Somatostatin induces hyperpolarization in pancreatic islet α cells by activating a G protein-gated K^+ channel

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Abstract Somatostatin inhibits glucagon-secretion from pancreatic α cells but its underlying mechanism is unknown. In mouse α cells, we found that somatostatin induced prominent hyperpolarization by activating a K^+ channel, which was unaffected by tolbutamide but prevented by pre-treating the cells with pertussis toxin. The K^+ channel was activated by intracellular GTP (with somatostatin), GTPγS or Gβγ subunits. It was thus identified as a G protein-gated K^+ (K_G) channel. RT-PCR and immunohistochemical analyses suggested the K_G channel to be composed of Kir3.2c and Kir3.4. This study identified a novel ionic mechanism involved in somatostatin-inhibition of glucagon-secretion from pancreatic α cells.

Key words: G protein-gated K^+ channel; Hyperpolarization; Pancreatic α cell; Somatostatin; Patch clamp; Immunohistochemistry

1. Introduction

Various neurotransmitters and hormones are known to inhibit secretion of hormones from pancreatic islets: i.e. noradrenaline, somatostatin and galanin inhibit insulin-secretion from β cells [1], while somatostatin, but neither noradrenaline nor galanin, inhibits glucagon-secretion from α cells (for review, see [2]). It was shown that noradrenaline, somatostatin, and galanin hyperpolarize the membrane in β cells and thus inhibit insulin-secretion [1]. The agonist-induced hyperpolarization in β cells may be mediated by activation of the ATP-sensitive K^+ (K_{ATP}) channel [3–5], although discrepancies exist regarding the properties of the agonist-activated K^+ channel in β cell lines and native β cells [6]. On the other hand, to our knowledge, the ionic mechanism responsible for somatostatin-inhibition of glucagon-secretion from α cells has not been determined. Because glucagon released from α cells enhances insulin-secretion from β cells, it is important to elucidate the mechanisms which regulate glucagon-secretion for understanding the physiological control of insulin-secretion.

In this study, we have examined the effects of somatostatin on the electrical activity of pancreatic α cells isolated from adult mice using the patch clamp technique. We found that in α cells somatostatin activated a G protein-gated K^+ (K_G) channel via G protein βγ subunits and induced hyperpolarization of the membrane. RT-PCR and immunohistochemical analyses of pancreatic islets suggested the K_G channel to be composed of Kir3.2c and Kir3.4. This study for the first time identified a ionic mechanism by which somatostatin inhibits glucagon-secretion from pancreatic α cells.

2. Materials and methods

2.1. Preparation and culture of islet cells

Pancreatic islets were isolated by the collagenase perfusion method from adult female DDY mice [7] under deep anesthesia with pentobarbital (80 mg/kg). Islets were then dissociated by EDTA and dispase (Godo Shusei Industries, Ltd., Tokyo, Japan) solution. Dissociated cells were plated onto collagen-coated glass slips and maintained for 1–6 days in RPMI tissue culture medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum and antibiotic-antimycotic (Life Technologies). The cells were grown in 5% CO_2 at 37°C.

2.2. Electrophysiological recordings

Whole cell and single-channel currents of cultured cells were measured by the patch clamp technique using a patch clamp amplifier (Axon 200A, Axon Instruments, Inc, Foster City, CA, USA). In whole cell current recording, the bathing solution contained (in mM): 130 NaCl, 5 KCl, 1.8 $CaCl_2$, 0.53 $MgCl_2$, and 5.5 HEPES-NaOH, pH 7.4. NaCl was replaced by equimolar KCl in 100 mM K^+-containing bathing solution. The pipette solution contained (in mM): 145 KCl, 3 K_2ATP, 4.3 $MgCl_2$, 1 EGTA, 0.1 GTP, and 5 HEPES-KOH, pH 7.3. The pipette solution used for the perforated patch clamp technique was as follows (in mM): 30 KCl, 110 L-Aspartic Acid, and 5 HEPES (pH was adjusted to 7.3 by adding KOH). Nystatin (100–200 μg/ml; Sigma, St. Louis, USA) was also added to the solution. In single-channel recording, the high K^+ bath solution contained (in mM): 145 KCl, 2 $MgCl_2$, 5 EGTA, and 5 HEPES-KOH, pH 7.3, and the pipette solution contained (in mM): 145 KCl, 1 $MgCl_2$, 1 $CaCl_2$, and 5 HEPES-KOH, pH 7.3. βγ subunit of pertussis toxin-sensitive G proteins was purified from the bovine brain as described [8] and stored at −80°C. Data were stored on video tapes using a PCM data recording system and subsequently replayed for computer analysis. Data were expressed as means ± S.E.

2.3. PCR analysis of mRNA

The cDNAs of isolated islet cells were synthesized as described previously [9]. Primers for PCR reaction were located in nucleotides −57 to −34 and 350 to 372 of mouse insulin cDNA, 14 to 33 and 463 to 482 of mouse glucagon cDNA, 92 to 111 and 327 to 346 of rat somatostatin cDNA, 435 to 457 and 1078 to 1100 of rat Kir3.1 cDNA, 914 to 933 and 1410 to 1431 of mouse Kir3.2a cDNA, 286 to 313 and 1471 to 1488 of mouse Kir3.2b cDNA, 954 to 973 and 1263 to 1282 of rat Kir3.2c cDNA, 611 to 630 and 1048 to 1067 of mouse Kir3.3 cDNA, 989 to 1006 and 1276 to 1294 of rat Kir3.4 cDNA. PCR amplification was performed for 30 cycles of denature at 94°C for 45 s, annealing for 1 min, and extension at 72°C for 1 min and then at 72°C for 8 min. The annealing temperature is 55°C for Kir3.1, insulin and glucagon, 59°C for Kir3.2a, 3.2b, 3.2c and somatostatin and 65°C for Kir3.3. Because the products from the first PCR reactions were not enough to be visualized, 2 μl of the first PCR products was used and amplified with the same primers except for Kir3.2a and the same condition as in the first reaction. For Kir3.2a, the second PCR was performed using the forward primer of 954–973

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of mouse Kir3.2a cDNA. The products were electrophoresed on a 3% agarose gel.

2.4. Immunohistochemistry

Properties of the polyclonal antibodies for Kir3.1 (aG1C-1), Kir3.2a (aG2A-5), Kir3.2b (aG2B-2), Kir3.2c (aG2C-3) and Kir3.4 (aG4N-10) were described previously [10]. The polyclonal antibody for Kir3.3 (aG3) was raised in rabbit against a synthetic peptide corresponding to the amino acid residues 2–15 (AQENAAFSPGSEEP) in the N-terminal region of mouse Kir3.3. The antiserum (aG3) was purified as described previously [10]. Female DDY mice (8 weeks) were anesthetized deeply with pentobarbital (80 mg/kg) and perfused with PBS and then with 50 ml of 4% paraformaldehyde in 0.1 M sodium phosphate, pH 7.4 (PAF solution). After perfusion, pancreases were enucleated, fixed again with PAF solution for 48 h, dehydrated with 30% sucrose, and frozen. Sections (10 μm) were cut on a cryostat and thaw-mounted on gelatin-coated slides. On the other hand, cultured cells on glasses were rinsed with PBS and then fixed with PAF solution. The samples of tissue sections or cultured cell preparations were washed with PBS, treated with PBS containing 0.1% Triton X-100 (PBST) and goat serum and then incubated with each antibody (1:500–1:1000) in PBST with 1% (w/v) bovine serum albumin at 4°C overnight. The samples were washed with PBS at room temperature and visualized with fluorescein isothiocyanate (FITC)-labeled anti-rabbit or guinea pig IgG (EY Laboratories, San Mateo, CA, USA) and Texas Red-labeled anti-mouse IgG (Protos Immunoresearch, San Francisco, CA, USA). The samples were examined with a confocal microscope (MRC-1024, Bio-Rad, Hertfordshire, England). For control experiments, antibodies preabsorbed with excess antigenic peptides were used. In the immunocytochemistry for cultured cells, the nuclei were visualized using DAPI (Sigma).

3. Results

Isolated single cells and clusters of 3–20 cells were obtained after culturing dissociated pancreatic islets for 1–6 days. To identify α and β cells in this preparation, the cells were immunostained with anti-insulin (green in Fig. 1Aa) and anti-glucagon (red in Fig. 1Ab) antibodies. As shown in Fig. 1Aa, 64% (16/25) of clusters contained only insulin-positive β cells. One or two glucagon-positive cells were detected in the remaining clusters. On the other hand, 26.3% (5/19 cells) of isolated single cells were glucagon-positive (Fig. 1Ab) and one cell was somatostatin-positive (not shown). The glucagon-positive α cells were ~10 μm in diameter, while the insulin-positive β cells were ~15 μm in diameter. The cell capacitance of α cells measured electrophysiologically was 3.3 ± 0.2 pF ($n = 6$), while that of β cells was 5.0 ± 0.6 pF ($n = 6$). Because it was reported that only α cells showed spontaneous action potentials in glucose-free bathing solution [11–13], for the patch clamp experiments of α cells we chose isolated single small cells which showed spontaneous action potentials under glucose-free conditions. Furthermore, we con-

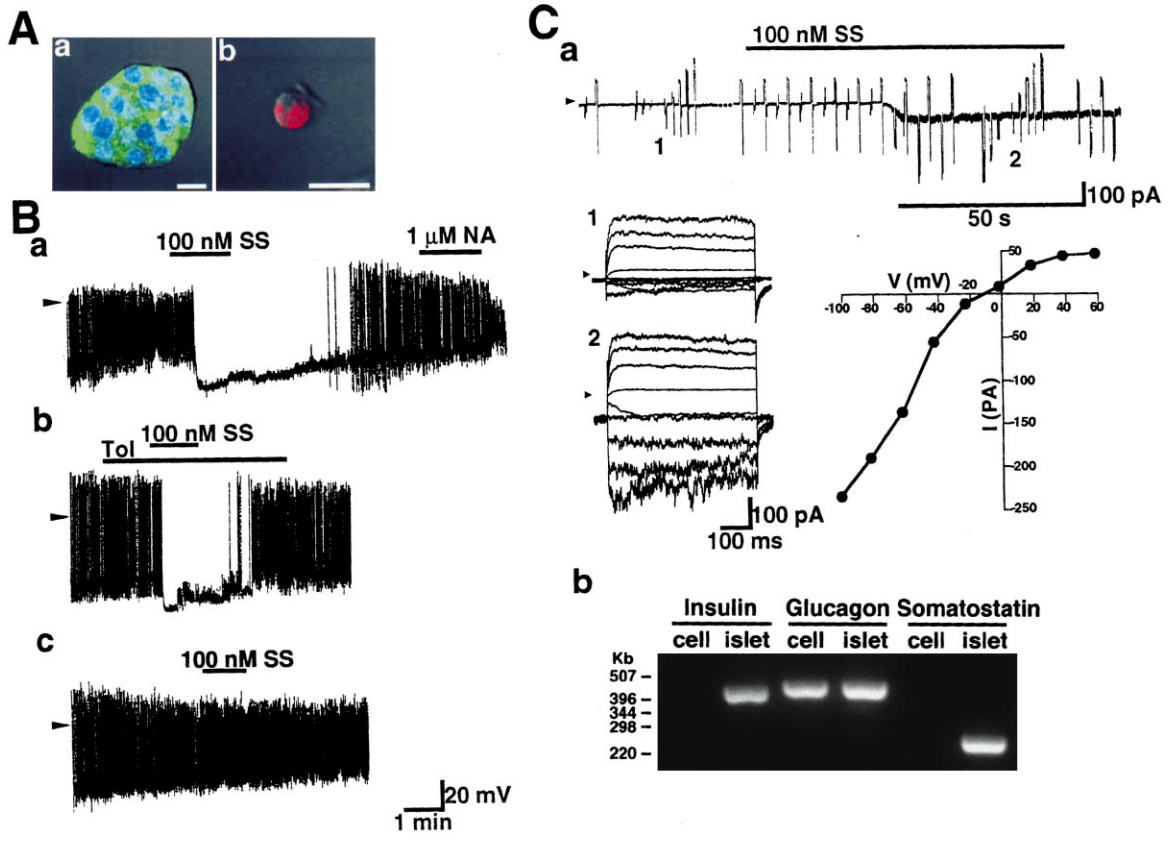

Fig. 1. Hyperpolarization is the response to somatostatin in α cells. A: Photomicrographs illustrating an insulin immunoreactive cluster cell (a, green) and a single glucagon immunoreactive cell (b, red). The nuclei were stained by DAPI (a, blue). Scale bar: 10 μm. B: Effect of somatostatin (SS) on electrical activity in α cell recorded with the perforated patch method under current clamp. Somatostatin but not noradrenaline (NA) inhibited electrical activity in α cell (a). Tolbutamide (500 μM, Tol) did not block hyperpolarization by 100 nM somatostatin (b). Somatostatin (100 nM) had no effect on action potentials in cells pre-treated overnight with pertussis toxin (100 ng/ml) (c). Arrowheads indicate the zero voltage level. C: Whole cell currents were measured under voltage clamp steps from −100 to +60 mV with 20 mV steps (number 1 and 2). The pipette solution contained 3 mM ATP and 100 μM GTP. Somatostatin induced tolbutamide insensitive inwardly rectifying currents (a, number 2). The holding potential was −40 mV. Zero current level is indicated by arrowheads. The I/V curve of the difference current is shown. RT-PCR was performed upon the recorded cell cDNA using specific primers for insulin, glucagon and somatostatin (b, cell). The cDNAs synthesized from mouse pancreatic islet was used as a control template (b, islet).

firmed the expression of glucagon by RT-PCR in each cell which had been used for the experiments in this study as shown in Fig. 1Cb. Cells which did not show spontaneous action potentials in glucose-free solution and had a resting potential of ~−60 mV ($n=20$) and depolarized with generation of spontaneous action potentials when glucose (11 mM) was added to the bath were identified as β cells and not studied further here.

Under perforated patch whole cell current clamp we could record spontaneous action potentials in α cells (Fig. 1Ba). When somatostatin (100 nM) was added to the bath, the cells were hyperpolarized to −67.2 ± 2.3 mV ($n=3$) and the spontaneous action potentials ceased. After wash-out of somatostatin, the membrane depolarized gradually to the control level and the action potentials resumed. Noradrenaline (1 μM) did not affect the membrane potential of the α cell. The same results were obtained in all three α cells examined. The somatostatin-induced hyperpolarization in α cells was not affected by tolbutamide (500 μM), an inhibitor of ATP-sensitive K^+ channels (Fig. 1Bb, $n=3$), while that in β cells was diminished by this drug (not shown). In Fig. 1Bc, the cell was pre-treated with pertussis toxin, an inhibitor of $G_{i/o}$ proteins [14–16]. In the pre-treated α cells ($n=3$), although the spontaneous action potentials were recorded as in the non-treated cells, somatostatin did not induce any hyperpolarization nor inhibit action potential firing.

In Fig. 1C, the effect of somatostatin on the membrane current of an α cell was examined under whole cell voltage clamp condition. The bathing solution contained 100 mM K^+ and the cell was held at −40 mV. A set of two successive voltage steps (500 ms in duration) to −90 mV and +10 mV separated by 3 s were applied to the cell every 6.4 s. Somatostatin increased inward currents at the holding potential and also during the voltage pulses to −90 mV and induced small outward currents during the steps to +10 mV. Fig. 1Ca-1 and Ca-2 depict families of membrane currents evoked by voltage steps to various potentials in control and during application of somatostatin (100 nM), respectively. The current-voltage relationship measured at the pulse end of the somatostatin-induced current is shown in the lower right panel of Fig. 1Ca. The current exhibited an inwardly rectifying property with a reversal potential of ~−10 mV, which is close to the K^+ equilibrium potential (E_K) under these experimental conditions. Noradrenaline (1 μM) and galanin (50 nM) had no effect in similar experiments (not shown). The identity of the cell in Fig. 1C as an α cell was confirmed by the expression of glucagon by RT-PCR of the cell cDNA (Fig. 1Cb). The same results were obtained in all six α cells examined. The inwardly rectifying K^+ current was not induced by somatostatin in α cells treated with pertussis toxin ($n=3$). These results suggest that pertussis toxin-sensitive G proteins are involved in somatostatin activation of an inwardly rectifying K^+ channel in α cells.

We examined the effects of G protein-stimulation on the α cell-K^+ channel activity (Fig. 2). Somatostatin (100 nM) was included in the pipette solution. In cell-attached patches, brief openings of a K^+ channel whose current amplitude was ~2 pA at −60 mV were recorded (Fig. 2Aa). Upon formation of an inside-out patch, the activity of the small K^+ channel disappeared and openings of a large K^+ channel (~5 pA at −60 mV) abruptly appeared (Fig. 2Ab). The large conductance K^+ channel was probably an ATP-sensitive K^+ channel because

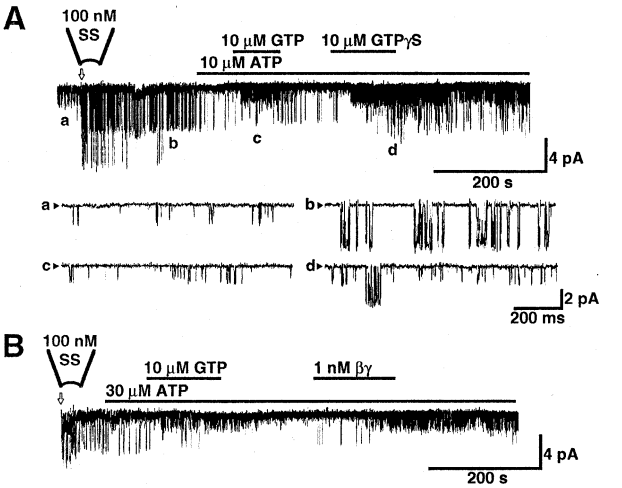

Fig. 2. G protein activators stimulate K_G channel in α cell. These records were obtained from two α cells which showed action potentials when bathed in normal bathing solution without added glucose. The bath solution was then changed to high K^+ solution and the membrane potential of the cell-attached membrane patch was voltage-clamped to −60 mV. A: After formation of an inside-out patch (open arrow), spontaneous K_{ATP} channel activity (b) was observed and reduced by the application of ATP. In the presence of somatostatin in the pipette solution, the application of GTP (c), GTPγS (d) and βγ subunits of G proteins (B) induced channel openings quite distinct from those of K_{ATP} channels.

channel openings were reduced when ATP (10 μM) was added to the bath. The addition of GTP (10 μM) induced openings of the small conductance K^+ channel (Fig. 2Ac). GTPγS (10 μM) caused openings of the small conductance K^+ channel to be sustained in the patch (Fig. 2Ad). The small conductance K^+ channel was also activated irreversibly by adding the βγ subunits of G proteins purified from bovine brain to the bath (Fig. 2B). Therefore, the somatostatin-activated K^+ channel in pancreatic α cells was identified as a G protein-gated K^+ (K_G) channel.

Recently four cDNAs of Kir subunits encoding mammalian K_G channels have been isolated. They are designated Kir3.1/GIRK1 [17,18], Kir3.2/GIRK2 [19], Kir3.3/GIRK3 [19] and Kir3.4/GIRK4 [20]. Kir3.2 has at least three splicing variants, i.e. Kir3.2a, Kir3.2b and Kir3.2c [21–23]. Functional K_G channels are believed to be heterotetramers of Kir3.0 subunits; neuronal K_G channels are composed of Kir3.1 and Kir3.2 [21], while the cardiac one is composed of Kir3.1 and Kir3.4 [20]. To examine which Kir3.0 subunits are expressed in the pancreatic islet cells, we performed RT-PCR and immunohistochemistry analyses of Kir3.0 subunits (Fig. 3). Only the PCR products of Kir3.2c and Kir3.4 were detected in pancreatic islets, although the products of all Kir3.0 subunits examined were detected in mouse cerebellum (Fig. 3A). The cellular distribution of Kir3.0 subunits was examined by immunostaining islets using polyclonal antibodies specific to each subunit [10]. The immunoreactivities of aG2A-5, aG2C-3 and aG4N-10 antibodies were clearly detected in the islets, while aG1C-1, aG2B-2 and aG3 immunoreactivity were not (Fig. 3B). Kir3.2a and Kir3.2c are splicing variants derived from the same gene and Kir3.2c is 11 amino acid residues longer than Kir3.2a. The 11 amino acid residues at the C-terminal tail in Kir3.2c are the only divergent part between the two clones [10]. Thus, the antibody against Kir3.2a (aG2A-5) recognizes both Kir3.2a and Kir3.2c, while aG2C-3

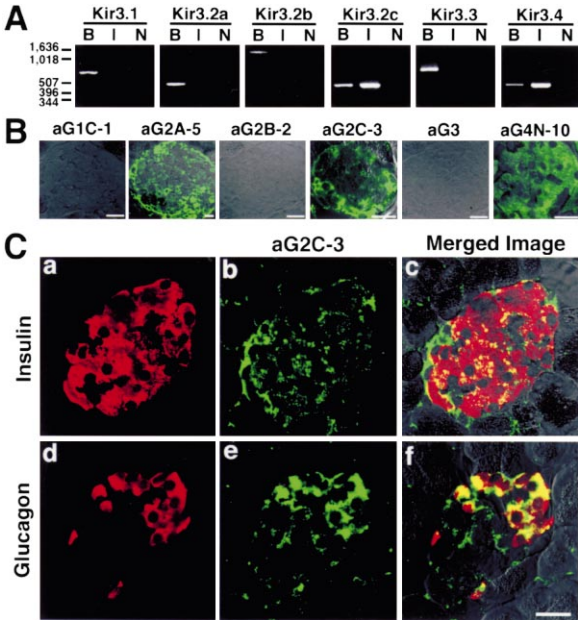

Fig. 3. Expression and distribution of Kir3.0 subunits in the mouse islet. A: RT-PCR analysis of six Kir3.0 subunits in an islet isolated from mouse pancreas. Islet expressed mRNAs of Kir3.2c and Kir3.4. B, cerebellum; I, islet; N, water. B: Immunohistochemical analysis of Kir3.0 in the mouse pancreas. Sections (10 μm) were stained with affinity-purified rabbit anti-mouse Kir3.1 antibody (aG1C-1), anti-mouse Kir3.2a and Kir3.2c common antibody (aG2A-5), anti-mouse Kir3.3 antibody (aG3) and anti-rat Kir3.4 antibody (aG4N-10), and with affinity-purified guinea pig anti-mouse Kir3.2b antibody (aG2B-2) and anti-mouse Kir3.2c antibody (aG2C-3), followed by FITC-conjugated anti-rabbit or anti-guinea pig IgG. These photographs were overlaid with the transmission images. Scale bar: 20 μm. C: The islet was double-stained with aG2C-3 (green) and anti-insulin or glucagon antibody (red), followed by FITC-conjugated anti-guinea pig IgG and Texas Red-labeled anti-mouse or rabbit IgG. Panels c and f show triple exposures of fluorescent images and transmission image. Scale bar (shown in panel f): 20 μm.

antibody reacts with only Kir3.2c [10]. Because Kir3.2a mRNA was not detected in RT-PCR analysis, the aG2A-5 antibody probably recognized Kir3.2c proteins in the islet. Consistently, the immunoreactivities of aG2A-5 and aG2C-3 distributed similarly in the peripheral regions of each islet. On the other hand, the Kir3.4 immunoreactivity (aG4N-10) distributed diffusely in all types of islet cells. All of these immunoreactivities disappeared when each antibody was preabsorbed with respective immunogenic peptide (not shown).

Fig. 3C shows the double staining of aG2C-3 and either anti-insulin or anti-glucagon antibody. Anti-insulin antibody detected many of the islet cells except those in the peripheral region (red in Fig. 3Ca). Because the aG2C-3 immunoreactivity existed mainly in the cells at peripheral region of the islet (green in Fig. 3Cb), the merged image did not produce much yellow signal (Fig. 3Cc). On the other hand, anti-glucagon antibody stained peripheral cells (red in Fig. 3Cd) similar to aG2C-3 (green in Fig. 2Ce) and the merged image of anti-glucagon and aG2C-3 produced prominent yellow signals (Fig. 3Cf). Thus, pancreatic α cells may express Kir3.2c and Kir3.4 subunits of K_G channels. The remaining green signals (aG2C-3) in Fig. 3Cf may be due to δ cells, since δ cells stained by anti-somatostatin antibody also exhibited aG2C-3 immunoreactivity (not shown).

In Fig. 4A, the currents flowing through the α cell-K_G channel were recorded at various membrane potentials in the cell-attached patch. Somatostatin (100 nM) was present in the pipette solution. Short spike-like openings of inward currents were observed at potentials more negative than 0 mV (= E_K in this experimental condition), while only small outward currents were recorded at potentials more positive than E_K. Fig. 4Ab shows the current-voltage relationship of the unitary current. The channel exhibited an inwardly rectifying property and its unitary conductance in the inward direction was 30.3 ± 0.7 pS (n = 3). The open time histogram at −60 mV could be fitted by a single exponential with a time constant of 0.20 ± 0.02 ms (n = 3).

Because the K_G channel in α cells seemed to be composed of Kir3.2c and Kir3.4, we co-transfected Kir3.2c and Kir3.4 with m_2 receptor in HEK293T cells. In the HEK293T cells, we could record K_G channel activity with acetylcholine in the pipette (Fig. 4Ba). The K_G channel showed an inwardly rectifying property and the unitary conductance for inward currents was 32.6 ± 0.5 pS (n = 3) (Fig. 4Bb). The open time histogram could be fitted by a single exponential with a time constant of 0.28 ± 0.03 ms (n = 3) (Fig. 4Bc). Therefore, the K_G channel recorded in the HEK293T cells co-transfected with Kir3.2c and Kir3.4 exhibited conductance and kinetic properties identical to those of the α cell-K_G channel.

4. Discussion

Glucagon secreted from α cells strongly stimulates insulin-secretion from β cells in the mammalian islet of Langerhans. Therefore, alteration of glucagon-secretion from α cells is an important element for physiological control of insulin-secretion. Although it is well known that somatostatin released from δ cells inhibits the secretion of glucagon from pancreatic α cells [2], its underlying mechanism is still unknown. Two possible mechanisms have been proposed [2]: (1) interference with second messenger systems and the exocytosis machinery, or (2) alterations of membrane ion conductances. For the first possibility, because somatostatin receptors are coupled to G_i

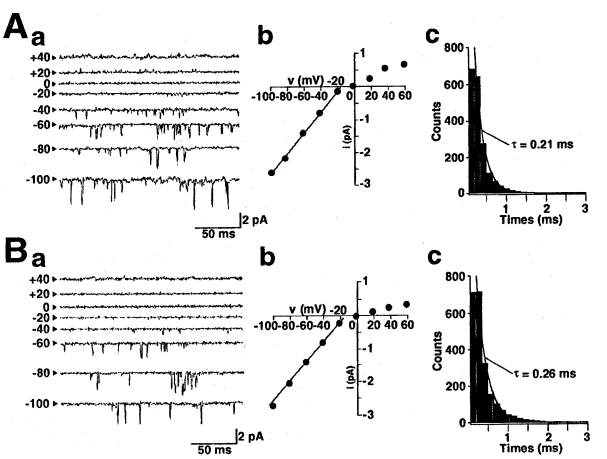

Fig. 4. Comparison of properties of K_G channels in an α cell (A) and Kir3.2c/Kir3.4 transfected in HEK293T cells (B). The single channel current-voltage relationship in the cell-attached patches (a and b) and open time histogram at the −60 mV membrane potential (c). The bath solution contained 150 mM K^+. The pipette solution contained 100 nM somatostatin (A) or 10 μM acetylcholine (B). Arrowhead indicates the zero current level.

proteins [24], the hormone may inhibit adenylyl cyclase and reduce cAMP level in α cells. The reduced level of cAMP may result in a decrease of glucagon-release from α cells. However, there is no report available in this context. For the second possibility, this study for the first time showed that somatostatin produces a prominent hyperpolarization of α cell membrane and inhibits its spontaneous action potentials. Because it is known that secretion of glucagon from α cells is induced mainly by an increase of intracellular Ca^{2+} due to the Ca^{2+} influx through L-type Ca^{2+} channels during spontaneous action potentials [2], the somatostatin-induced hyperpolarization and inhibition of spontaneous action potentials would result in inhibition of glucagon-secretion by preventing activation of voltage-dependent Ca^{2+} channels. Consistent with this idea, it was also reported that GABA, co-released with insulin, from β cells inhibits glucagon-secretion from α cells by activating $GABA_A$ receptor chloride channels [25]. Activation of the chloride channel resulted in hyperpolarization and cessation of action potentials, which would stop the Ca^{2+} inflow across the membrane. Therefore membrane hyperpolarization would be a general mechanism for various hormones to decrease glucagon-secretion from pancreatic α cells. Further studies using e.g. Ca^{2+}-imaging and measurement of hormone-secretion are, however, needed to evaluate the significance of hyperpolarization in regulation of glucagon-release from α cells. It is also necessary to examine the possible involvement of interference of second messenger systems and the exocytosis machinery in agonist-induced inhibition of glucagon-secretion.

RT-PCR and immunostaining analyses indicated that Kir3.2c and Kir3.4 proteins were expressed in α cells. The distribution of Kir3.2c was rather restricted to α and δ cells, while Kir3.4 was expressed in all types of islet cells. Although Kir3.2c was first cloned from a β cell line, human pancreas or insulinoma [26–28], it could not form a functional K^+ channel responding to G protein-stimulation when expressed alone in mammalian cell lines or in *Xenopus* oocyte [10,28]. Similarly, it was also shown that Kir3.4 alone with m2 receptor expressed in *Xenopus* oocytes forms a minimal functional current with very fast open-close kinetics [29]. Ferrer et al. [28] showed that co-injection of Kir3.2c and Kir3.4 with m2 receptors results in prominent expression of carbachol-activated strong inwardly rectifying currents. Consistently, we could record a K_G channel activity in HEK293T cells co-transfected with Kir3.2c, Kir3.4 and m2 receptor cDNAs. The conductance and kinetic properties of the expressed K_G channel in HEK cells were identical to those of the α cell channel activated by somatostatin. Therefore, it seems likely that the K_G channels in pancreatic α cells are composed of Kir3.2c and Kir3.4.

In conclusion, this study for the first time identified an ionic mechanism by which somatostatin inhibits glucagon-secretion from pancreatic α cells. Further studies on regulation of glucagon-secretion are important to elucidate physiological control of insulin-secretion from β cells.

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