. Therefore, we concluded that ACh- and His-induced currents are carried mainly by K^+.

Fig. 5, *A* and *B*, shows the effect of the K^+ channel blocker, TEA (Findlay, Dunne, Ullrich, Wollheim, and Petersen, 1985), on the ACh- and His-induced currents. In Fig. 5 *A*, TEA (5 mM) completely inhibited the outward current induced by ACh (1 μM). TEA also abolished the effect of His (1 μM) (Fig. 5 *B*). Around 6 min after the washout of TEA and His, a second application of His (1 μM) induced an outward

current in the same cell. These results are consistent with the notion that the outward currents induced by ACh and His are due to the openings of K^+ channels.

In Fig. 5 *C*, charybdotoxin (100 nM), a blocker of the Ca^{2+}-activated K^+ channel (Miller, Moczydlowsky, Latorre, and Phillips, 1985; Smith, Phillips, and Miller, 1986), was added to the bath solution during the application of ACh. The toxin completely inhibited the ACh- and His-induced outward currents. These observations may indicate that the ACh- and His-induced K^+ currents are flowing through a charybdotoxin-sensitive Ca^{2+}-activated K^+ channel ($I_{K.Ca}$), and suggest that intracellular Ca^{2+} increases in response to these secretagogues in JR-1 cells.

ACh and His Increase Intracellular Ca^{2+} in JR-1 Cells

In Fig. 6, we examined the effects of ACh and His on the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) of JR-1 cells using the fluorescent Ca^{2+} indicator, indo-1/AM. The resting $[Ca^{2+}]_i$ of these cells was estimated at 120 ± 10 nM (n = 5). As shown in Fig. 6 *A*, the application of ACh (100 μM) to the bathing solution resulted in a rapid

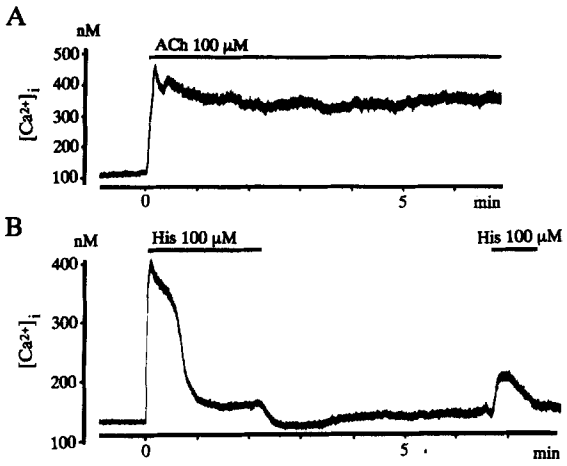

FIGURE 6. Effects of ACh (*A*) and His (*B*) on the concentration of intracellular Ca^{2+} in JR-1 cells. Intracellular Ca^{2+} concentration was estimated by the ratio of the fluorescence at 410- and 480-nm wavelength measured with the fluorescent Ca^{2+} indicator indo-1/AM. The protocols superfusing ACh and His are indicated by the bars above each trace.

increase in $[Ca^{2+}]_i$ to 450 ± 20 nM (n = 4). After the initial increase, $[Ca^{2+}]_i$ gradually decreased to a steady level that was ~70% of the peak level. This steady elevation of $[Ca^{2+}]_i$ was maintained during the continuous application of 100 μM ACh. The ACh-induced increase in $[Ca^{2+}]_i$ was inhibited by atropine (1 μM; not shown).

In Fig. 6 *B*, His (100 μM) also increased $[Ca^{2+}]_i$ to 400 ± 20 nM (n = 3). However, the His-induced increase in $[Ca^{2+}]_i$ was transient and decreased to ~10% of the peak value within 1 min of the continuous application of the agonist. 4 min after washout of His from the bath, His was reapplied to the bathing solution. The His-induced elevation of $[Ca^{2+}]_i$ during the second application reached only ~20% of the peak value of the first application. The His-induced increase in $[Ca^{2+}]_i$ was prevented by chlorphenilamine maleate (1 μM; not shown). Similar results were obtained using 1–10 μM His and ACh. These findings indicate that both ACh and His increase $[Ca^{2+}]_i$ with a similar time course as they elicit $I_{K.Ca}$. Indeed, the ACh-induced elevation of $[Ca^{2+}]_i$ persisted, while the His-induced elevation faded within 1 min.

In Fig. 7, we examined the effects of intracellular and extracellular Ca^{2+} on the agonist-induced K^+ current. When the concentration of EGTA in the pipette solution was increased from 0.15 to 10 mM to buffer $[Ca^{2+}]_i$, both ACh and His induction of the K^+ current were abolished. This is in accord with the notion that both ACh and His induce $I_{K.Ca}$ by increasing $[Ca^{2+}]_i$ in JR-1 cells.

When ACh (1 μM) was applied to a cell in the Ca^{2+}-free bathing solution (with 0.15 mM EGTA in the pipette solution), ACh (1 μM) induced a transient increase of $I_{K.Ca}$ (Fig. 7 *B*). His (1 μM) further added to the Ca^{2+}-free bathing solution did not affect the membrane current. In the continuous presence of ACh, when extracellular Ca^{2+} ($[Ca^{2+}]_o$) was increased from 0 to 1.8 mM, the $I_{K.Ca}$ resumed immediately and remained sustained. The current disappeared on washout of ACh from the bath. These results suggest that at least two different mechanisms underlie the ACh induction of $I_{K.Ca}$: the initial transient activation of $I_{K.Ca}$ may reflect the ACh-induced

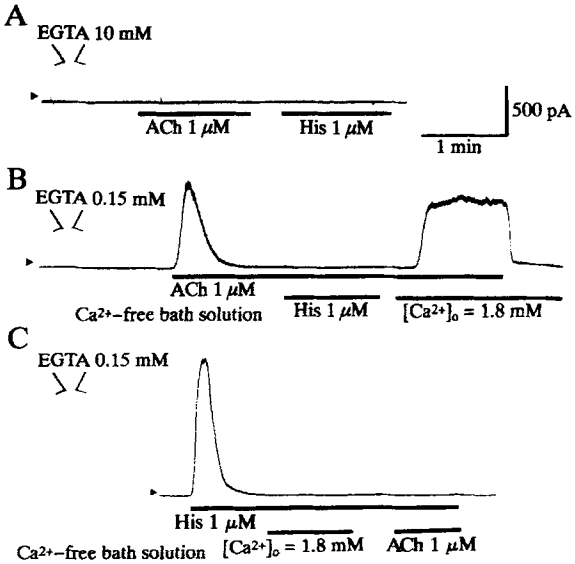

FIGURE 7. Effect of removal of intra- and extracellular Ca^{2+} on the ACh and His actions. HP = 0 mV. (*A*) The pipette solution contained 10 mM EGTA and 1.8 mM $[Ca^{2+}]_o$. In this condition, the responses to ACh and His were abolished. (*B*, *C*) With the pipette solution containing 0.15 mM EGTA, cells were superfused with Ca^{2+}-free bathing solution. At the end of the experiments, $[Ca^{2+}]_o$ was increased from 0 to 1.8 mM. The protocols superfusing various substances are indicated by the bars beneath each current trace.

Ca^{2+} release from intracellular Ca^{2+} storage sites, while the sustained activation of $I_{K.Ca}$ may be due to the ACh-induced Ca^{2+} influx across the cell membrane.

When His was applied to the cell before ACh in the Ca^{2+}-free bathing solution, His also elicited a brief transient increase of $I_{K.Ca}$ (Fig. 7 *C*). Further application of ACh to the cell did not induce $I_{K.Ca}$. In the continuous presence of His, an increase in $[Ca^{2+}]_o$ from 0 to 1.8 mM did not restore $I_{K.Ca}$. This is probably due to the desensitization of the His response. Hence, we could not identify the Ca^{2+} influx–dependent component of the His response.

IP_3 as the Possible Second Messenger of the ACh and His Activation of the Ca^{2+}-activated K^+ Current

In various cells, receptor-dependent intracellular Ca^{2+} mobilization is mediated by IP_3 (Nishizuka, 1984; Berridge and Irvine, 1989; Marty, 1991; Neher, 1992;

Petersen, 1992). To evaluate whether the IP_3-Ca^{2+} mobilization pathway is involved in the action of ACh and His in JR-1 cells, we examined the effects of IP_3 and IP_4 on membrane currents of these cells (Fig. 8). Cells were held at 0 mV, and the holding current was continuously monitored. The pipette solution contained IP_3 (100 μM or 1 mM) and/or IP_4 (100 μM or 1 mM).

In the control bathing solution (1.8 mM $[Ca^{2+}]_o$), an outward K^+ current was evoked abruptly 1–2 min after the formation of the whole cell clamp configuration,

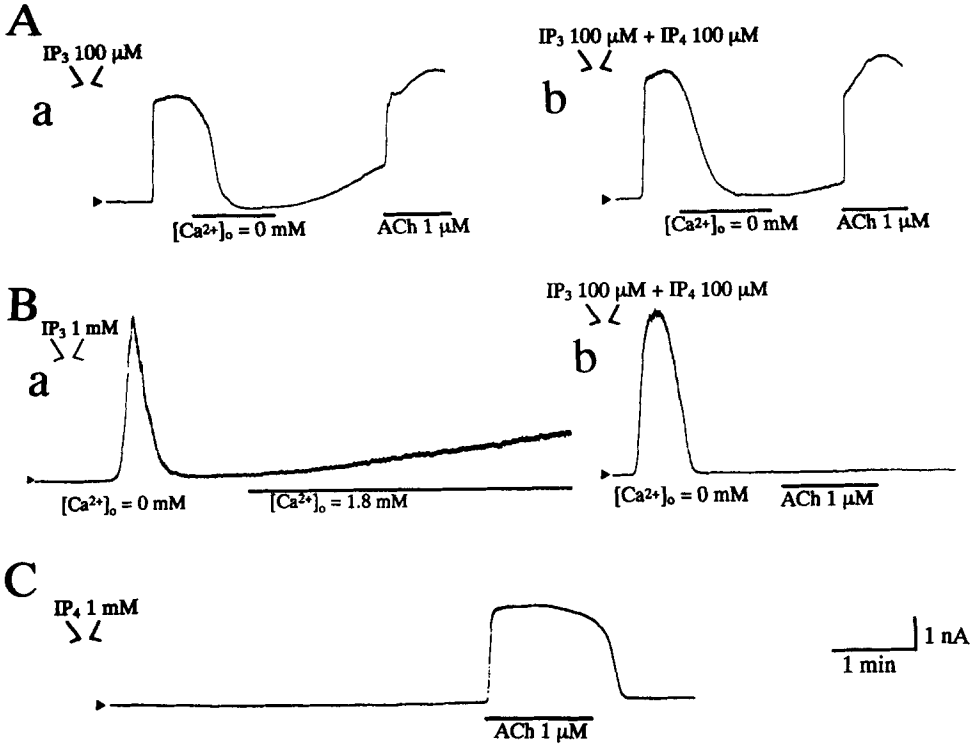

FIGURE 8. Effects of IP_3 and IP_4 on the membrane currents of JR-1 cells. HP = 0 mV. Arrowheads indicate the zero current level. The patch pipettes contained IP_3, IP_3 + IP_4 (*A*, *B*), or IP_4 (*C*). (*A*) In the control bathing solution. The Ca^{2+}-free bathing solution was applied to the cells as indicated. (*B*) In the Ca^{2+}-free bathing solution. The control bathing solution was superfused after the transient increase of $I_{K.Ca}$ faded in the left trace. (*C*) In the control bathing solution. The protocols perfusing various substances are indicated by the bars beneath each current trace.

when the pipette solution contained either IP_3 (100 μM) (Fig. 8 *A,a*) or IP_3 (100 μM) + IP_4 (100 μM) (Fig. 8 *A,b*). The current was sustained. Thus, IP_3 and IP_3 + IP_4 were likely to evoke not only the Ca^{2+} release from intracellular storage sites but also Ca^{2+} influx across the cell membrane provided that $[Ca^{2+}]_o$ was continuously present.

The current density at 0 mV was 51.5 ± 17.6 μA/cm^2 (n = 4) with IP_3 pipettes and 49.8 ± 16.4 μA/cm^2 (n = 3) with IP_3 + IP_4 pipettes, respectively. These values were not significantly different from the control values of ACh- and His-induced outward

currents. The currents induced by IP_3 and IP_3 + IP_4 were suppressed by 5 mM TEA and 100 nM charybdotoxin (not shown). When the pipette solution contained 10 mM EGTA, neither IP_3 nor IP_3 + IP_4 could affect the membrane current (n = 5 for each condition; not shown). During the steady activation of the current by intracellular IP_3 or IP_3 + IP_4, ACh (1 μM) or His (1 μM) did not further increase the outward current (not shown).

The sustained phase of the IP_3- or IP_3 + IP_4-induced outward currents was abolished when Ca^{2+} was omitted from the bathing solution (Fig. 8 *A,a* and *b*), indicating that the sustained activation of $I_{K.Ca}$ is due to Ca^{2+} influx across the cell membrane. However, contrary to the effect of ACh (Fig. 7 *B*), when Ca^{2+} was readded to the bathing solution, the outward current did not resume immediately. The Ca^{2+} influx–related $I_{K.Ca}$ increased with a slow time course in the presence not only of IP_3 (Fig. 8 *A,a*) but also of IP_3 + IP_4 (Fig. 8 *A,b;* half-recovery time of ~2–5 min). Further application of ACh (1 μM) caused immediate recovery of the sustained activation of $I_{K.Ca}$ in both cases. Similar findings were obtained in two groups of five cells tested with IP_3 or IP_3 + IP_4 in pipettes.

In the Ca^{2+}-free bathing solution, IP_3 (100 μM–1 mM; Fig. 8 *B,a*) or IP_3 (100 μM) + IP_4 (100 μM; Fig. 8 *B,b*) also evoked an outward current 1–2 min after the formation of the whole cell clamp mode. The response was transient and may reflect the Ca^{2+} release from storage sites induced by IP_3 or IP_3 + IP_4. This transient activation of $I_{K.Ca}$ was similar to the ACh response in the Ca^{2+}-free bathing solution. After the transient $I_{K.Ca}$ activation, ACh (1 μM) did not affect the membrane current in the continuous presence of the Ca^{2+}-free bathing solution (Fig. 8 *B,b*). Upon increasing $[Ca^{2+}]_o$ to 1.8 mM, the K^+ current again increased with a slow time course (Fig. 8 *B,a*), which was accelerated by ACh (not shown). Similar observations were obtained in three other cells for each case. Thus, the outward currents evoked by IP_3 and IP_3 + IP_4 did not differ from each other significantly in their time course, responses to removal and reapplication of $[Ca^{2+}]_o$, and response to ACh.

IP_4 (100 μM–1 mM) alone applied into the cells through the pipette did not affect the holding current of the JR-1 cells (Fig. 8 *C*). ACh added to the bathing solution induced a full activation of $I_{K.Ca}$. Upon washout of ACh, the current returned to its control level. Intracellular application of cAMP (1 mM), cGMP (1 mM), and diacylglycerol (100 μg/ml) did not affect either of the membrane currents present in JR-1 cells (not shown).

The above results suggest that IP_4 alone does not play a significant role in Ca^{2+} handling by JR-1 cells. When IP_3 was loaded into the cells through pipettes, not only Ca^{2+} release from intracellular storage sites but also Ca^{2+} influx across cell membrane were induced in the continuous presence of $[Ca^{2+}]_o$. Thus, these IP_3-mediated events might be involved in the ACh induction of $I_{K.Ca}$ in JR-1 cells. However, it should also be noted that after a brief exposure of cells to the Ca^{2+}-free bathing solution, ACh, but neither intracellular IP_3 nor IP_3 + IP_4, induced immediate recovery of the sustained Ca^{2+} influx–related $I_{K.Ca}$.

To further define the properties of the agonist-dependent Ca^{2+} mobilization in JR-1 cells, we examined the effects of heparin, an inhibitor of IP_3-dependent Ca^{2+} release (Kobayashi, Kitazawa, Somlyo, and Somlyo, 1989), and of the monoclonal antibody to the IP_3 receptor, mAb18A10 (Nakade et al., 1991), on the ACh and His induction of $I_{K.Ca}$ (Fig. 9).

With the pipette containing the control internal solution, a full amplitude of $I_{K.Ca}$ was induced within 2–3 s in response to ACh (1 μM) and sustained during the application of the agonist (Fig. 9 *A*). When 10 U/ml heparin was added to the pipette solution, ACh (1 μM) induced the current by only approximately one-tenth of the control value after a delay of ~10–15 s (Fig. 9 *B,a*). Subsequent application of His did not induce any current. The ACh response was reproducible. When 50 U/ml heparin was added to the pipette solution, both ACh and His responses were completely prevented (Fig. 9 *B,b*).

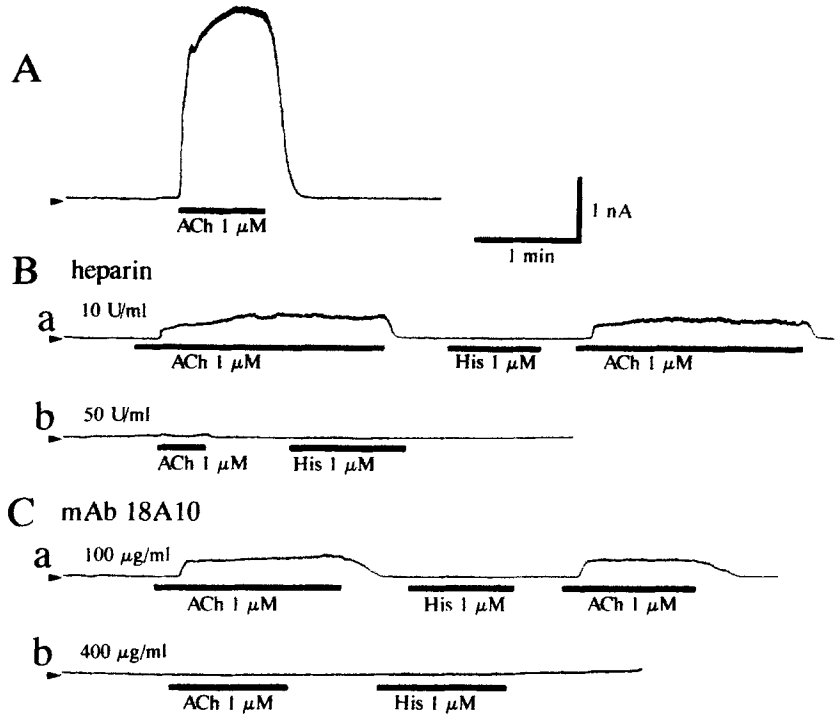

FIGURE 9. Effects of heparin and the monoclonal antibody to the IP_3 receptor channel, mAb18A10, on the ACh and His induction of $I_{K.Ca}$. (*A*) ACh induction of $I_{K.Ca}$ in the control condition. (*B*) The pipette solution contained 10 (*a*) or 50 U/ml (*b*) heparin. (*C*) The pipette solution contained 100 (*a*) or 400 μg/ml (*b*) mAb18A10. HP = 0 mV. The arrowheads indicate the zero current level. The protocols perfusing ACh or His are indicated by the bars beneath each current trace.

The monoclonal antibody, 18A10, reacts with the 12 amino acid residues located near the putative Ca^{2+} channel region of the IP_3 receptor. It is postulated that binding of this antibody may cause partial occlusion or a conformational change of the channel (Nakade et al., 1991). Similarly to heparin, mAb18A10 also inhibited the ACh and His responses in a concentration-dependent manner (Fig. 9 *C*). With 100 μg/ml of the antibody in the pipette solution, ACh (1 μM) induced $I_{K.Ca}$ by approximately one-tenth of the control value after a delay (~10–15 s) (Fig. 9 *C,a*). His did not induce $I_{K.Ca}$. When the concentration of the antibody was increased to

400 μg/ml, both the ACh and His responses were completely inhibited (Fig. 9 *C,b*). With the boiled antibody (at 100°C for 20 min) in the pipette, ACh and His induced the K^+ current at 0 mV by 48.3 ± 14.2 μA/cm^2 (n = 4) and 46.7 ± 11.6 μA/cm^2 (n = 3), respectively (not shown). These values are not significantly different from control values. Thus, the denatured antibody did not affect the ACh and His responses.

Since IP_3 is generated from membrane phospholipids by phospholipase C (PLC) in various cells (Nishizuka, 1984; Berridge and Irvine, 1989), we examined the effects of the PLC inhibitor, neomycin, on the ACh and His induction of $I_{K.Ca}$ (Fig. 10). Neomycin (1–100 μM) suppressed the ACh induction of $I_{K.Ca}$ in a concentration-dependent manner. When 1 μM neomycin was added to the pipette solution, the ACh response was not affected during the first minute after the formation of the

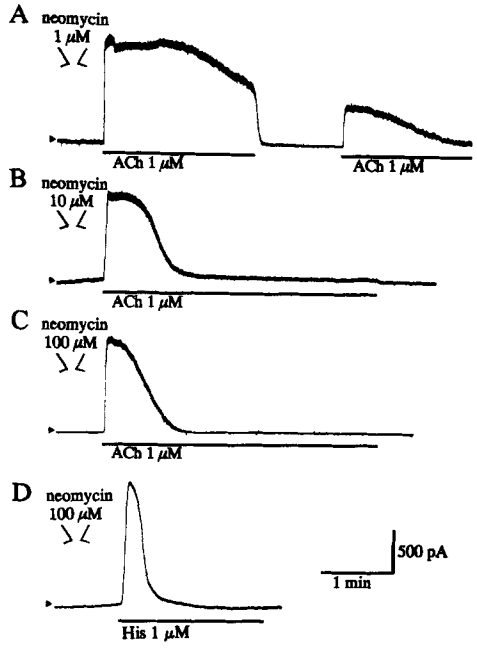

FIGURE 10. Effect of neomycin on the ACh and His induction of $I_{K.Ca}$. The pipette solution contained 1 (*A*), 10 (*B*), and 100 μM (*C, D*) neomycin. The protocols for perfusing ACh are indicated by the bars beneath each current trace. HP = 0 mV. Arrowheads indicate the zero current level.

whole cell configuration, but gradually became suppressed (Fig. 10 *A*). The suppression of the ACh-induced current was accelerated when the concentration of neomycin was increased to 10 or 100 μM (Fig. 10, *B* and *C*). However, even when 100–200 μM neomycin was added to the pipette solution, the initial transient activation of $I_{K.Ca}$ by ACh was not markedly affected. The His response was also not altered to a large extent by 100–200 μM neomycin (Fig. 10 *D*). We could not examine the effect of higher concentrations of neomycin, since neomycin at concentrations >300 μM made cells leaky. These results suggest that PLC is involved in the ACh-induced sustained activation of $I_{K.Ca}$ in JR-1 cells. The initial Ca^{2+} release phase of the ACh and His responses was relatively resistant to neomycin.

Involvement of GTP-binding Proteins in the ACh and His Activation of the K^+ Current in JR-1 Cells

In many cell types, the receptor-dependent activation of PLC involves a certain population of G proteins (Nishizuka, 1984; Berridge and Irvine, 1989). To explore whether G proteins are involved in the ACh and His induction of $I_{K.Ca}$ in JR-1 cells, we examined the effects of GDPβS and GTPγS on the ACh and His responses (Figs. 11 and 12).

GDPβS (200 μM–2 mM) was added to the pipette solution (Fig. 11). In the presence of 200 μM GDPβS, the ACh response became transient, while the His response was apparently unaffected (Fig. 11 *A*). When the concentration of GDPβS was increased to 1 mM, the initial transient increase of $I_{K.Ca}$ by ACh and the His-induced $I_{K.Ca}$ were attenuated and finally abolished at 2 mM GDPβS (Fig. 11, *B* and *C*). The peak current induced by ACh (1 μM) and His (1 μM) at 0 mV was

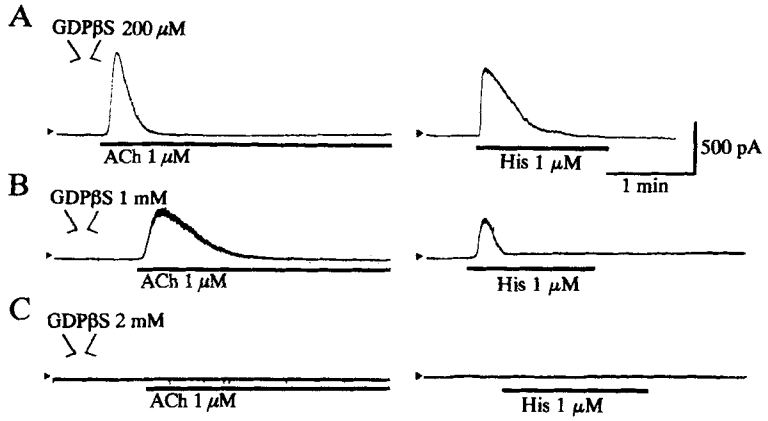

FIGURE 11. Effects of GDPβS on the ACh and His induction of $I_{K.Ca}$. The pipette solution contained 200 μM (*A*), 1 mM (*B*), and 2 mM (*C*) GDPβS. ACh (1 μM) or His (1 μM) was applied to the cells as indicated by the bars beneath each current trace. Arrowheads indicate the zero current level. HP = 0 mV.

54.2 ± 31.1 μA/cm^2 (n = 10) and 52.9 ± 33.7 μA/cm^2 (n = 10) in control cells, 14.8 ± 5.6 μA/cm^2 (n = 5) and 13.9 ± 8.5 μA/cm^2 (n = 5) in 200 μM GDPβS-loaded cells, and 7.6 ± 2.5 μA/cm^2 (n = 5) and 7.6 ± 4.0 μA/cm^2 (n = 5) in 1 mM GDPβS-loaded cells, respectively.

Fig. 12 compares the effects of various concentrations of ACh on the induction of $I_{K.Ca}$ in cells loaded with GTP vs. GTPγS. The pipette solution contained either GTP (100 μM) (*A*) or GTPγS (300 μM) (*B*). In GTP-loaded cells, 3 and 10 nM ACh did not significantly induce $I_{K.Ca}$. At 30 nM ACh, a minimal increase of $I_{K.Ca}$ was recorded. 100 nM of ACh was needed to evoke the full size of $I_{K.Ca}$. Initiation of this effect was abrupt, probably due to the triggering of intracellular release of Ca^{2+} from intracellular storage sites. The Ca^{2+} influx, subsequent to the release, may have been responsible for the sustained activation of $I_{K.Ca}$. Similar results were obtained in four other cells.

In GTPγS-loaded cells, a minimal gradual increase of $I_{K.Ca}$ was already observed at 3 nM ACh. 10 nM ACh induced a transient increase of $I_{K.Ca}$. The current decreased to a lower level during the application of 10 nM ACh. When the ACh concentration was increased to 30 nM, 100 nM, 300 nM, and 1 μM, the amplitudes of the initial transient $I_{K.Ca}$ and the sustained current became larger. Thus, GTPγS increased the sensitivity of induction of $I_{K.Ca}$ by ACh. The results on the effects of GDPβS and GTPγS suggest that a population of G proteins is involved in the ACh and His induction of $I_{K.Ca}$ in JR-1 cells.

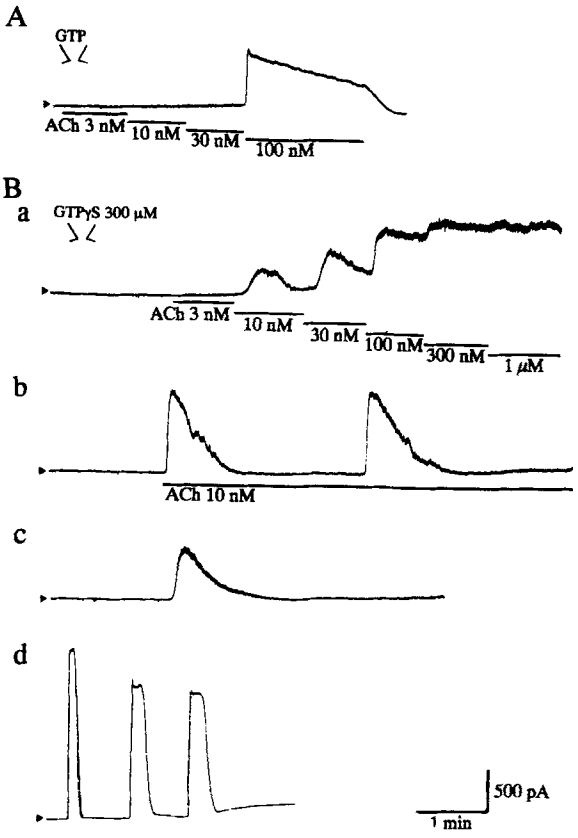

FIGURE 12. Effect of intracellular GTPγS on the ACh induction of $I_{K.Ca}$. (*A*) The control pipette solution contained 100 μM GTP. (*B*) The pipette solution contained 300 μM GTPγS instead of GTP. The protocols perfusing ACh are indicated by the bars beneath each current trace. Arrowheads indicate the zero current level. HP = 0 mV. *a* and *b* were obtained from the same cell. In the cells in *b* and *c*, $I_{K.Ca}$ was evoked spontaneously.

In GTPγS-loaded cells, an oscillatory activation of $I_{K.Ca}$ was sometimes observed either in the presence of low concentrations of ACh (Fig. 12 *B,b*) or in the absence of agonists (Fig. 12 *B,c* and *d*). The oscillation of $I_{K.Ca}$ has never been observed in GTP-loaded cells (n = 50). The current trace shown in Fig. 12 *B,b* was obtained from the same cell as in *a*. 5 min after washout of ACh, 10 nM ACh was reapplied to the cell. ACh (10 nM) evoked a full-sized $I_{K.Ca}$, which decreased to the baseline level spontaneously. In the continuous presence of 10 nM ACh, the transient activation of $I_{K.Ca}$ was evoked around every 3 min. GTPγS (300 μM) alone did not evoke the sustained increase of $I_{K.Ca}$ in the absence of ACh (n = 10), but induced a transient or oscillatory increase of $I_{K.Ca}$ (Fig. 12 *B,c* and *d*).

Fig. 13 shows the lack of effect of pertussis toxin (PTX) and *N*-ethylmaleimide (NEM) on the ACh induction of $I_{K.Ca}$. JR-1 cells were incubated in culture media containing 500 ng/ml PTX for 6–12 h before these experiments. In the PTX-pretreated cells, His (1 μM) and ACh (1 μM) induced $I_{K.Ca}$ as effectively as in control cells (Fig. 13 *A*). The current density of the peak $I_{K.Ca}$ at 0 mV in PTX-pretreated cells was 57.6 ± 22.3 μA/cm^2 (n = 5, P = 0.830 compared with the control value of 54.2 ± 31.1 μA/cm^2, n = 10) in the presence of ACh and 65.9 ± 26.6 μA/cm^2 (n = 5, P = 0.467 compared with the control value of 52.9 ± 33.7 μA/cm^2, n = 10) in the presence of His. In Fig. 13 *B*, we examined the effects of NEM (50 μM), which uncouples the receptors from the PTX-sensitive G proteins (Katada, Kurose, Oinuma, Hoshino, Shinoda, Asanuma, and Ui, 1986; Kitamura and Nomura, 1987; Ueda, Misawa, Katada, Ui, Takagi, and Satoh, 1990), on the ACh induction of $I_{K.Ca}$. After washout of the first application of ACh, NEM was added to the bathing solution for 7 min. The second application of ACh (1 μM) induced $I_{K.Ca}$ of similar magnitude (56.7 ± 22.6 μA/cm^2, n = 5, P = 0.877) when compared with the control value. These results indicate that the G proteins involved in the ACh and His induction of $I_{K.Ca}$ in JR-1 cells are PTX insensitive.

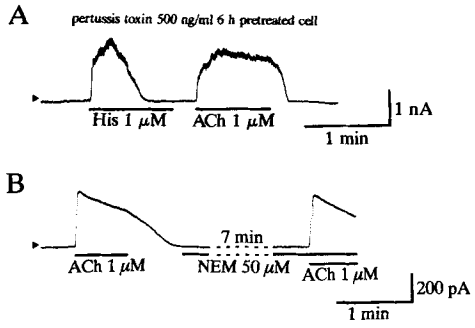

FIGURE 13. Effects of PTX and NEM on the ACh or His induction of $I_{K.Ca}$. (*A*) The cell was pretreated with 500 ng/ml PTX for 6 h. (*B*) The cell was treated with 50 μM NEM for 8 min before the second application of ACh. The protocols perfusing ACh, His, and NEM are indicated by the bars beneath each current trace. The arrowheads indicate the zero current level. HP = 0 mV in *A* and −40 mV in *B*.

DISCUSSION

The major findings of this study are as follows: (*a*) ACh and His induce an outward current in JR-1 cells, a mucin-producing epithelial cell line derived from human gastric cancer, predominantly via functional m_3-muscarinic ACh and H_1-His receptors. (*b*) This current is a charybdotoxin-sensitive, Ca^{2+}-dependent K^+ channel current ($I_{K.Ca}$). (*c*) The ACh-induced activation of $I_{K.Ca}$ persists, while the His response desensitizes rapidly. (*d*) The IP_3-Ca^{2+} mobilization mediated by PTX-insensitive G proteins may be involved in the signaling. (*e*) Ca^{2+} influx across the cell membrane in addition to Ca^{2+} release from the storage sites was induced when IP_3 or IP_3 + IP_4 was loaded into JR-1 cells through pipettes. (*f*) After a brief exposure of cells to Ca^{2+}-free bathing solution, ACh, but not intracellular IP_3 or IP_3 + IP_4, induced immediate recovery of the Ca^{2+} influx–related $I_{K.Ca}$.

IP_3 as a Common Intracellular Mediator for Activation of a Ca^{2+}-activated K^+ Channel by ACh and His in JR-1 Cells

The time course of activation of $I_{K.Ca}$ as well as the increase in $[Ca^{2+}]_i$ differ between ACh and His (Figs. 2 and 6): the ACh response persisted, while the His response was transient even in the continuous presence of the agonist. The ACh activation of $I_{K.Ca}$ was composed of the initial Ca^{2+} release–related component and the sustained Ca^{2+} influx–related component. On the other hand, we could not identify the Ca^{2+} influx–related component of the His-activated $I_{K.Ca}$. There may be two possible explanations for this observation: (*a*) the H_1-His receptor may rapidly desensitize in the continuous presence of the agonist, or (*b*) the H_1 receptor may not be coupled to the Ca^{2+} influx mechanism.

In both ACh and His, the agonist-induced current was a voltage- and time-independent K^+ current which could be inhibited by charybdotoxin, TEA, and high concentrations of intracellular EGTA. Thus, these two agonists may activate the same population of $I_{K.Ca}$ in JR-1 cells. Consistent with this concept, the ACh and His induction of $I_{K.Ca}$ were not additive, but saturative (Fig. 2 *B,a*). In Ca^{2+}-free bathing solution, prior application of ACh prevented the activation of $I_{K.Ca}$ upon subsequent application of His and vice versa (Fig. 7, *B* and *C*). This observation also indicates that ACh and His cause Ca^{2+} release from the same population of intracellular Ca^{2+} storage sites in JR-1 cells. Furthermore, heparin, an IP_3-dependent Ca^{2+} release channel inhibitor, and mAb18A10, a monoclonal antibody for the IP_3 receptor channel, inhibited the ACh and His induction of $I_{K.Ca}$ in a concentration-dependent manner. These results suggest that ACh and His share common intracellular messengers to induce $I_{K.Ca}$. Therefore, it is unlikely that the H_1 receptor is coupled only to the Ca^{2+} release and not to the Ca^{2+} influx mechanism, although we cannot rule out this possibility completely. Thus, the rapid fade of the His response may be due to the desensitization of the H_1-His receptor.

IP_3 as well as IP_3 + IP_4, applied into the cells through pipettes, evoked a regenerative and sustained activation of $I_{K.Ca}$ in Ca^{2+}-containing bathing solution and a transient activation of $I_{K.Ca}$ in Ca^{2+}-free bathing solution (Fig. 8). These effects of IP_3 and IP_3 + IP_4 are similar to those of ACh (Figs. 2 and 7). The effects of IP_3 and IP_3 + IP_4 on $I_{K.Ca}$ did not differ significantly from each other in the time course, responses to removal and reapplication of $[Ca^{2+}]_o$, and response to ACh (Fig. 8, *A* and *B*). IP_4 alone did not affect the membrane current of JR-1 cells (Fig. 8 *C*). However, high amounts of IP_4 might have been generated in the cells loaded with IP_3. Therefore, although we could not detect a significant difference in the responses between IP_3-loaded and IP_3 + IP_4-loaded cells, we cannot exclude the possibility that IP_4 may also be involved in the activation of $I_{K.Ca}$ in JR-1 cells loaded with IP_3. However, it is evident that IP_4 alone does not mediate the ACh and His responses. Cyclic nucleotides and diacylglycerol applied into cells did not induce $I_{K.Ca}$ (not shown). In contrast, IP_3 alone applied into cells could induce not only Ca^{2+} release from storage sites but also Ca^{2+} influx across the cell membrane in Ca^{2+}-containing bathing solution. Furthermore, heparin and mAb18A10 (inhibitors of the IP_3-dependent Ca^{2+} release channel) inhibited both the ACh and His responses.

Therefore, it is likely that IP_3 is the primary second messenger mediating the ACh and His activation of $I_{K.Ca}$ in JR-1 cells.

Although IP_3 is probably involved in both the Ca^{2+} release–related and Ca^{2+} influx–related components of ACh-induced $I_{K.Ca}$, the Ca^{2+} release–related component was more resistant to GDPβS and neomycin than the Ca^{2+} influx–related component (Figs. 10 and 11). Two possibilities may explain this observation: (*a*) The G protein–PLC systems that are coupled to the Ca^{2+} release and to the Ca^{2+} influx are different in JR-1 cells, and the system coupled to Ca^{2+} release is more resistant to GDPβS and neomycin. (*b*) The Ca^{2+} release mechanism is much more sensitive to IP_3 than the Ca^{2+} influx mechanism. Thus, even though GDPβS and neomycin suppress ACh-induced generation of IP_3 by inhibiting G proteins or PLC, the residual amount of IP_3 may be sufficient to induce Ca^{2+} release but not Ca^{2+} influx.

In regard to the first possibility, we need to further assume the compartmentalization of IP_3 in JR-1 cells to explain the results obtained using GDPβS and neomycin. Since GTPγS preferentially enhanced the Ca^{2+} release–related component over the Ca^{2+} influx–related component (Fig. 12; see also below), the sensitivities of the Ca^{2+} release and Ca^{2+} influx to IP_3 seem to be differently regulated in JR-1 cells. Thus, the second possibility seems more likely than the first one. However, further studies are needed to discriminate between these possibilities.

Mechanism of Ca^{2+} Influx in JR-1 Cells

Ca^{2+} influx after stimulation of the phosphoinositide signal pathway is a widespread and physiologically important phenomenon (Neher, 1992; Petersen, 1992). Several mechanisms may underlie this process. In lacrimal gland acinar cells, it was shown that IP_3 and IP_4 synergically evoke Ca^{2+} influx (Morris, Gallacher, Irvine, and Petersen, 1987). Lückhoff and Clapham (1992) described an intracellular Ca^{2+}-activated divalent cation–selective channel whose open probability is increased by IP_4. On the other hand, it has also been suggested that the decisive factor for Ca^{2+} entry is not the second messenger generated by the agonist, but rather the filling state of the intracellular Ca^{2+} stores (Putney, 1990). Recently, Hoth and Penner (1992) identified a Ca^{2+} current in mast cells that is activated by depletion of intracellular Ca^{2+} stores caused by EGTA, IP_3, or ionomycin. These two types of mechanisms, i.e., the second messenger–mediated Ca^{2+} influx and the depletion of Ca^{2+} store site-mediated one are supposed to exist in parallel in certain types of cells, such as liver cells (Kass, Llopis, Chow, Duddy, and Orrenius, 1990), and to play key roles in the agonist-dependent regulation of Ca^{2+} entry (Neher, 1992). This study, using $I_{K.Ca}$ as an indicator for monitoring the alteration of $[Ca^{2+}]_i$, suggests that IP_3 (possibly but not necessarily with IP_4) may be the primary intracellular second messenger involved in the agonist-dependent Ca^{2+} mobilization in JR-1 cells. However, either IP_3 alone or IP_3 + IP_4 could not mimic all of the properties of ACh induction of the Ca^{2+} influx–related component of $I_{K.Ca}$.

In the Ca^{2+}-free bathing solution, ACh evoked a transient increase of $I_{K.Ca}$, which may reflect Ca^{2+} release from storage sites. The Ca^{2+} influx–related increase of $I_{K.Ca}$ in the ACh response resumed immediately when $[Ca^{2+}]_o$ was increased from 0 to 1.8 mM (Fig. 7 *B*), which might be consistent with the notion that depletion of Ca^{2+} in intracellular stores facilitates Ca^{2+} influx. However, when stored Ca^{2+} was released by

the intracellular application of IP_3 or IP_3 + IP_4 in Ca^{2+}-free bathing solution, the $I_{K.Ca}$ did not resume immediately upon increasing $[Ca^{2+}]_o$ from 0 to 1.8 mM (Fig. 8, *A* and *B*). These observations may indicate that depletion of Ca^{2+} stores and the presence of intracellular IP_3 (with or without IP_4) are not sufficient to induce Ca^{2+} influx across the cell membrane in JR-1 cells.

The inability of IP_3 (with or without IP_4) to immediately increase Ca^{2+} influx across the cell membrane upon restoration of $[Ca^{2+}]_o$ could be due to the relative depletion of intracellular Ca^{2+} stores which should be refilled before activation of Ca^{2+} influx. ACh might be able to speed this process. However, the refilling of the stores detected by the amplitude of ACh-induced Ca^{2+} release–related $I_{K.Ca}$ occurred with a half-recovery time of ~1 min in JR-1 cells in Ca^{2+}-containing bathing solution (not shown). Furthermore, even in the continuous presence of high concentrations of IP_3 or IP_3 + IP_4, ACh clearly accelerated the reactivation of the Ca^{2+} influx–related $I_{K.Ca}$ after brief exposure to Ca^{2+}-free bathing solution (Fig. 8 *A*). In this condition, it could be assumed that the intracellular stores are kept depleted by intracellular IP_3 or IP_3 + IP_4. Thus, acceleration of the refilling process may not be the mechanism through which ACh immediately restores $I_{K.Ca}$ upon restoration of $[Ca^{2+}]_o$. However, one might still raise the possibility that ACh accelerates the refilling process to a speed of the order of microseconds to milliseconds even in the presence of intracellular IP_3 or IP_3 + IP_4. It is difficult to test such a possibility in this study.

In the continuous presence of $[Ca^{2+}]_o$, IP_3 (with or without IP_4) could evoke not only the Ca^{2+} release–related $I_{K.Ca}$ but also the Ca^{2+} influx–related sustained $I_{K.Ca}$ with a similar time course as the ACh response (Fig. 8 *A*). Therefore, an increase of $[Ca^{2+}]_i$ released from storage sites by IP_3, not the depletion of $[Ca^{2+}]$ in the sites, might be a prerequisite for IP_3 to induce rapid Ca^{2+} influx. In other words, IP_3-induced Ca^{2+} influx might have a Ca^{2+}-induced Ca^{2+} influx property. However, in GDPβS (200 μM)–loaded cells, the Ca^{2+} influx–related component of ACh-induced $I_{K.Ca}$ was selectively inhibited without significant alteration of the Ca^{2+} release–related component (Fig. 11 *A*). Similar results were obtained when neomycin (100–200 μM) was applied into the cells (Fig. 10). It was also observed, when GTPγS was loaded to the cells, that the Ca^{2+} release–related component of $I_{K.Ca}$ was enhanced much more efficiently than the Ca^{2+} influx–related one (Fig. 12). If Ca^{2+} influx is triggered by Ca^{2+} released from stores, the Ca^{2+} influx–related component should be suppressed or enhanced somehow in parallel with the Ca^{2+} release–related component. Thus, the release of Ca^{2+} from stores, the resultant transient increase of $[Ca^{2+}]_i$, and the depletion of $[Ca^{2+}]$ in stores may not be sufficient to induce Ca^{2+} influx in JR-1 cells. It seems likely that the second messengers generated by G protein–mediated activation of PLC, i.e., IP_3 and possibly IP_4, are essential to induce the sustained Ca^{2+} influx after the transient release of Ca^{2+} from stores. It is possible that, in the presence of a certain amount of IP_3, a transient increase of $[Ca^{2+}]_i$ caused by either ACh or IP_3 itself triggers Ca^{2+} influx. However, this mechanism still cannot explain the immediate acceleration by ACh of the IP_3 or IP_3 + IP_4-induced slow recovery of $I_{K.Ca}$ upon restoration of $[Ca^{2+}]_o$ (Fig. 8 *A*).

Therefore, we suggest that ACh can enhance Ca^{2+} influx through a process that does not include depletion or refilling of Ca^{2+} stores and a transient increase of $[Ca^{2+}]_i$. An unidentified mechanism may be involved in the ACh-induced Ca^{2+} influx

across the cell membrane at least in the recovery after exposure to Ca^{2+}-free bathing solution. Through such a mechanism, ACh may facilitate the Ca^{2+} influx–related $I_{K.Ca}$ by acting on the putative Ca^{2+} influx channel itself or on some other sites that indirectly facilitate Ca^{2+} influx. However, the following questions remain: (*a*) Is intracellular IP_3 (with or without IP_4) essential for this unidentified mechanism to trigger the immediate activation of Ca^{2+} influx during the recovery after exposure to Ca^{2+}-free solution? (*b*) Is this unidentified mechanism involved in the ACh response in the Ca^{2+}-containing bathing solution? (*c*) Can the regenerative and sustained activation of $I_{K.Ca}$ evoked by IP_3 or IP_3 + IP_4 in the continuous presence of $[Ca^{2+}]_o$ explain most of the ACh response? Further studies are needed to clarify the mechanism underlying ACh-induced facilitation of Ca^{2+} influx in JR-1 cells.

Heparin and mAb18A10 inhibit not only the Ca^{2+} release–related component but also the Ca^{2+} influx–related component of ACh-induced $I_{K.Ca}$ in a concentration-dependent fashion (Fig. 9). Boiled antibody did not affect the ACh response. With low concentrations of heparin and mAb18A10 in the pipette solution, ACh (1 μM) induced a small, sustained activation of $I_{K.Ca}$ with a delay of 10–15 s (Fig. 9). Since the 1 μM ACh-induced Ca^{2+} release can be triggered within 2–3 s upon application of the agonist in the control condition, the observed delay may indicate that the Ca^{2+} release from the stores was completely suppressed by low concentrations of heparin and mAb18A10. The small, sustained current may be caused by the remaining Ca^{2+} influx across the cell membrane. It is thus very likely that heparin and mAb18A10 inhibit the Ca^{2+} release component of ACh-induced $I_{K.Ca}$ by blocking the function of the IP_3-dependent Ca^{2+} channels located on the intracellular Ca^{2+} stores in JR-1 cells. The question that remains is how heparin and mAb18A10 suppress the Ca^{2+} influx component of ACh-induced $I_{K.Ca}$. The Ca^{2+} influx can be suppressed by these substances either through direct suppression of a receptor-operated putative Ca^{2+} channel or through inhibition of Ca^{2+} release from stores if a transient increase of $[Ca^{2+}]_i$ is the prerequisite for ACh to induce Ca^{2+} influx. Although we cannot discriminate between these possibilities in this study, we also could not obtain evidence to support the notion that the filling state of Ca^{2+} in the stores is the primary determinant of Ca^{2+} influx in JR-1 cells. Since ACh could immediately enhance the Ca^{2+} influx after a brief exposure to Ca^{2+}-free bathing solution in the continuous presence of IP_3 or IP_3 + IP_4, we presently assume that heparin and mAb18A10 directly inhibit the agonist-operated Ca^{2+}-permeable channel at the cell membrane.

Ca^{2+} Oscillation in the GTPγS-loaded JR-1 Cells

In the presence of intracellular GTPγS, an oscillatory activation of $I_{K.Ca}$ was observed either in the presence or absence of ACh. Such oscillation of $I_{K.Ca}$ has never been evoked with the control internal solution. Although we have not examined the mechanism underlying the oscillation of $I_{K.Ca}$, several processes may be responsible for the oscillation of $[Ca^{2+}]_i$: (*a*) $[Ca^{2+}]_i$ could increase gradually either in the absence or presence of agonist to a threshold level necessary to trigger the regenerative release of Ca^{2+} from the store to activate $I_{K.Ca}$; (*b*) $[Ca^{2+}]_i$ could return to the subthreshold level after the activation of $I_{K.Ca}$; and (*c*) the Ca^{2+} store could be refilled. These three processes could occur repeatedly in sequence (Petersen, 1992). There-

fore, the threshold for the process(es) necessary for triggering of Ca^{2+} release and for Ca^{2+} influx should be significantly different for $[Ca^{2+}]_i$ to oscillate. Otherwise, $[Ca^{2+}]_i$ would not return to its subthreshold level because of a significant Ca^{2+} influx. Intracellular GTPγS seems to facilitate Ca^{2+} release more effectively than Ca^{2+} influx (Fig. 12, *A* and *B,a*), which may generate the above conditions in JR-1 cells. If only the second messengers, i.e., IP_3 or IP_3 + IP_4, mediate both the ACh-induced Ca^{2+} release and Ca^{2+} influx, GTPγS may somehow accelerate both components in parallel. As discussed above, we hypothesized that muscarinic receptors send unidentified signals in addition to IP_3 (with or without IP_4) to the Ca^{2+} influx mechanism. The unidentified signals may not be stimulated by GTPγS. Alternatively, GTPγS might increase the sensitivity to $[Ca^{2+}]_i$ of Ca^{2+} release channels at the store. Further studies are needed to elucidate how intracellular GTPγS preferentially increases the sensitivity of Ca^{2+} release in response to ACh.

Physiological Functional Role of the Ca^{2+}-activated K^+ Channel in JR-1 Cells

JR-1 cells secrete mucin in response to ACh and His. Other mucin-secreting subclones have been developed from human colonic adenocarcinoma (i.e., HT29 [Augeron and Laboisse, 1984; Huet, Sahuquillo, Coudrier, and Louvard, 1987] and T84 [Dharmsathaphorn, Mandel, Masui, and McRoberts, 1984; Madara and Dharmsathaphorn, 1985; McCool, Marcon, Forstner, and Forstner, 1990]) and from human small intestine (i.e., intestine 407 [Henle and Deinhardt, 1957]). In these intestinal cell lines, carbachol stimulates secretion of mucin and electrolytes, such as K^+ and Cl^-. Increase of $[Ca^{2+}]_i$ is supposed to mediate the response in these cells (Yada and Okada, 1984; Roumagnac and Laboisse, 1987; Phillips, Huet, Bilbo, Podolsky, Louvard, and Neutra, 1988; McCool et al., 1990). It was also shown that agonist-dependent mucin secretion in gastric epithelial cells was mediated by an increase of $[Ca^{2+}]_i$ (Seidler and Sewing, 1989). The present results showed that ACh and His increase $[Ca^{2+}]_i$ in JR-1 cells, which results in an increase of K^+ efflux by activation of $I_{K.Ca}$. The activation of $I_{K.Ca}$ hyperpolarizes the cell membrane, which should provide a favorable electrical gradient for Cl^- efflux and Ca^{2+} influx across the cell membrane. Therefore, the activation of $I_{K.Ca}$ may be a mechanism that facilitates the secretion of Cl^- and also serves as a positive feedback mechanism for agonist-induced increase of $[Ca^{2+}]_i$. We showed that the ACh-induced activation of $I_{K.Ca}$ persisted, while the His activation quickly desensitized. Therefore, in the ACh response, this positive feedback mechanism could effectively be activated. This may explain the former report that ACh is much more effective than His in mucin secretion and cell proliferation (Keates and Hanson, 1990; McCool et al., 1990).

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