Bradykinin-activated transmembrane signals are coupled via \( N_o \) or \( N_i \) to production of inositol 1,4,5-trisphosphate, a second messenger in NG108-15 neuroblastoma–glioma hybrid cells

(GTP-binding proteins/pertussis toxin/adenylate cyclase/ion channels/phosphatidylinositol turnover)

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ABSTRACT The addition of bradykinin to NG108-15 cells results in a transient hyperpolarization followed by prolonged cell depolarization. Injection of inositol 1,4,5-trisphosphate or \( Ca^{2+} \) into the cytoplasm of NG108-15 cells also elicits cell hyperpolarization followed by depolarization. In contrast, injection of inositol 1,4,5-trisphosphate or \( Ca^{2+} \) into the cytosol did not alter \( M \) channel activity. Incubation of NG108-15 cells with pertussis toxin inhibits bradykinin-dependent cell hyperpolarization and depolarization. Bradykinin stimulates low \( K_m \) GTPase activity and inhibits adenylate cyclase in NG108-15 membrane preparations but not in membranes prepared from cells treated with pertussis toxin. Reconstitution of NG108-15 membranes from cells treated with pertussis toxin with nanomolar concentrations of a mixture of highly purified \( N_o \) and \( N_i \) [guanine nucleotide-binding proteins that have no known function (\( N_o \)) or inhibit adenylate cyclase (\( N_i \))] restores bradykinin-dependent activation of GTPase and inhibition of adenylate cyclase. These results show that [bradykinin-receptor complex] interacts with \( N_o \) or \( N_i \) and suggest that \( N_o \) and/or \( N_i \) mediate the transduction of signals from bradykinin receptors to phospholipase C and adenylate cyclase.

In this report we show that pertussis toxin partially inhibits the effects of BK on NG108-15 cells and that activation of BK receptors by BK results in activation of GTPase of \( N_i \) (11) or \( N_o \) (12) [guanine nucleotide-binding proteins that have no known function (\( N_o \)) or inhibit adenylate cyclase (\( N_i \))], which are substrates for pertussis toxin (13, 14). The results suggest that \( N_i \) or \( N_o \) are involved in signal transduction initiated by [BK-receptor] complexes that result in activation of phospholipase C and inhibition of adenylate cyclase.

METHODS AND MATERIALS

BK was obtained from Sigma or Calbiochem; \( \text{InsP}_3 \) and inositol 2-phosphate were obtained from Sigma; [D-Ala\(_2\),Met\(_4\)]enkephalinamide was from Calbiochem. Pertussis toxin was a gift from R. Sekura (National Institute of Child Health). \( 4\text{CaCl}_2 \) was obtained from Amersham, and \( \gamma\text{PIGTP} \) was from New England Nuclear.

NG108-15 cells used for electrophysiological studies were cultured in polyornithine-coated 35-mm Petri dishes and were treated with 10 \( \mu \text{M} \) prostaglandin \( E_1 \) and 1 \( \mu \text{M} \) theophylline for 1–3 weeks before use (15).

RESULTS

Cell Responses to BK, \( \text{InsP}_3 \), or \( Ca^{2+} \). Addition of BK by iontophoresis to the external surface of an NG108-15 cell resulted in cell membrane hyperpolarization followed by depolarization (Fig. 1A) (8). Injection of \( \text{InsP}_3 \) (Fig. 1B) or \( Ca^{2+} \) (Fig. 1D) into the cytoplasm of a cell also resulted in cell hyperpolarization followed by depolarization. Injection of inositol 2-phosphate into the cytoplasm had little or no effect on the cell membrane potential (Fig. 1C). These results suggest that the responses of NG108-15 cells to BK are dependent on an increase in cellular levels of \( \text{InsP}_3 \), apparently due to activation of the phospholipase C that catalyzes the formation of \( \text{InsP}_3 \) and diacylglycerol from phosphatidylinositol 4,5 bisphosphate followed by an \( \text{InsP}_3 \)-dependent release of stored \( Ca^{2+} \) into the cytoplasm.

The relation between the amount of iontophoretic current used for intracellular \( \text{InsP}_3 \) injection and the magnitude of the hyperpolarizing and depolarizing cell responses is shown in Fig. 2A. Both responses of cells increased as the quantity of \( \text{InsP}_3 \) iontophoretic currents (\( \mu \text{sec} \)) increased up to about 65 nC. With some cells, injection of \( \text{InsP}_3 \) with relatively high amounts of iontophoretic current, such as 220 nC, elicited a depolarizing cell response but little or no hyperpolarizing response (Fig. 2 B and C). The mean hyper-

Abbreviations: BK, bradykinin; \( \text{InsP}_3 \), inositol 1,4,5-trisphosphate; \( \text{Et}_{2}\text{NCl} \), tetraethylammonium chloride; \( N_i \) or \( N_o \), guanine nucleotide-binding proteins that couple some species of activated receptors with adenylate cyclase stimulation or inhibition, respectively; \( N_{\text{re}} \), an \( N \) protein of unknown function.
Fig. 1. Typical membrane potential changes of NG108-15 cells evoked by extracellular BK or intracellular injection of InsP$_3$ or Ca$^{2+}$. (A) Cell hyperpolarization followed by depolarization elicited by extracellular application of BK. The potential changes were recorded with an intracellular microelectrode filled with 3 M KCl. A second microelectrode filled with 0.1 mM BK dissolved in 0.1 mM HCl was located extracellularly close to the cell surface. BK was applied at zero time by iontophoresis (50 nA for 1 sec). (B) Cell hyperpolarizing and depolarizing responses induced by intracellular injection of InsP$_3$. The recording electrode (3 M KC1) and a micropipette filled with 1 mM InsP$_3$, dissolved in H$_2$O were inserted into an NG108-15 cell. At zero time +100 nA was passed through the InsP$_3$ pipette for 0.5 sec. (C) One mM inositol 2-phosphate dissolved in H$_2$O was injected intracellularly (~100 nA for 1 sec). (D) Ca$^{2+}$ was injected into the cytoplasm of an NG108-15 cell from a micropipette filled with a solution containing 0.5 M CaCl$_2$ by iontophoresis with 100 nA for 1 sec at zero time. The upward and/or downward deflections of the traces between 0 and 2 sec are due to the iontophoretic current rather than the compound applied.

Fig. 2. The relation between the amount of iontophoretic current used for intracellular InsP$_3$ injection and the magnitudes of the hyperpolarizing and depolarizing cell responses. (A) Typical examples of hyperpolarizing and depolarizing responses of NG108-15 cells evoked by intracellular injection of InsP$_3$ for 2 sec; the amount of current in nC is indicated in each panel. (B) An example of InsP$_3$ intracellular injection by iontophoresis using a large amount of current (~220 nC), which elicited primarily cell depolarization. The discontinuities in the trace at approximately 10–15 sec suggest some cell hyperpolarization. At 30–60 sec passive hyperpolarizing currents were injected to test input membrane resistance. (C) The mean changes in membrane potential during the hyperpolarizing and depolarizing phases of NG108-15 cell responses to intracellular injection of InsP$_3$, with different amounts of iontophoretic current (nC). Each point represents the mean of values obtained from 5–19 cells; each cell was used for only one determination. The hyperpolarization and depolarization values shown are the maximum membrane potential deflections at 5–20 and 30–45 sec, respectively, after injection of InsP$_3$.
FIG. 3. Effects of Et4N+ (A and C) or Co2+ (B and D) on the responses of NG108-15 cells to extracellular BK (A and B) or intracellular InsP3 (C and D). (A) NG108-15 cells were incubated for 20–30 min in a 35-mm Petri dish containing 2 ml of Dulbecco's modified Eagle's medium supplemented with 5 mM Et4N+. At zero time 2 μl of a solution containing 10 μM BK dissolved in 150 mM NaCl was added to the surface of the medium close to the cell being tested. Action potentials were evoked by passing depolarizing pulses of current (0.2 nA for 60 msec) through the intracellular recording microelectrode. (B) NG108-15 cells were incubated in 2 mM Co2+/20 mM Tris-HCl, pH 7.2/150 mM NaCl/5.4 mM KCl/0.8 mM MgCl2/1.8 mM CaCl2/20 mM glucose for approximately 10–20 min. Hyperpolarizing currents (0.5 nA for 60 msec) were passed through the intracellular recording microelectrode but failed to evoke offspikes. BK was applied at zero time in a 2-μl drop as described above. (C) NG108-15 cells were incubated in the presence of 5 mM Et4N+ as described above. InsP3 (0.1 mM) was injected into the cytoplasm by iontophoresis (-100 nA for 1 sec). (D) Cells were incubated in the presence of 2 mM Co2+ in the medium as described above for 10–20 min. InsP3 was injected intracellularly at zero time (-200 nA for 1 sec).

BK-Dependent Efflux of 45Ca2+. As reported (8), BK stimulates 45Ca2+ influx into NG108-15 cells. The effect of BK on 45Ca2+ efflux from NG108-15 cells is shown in Fig. 5. Cells were incubated in the presence of 1.8 mM 45CaCl2 for 8 min to promote 45Ca2+ uptake and washed for 3 min, and then dishes were perfused with control medium or medium with BK. BK stimulated the rate of 45Ca2+ efflux approximately 3-fold during the first 20 sec, but by 80 sec the rate of 45Ca2+ efflux had returned to the control value.

Effects of Pertussis Toxin on Cell Responses to BK. Both the hyperpolarizing and depolarizing responses of NG108-15 cells to BK were inhibited by treatment of the cells with pertussis toxin (200 ng per ml, 15 hr) (Fig. 6). The maximum inhibition by pertussis toxin was obtained with 1 μM BK; higher concentrations of BK resulted in less inhibition by pertussis toxin. The maximum inhibition of hyperpolarization (72%) was obtained with 1000 ng of pertussis toxin per ml, the highest concentration tested; 50% of the maximum observed inhibition was obtained with 35–100 ng of pertussis toxin per ml of medium. These results suggest that GTP-binding regulatory proteins such as N4 (13, 14) or N9 (12) mediate the hyperpolarizing cell response to BK. The results also are consistent with the suggestion that pertussis toxin acts primarily to decrease receptor affinity for ligands (17).

Because all known GTP-binding regulatory proteins also display hormone-stimulated GTPase activities, we measured the effect of BK on GTPase activity of NG108-15 cell membranes. The data shown in Table 1 demonstrate that BK or the opioid peptide [D-Ala2, Met5]enkephalinamide stimulated the activity of a low Km GTPase in NG108-15 membranes and that the stimulatory effects of BK or [D-
NG108-15 cells by the addition of a mixture of bovine brain Nα and Nβ (approximately 70% Nα and 30% Nβ) estimated to be >95% pure for 15 hr at 30°C in the standard assay mixture (18) without radioactive GTP and then were incubated for an additional 15 min in the presence of approximately 60,000 cpm (1 nM) [γ-32P]GTP. *P* released in the absence of BK or [D-Ala2, Met5]enkephalinamide has been subtracted. Mean values (three experiments) are shown. (B) Low K<sub>m</sub> GTPase activity of membranes prepared from untreated NG108-15 cells is shown as a function of BK concentration. () BK with 10 μM [D-Ala2, Met5]enkephalinamide; () BK without [D-Ala2, Met5]enkephalinamide. The ordinate scale is pmols of P<sub>i</sub> released per min/mg of protein. (C) Adenylate cyclase activity of membranes prepared from untreated NG108-15 cells, assayed as described (20), is shown as a function of BK concentration in the absence () or presence () of 10 μM [D-Ala2, Met5]enkephalinamide.

**DISCUSSION**

Exposure of NG108-15 cells to BK elicits increases in InsP<sub>3</sub> and diacylglycerol levels (8); in turn, InsP<sub>3</sub> stimulates the release of stored Ca<sup>2+</sup> into the cytoplasm (10), thereby activating Ca<sup>2+</sup>-dependent K<sup>+</sup> channels and increasing the rate of K<sup>+</sup> efflux. Cell hyperpolarization results from the increase in K<sup>+</sup> efflux.

The observations that support these conclusions are: (i) injection of InsP<sub>3</sub> or Ca<sup>2+</sup> into the cytoplasm of NG108-15 cells results in cell hyperpolarization followed by depolarization; (ii) hyperpolarization of cells elicited by BK, InsP<sub>3</sub>, or Ca<sup>2+</sup> is accompanied by a decrease in membrane resistance—i.e., an increase in cell permeability to ions; (iii) the reversal potential for BK-dependent hyperpolarization is approximately −80 mV, which is close to the equilibrium potential for K<sup>+</sup>; (iv) the amplitudes of the hyperpolarizing responses of 108CC-25 (23) and NG108-15 cells (unpublished results) to BK are dependent on the concentration of extracellular K<sup>+</sup>; and (v) BK-dependent hyperpolarization is blocked by Et4N<sup>+</sup>, which is known to inhibit some Ca<sup>2+</sup>-dependent K<sup>+</sup> channels (10).

The long-lasting depolarization evoked by BK is associated with an increase in membrane resistance—i.e., cell permeability to ions decreases. Other species of receptors have been shown to mediate cell depolarization by decreasing cell permeability, such as muscarinic acetylcholine receptors (24) and receptors for luteinizing hormone-releasing hormone (25–27), substance P (28, 29), thyrotropin-releasing hormone (30, 31), neurotensin (32), somatostatin (33), angiotensin (34), prostaglandin D<sub>2</sub> (35), and the small cardioactive
peptide (36). Cell depolarization mediated by muscarinic acetylcholine receptors has been attributed to inhibition of M channels for K⁺, which decreases K⁺ efflux from cells (24). Recent results obtained with voltage-clamp conditions show that NG108-15 cells have M channels that are inhibited by BK (Higashida and Brown, unpublished data).

In contrast, injection of InsP₃ or Ca²⁺ into the cytoplasm of NG108-15 cells results in cell hyperpolarization followed by a long-lasting depolarization that is accompanied by a decrease in membrane resistance—i.e., an increase in cell permeability to ions. Injection of InsP₃ or Ca²⁺ into the cytoplasm did not affect the activity of M channels; therefore, elevation of cytosolic levels of InsP₃ or Ca²⁺ elicited most, but not all, of the cell responses to extracellular BK. Because BK stimulates the formation of InsP₃ in NG108-15 cells, part of the BK-induced depolarization probably also is due to an increase in cell permeability to ions. The most likely candidates for the ion channels that are activated during the depolarizing phase are the Ca²⁺-dependent cation channel (37) and a Ca²⁺ channel.

We find that BK-dependent cell hyperpolarization and depolarization are inhibited by pertussis toxin, which suggests that a GTP-binding regulatory protein such as Nₐ or Nᵢ plays a role in the signal transduction process. BK was shown to stimulate a low Kᵦᵣ GDPase and to inhibit adenylate cyclase in NG108-15 membrane preparations, and these effects of BK were blocked by pertussis toxin. Reconstitution of membranes from pertussis toxin-treated NG108-15 cells with nanomolar concentrations of a mixture of highly purified bovine brain Nₐ and Nᵢ restored BK-dependent activation of low Kᵦᵣ GDPase and inhibition of adenylate cyclase. BK- and [D-Ala²,Met⁵]enkephalinamide-dependent inhibitions of adenylate cyclase were additive, whereas norepinephrine- and [D-Ala²,Met⁵]enkephalinamide-dependent inhibitions were not additive, which suggest that BK and opiates inhibit adenylate cyclase by different mechanisms. Because the [βγ] subunits of the N proteins are functionally interchangeable (38), activation of any N protein, with dissociation of [βγ] complexes to α and [βγ], will result in inhibition of adenylate cyclase by mass action, since the released [βγ] subunits will combine with the free α subunit of activated Nᵢ, the N protein that stimulates adenylate cyclase. In addition, our results suggest that Nₐ or Nᵢ are involved in the transduction of signals from BK receptors to Ca²⁺ mobilization, presumably via activation of phospholipase C, as has been suggested for several other species of receptors (39–42).