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Research Article

The mechanisms of corticotropin-releasing hormone (CRH) induced excitation of ACTH-secreting adenoma cells were investigated using the perforated whole-cell clamp technique and intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) measurement. CRH depolarized ACTH-secreting adenoma cells by activating a nonselective cation current that showed slight inward rectification. This channel did not seem to be a member of the Ca^{2+} -activated cation currents because it was activated even when the $[\text{Ca}^{2+}]_i$ was chelated below 50 nM. The activation of the current was induced by protein kinase A-mediated pathways. By $[\text{Ca}^{2+}]_i$ measurement, CRH increased $[\text{Ca}^{2+}]_i$ of these cells dependently on voltage-gated Ca^{2+} current. This CRH-induced $[\text{Ca}^{2+}]_i$ increase was abolished in Na^{+} -free extracellular solution, but was not abolished by the addition of 5 μM tetrodotoxin to the extracellular solution. CRH-induced ACTH secretion from the cultured adenoma cells was also abolished in Na^{+} -free extracellular solution, but not in tetrodotoxin-containing extracellular solution. These data indicate that a Na^{+} current (maybe the nonselective cation current) other than voltage-gated Na^{+} current plays an important role in CRH-induced $[\text{Ca}^{2+}]_i$ increase and ACTH secretion. CRH also activated a nonselective cation current in nonadenoma human corticotrophs, suggesting that the activation of a nonselective cation current is a physiological mechanism of CRH-induced excitation in human corticotrophs.

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Corticotropin-releasing Hormone Excites Adrenocorticotropin-secreting Human Pituitary Adenoma Cells by Activating a Nonselective Cation Current

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Abstract

The mechanisms of corticotropin-releasing hormone (CRH)-induced excitation of ACTH-secreting adenoma cells were investigated using the perforated whole-cell clamp technique and intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) measurement. CRH depolarized ACTH-secreting adenoma cells by activating a nonselective cation current that showed slight inward rectification. This channel did not seem to be a member of the Ca^{2+} -activated cation currents because it was activated even when the $[\text{Ca}^{2+}]_i$ was chelated below 50 nM. The activation of the current was induced by protein kinase A-mediated pathways. By $[\text{Ca}^{2+}]_i$ measurement, CRH increased $[\text{Ca}^{2+}]_i$ of these cells dependently on voltage-gated Ca^{2+} current. This CRH-induced $[\text{Ca}^{2+}]_i$ increase was abolished in Na^+ -free extracellular solution, but was not abolished by the addition of 5 μM tetrodotoxin to the extracellular solution. CRH-induced ACTH secretion from the cultured adenoma cells was also abolished in Na^+ -free extracellular solution, but not in tetrodotoxin-containing extracellular solution. These data indicate that a Na^+ current (maybe the nonselective cation current) other than voltage-gated Na^+ current plays an important role in CRH-induced $[\text{Ca}^{2+}]_i$ increase and ACTH secretion. CRH also activated a nonselective cation current in nonadenoma human corticotrophs, suggesting that the activation of a nonselective cation current is a physiological mechanism of CRH-induced excitation in human corticotrophs. (*J. Clin. Invest.* 1996. 98:2033–2041.) Key words: corticotropin-releasing hormone • corticotroph • nonselective cation current • protein kinase A • adrenocorticotropin secretion

Introduction

Corticotropin-releasing hormone (CRH)¹ is one of the major regulatory hormones of the neuroendocrine response to stress

(1, 2). Secreted from the hypothalamic CRH-neurons into a pituitary portal system in response to stress, CRH stimulates ACTH secretion from corticotrophs in the anterior pituitary (2). The mechanism of CRH-induced ACTH secretion has been investigated using rat corticotrophs and AtT-20 cells, an ACTH-secreting mouse pituitary cell line, and ACTH-secreting human pituitary adenoma cells. CRH stimulates ACTH secretion through the activation of adenylyl cyclase (3, 4) and through the increase in intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) (5, 6). CRH-induced $[\text{Ca}^{2+}]_i$ increase is due to the Ca^{2+} influx through voltage-gated Ca^{2+} channels, which is stimulated by CRH through protein kinase A (PKA)-mediated pathways (5, 7). In addition to CRH-induced activation of voltage-gated Ca^{2+} channels, CRH depolarizes ACTH-secreting cells and increases action potential frequency that is accompanied by $[\text{Ca}^{2+}]_i$ transients (7). These suggest that the increased frequency of action potential augments Ca^{2+} influx through the voltage-gated Ca^{2+} channels that are open during action potentials. To understand the significance of the CRH-induced excitation in ACTH secretion, it is necessary to investigate the mechanism of CRH-induced excitation and its relation to $[\text{Ca}^{2+}]_i$ increase and ACTH secretion. We investigated these ionic mechanisms by electrophysiological experiments using perforated whole-cell clamp on human ACTH-secreting adenoma cells and nonadenoma human corticotrophs. We found that CRH excited ACTH-secreting adenoma cells by activating a nonselective cation current through the PKA-mediated pathway. This CRH-induced activation of a cation current played an important role in CRH-induced $[\text{Ca}^{2+}]_i$ increase and ACTH secretion. This is the first report that showed CRH-induced activation of a nonselective cation current in vertebrate cells, including human corticotroph.

Methods

Drugs. CRH, PKC(19-36), a protein kinase C (PKC) inhibitor peptide, and PKI(5-24), a PKA inhibitor peptide, were purchased from Peninsula Laboratories Inc. (Belmont, CA). 8-bromoadenosine 3',5'-cyclic monophosphate (8Br-cAMP), forskolin, and nystatin were purchased from Sigma Chemical Co. (St. Louis, MO). *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinoleinsulfonamide (H89) was purchased from Seikagaku-kogyo Co., Ltd. (Tokyo, Japan), and dispase from Godo Shusei Co., Ltd. (Tokyo, Japan). Fura 2/AM was purchased from Molecular Probes, Inc. (Eugene, OR).

Cell preparation. ACTH-secreting pituitary adenomas were obtained with informed consent from three patients operated for ACTH-secreting pituitary adenoma by transsphenoidal surgery (patients 1–3). The use of surgically removed human pituitary tissues obtained during surgery as experimental materials is permitted by the Investigation Committee of Hypothalamo-Pituitary Diseases, The Ministry of Health and Welfare of Japan, and The Ethical Committee of University of Tokyo School of Medicine (Tokyo, Japan). Preoperative serum ACTH levels of these patients were 61 pg/ml in patient 1, 53 pg/ml in patient 2, and 90 pg/ml in patient 3. All three patients re-

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1. Abbreviations used in this paper: $[\text{Ca}^{2+}]_i$, Ca^{2+} concentration; CRH, corticotropin-releasing hormone; PKA (or PKC), protein kinase A (or C); PKI, a PKA inhibitor peptide; TMA, tetramethylammonium; TTX, tetrodotoxin.

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sponded to 100 µg intravenous CRH administration, peak ACTH value was 132 pg/ml in patient 1, 155 pg/ml in patient 2, and 210 pg/ml in patient 3. The excised adenoma tissues were minced into small pieces (< 1 mm) and were digested with 1,000 U/ml dispase. For investigating the hormonal release, cells were seeded on 24-well dishes at a density of 10⁵ cells/well. For [Ca²⁺]_i measurements, cells were seeded on 22-mm round cover glasses and placed in 35-mm culture dishes. For electrophysiological experiments they were seeded on 35-mm culture dishes. Cells were cultured in DMEM containing 10% heat-inactivated FCS, and kept in humidified air containing 5% CO₂ at 37°C. All the experiments were carried out using cells cultured for 1 to 2 wk.

Nonadenoma human anterior pituitary cells were obtained by transsphenoidal surgery from another patient suffering from Cushing's disease. As part of the surgical procedure, it is sometimes inevitable to remove some small pieces of normal tissue from the anterior pituitary to reveal the adenoma tissue. The patient had been informed about this possibility in advance and he gave his consent that such normal tissue could be used for this experiment in the case that it had to be removed when looking for the adenoma. The normal pituitary tissue was digested and seeded on 35-mm tissue culture dishes by the same method as stated above. Cells were cultured in the same manner as described for the ACTH-secreting adenoma cells. After electrophysiological experiments, cells that responded to CRH application were stained for human ACTH and were confirmed to be ACTH-secreting cells (see *Cell identification*, below).

Electrophysiology. The perforated whole-cell clamp technique was used (8). The standard patch electrode solution contained the following (in mM): 95 K aspartate, 47.5 KCl, 1 MgCl₂, 0.1 EGTA (tetramethylammonium [TMA] salt, pH 7.2), and 10 Hepes. The standard external solution was as follows (in mM): 128 NaCl, pH 7.4, 5 KCl, 1 MgCl₂, 2.5 CaCl₂, and 10 Hepes. The Na⁺-free solution was made by replacing Na⁺ of the standard solution with isoosmotic TMA⁺ or isoosmotic choline⁺. Other cation-substituted solutions except for Na⁺-free solution were made by replacing Na⁺ and K⁺ in the standard solution with isoosmotic cation (Li⁺ or NH₄⁺). Other changes in the composition of the extracellular and internal solutions are noted. During the experiments, the extracellular solution was continuously perfused by a peristaltic pump. Agonists were applied by changing the perfusing solution. It took ~ 2 min to change the bath solution with this peristaltic pump system. The liquid junction potentials between the standard extracellular solution and other solutions used (internal and external) were measured using a 3 M KCl electrode as a reference, and all the data were corrected for the liquid junction potential (−8 to 2 mV). An amplifier (EPC-7; List Biological Labs, Inc., Campbell, CA) was used for recording the membrane current and potential. All experiments were performed at room temperature (22–25°C). Glass capillaries of 1.5 mm diameter with a filament were used to make patch electrodes. The resistance of the patch electrodes were between 5 and 8 MΩ. Details of the perforated whole-cell clamp technique have been reported elsewhere (9). Current clamp recordings were started after the series resistance fell below 50 MΩ. Voltage clamp recordings were made after the series resistance fell below 20 MΩ. Since the amplitude of the current was < 150 pA, the errors caused by series resistance were ignored.

Microinjection of peptides. PKI(5-24) and PKC(19-36) were injected into the cell by microinjection. The details of the method for microinjection have already been reported (10). The peptides were dissolved in 150 mM KCl at the concentration of 0.1 mM. The peptide solution was microinjected through microcapillaries with a tip resistance of 20 MΩ by pressure injection (110 hPa, 0.1 s). At the time of injection, a slight swelling of the cell was observed. The volume of the injected solution was estimated approximately by the decrease of the solution after multiple injections. The injected solution was ~ 10–30 fl per cell, which was ~ 1/100 of the cell volume (500–1,500 fl). Microinjection was performed 20 min before patch clamping. The cells with input resistance of more than 1 GΩ after the microinjection were used for the experiments.

[Ca²⁺]_i measurement. Cells were loaded with fura 2 by incubating with 2 µM fura 2/AM in Hanks' balanced salt solution containing 0.1% bovine serum albumin for 40 min at room temperature. Ca²⁺ measurements were performed on a Nikon Diaphot microscope (Nikon, Tokyo, Japan). Each cell was excited at 340 and 380 nm alternately at a frequency of 100 Hz with CAM220 (Nihonbunko, Tokyo, Japan). A band filter was used to monitor the fluorescent emission at 510 nm. The cytosolic free Ca²⁺ concentration was determined from the equation $[Ca^{2+}]_i = K(R - R_{min}) / (R_{max} - R)$ (11). In this equation, K represents $K_d(F_{min}/F_{max})$, where K_d is the dissociation constant of fura 2 (130 nM at 25°C), and F_{max}/F_{min} is the ratio of Ca²⁺-free and -bound fura 2 fluorescence at 380 nM. R_{min} is the 340/380 fluorescence ratio of Ca²⁺-free fura 2, and R_{max} is the 340/380 ratio of Ca²⁺-bound fura 2. Calibration was performed on every cell by permeabilizing the cell to Ca²⁺ with 2 µM digitonin. Cells were first permeabilized in Ca²⁺-free saline (5 mM EGTA, 150 mM KCl, and 10 mM Hepes, pH 7.2), for determination of R_{min} and F_{min} , and then in high Ca²⁺ saline (2.5 mM CaCl₂, 150 mM KCl, and 10 mM Hepes, pH 7.4) for determination of R_{max} and F_{max} . The [Ca²⁺]_i traces shown in the figures were filtered with a bandwidth of 1 Hz to reduce the noise. Agonists were applied by changing the bath solution with a peristaltic pump. In the [Ca²⁺]_i measurement experiment, it took ~ 30 s to change the bath solution in this peristaltic pump system.

Hormonal release study. Adenoma cells cultured in 24-well dishes were washed twice with serum-free DMEM containing 0.1% BSA. They were incubated with the same medium containing 100 nM CRH for 2 h with or without 2 µM nitrendipine or 10 µM forskolin. Some cells were pretreated with 5 µM H89, a PKA inhibitor, for 1 h. After H89 pretreatment, cells were treated with or without 100 nM CRH for 2 h. For the experiments using Na⁺-free extracellular solution, cells were incubated in Na⁺-free extracellular solution (see above) containing 100 mg/dl glucose with or without 100 nM CRH for 2 h. After the incubation with various agents, the solution was collected and stored at −20°C until the hormonal assay. ACTH was assayed using a radioimmunoassay kit (Daiichi Radioisotope Laboratories, Tokyo, Japan).

Cell identification. At an early stage of the experiments, the cells were stained against human ACTH immunocytochemically to identify ACTH-secreting adenoma cells. Cells ($n = 7$) that showed membrane hyperpolarizations in response to CRH application (see below) were fixed by 2% formaldehyde in phosphate buffer and stained for human ACTH by using a human ACTH immunostaining kit (DAKO SA, Glostrup, Denmark). All these cells stained positive for ACTH. In the subsequent experiments, data were obtained from cells that satisfied the criteria for pituitary adenoma cells obtained from our experience, which is round-shaped cells with a diameter of 10–20 µm that have a smooth glittering surface under a scanning light microscope. These characteristics were apparently different from the characteristics of the spindle-shaped fibroblast-like cells that grew in the adenoma cell culture.

Results

Membrane depolarization by CRH. Fig. 1 A shows a potential record from a human ACTH-secreting adenoma cell under current clamp in the standard extracellular solution. The pipette solution was the standard internal solution. The resting membrane potential of this cell was ~ −52 mV and this cell exhibited spontaneous action potentials. Spontaneous action potentials were observed in 84% of the examined cells ($n = 50$). Application of 1 nM CRH induced depolarization to the level of −40 mV and increased action potential frequency. When CRH was washed out from the bath solution, the membrane potential reversed to the basal level. CRH (10 nM)-induced depolarization was observed in 10 out of 10 cells from adenoma 1, 11 out of 12 cells from adenoma 2, and 8 out of 8

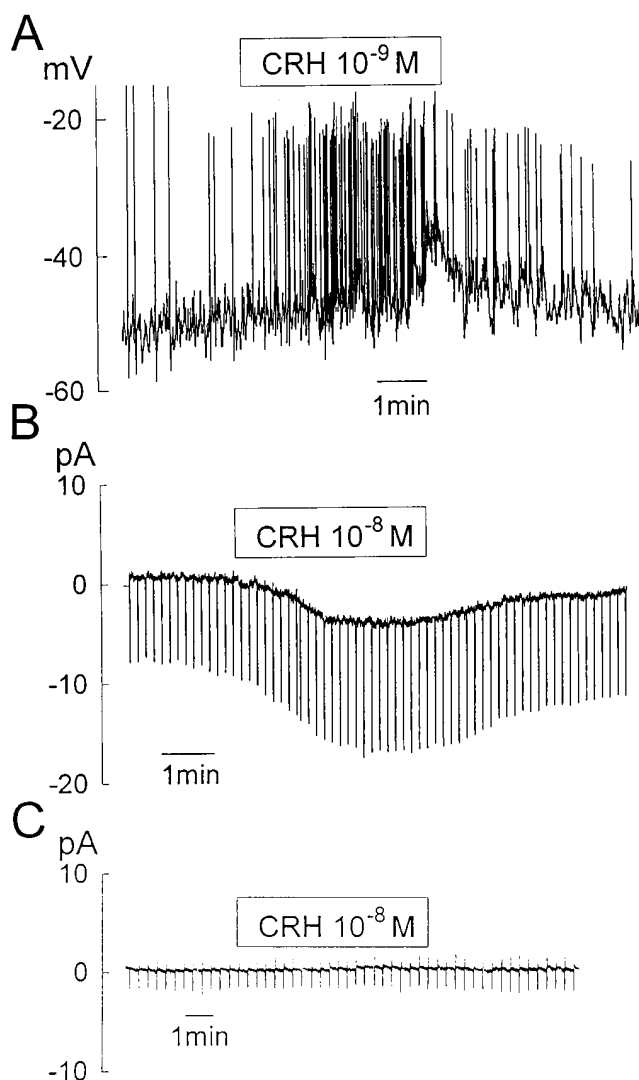


Figure 1. (A) Membrane depolarization caused by CRH in an ACTH-secreting pituitary adenoma cell. The cell exhibits spontaneous action potentials and the resting membrane potential was -52 mV. Application of 1 nM CRH depolarized the membrane and increased the action potential frequency. (B) The current record from another cell under voltage clamp at holding potential of -48 mV. Application of CRH (10 nM) induced inward current accompanied by membrane conductance increase. The membrane conductance was measured by applying a hyperpolarizing pulse of -60 mV every 10 s. (C) The current record of another cell under voltage clamp in Na^+ -free (TMA^+ -substituted) extracellular solution. The holding potential was -48 mV. Membrane conductance was monitored by applying hyperpolarizing pulse of -60 mV every 20 s. Application of CRH (10 nM) did not induce an inward current.

cells from adenoma 3. The mean value of depolarization induced by 10 nM CRH was 9.6 ± 4.5 mV (mean \pm SD, $n = 29$).

To examine the ionic mechanism of this CRH-induced depolarization, whole cell currents were recorded under voltage clamp. Fig. 1 B shows the membrane current of another adenoma cell recorded under voltage clamp at a holding potential of -48 mV. The membrane conductance was monitored by applying a 60 -mV hyperpolarizing pulse once every 10 s. Application of 10 nM CRH evoked an inward shift of the holding

current accompanied by an increase in membrane conductance. This inward current was reversible when CRH was washed out from the bath solution.

These results indicate that CRH-induced inward current is responsible for the CRH-induced depolarization. Therefore we investigated the extracellular Na^+ -dependency of the CRH-induced inward current because Na^+ was the major cation in the extracellular solution. Fig. 1 C shows a current record of another adenoma cell in Na^+ -free (TMA^+ -replaced) extracellular solution under voltage clamp. The membrane conductance was monitored by the method in Fig. 1 B. Application of CRH did not induce a noticeable current. This indicates that the CRH-induced inward current was extracellular Na^+ -dependent. Na^+ -free extracellular solution abolished CRH-induced current in 11 out of 11 cells examined. This inward current under voltage clamp seems to correspond to the depolarization observed under current clamp because CRH-induced depolarization was also abolished in Na^+ -depleted extracellular solution (data not shown).

Characteristics of the CRH-induced current. Fig. 2 A, left and center, shows membrane currents under voltage clamp obtained before (control) and during (CRH) the application of 10 nM CRH, respectively. We used the standard extracellular solution and the holding potential was -48 mV. The control current was subtracted from the current recorded during the CRH application to obtain CRH-induced current (Fig. 2 A, right). The I-V relation of CRH-induced current in Fig. 2 A, right is plotted against the membrane potential in Fig. 2 B, (○), where the data from two other cells are included (● and △). The CRH-activated current showed slight inward rectification. Because the I-V curve was nearly linear between -48 and -78 mV, the reversal potential of CRH-induced current was estimated by extrapolating the I-V curve in this segment, which fell between -20 and 0 mV. This reversal potential was between the equilibrium potential of Na^+ and K^+ , which suggests that the CRH-induced current is a nonselective cation current. The slope conductance between -48 and -78 mV of the CRH (10 nM)-induced current was 97 ± 42 pS (mean \pm SD, $n = 6$) in adenoma 1, 185 ± 73 pS in adenoma 2, and 238 ± 37 pS in adenoma 3. Fig. 2 C shows the concentration dependency of the CRH-induced conductance in adenoma 3. Maximum response was obtained at > 10 nM. Because the I-V relation showed little voltage dependency, a ramp test potential was used for further experiments.

Fig. 3 A shows the I-V relationship of the membrane current obtained by a ramp test potential over -120 to -50 mV in 800 ms from the holding potential of -48 mV. When the I-V relations of the cell before and during CRH application were extrapolated, the extrapolating lines crossed between -20 and 0 mV. These indicate that the reversal potential measured approximately by ramp test protocol was close to the reversal potential estimated by pulse step protocol. The reversal potential of the CRH-induced current estimated by ramp pulse was -10.5 ± 7.9 mV ($n = 6$).

The ion selectivity of the channel responsible for the CRH-induced inward current was investigated. In these experiments, the extracellular Na^+ was replaced with other cations (Li^+ , NH_4^+ , or TMA^+). Fig. 3, B–D shows the effect of 10 nM CRH on the membrane current under voltage clamp in Li^+ -, NH_4^+ -, and TMA^+ -extracellular solution. The holding potential was -48 mV. CRH induced a cation current in Li^+ - and NH_4^+ -extracellular solution. However in TMA^+ -extracellular solu-

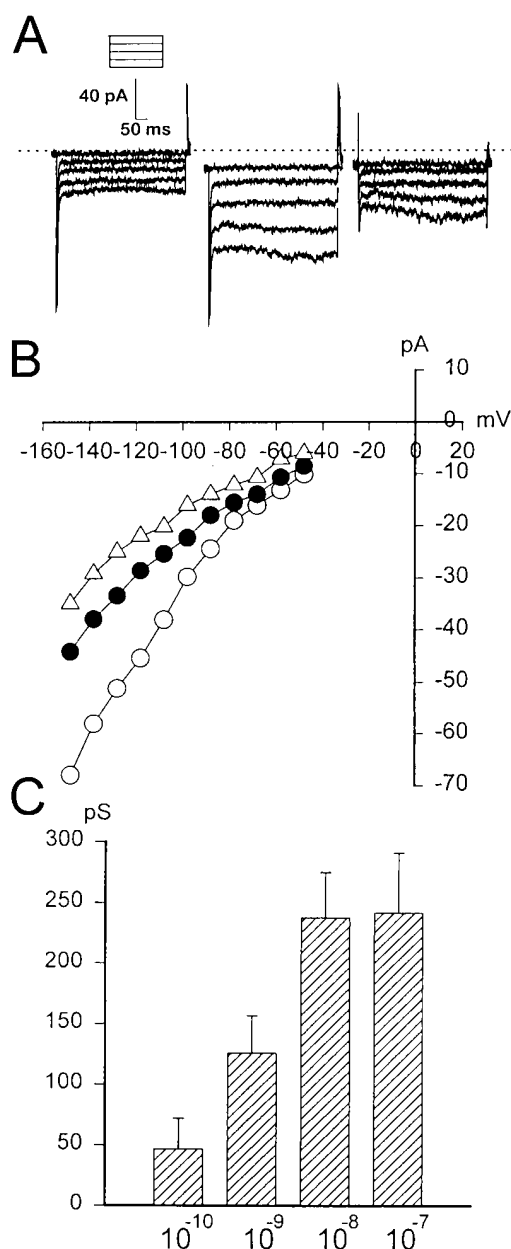


Figure 2. CRH-induced cation conductance. (A) Membrane currents under voltage clamp evoked by step pulse potentials before (left) and after (center) the application of 10 nM CRH. We used the standard extracellular solution and the holding potential was -48 mV. The test pulses were to -48 , -68 , -88 , -108 , and -128 mV. The control current (left) was subtracted from that recorded during the CRH application (center) to obtain the CRH-induced current (right). The broken line is zero current level. (B) I-V relation of CRH-induced current (○) in (A), where I-V curves from two other cells are included (● and △). (C) Dose dependency of CRH-induced conductance. CRH-induced conductance was estimated by dividing the current difference of the CRH-induced current at -48 and -78 mV by the potential difference (30 mV).

tion, CRH did not activate a current. These indicate that the CRH-stimulated channel is permeable to Li^+ and NH_4^+ , but not to TMA^+ . In addition to TMA^+ , choline $^+$ was used as an indifferent monovalent cation to substitute Na^+ in the extracellular solution. CRH did not activate a current in choline $^+$ -

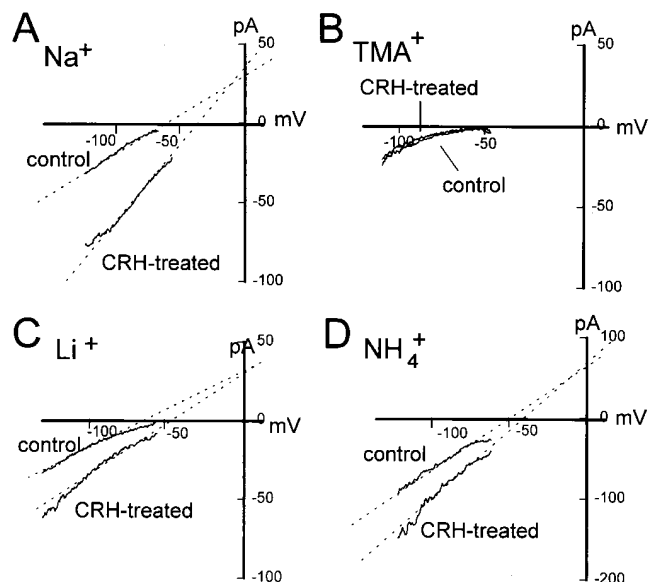


Figure 3. Ion selectivity of CRH-induced current. I-V relationships were obtained before and during application of 10 nM CRH using the ramp pulse protocol. Holding potential was -48 mV, and dwell time of the ramp pulse from -150 to 20 mV was 1 s. Part of the current traces are shown. Standard extracellular solution was the extracellular solution in A, isoosmotic TMA^+ solution in B, Li^+ solution in C, and NH_4^+ solution in D. Broken lines were drawn by eye. Control indicates current record before the application of CRH, CRH-treated indicates current record after the application of CRH.

extracellular solution either (data not shown). Similar results were obtained in more than three other cells in each cation-exchanged extracellular solution.

To investigate the permeability of the CRH-induced current for Cl^- , the extracellular Cl^- was reduced to 15 mM by substituting NaCl with isoosmotic sodium methanesulfonate. Application of CRH induced a cation current in the low Cl^- solution (data not shown). The reversal potential of the CRH-induced current was -8.3 ± 7.8 mV ($n = 5$) and was close to that of the CRH-induced current obtained in the standard extracellular solution containing 140 mM Cl^- . These indicate that the channel for the CRH-induced current is not permeable to Cl^- .

To estimate the permeability ratio for Na^+ and K^+ of the CRH-induced conductance, the reversal potential of the CRH (10 nM)-induced current was measured using a solution containing only Na^+ as a cation for extracellular solution and a solution containing only K^+ as a cation for intracellular solution. The reversal potential was -8.8 ± 6.8 mV ($n = 5$). If we hypothesize that Na^+ and K^+ flow are independent, the permeability ratio of CRH-induced current to K^+ and Na^+ can be calculated by the Goldman-Hodgkin-Katz equation (12, 13): $E_{\text{rev}} = (RT/F) \ln[(P_{\text{K}}[\text{K}]_o + P_{\text{Na}}[\text{Na}]_o)/(P_{\text{K}}[\text{K}]_i + P_{\text{Na}}[\text{Na}]_i)]$. Where E_{rev} is the reversal potential of CRH-induced current, P_{K} and P_{Na} are permeability for K^+ and Na^+ , respectively, and $[\text{K}]_o$ and $[\text{Na}]_o$ stand for extracellular concentration of K^+ and Na^+ , respectively. $[\text{K}]_i$ and $[\text{Na}]_i$ stand for intracellular concentration of K^+ and Na^+ , respectively. R is the gas constant, T the absolute temperature, and F the Faraday's constant. RT/F is nearly 25.69 at 25°C . In this equation, $[\text{K}]_o$ and $[\text{Na}]_i$ were 0 mM, $[\text{Na}]_o$ was 143 mM, and $[\text{K}]_i$ was 144 mM. Permeability ratio of CRH-induced current to K^+ and Na^+ ($P_{\text{K}}/P_{\text{Na}}$) was cal-

culated to be ~ 1.4 . However, because the I-V curve of the CRH-induced current showed slight inward rectification, the reversal potential estimated by extrapolating the I-V relations between -48 and -68 mV may be slightly more hyperpolarized to the true reversal potential. Therefore, P_K/P_{Na} may be a little smaller than calculated, meaning that the CRH-induced current may be a little more permeable to Na^+ than calculated.

Extracellular Na^+ -dependency of the CRH-induced $[Ca^{2+}]_i$ increase. Application of 10 nM CRH increased $[Ca^{2+}]_i$ of these cells (Fig. 4 A) in the standard extracellular solution.

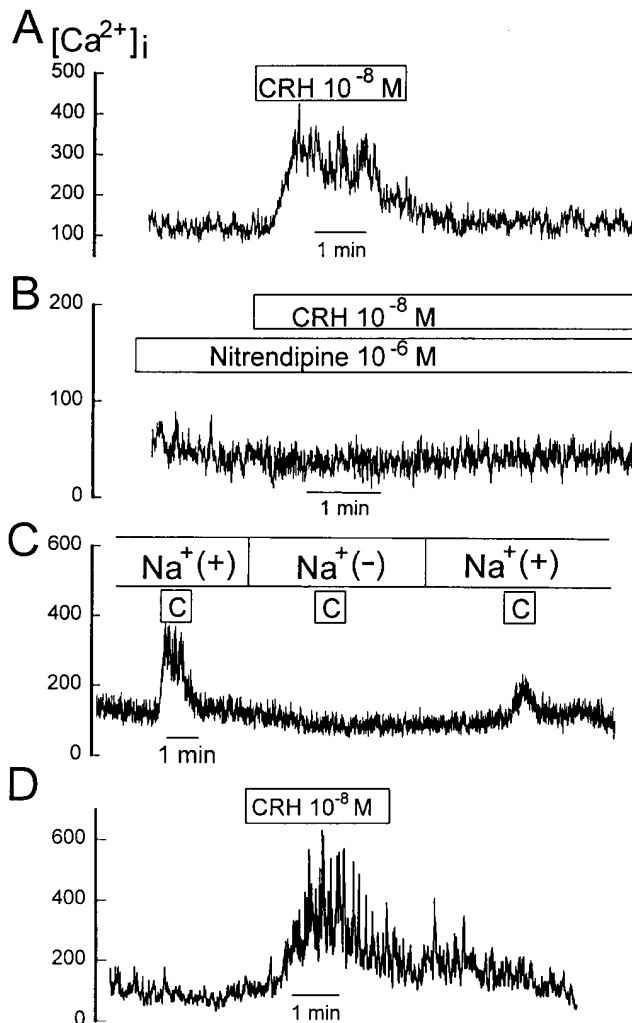


Figure 4. CRH-induced $[Ca^{2+}]_i$ increase in ACTH-secreting adenoma cells. (A) Application of 10 nM CRH increased $[Ca^{2+}]_i$ in the standard extracellular solution. (B) CRH-induced $[Ca^{2+}]_i$ increase was not observed in the standard extracellular solution containing 1 μ M nitrendipine (C) extracellular Na^+ dependency of CRH-induced $[Ca^{2+}]_i$ increase. Application of CRH (10 nM) increased $[Ca^{2+}]_i$ in a cell in the standard extracellular solution. After changing the extracellular solution with Na^+ -free extracellular solution, CRH (10 nM) was again applied. CRH did not increase $[Ca^{2+}]_i$ in Na^+ -free solution. However, when the extracellular solution was changed to the standard extracellular solution again, the increase in $[Ca^{2+}]_i$ by CRH (10 nM) was observed. $Na^+(+)$ indicates that the extracellular solution is the standard extracellular solution and $Na^+(-)$ indicates that the extracellular solution was Na^+ -free. (D) CRH increased $[Ca^{2+}]_i$ in the standard extracellular solution containing 5 μ M TTX.

This increase was a reversible phenomenon. Fig. 4 B shows the effect of nitrendipine on CRH-induced $[Ca^{2+}]_i$ change. Application of nitrendipine (1 μ M) decreased $[Ca^{2+}]_i$ by itself and further application of CRH (10 nM) together with nitrendipine (1 μ M) did not increase $[Ca^{2+}]_i$, indicating that Ca^{2+} influx through L-type voltage-gated Ca^{2+} channel is responsible for the $[Ca^{2+}]_i$ increase. Similar results were obtained in six cells in adenoma 1, and four cells in adenoma 2.

The extracellular Na^+ -dependency of the CRH-induced $[Ca^{2+}]_i$ increase was examined. Fig. 4 C shows the $[Ca^{2+}]_i$ of another adenoma cell. Application of CRH increased $[Ca^{2+}]_i$ of the cell in the standard extracellular solution. After changing the extracellular solution to Na^+ -free solution, CRH (10 nM) was applied in the Na^+ -free extracellular solution. CRH did not increase $[Ca^{2+}]_i$ in this solution. The lack of CRH response in Na^+ -free solution was not due to the CRH receptor desensitization because CRH-induced $[Ca^{2+}]_i$ increase was observed again when the extracellular solution was returned to the standard extracellular solution. These data indicate that the CRH-induced $[Ca^{2+}]_i$ increase was extracellular Na^+ dependent. Similar results were obtained in four other cells. To see that the extracellular Na^+ -dependency of the CRH-induced $[Ca^{2+}]_i$ increase was not related to Na^+ -action potentials, the standard extracellular solution containing 5 μ M tetrodotoxin (TTX) was used. In this condition, CRH increased $[Ca^{2+}]_i$ of these cells (Fig. 4 D), indicating that abolishment of TTX-sensitive Na^+ action potentials is not the cause of the abolishment of CRH-induced $[Ca^{2+}]_i$ increase in the Na^+ -free extracellular solution.

PKA-mediated activation of CRH-induced cation current. Fig. 5 A shows the membrane current before and after the application of 10 μ M forskolin under voltage clamp. A ramp test potential from -120 to -50 mV in 800 ms was applied to ob-

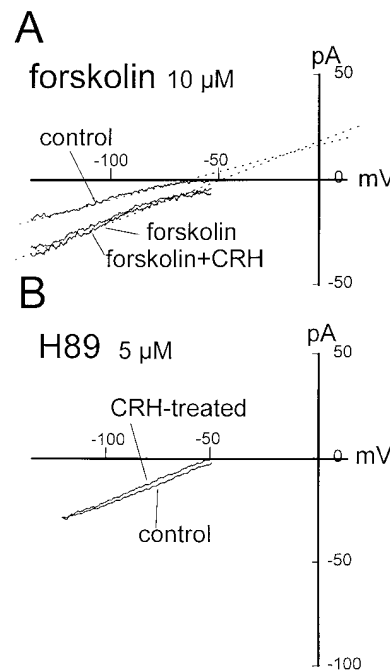


Figure 5. Relationship between CRH-induced current and cAMP. (A) Effect of forskolin on the membrane current. Forskolin was applied to cells with or without 10 nM CRH. Broken lines were drawn by eye. Control indicates current record before the application of forskolin, forskolin indicates current record after the application of forskolin, and forskolin + CRH indicates current record after the combined application of forskolin and CRH. (B) Effect of H89 on the CRH-induced current. Application of CRH (10 nM) did not change the membrane current in a cell pre-

treated with H89 (5 μ M) for 30 min. Extracellular solution and ramp pulse protocol were the same as in Fig. 3 A. Control indicates current record before the application of CRH, and CRH-treated indicates current record after the application of CRH.

tain the I-V relationship from the holding potential of -48 mV. Forskolin ($10\text{ }\mu\text{M}$) induced an inward current whose reversal potential was between the equilibrium potential of Na^+ and K^+ , suggesting that the induced current was a nonselective cation current. After maximal response was achieved by forskolin, CRH (10 nM) was applied together with forskolin, which did not induce additional change in the membrane current ($n = 5$). These data indicate that forskolin induced a cation current and occluded the effect of CRH on the membrane current. These suggest that CRH activates the cation current through the activation of an adenyl cyclase. When a cell-permeable cAMP analogue, 8Br-cAMP (100 nM), was applied to the cell, similar cation current was induced and the effect of CRH on membrane current was occluded by this compound, as was the case for forskolin (data not shown).

To investigate whether PKA is involved in the CRH-induced activation of the cation current, a PKA-inhibitor H89 ($14\text{ }\mu\text{M}$) was used. Fig. 5 B shows the membrane currents before and after the application of CRH (10 nM) in a cell pretreated with $5\text{ }\mu\text{M}$ H89 for 30 min. CRH did not induce the cation current in H89-treated cells ($n = 7$). Because H89 was not a specific inhibitor for PKA, a specific PKA-inhibitor PKI(5-24) was injected into the cell by microinjection technique. As a control peptide, a specific PKC-inhibitor PKC(19-36) was used. Fig. 6 A shows the effect of CRH on the membrane current of a cell microinjected with PKI(5-24). CRH did not induce any change on the membrane current. Microinjection of PKC(19-36) did not

abolish the CRH-induced activation of the cation current (Fig. 6 B). Fig. 6 C summarizes the results of these experiments. The CRH response in PKI(5-24)-injected cells was profoundly attenuated compared with the response in PKC(19-36)-injected cells, which was not significantly different from that in untreated cells.

Independency of CRH-induced cation current on $[\text{Ca}^{2+}]_i$. There are a group of nonselective cation currents that are activated by the increase in $[\text{Ca}^{2+}]_i$ in various kinds of cells (15–17). Because CRH increased $[\text{Ca}^{2+}]_i$ of these ACTH-secreting adenoma cells, it is possible that the CRH-induced cation current is one of the Ca^{2+} -activated cation currents. To investigate this, we loaded the cell with cell-permeable Ca^{2+} chelator, BAPTA/AM, to clamp the $[\text{Ca}^{2+}]_i$ lower than 100 nM , at which Ca^{2+} -activated cation currents are activated (18). Fig. 7 A shows the time course of the $[\text{Ca}^{2+}]_i$ of a cell incubated for 100 min in the standard extracellular solution containing BAPTA/AM ($10\text{ }\mu\text{M}$) and 0.1% BSA. The initial $[\text{Ca}^{2+}]_i$ was $\sim 250\text{ nM}$ and decreased gradually during the incubation.

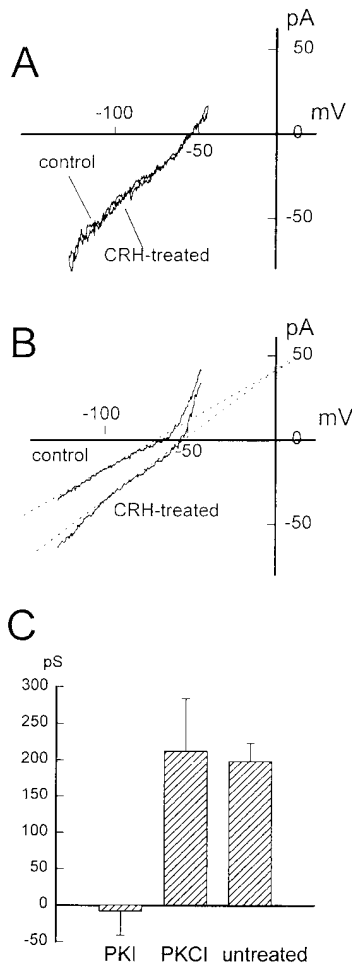


Figure 6. Effect of microinjected PKI(5-24) on the CRH-induced current. (A) Cells had been injected with PKI(5-24) by microinjection and was applied with CRH (10 nM). Application of CRH did not change membrane current. (B) Cell had been injected with PKC(19-36) by microinjection and was applied with CRH (10 nM). CRH induced a cation current. Control indicates current record before the application of CRH, and CRH-treated indicates current record after the application of CRH. (C) The CRH-induced conductance in cells injected with PKC(19-36) or PKI(5-24), and in untreated cells. PKCI, the data obtained from cells injected with PKC(19-36); PKI, the data obtained from cells injected with PKI(5-24); untreated, data obtained from untreated cells. The bar indicates 1 SD. $*P < 0.01$; data were analyzed by ANOVA.

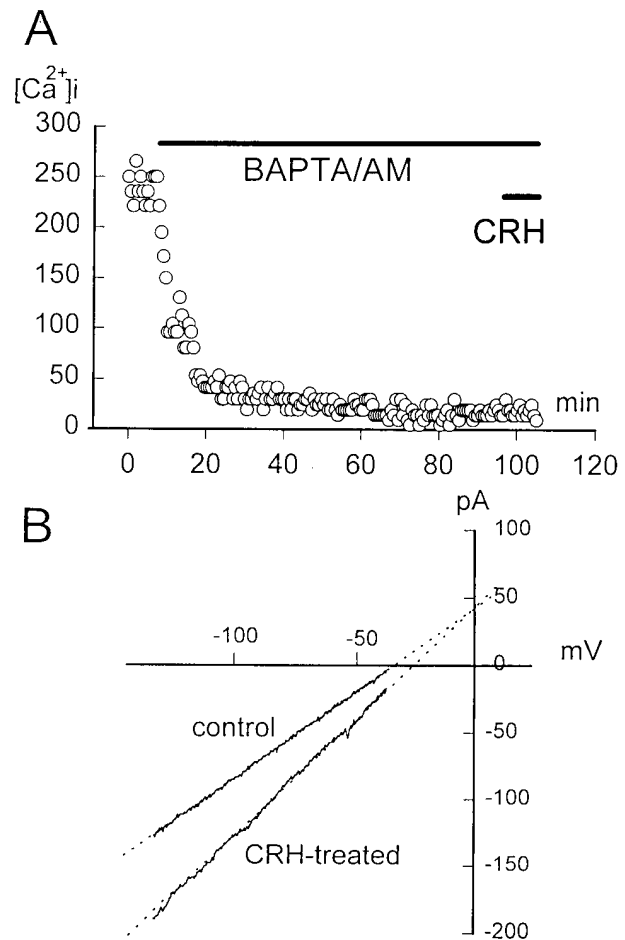


Figure 7. (A) Time course of $[\text{Ca}^{2+}]_i$ of a cell during incubation in the standard extracellular solution containing BAPTA/AM ($10\text{ }\mu\text{M}$) and 0.1% BSA over 100 min. BAPTA/AM indicates incubation with BAPTA/AM. CRH indicates application of CRH (10 nM). (B) Membrane currents evoked by a ramp test potential before and after the application of CRH (10 nM) of the BAPTA/AM-treated cell (for 100 min) in Na^+ -free extracellular solution. Control, current record before the application of CRH; CRH-treated, current record after the application of CRH.

Finally, it reached ~ 30 nM at 100 min. Application of CRH (10 nM) did not increase $[Ca^{2+}]_i$ at this point. When the results from five experiments were summarized, the value of $[Ca^{2+}]_i$ after 100 min BAPTA/AM treatment was 25 ± 10 nM and ranged between 15 and 45 nM. It did not increase after the application of CRH (10 nM) and remained around 27 ± 17 nM, ranging between 20 and 50 nM. Therefore, treating the cell with BAPTA/AM for 100 min was enough to clamp $[Ca^{2+}]_i$ lower than 50 nM even when CRH is to be applied. We investigated the effect of CRH (10 nM) on the membrane current after treating the cell with BAPTA/AM as mentioned above. In addition to the treatment by BAPTA/AM, we used Ca^{2+} -free extracellular solution to avoid the local $[Ca^{2+}]_i$ increase beneath the plasma membrane due to the Ca^{2+} influx through the voltage-gated Ca^{2+} channels. Fig. 7 B shows the membrane current evoked by a ramp test potential before and after the application of CRH (10 nM) on a BAPTA/AM-treated cell. CRH induced a cation current in this cell. Application of CRH induced a cation current in the BAPTA/AM-treated (100 min) cells in Ca^{2+} -free extracellular solution in six out of six cells, including this cell. These data suggest that the cation current induced by CRH is not a member of the Ca^{2+} -activated cation currents.

Hormone release study. To estimate the role of CRH-induced cation current in CRH-induced hormone secretion, human ACTH secretion from adenoma cells was measured. Adenoma cells were incubated in Na^+ -free extracellular solution, and the effect of CRH application on the ACTH secretion was examined. Fig. 8 shows the effects of CRH on ACTH secretion from primary cultured cells of adenoma 1. CRH increased ACTH secretion in Na^+ -containing extracellular solution with a concentration dependency. CRH (100 nM) increased the secretion to the level of $300 \pm 45\%$ (mean \pm SD, $n = 4$) of the control. The CRH-induced increase of ACTH secretion was abolished in Na^+ -free (TMA^+) extracellular solution, indicating that CRH-induced increase of ACTH secretion was dependent on extracellular Na^+ . These data suggest that CRH-induced activation of Na^+ -permeable (nonselective) cation

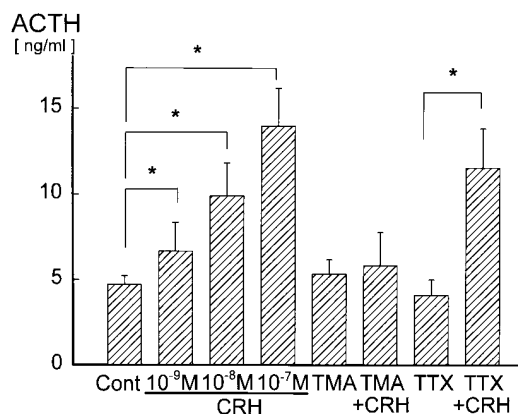


Figure 8. CRH-induced ACTH secretion. Cont, control extracellular medium; TMA^+ , Na^+ -free extracellular medium; TMA^+ + CRH, Na^+ -free extracellular medium containing 10 nM CRH; TTX, control extracellular medium containing 5 μM TTX; TTX + CRH, control extracellular medium containing 5 μM TTX and 10 nM CRH. Data are shown as mean \pm SD of data from four wells. Bar indicates 1 SD. $*P < 0.05$. The data were analyzed by ANOVA and found to be significantly different ($P < 0.01$) and difference between each pair was analyzed by post test.

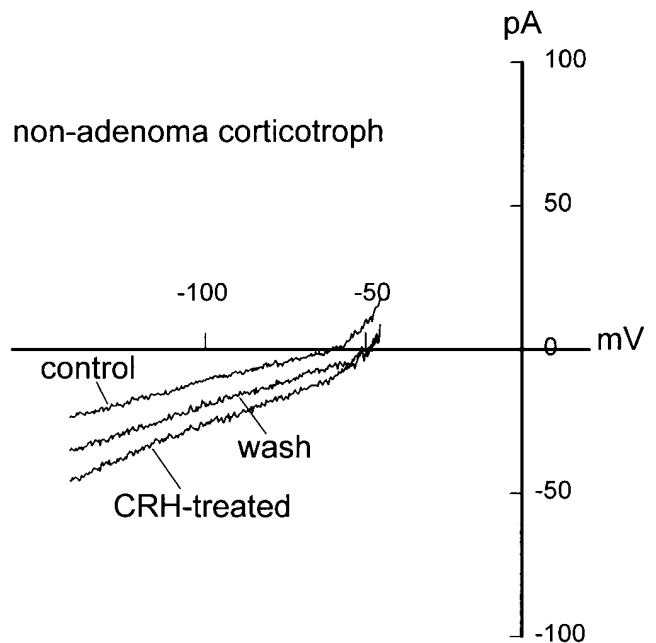


Figure 9. Effect of CRH on a nonadenoma cell. Resting potential was -58 mV. Application of CRH (10 nM) induced a current with reversal potential ~ 0 mV. Holding potential and ramp pulse protocols were the same as those in Fig. 3 A. Control, current record before the application of CRH; CRH-treated, current record after the application of CRH; and wash, current record after washing out CRH from the extracellular solution.

channel play an important role in the mechanism of CRH-induced ACTH secretion.

Activation of a nonselective cation current in nonadenoma corticotroph by CRH. To investigate whether the activation of the nonselective cation current was specific in adenoma cells or a common feature in corticotrophs, nonadenoma anterior pituitary cells were used. These cells were primary cultured from a small fragment of the nonadenoma anterior pituitary tissue that had to be resected to make approach to the adenoma during the transsphenoidal procedure (see *Cell preparation*, Methods). It was difficult to distinguish between corticotrophs and other cell types morphologically. Therefore, we randomly challenged the cells with CRH (10 nM) under voltage clamp. In 4 out of 28 cells, application of CRH activated a cation current. Fig. 9 shows representative current records evoked by a ramp test potential before and after the application of CRH. The reversal potential was close to 0 mV, suggesting that the current was a nonselective cation current. All the four cells were stained positive for human ACTH and only 1 of the 24 cells that did not respond to CRH were stained positive for human ACTH. These indicate that CRH activates a nonselective cation current in at least some of the nonadenoma corticotrophs.

Discussion

CRH is the major regulatory peptide of the neuroendocrine response to stress (2). It is secreted from hypothalamic CRH neurons and excites ACTH-secreting cells in the anterior pituitary. The mechanism of this excitation was investigated by using human ACTH-secreting adenoma cells in this study. These

adenoma cells were excited by the application of CRH and secreted ACTH *in vitro*. The excitation was caused by CRH-induced depolarization through the activation of a nonselective cation current. The I-V curve of this cation current showed slight inward rectification. This channel was permeable to other cations such as Li^+ and NH_4^+ , but was not permeable to TMA^+ . The inward current was abolished when the extracellular solution was Na^+ -free (TMA^+ -substituted) and the intracellular solution contained high K^+ . Because the cation current was permeable to both Na^+ and K^+ , the activation of the current in Na^+ -free (TMA^+ -substituted) extracellular solution with high K^+ intracellular solution was expected to induce an outward current. Interestingly, the expected outward current carried by K^+ was also not observed. This absence of the expected outward current may be explained by the inward rectification of the CRH-induced nonselective cation current. The activation of a nonselective cation current by CRH has not been reported in vertebrate cells, including human corticotrophs.

In several cells, including cardiac and pancreas acinar cells, nonselective cation channels are activated by the increase in $[\text{Ca}^{2+}]_i$ (15–17). Because CRH increased $[\text{Ca}^{2+}]_i$ in these adenoma cells, we speculated that CRH-induced activation of the cation channel was mediated by CRH-induced $[\text{Ca}^{2+}]_i$ increase. However, CRH-induced activation of the nonselective cation current was observed even when $[\text{Ca}^{2+}]_i$ was chelated lower than 50 nM by incubation with BAPTA/AM in Ca^{2+} -free extracellular solution. Most of the Ca^{2+} -activated cation currents are activated when the $[\text{Ca}^{2+}]_i$ was higher than 100 nM. Therefore, these data suggest that CRH-activated nonselective cation current is not a member of the Ca^{2+} -activated cation currents.

The activation of this current was mimicked by forskolin and 8Br-cAMP. Microinjection of PKA inhibitor peptide into the cell abolished the activation of the CRH-induced current. These data indicate that the mechanism of CRH-induced activation of the nonselective cation current was mediated by PKA. Modulation of ionic channels through PKA-mediated phosphorylation is reported for voltage-gated Ca^{2+} and K^+ channels in several types of cells (19, 20). In human growth hormone-secreting adenoma cells, GHRH excited the cells by activating a nonselective cation current through a PKA-mediated mechanism (21). The activation of the nonselective cation current may be a common mechanism of excitation in human anterior pituitary cells for agonists that stimulate cAMP production.

The excitation of the corticotrophs by CRH has been investigated in rat pituitary (22). There were obvious differences between rat corticotrophs and these human ACTH-secreting adenoma cells in the mechanism of CRH-induced excitation. (a) The excitation in rat corticotrophs are suggested to be due to the inhibition of K^+ conductance, and not by an increase in cation conductance. (b) The excitation by CRH was not completely blocked by H89 in rat corticotrophs, whereas it was almost abolished by H89 or PKI(5-24) in human ACTH-secreting adenoma cells. The differences in the mechanism of CRH-induced excitation in the ACTH-secreting cell may be due to the difference of species or the difference between normal cells and adenoma cells. The former may be the case because CRH also induced a cation current in nonadenoma human corticotrophs. The combination of the activation of a nonselective cation current and inhibition of K^+ conductance is a very commonly observed response to neurotransmitters and hormones and typically the two components differ in mag-

nitude among cells (23–25). It may simply be that the balance between these two pathways is different between rat and human corticotrophs.

CRH-induced depolarization was previously reported in human ACTH-secreting adenoma cells by Mollard et al. (26). In their report, application of CRH depolarized the membrane with an increase in input resistance. They speculated that the depolarization is due to the inhibition of K^+ conductance. In the adenoma cells in this article, the excitation was due to the activation of a nonselective cation current. The difference in the mechanism of excitation is very interesting because it suggests an unknown variety of mechanisms of tumorigenesis in human ACTH-secreting adenomas. However, the difference may be due to the difference of experimental technique. In the report of Mollard et al. (26), they employed an intracellular recording technique. In this report, we used the perforated whole-cell clamp. Further studies are needed to understand the relation between the CRH-induced inhibition of membrane conductance in their study and CRH-induced nonselective cation conductance revealed in our study.

In these ACTH-secreting cells, CRH-induced $[\text{Ca}^{2+}]_i$ increase was dependent on Ca^{2+} influx through voltage-gated Ca^{2+} channels. CRH-induced $[\text{Ca}^{2+}]_i$ increase was dependent on extracellular Na^+ and was not dependent on TTX-sensitive Na^+ channel activities. These indicate that the Na^+ -dependent nonselective cation current is important in CRH-induced $[\text{Ca}^{2+}]_i$ increase. The CRH-induced depolarization together with increased action potential frequency activates voltage-gated Ca^{2+} channels. The Ca^{2+} influx through voltage-gated Ca^{2+} channels increases $[\text{Ca}^{2+}]_i$ of these cells. As $[\text{Ca}^{2+}]_i$ is closely related to ACTH secretion rate in ACTH-secreting cells (27, 28), the CRH-induced $[\text{Ca}^{2+}]_i$ increase due to membrane depolarization stimulates ACTH secretion. Therefore, CRH-induced depolarization may play a major role in CRH-induced ACTH secretion. This speculation is supported by the following observations: (a) both the CRH-induced cation current (which causes depolarization) and CRH-induced ACTH secretion were abolished in Na^+ -free extracellular solution; (b) CRH-induced $[\text{Ca}^{2+}]_i$ increase was abolished in Na^+ -free extracellular solution; (c) both the CRH-induced ACTH secretion and CRH-induced $[\text{Ca}^{2+}]_i$ increase were not abolished by TTX. These indicate that extracellular Na^+ -dependent depolarization is crucially important in CRH-induced ACTH secretion. However, the significance of the CRH-induced nonselective cation current in CRH-induced ACTH secretion was not examined in nonadenoma corticotrophs because the amount of the nonadenoma tissue available was limited. This question needs further study to solve.

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