Secretagogue-induced Exocytosis Recruits G Protein-gated K⁺ Channels to Plasma Membrane in Endocrine Cells

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Stimulation-regulated fusion of vesicles to the plasma membrane is an essential step for hormone secretion but may also serve for the recruitment of functional proteins to the plasma membrane. While studying the distribution of G protein-gated K⁺ (K₉) channels in the anterior pituitary lobe, we found K₉ channel subunits Kir3.1 and Kir3.4 localized on the membranes of intracellular dense core vesicles that contained thyrotropin. Stimulation of these thyrotroph cells with thyrotropin-releasing hormone provoked fusion of the vesicles to the plasma membrane, which could form a new type of negative feedback control loop for hormone secretion in the endocrine system.

The G protein-gated K⁺ (K₉) channel, a member of the inwardly rectifying K⁺ (Kir) channel family, is directly activated by the pertussis toxin-sensitive G proteins. This system was first discovered in the muscarinic deceleration of the heartbeat (1). More recently, it is considered to play an essential role in the hormone-mediated inhibitory regulation of neural excitability (1, 2). Electrophysiological studies have revealed that a variety of inhibitory receptors, including M₂-muscarinic, A₁-adrenergic, D₂-dopamine, α₂-adrenergic, serotonin, γ-aminobutyric acid type B, opioid, and somatostatin receptors in the brain, are coupled to K₉ channels (3). In endocrine organs such as the anterior pituitary lobe and pancreatic islet, it was also reported that some neurotransmitters including dopamine and somatostatin hyperpolarize the membrane by activating K₉ channels, which results in the inhibition of hormone secretion.

The main subunit of K₉ channels has been cloned from the heart and designated GIRK1/Kir3.1 (4, 5). In the brain and heart, Kir3.1 forms functional K₉ channels by assembling with other Kir3.0 subunits such as GIRK2/Kir3.2, GIRK3/Kir3.3 (6, 7), and GIRK4/CIR/Kir3.4 (8). Because Kir3.1 mRNA was detected in the anterior pituitary lobe (9) and pancreatic islet (10), this subunit may also contribute to the formation of K₉ channels in endocrine cells. The anterior pituitary lobe contains several kinds of endocrine cells: lactotrophs, somatotrophs, corticotrophs, and thyrotrophs. Although electrophysiological experiments have shown that somatostatin or dopamine activate K₉ currents in lactotrophs (9) and in several cell lines derived from the anterior pituitary lobe, such as GH3 (5) and AtT20 (6), the cellular and subcellular localizations of K₉ channels in vivo pituitary endocrine cells have not been examined.

In this study, using a polyclonal antibody specific to Kir3.1, we found that this subunit was expressed only in thyrotroph cells and, surprisingly, was localized predominantly on intracellular secretory vesicles. Kir3.4 was co-localized on the vesicles with Kir3.1. Thyrotropin-releasing hormone (TRH) stimulation of thyrotrophs caused fusion of the vesicles to the cell membrane, increase of cell capacitance, and enhancement of dopamine- or somatostatin-induced K₉ current. These data indicate a novel mechanism for the rapid insertion of functional ion channels into the plasma membrane, which could form a new type of negative feedback control loop for thyrotrophs by tuning up their inhibitory regulatory signaling system in response to the stimulatory signal.

EXPERIMENTAL PROCEDURES

Preparation of Polyclonal Antibodies—The polyclonal antibody for Kir3.1 (aG1C-1) was raised in rabbit against a synthetic peptide corresponding to amino acid residues 488–501 (LPAAKRMNMDKDTFT) of Kir3.1/GIRK1 (15, 16). We have also developed the antibody to Kir3.4 (aG4N-10) in rabbit using the antigenic peptide, DSRNAMNQD-(aG4N-10) in rabbit using the antigenic peptide, DSRNAMNQD-MEIGV, which corresponds to amino acids 4-17 of Kir3.4 (17). These antibodies have been successfully used for immunoprecipitation and Western blotting analyses of Kir3.1 protein (15, 16) and Kir3.4 protein in the brain and heart. Furthermore, in the rat heart, the immunoreactivities were detected with the antibodies only in the atrium but not in the ventricle (data not shown). This finding coincides with the expression of the cardiac K₉ channel, which is composed of the subunits Kir3.1 and Kir3.4 in the heart atrium (13).

Immunohistochemical Study—Immunohistochemistry was performed according to a method described elsewhere (15). Rats were anesthetized with sodium pentobarbital and perfused transcardially with 4% (w/v) paraformaldehyde, 0.5% (w/v) glutaraldehyde, and 0.2% (w/v) picric acid, 0.1 M phosphate buffer (pH 7.4). The anterior pituitary lobe was removed,

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‡ The abbreviations used are: K₉, G protein-gated potassium channel; Kir, inwardly rectifying potassium channel; TSH, thyroid-stimulating hormone (thyrotropin); TRH, thyrotropin-releasing hormone; AC/TH, adrenocorticotropic hormone; RT-PCR, reverse transcriptase-polymerase chain reaction; Cᵣ, membrane capacitance; Gᵣ, membrane conductance; Iₑ, membrane current.

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postfixed in a fixative containing 4% paraformaldehyde and 0.2% picric acid for 48 h at 4 °C, and transferred to 0.1 M phosphate buffer containing 15% (w/v) sucrose and 0.1% (w/v) sodium azide at 4 °C. Sections were cut with a cryostat at 20 μm of thickness and stored at 4 °C in the same solution until used. After rinsing, the sections were placed in free-floating state and washed in 0.1 M phosphate buffer containing 0.2% (w/v) saline containing 0.3% (v/v) Triton X-100 and 1% (w/v) bovine serum albumin and incubated for 3 days at 4 °C. After incubation with biotinylated goat anti-rabbit IgG, staining was accomplished using the avidin-biotin complex method (Vectastain Elite kit, Vector Laboratories, Burlingame, CA), with nickel-diaminobenzidine as the chromogen. Control procedures consisted of preabsorbing the HRP secondary antibody with a saturating concentration of the antigenic peptide.

For double immunofluorescent staining, sections were first incubated with aG1C-1 and a monoclonal antibody for each pituitary hormone, i.e., anti-prolactin monoclonal antibody (QED Bioscience Inc., San Diego, CA), anti-growth hormone monoclonal antibody (Quartett, Berlin, Germany), anti-luteinizing hormone monoclonal antibody (Quartett, San Mateo, CA) and Texas Red-labeled anti-mouse IgG (Protos Immunofluorescent anti-rabbit IgG (E. Y. Laboratories, YLEM, Rome, Italy), or anti-thyrotropin (TSH) monoclonal antibody (YLEM). The sections were washed thoroughly and incubated with fluorescein isothiocyanate-labeled goat anti-rabbit IgG (E. Y. Laboratories, YLEM, Rome, Italy), or anti-thyrotropin (TSH) monoclonal antibody (Quartett, Berlin, Germany), anti-luteinizing hormone monoclonal antibody (Quartett, Berlin, Germany), anti-growth hormone monoclonal antibody (Quartett, Berlin, Germany), and anti-thyrotropin monoclonal antibody (YLEM, Rome, Italy), or anti-thyrotropin (TSH) monoclonal antibody (Quartett). The sections were washed thoroughly and incubated with fluorescein isothiocyanate-labeled anti-rabbit IgG (E. Y. Laboratories, San Mateo, CA) and Texas Red-labeled anti-mouse IgG (Protos Immunofluorescent anti-rabbit IgG (E. Y. Laboratories, YLEM, Rome, Italy), or anti-thyrotropin (TSH) monoclonal antibody (Quartett).

Electron Microscopic Study—For electron microscopy, sections were processed according to the method described elsewhere (18). TRH stimulation involved the injection of 10 μg of TRH into the femoral vein 10 min before the perfusion, which was enough to stimulate the thyrotroph in vivo (19). The vibratome sections were dehydrated in a graded series of ethanol and incubated in a mixture of ethanol and LR Gold resin (London Resin, Berkshire, U. K.) followed by flat embedment in fresh LR Gold containing 0.1% (v/v) benzyl on silicon-coated glass slides. The embedded sections were polymerized for 6 h in an ultraviolet cryochamber (Pelco, Ted Pella Inc., Redding, CA) at -20 °C. Small blocks containing anterior pituitary lobe were cut out and glued to black resin. Ultrathin sections were cut using an ultramicrotome (Ultracut UCT, Leica, Vienna, Austria) and mounted on collodion and carbon-coated nickel grids (thin bar grid, Nishin EM Co. Ltd., Tokyo, Japan). The nickel grids with ultrathin sections were incubated with 3% (v/v) normal goat serum in 0.1 M phosphate-buffered saline containing 0.2% bovine serum albumin, 0.2% (w/v) saponin, and 0.05% (w/v) NH4Cl for 30 min at room temperature. The grids were then incubated with aG1C-1 (1:100) or aG4N-10 (1:50) in the same buffer for 2 h and then with a 15-nm immunogold conjugated goat anti-rabbit IgG (British BioCell International Inc., Cardiff, U. K., diluted 1:40) for 1.5 h at room temperature. In the case of double immunolabeling, the grids were incubated with aG1C-1 (1:100) or aG4N-10 (1:50) in the same buffer for 2 h and then with a 15-nm immunogold conjugated goat anti-rabbit IgG (British BioCell International Inc., Cardiff, U. K., diluted 1:40) for 1.5 h at room temperature. In the case of double immunolabeling, the grids were incubated with aG1C-1 (1:100) or aG4N-10 (1:50) in the same buffer for 2 h and then with a 15-nm immunogold conjugated goat anti-rabbit IgG (British BioCell International Inc., Cardiff, U. K., diluted 1:40) for 1.5 h at room temperature. This adjustment gave whole cell capacitance values of 9.0–13.4 pF.

Cell Capacitance Measurement—Simultaneous measurements of changes in membrane capacitance (Cm), conductance (Gm), and membrane current (Im) were carried out using a patch-clamp amplifier (EPC-7, List Electronics) and a two-phase lock-in amplifier (NF5610B, NF Electronic Circuit Design) as described previously (20). The capacitance of the whole cell, originating from the capacitance of the plasma membrane, were minimized by adjusting the capacitance and the time constant of the capacitance cancellation circuit in the patch-clamp amplifier while applying voltage pulses with an amplitude of 10 mV.

This adjustment gave whole cell capacitance values of 9.0–13.4 pF and series resistance values of 5–9 MΩ. After the cancellation, a 600-Hz, 3.4-mV peak to peak sine-wave voltage was superimposed on the holding potential of -100 mV. The resulting current output was fed into the lock-in amplifier. The phase offset of the lock-in amplifier was adjusted so that when the capacitance of the cancellation circuit was modified to calibrate Cm, there was no change in the output for Gm (closed circles at the Cm trace in Fig. 3A).

RESULTS AND DISCUSSION

Kv1 channels are heteromultimeric proteins composed of the subunits Kir3.1 + Kir3.4 in the heart (17) and Kir3.1 + Kir3.2 in the brain (25, 26). The Kir3.1 subunit may therefore be common to the Kv1 channels of various tissues (8, 9). We examined the distribution of Kir3.1 immunoreactivity in sections of rat pituitary using a specific polyclonal antibody (aG1C-1) (15, 16) (Fig. 1). The Kir3.1-positive cells were scattered in the anterior pituitary lobe and made up ~3% of the anterior pituitary cells (Fig. 1A and B). The Kir3.1-positive cells had a polygonal or stellate shape between 20 and 30 μm in size. To identify which types of pituitary cells express Kir3.1, sections were double-stained for Kir3.1 and various pituitary hormones. Fig. 1, A and B, shows that Kir3.1 immunoreactivity (green) was detected only in the cells that were stained with anti-TSH antibody (red). In 176 TSH-positive cells (i.e., thyrotrophs, 149 cells (~85%) were also Kir3.1-positive. No Kir3.1 immunoreactivity was detected in cells stained with anti-prolactin, anti-growth hormone, anti-luteinizing hormone, or anti-ACTH antibodies. Prominent orange-yellow signals generated by the double staining of Kir3.1 and red (TSH) suggest that they may be localized in close proximity in thyrotrophs (Fig. 1D).
The subcellular localization of Kir3.1 was examined using immunoelectron microscopy (Fig. 1, E, F, and H). The immunoreactive signals (gold particles) were detected predominantly on secretory vesicles and rarely on the plasma membrane of Kir3.1-positive pituitary cells (Fig. 1E). The cells were ~30 μm in diameter and polygonal in shape. The reactive intracellular secretory vesicles were ~100 nm in diameter. These morphological features of Kir3.1-positive cells are those of the thyrotroph (27). Furthermore, with double immunolabeling under the electron microscope, we found that Kir3.1 (detected by 15-nm gold particles) and TSH (10-nm gold particles) were localized in the same secretory vesicles (Fig. 1E, inset). In contrast, the adjacent cell, which contained large 200–300-nm-diameter electron dense vesicles, did not show any immunoreactivity to αG1C-1.

Fig. 1, F and H, depicts examples of Kir3.1 immunoreactivity in the thyrotrophs obtained from TRH-administered rats. TRH stimulation caused an increase in the detection of gold particles on the plasma membrane (Fig. 1F, arrows). The number of gold particles detected on the plasma membrane in the control was 0.25 ± 0.25% (mean ± S.E., n = 15 cells) of the total number of particles that existed within 1.5 μm of the plasma membrane. This number increased to 7.56 ± 1.80% (n = 15 cells) after TRH stimulation (Fig. 1G). Furthermore, as indicated by the arrowheads in Fig. 1H, after TRH stimulation various stages of fusion of the gold particle-positive vesicles with the plasma membrane were detected. These results suggest that the Kᵦ channels on the secretory vesicles of thyrotrophs could be translocated to the plasma membrane during TRH-induced exocytosis.

It is known that dopamine-D₂ and somatostatin receptors can couple to Kᵦ channels via pertussis toxin-sensitive G proteins in pituitary cells (2). Therefore, TRH-induced recruitment of the Kᵦ channels to the plasma membrane of thyrotrophs may in turn facilitate the effect of inhibitory transmitters such as dopamine and somatostatin. To examine this possibility, electrophysiological techniques were applied to dissociated pituitary cells (Fig. 2). From 82 cells examined, we observed three distinct types of cells in terms of their responses to bromocriptine, somatostatin, and TRH (Types I–III). Fig. 2A depicts a representative cell current record of the response of Type I cells (n = 6). The dopamine receptor agonist bromocriptine induced a small inwardly rectifying K⁺ (Kir) current, which was markedly enhanced upon the addition of TRH to the bath. The enhanced bromocriptine-induced Kir current was inhibited by sulpiride, an antagonist for the dopamine receptor. After ~10 min of wash-out of bromocriptine and sulpiride, somatostatin induced a small Kir current similar to that induced by bromocriptine under control conditions. The somatostatin-induced Kir current was also enhanced by the addition of TRH. In the six Type I cells, TRH increased the bromocriptine-induced Kᵦ current by 6.0 ± 0.5-fold (mean ± S.E.) and the somatostatin-induced Kᵦ current by 7.0 ± 0.8-fold, measured at the command pulse to ~100 mV, TRH alone, in the absence of bromocriptine or somatostatin, did not induce any appreciable Kir current (data not shown). Both bromocriptine- and somatostatin-induced currents reversed at approximately ~30 mV close to the equilibrium potential for K⁺ with 50 mM extracellular K⁺ and exhibited clear inward rectification (Fig. 2A, lower right panel). The currents activated slowly during hyperpolarizing command voltage pulses, which is a feature of Kᵦ channels containing Kir3.1 subunits (Fig. 2A, lower left panels) (17, 25). In RT-PCR analysis of cytoplasmic RNA aspirated from the cell presented in Fig. 2A, we detected transcripts of Kir3.1 and TSHβ as shown in lane 1 of Fig. 2D. The same results were obtained in the remaining five Type I cells (including the result shown in lane 4 of Fig. 2D). Thus, Type I cells are thyrotrophs that may possess Kᵦ channels containing the Kir3.1 subunit.

Because it is known that homomeric Kᵦ channels composed of Kir3.1 are not functional (17) but that heteromers of Kir3.1 and another Kir3.0 subunit form functional Kᵦ channels, we have tried to identify the Kir3.0 subunit that may assemble with Kir3.1 in the vesicular Kᵦ channels. Kir3.3 was not detected by RT-PCR in the mRNA from whole anterior pituitary...
lobe (data not shown). Therefore, the expression of Kir3.2 and Kir3.4 subunits was examined by single-cell RT-PCR. All six Type I cells expressed a Kir3.4 transcript, and one of them also expressed Kir3.2. Furthermore, we detected Kir3.4 immunoreactivity (15-nm gold particles) on the vesicles containing TSH (10-nm particles) with electron microscopic immunocytochemistry (Fig. 2E). Kir3.2 immunoreactivity was not detected on the vesicles (data not shown). These results suggest that Kir3.4 may be the subunit that makes up the vesicular K_C channel with Kir3.1.

In Type II cells (n = 21), bromocriptine or somatostatin induced a very small Kir current, which was not affected by TRH (Fig. 2B). Lanes 2 and 5 in Fig. 2D depict examples of RT-PCR analysis of Type II cell mRNA; lane 2 is from the cell shown in Fig. 2B. In the cytoplasmic RNAs of 10 cells of this type, we detected transcripts of Kir3.2 and/or Kir3.4 but not of Kir3.1. TSHβ mRNA was detected only in the cell shown in lane 2 but not in the remaining nine Type II cells. In Type III

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**FIG. 2.** Three types of cell current responses of dissociated pituitary cells to bromocriptine, somatostatin, and TRH. The external solution contained 50 mM K^+. Command pulses of 500 ms in duration to -100 and +40 mV were applied from the holding potential of -30 mV. A, perfusion of bromocriptine (10 nM) induced an inward current without any effect on the outward current. Addition of TRH (final concentration in the bath, 3 μM) drastically augmented the inward current, which was almost completely inhibited by the application of sulpiride (100 nM). After more than 10 min (double slashes) of wash-out, perfusion of somatostatin (0.1 μM) evoked a small inward current, and TRH application further enhanced this current. Lower left panel, superimposed whole cell current traces evoked by bromocriptine (traces 2-1 and 3-1) or somatostatin (5-4 and 6-4) before (2-1 and 5-4) and after TRH stimulation (3-1 and 6-4). Currents were recorded with voltage steps from -120 to +40 mV in 20-mV steps. Arrowheads indicate a current level of 0 in A–C. Bars in upper and lower left panels are 1 nA for 1 min and 100 pA for 500 ms, respectively. These scales are applicable also to B and C. Lower right panel, current-voltage relationships of bromocriptine- and somatostatin-induced currents after TRH administration. Open circles, bromocriptine-induced currents (trace 3-1). Closed circles, somatostatin-induced currents (trace 6-4). B, in a Type II cell, perfusion of bromocriptine induced a small inward current, which was not affected by TRH. Lower panels of B and C, superimposed whole cell currents evoked by bromocriptine before (trace 2-1) and after TRH stimulation (trace 3-1) with the same voltage steps as in A. C, in most of the dissociated pituitary cells (Type III cells), dopamine and TRH did not affect the membrane current. D, representative results of single-cell RT-PCR. The cytosols were sucked through the patch pipettes after current recording, and then RT-PCR was done. All of the Type I cells (n = 6) expressed the Kir3.1 and TSHβ transcripts (lanes 1 and 4). Lane 1 exhibits the transcripts obtained from the cell whose current record is shown in A. Type II (lanes 2 and 5) and Type III (lanes 3 and 6) cells did not express the Kir3.1 transcripts, although a few cells expressed the TSHβ mRNAs (lane 2). Lanes 5 and 3 illustrate transcripts obtained from the cells whose current recordings are shown in B and C, respectively. Lane P, PCR product of whole pituitary cDNA; lane C, PCR product of each cDNA. E, Kir3.4 immunoreactivity (detected by 15-nm gold particles) was also located on the TSH (10-nm particles)-containing vesicles. Scale bar = 0.25 μm.
response cells (n = 55), bromocriptine or somatostatin did not induce any Kir current, and TRH showed no appreciable effect (Fig. 2C). We could not detect any transcripts of Kir3.1, Kir3.2, or TSHβ in 10 Type III cells examined (lane 3 in Fig. 2D) from the cell shown in Fig. 2C). Two of the 10 Type III cells expressed Kir3.4 mRNA. In conclusion, TRH enhances bromocriptine or somatostatin activation of Kir3.4 channel specifically in thyrotrophs, which express Kir3.1 mRNA.

To examine whether the TRH-induced augmentation of Kir3.4 channel activity in Type I thyrotrophs is derived from the exocytosis of secretory vesicles, as was suggested by the electron microscopic examination (Fig. 1, E–H), we simultaneously measured Cm, Gm, and Im in Type I cells (Fig. 3A). The cells were bathed in 5 mM K+ bathing solution and held at −100 mV. A 600-Hz sine wave with a peak to peak amplitude of 3.4 mV was applied to the cell under voltage clamp. The application of bromocriptine caused some increase of Gm and of the small inward K+ current without any significant effect on Cm. When TRH was added in the continued presence of bromocriptine, the inward K+ current was markedly enhanced in parallel with the simultaneous increase of both Cm and Gm. The increase of Cm may reflect the fusion of secretory vesicles to the plasma membrane induced by TRH (28). In the continued presence of TRH, Cm, Gm, and Im fluctuated in parallel and slowly returned toward their basal levels. The decrease of Cm may represent endocytosis of membrane containing KG channels. This TRH-induced response was observed in 5 of 45 cells examined. In these 5 cells, expression of mRNAs of Kir3.1, Kir3.4, and TSHβ was confirmed by retrospective single-cell RT-PCR (data not shown). The remaining 40 cells did not express any Kir3.1 transcript. The simultaneous measurement of Cm, Gm, and Im indicates that TRH-induction augmented of KG channel activity in Type I cells is associated with the exocytosis of secretory vesicles.

TRH-induced TSH secretion may be composed of voltage-dependent and voltage-independent components, as is the case for prolactin secretion by TRH in the lactotroph (29). The increase in Cm measured in Fig. 3A must represent a voltage-independent component because these experiments were conducted under voltage clamp. The TRH-induced voltage-independent fusion of secretory vesicles causes an increase in the number of KG channels on the plasma membrane. The increased KG channels should then facilitate the hyperpolarization induced by dopamine and somatostatin, which may suppress the depolarization-induced secretion. TRH-induced recruitment of vesicular KG channels to the plasma membrane may act as a feedback control mechanism to avoid excessive stimulation of the cell (Fig. 3B).

At the crustacean neuromuscular junction, repetitive stimulation of motor nerves resulted in an increase of the number of active zones containing a voltage-dependent Ca2+ channel at the axonal terminus, which was implicated in the long-term facilitation of neurotransmitter release (30). The increase of active zones is thought to be the result of the translocation to the plasma membrane of a voltage-dependent Ca2+ channel localized on secretory vesicles (31). Similarly, it was shown that the number of postsynaptic γ-aminobutyric acid type A receptors was rapidly increased by insulin (32) or in an experimental model of temporal lobe epilepsy (33). The stimulation-induced increase of γ-aminobutyric acid type A receptors may underlie the long-term modification of synaptic transmission in hippocampal inhibitory synapses (32, 33). These previous studies require a mechanism that enables stimulation to translocate ion channels from an intracellular pool to the plasma membrane. This study provides, for the first time, clear evidence to indicate that the localization of ion channels on secretory vesicles actually plays a role in the physiological regulation of membrane excitability. Further studies are needed to elucidate the molecular mechanisms localizing ion channels on secretory vesicles. This line of study may provide novel insights into the stimulation control of cellular excitability, including long-term modification of synaptic transmission.

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