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Pertussis toxin-insensitive G protein mediates substance P-induced inhibition of potassium channels in brain neurons

(inward rectification/cholinergic neurons/nucleus basalis/brain neuron culture/guanosine 5′-[γ-thio]triphosphate)

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ABSTRACT **Substance P excites neurons by suppressing inward rectification channels. We have investigated whether the substance P receptor interacts with the inward rectification channels through a guanine nucleotide-binding protein (G protein) by using dissociated cultured neurons from the nucleus basalis of newborn rats. During intracellular application of guanosine 5′-[γ-thio]triphosphate and 5′-guanylyl imidodiphosphate, hydrolysis-resistant GTP analogues that irreversibly stimulate G proteins, substance P application almost irreversibly suppressed the inward rectification channels. Pretreatment with pertussis toxin did not significantly influence substance P action. Intracellular application of cAMP and 3-isobutyl-1-methylxanthine or of 9-(tetrahydro-2-furyl)adenine (SQ 22,536), an inhibitor of adenylate cyclase, did not alter the substance P-induced response. We conclude that the inhibition of inward rectification channels by substance P is mediated through a G protein. However, the effect is not mediated through adenylate cyclase or the cAMP system. This G protein, which is insensitive to pertussis toxin, could be an unidentified G protein.**

The nucleus basalis of Meynert of the forebrain is one of the major sources of acetylcholine in the brain and plays a crucial role in cognition; its disfunction leads to the development of Alzheimer disease (1). Recently we found that substance P produces excitation in cultured cholinergic neurons from the nucleus basalis (2) and that this substance P effect is largely caused by an inhibition of the inwardly rectifying K^+ channels (3). In agreement with these physiological findings, Bolam *et al*. (4) and Beach *et al*. (5) reported that nucleus basalis neurons are innervated by substance P-containing nerve terminals. Thus, substance P may play an important role in regulating activity of the cholinergic neurons of the nucleus basalis. The present work was designed to further examine the mechanism of the substance P effect on these neurons.

Certain transmitters exert their inhibitory effects by enhancing the activity of resting K^+ channels, and there is now strong evidence that a guanine nucleotide-binding protein (G protein) mediates the signal transduction of these inhibitory effects (6–11). On the other hand, some neurotransmitters, including substance P, produce an excitatory influence by suppressing the resting K^+ conductance. However, almost nothing is known about the role of G proteins in the link between these excitatory receptors and the K^+ channels. We now present data demonstrating that substance P receptors interact with the inwardly rectifying K^+ channels through a G protein; this G protein is resistant to pertussis toxin.

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MATERIALS AND METHODS

Culture Methods. The method of culturing neurons from the nucleus basalis was the same as that previously described (2), except for the following modifications. The culture medium contained 5% heat-inactivated rat serum (prepared in our laboratory) plus 10% heat-inactivated horse serum (GIBCO). Neurons were dissociated by using papain (12–20 units/ml) (12, 13) instead of trypsin. Newborn rats [2- to 4-day-old Long–Evans rats (Charles River Breeding Laboratories)] were used. Experiments were conducted on neurons cultured for 9–12 days.

Electrophysiology. The techniques were similar to those used in Stanfield *et al*. (3). We used the whole-cell version of the patch clamp technique. During the experiments the culture was superfused continuously with an oxygenated Krebs' solution containing 146 mM NaCl, 5 mM KCl, 2.4 mM $CaCl_2$, 1.3 mM $MgCl_2$, 5 mM Hepes-NaOH buffer, 11 mM glucose, and 1 μM tetrodotoxin (pH 7.4). In standard experiments the patch pipette was filled with an internal solution containing 120 mM potassium aspartate, 40 mM NaCl, 5 mM Hepes-KOH buffer, 0.5 mM EGTA-KOH, 0.25 mM $CaCl_2$, 3 mM $MgCl_2$, 2 mM Na_2ATP, 100 μM Na_3GTP (sometimes this component was excluded), and ≈8 mM KOH (pH 7.2). When a hydrolysis-resistant GTP analogue or a GDP analogue was added, GTP was omitted from the internal solution. The values of membrane potentials were corrected for a 9-mV liquid junction potential. Substance P was applied by pressure ejection from a glass pipette with tip diameter of 4–5 μm, placed ≈40 μm from the perimeter of the soma. Bath temperature was kept at 33–36°C (mean, 34°C).

Pertussis Toxin Treatment. The pretreatment with pertussis toxin was done according to procedures described by Holz *et al*. (14). Our pertussis toxin solution contained 14 μg of toxin per ml of 10 mM sodium phosphate buffer (pH 7.2)/50 mM NaCl/0.04% heat-inactivated bovin serum albumin. This solution was then added to the culture until the final toxin concentration was 500 ng/ml. Freshly dissolved pertussis toxin was used for each experiment. Before the toxin was added, culture medium was replaced with solution that had the same composition as the culture medium, except that it was devoid of serum, penicillin-streptomycin, and ascorbic acid. The toxin-treated cultures were incubated at 37°C for 15–22 hr. Control cultures were treated exactly the same as

Abbreviations: GTP[γS], guanosine 5′-[γ-thio]triphosphate; GDP-[βS], guanosine 5′-*O*-[β-thio]diphosphate; iBuMeXan, 3-isobutyl-1-methylxanthine; p[NH]ppG, 5′-guanylyl imidodiphosphate.

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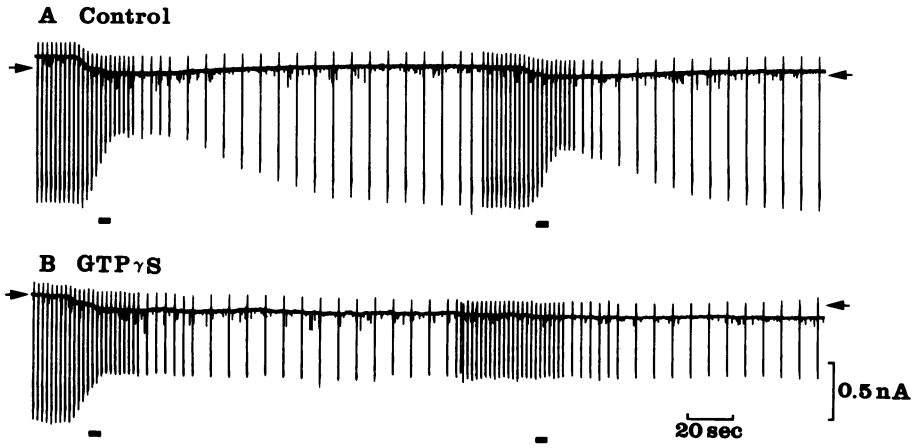

FIG. 1. Substance P-induced response persists in the presence of GTP[γS] in cultured nucleus basalis neurons. The whole-cell clamp was used for recording membrane currents. Hyperpolarizing rectangular-wave pulses (50 mV, 100 msec) were intermittently imposed. Substance P (300 nM) was pressure ejected during the time indicated by horizontal bars. Before application the drug pipette was kept in the air to avoid contaminating the superfusing solution with the drug. When the pipette approached the neuron, there appeared to be leakage of substance P before pressure was applied. The puff pipette was withdrawn from the bath into air as soon as ejection was complete. The interval between first and second applications of substance P was ≈3 min. Arrows indicate the zero current levels. In both *A* and *B*, records were taken 5 min after rupture of the patch. (*A*) Control neuron. Patch pipette contained 100 μM GTP. Holding potential, −74 mV. (*B*) Patch pipette contained 100 μM GTP[γS]. Holding potential, −74 mV. Small noise-like deflections on the base line are short-lasting inward currents of unknown origins; they could be spontaneously occurring Ca^{2+} spikes in dendrites.

the toxin-treated cultures, except pertussis toxin was omitted.

Sources of Chemicals. Suppliers are indicated in parentheses: papain [Cooper Biochemicals (Malvern, PA) or Pharmacia]; substance P (Peninsula Laboratories, San Carlos, CA); guanosine 5′-[γ-thio]triphosphate (GTP[γS]) and guanosine 5′-*O*-[β-thio]diphosphate (GDP[βS]) (Boehringer Mannheim); 5′-guanylyl imidodiphosphate (p[NH]ppG), 3-isobutyl-1-methylxanthine (iBuMeXan), and cAMP (Sigma); 9-(tetrahydro-2-furyl)adenine (SQ 22,536; gift of Squibb Institute); pertussis toxin (List Biological Labs, Campbell, PA); bovine serum albumin (fraction V; Calbiochem).

RESULTS

The average soma diameter of the cultured neurons was 24 μm. In a previous paper we had shown that such large cultured neurons from nucleus basalis are almost always cholinergic (2). Application of substance P to these cholinergic neurons depolarizes them in most cases (2).

Hydrolysis-Resistant GTP Analogues. When agonists bind to receptors that interact with G proteins, the G protein binds to GTP and becomes activated. The activation of the G protein ends when GTP is hydrolyzed by the G protein. In the presence of a nonhydrolyzable GTP analogue instead of GTP, the G protein will be irreversibly activated (6, 8, 10, 14, 15).

Fig. 1 shows the effects of a nonhydrolyzable GTP analogue GTP[γS] on the substance P response. Fig. 1*A* is control, in which the patch pipette contained the standard solution with 100 μM GTP, whereas in *B* the pipette solution contained 100 μM GTP[γS]. In both *A* and *B*, we started recording 5 min after rupture of the patch. We assumed that 5 min was long enough for the solution in the pipette to exchange with the cytoplasm.

The membrane potential was held at −74 mV under the voltage clamp condition, and recurring hyperpolarizing (50 mV, 100 msec) rectangular pulses were imposed. With the slow time base of Fig. 1, current pulses appear as brief spikes superimposed on the base-line currents. In the control cell (*A*), the application of substance P (300 nM) produced a considerable decline in the amplitude of the current pulses, reflecting a decrease in the conductance. The conductance then slowly increased, returning to the original level in about 2 min. Concomitant with the change in conductance, the base-line current level changed: the reduction of conductance was accompanied by an inwardly directed shift in the base-line current (≈150 pA), and the slow return of conductance coincides with the recovery of the base-line current.

Both the inward shift of the base-line current and the decrease in conductance are caused by a suppression of the inwardly rectifying K^+ conductance by substance P (3). The holding potential (−74 mV) is above the K^+ equilibrium

Table 1. Effect of GTP analogues on the substance P-induced response

GTP analogue in the patch pipette	*n*	First substance P-sensitive conductance, nS	Recovery from substance P effect, %	Second substance P-sensitive conductance, nS
Control with 100 μM GTP	6	6.0 ± 0.4	88 ± 8	4.0 ± 1.0
Control without GTP	3	6.7 ± 1.0	92 ± 4	4.4 ± 0.4
100 μM GTP[γS]	6	6.5 ± 1.0	2 ± 6	0.5 ± 0.2
350 μM GTP[γS]	2	12.3 ± 0.8	4 ± 4	0.0 ± 0.0
350 μM p[NH]ppG	3	7.3 ± 0.5	25 ± 16	1.6 ± 0.4

Values are mean ± SEM. Experimental protocol was as shown in Fig. 1*A*. Five minutes after rupture of the patch, the first application of substance P (300 nM) was given, followed 3 min later by the second application of substance P. Membrane conductance was measured with hyperpolarizing rectangular-wave pulses (50 mV, 100 msec); holding potential was always −74 mV. The conductance values were normalized to a standard-sized neuron (soma diameter, 24 μm). The substance P-sensitive conductance was obtained by subtracting the smallest conductance seen during the application of substance P from the conductance measured just before its application. Recovery from application of substance P was defined as the ratio X/Y; X was obtained by subtracting the smallest conductance measured during the first substance P application from the membrane conductance measured just before the second substance P application, and Y was the maximal substance P-sensitive conductance during the first substance P application. Nucleus basalis neurons were used.

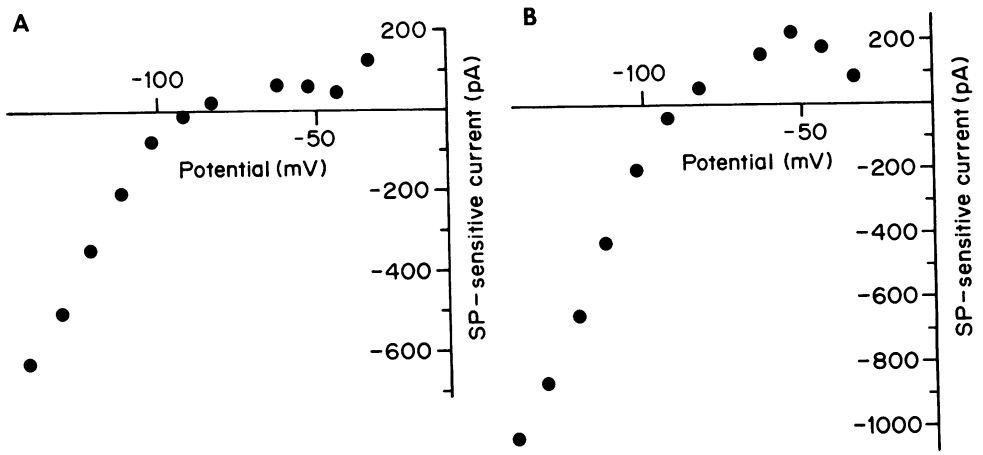

FIG. 2. Current–voltage relations in nucleus basalis neurons. The current plotted is the substance P (300 nM)-sensitive current, measured by subtracting current during application of substance P from current before application of substance P. (*A*) Control neuron. The patch pipette contained 100 μM GTP. (*B*) Patch pipette contained 100 μM GTP[γS].

potential (−85 mV), and hence when the K^+ conductance is decreased, less outward current is needed to bring the membrane to the holding potential, as indicated by the inward shift of the base-line current.

The second application of substance P, which followed the first application by 3 min, produced a response similar to the first one, except that the magnitude was slightly smaller, perhaps due to desensitization.

Fig. 1*B* shows an experiment in which the intrapipette solution contained 100 μM GTP[γS] instead of GTP. The intracellular application of GTP[γS] alone did not produce a noticeable change in membrane conductance. Application of substance P to this cell, however, produced an almost irreversible reduction in membrane conductance and an irreversible inward shift of the base-line current. The second application of substance P failed to produce any further change in membrane conductance or the base-line current.

The data of Fig. 1 can be quantified in the following way: in the control cell there was an almost complete recovery from the reduction of conductance (93% recovery) after 3 min of the first substance P application, and the second application produced a conductance change that is 74% of the first one. In the GTP[γS]-containing cell the conductance did not recover at all (−7.5% recovery), and the second application produced hardly any conductance change (0.4% of the first conductance change).

Table 1 summarizes results of the GTP[γS] experiments. The control neurons clearly differ from the GTP[γS]-containing neurons. We used two kinds of controls, one with 100 μM GTP and the other without GTP in the patch pipette; no difference seemed to exist between these two control groups. In both control groups, recovery from the first substance P effect was almost complete (88% or 92%). Even when no GTP is present in the patch pipette, local concentration of GTP remaining near the membrane is probably sufficient for G proteins to function (16). On the other hand, hardly any recovery occurred in the neurons infused with either 100 μM or 350 μM GTP[γS] (2% or 4% recovery). We also used another hydrolysis-resistant GTP analogue, 5'-guanylyl imidodiphosphate (p[NH]ppG), which had an effect similar to that of GTP[γS], although not as strong as the latter (Table 1). This result agrees with the fact that the affinity to G proteins (G_s or G_i) of p[NH]ppG is less than that of GTP[γS] (15, 17). We tested the effect of a GDP derivative, GDP[βS], which prevents the reaction of G proteins with GTP (18). However, the intracellular application of GDP[βS], even at 500 μM, did not produce significant inhibitory effects on substance P-sensitive conductance (data not shown). This result could relate to the fact that GDP[βS] has a smaller affinity to G proteins than has GDP or GTP (19).

Fig. 2 shows that substance P suppresses the inwardly rectifying channels, in agreement with the data of Stanfield *et al.* (3). Rectangular-wave pulses of various voltages were applied both before the application of substance P and while substance P effect was at its peak. Subtraction of the two sets of record yields the substance P-sensitive current. This difference represents the ionic conductance affected by substance P. In Fig. 2 we plot substance P-sensitive current as a function of membrane potential in a control neuron (*A*) and in a GTP[γS]-containing neuron (*B*). In both neurons the substance P-sensitive current reveals inwardly directed rectification, with a reversal potential close to the potassium

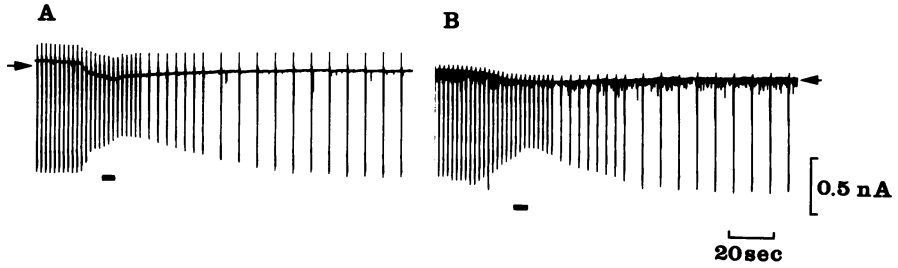

FIG. 3. Pertussis toxin had little or no effect on substance P response. Nucleus basalis neurons cultured for 10 days and whole-cell recording were used. See Fig. 1 legend for the general protocols. Arrows indicate the zero current levels. Substance P (300 nM) was pressure ejected during the time indicated by horizontal bars. Hyperpolarizing rectangular-wave pulses (50 mV, 100 msec) were intermittently imposed. (*A*) Control neuron. Substance P application produced a reduction in conductance and an inward shift of the base-line current. Holding potential, −74 mV. (*B*) Pretreated with pertussis toxin (500 ng/ml) for 18 hr. Substance P produced a response similar to that of the control neuron. Holding potential, −74 mV.

Table 2. Pertussis toxin pretreatment

	n	Membrane conductance before substance P application, nS	Substance P-sensitive conductance, nS	Inward current, pA
Control	13	16.3 ± 1.3	6.2 ± 0.7	94 ± 16
Pertussis toxin-treated	13	21.0 ± 3.1	5.4 ± 0.7	62 ± 5

Values are mean ± SEM. The membrane conductance was measured by imposing hyperpolarizing rectangular-wave pulses (50 mV, 100 msec) from a holding potential of −74 mV, and was normalized to the standard-sized cell of 24-μm soma diameter. Value of the inward current represents the maximal magnitude of the inwardly directed shift of the base-line current. Nucleus basalis neurons were used.

equilibrium potential. The conclusion from the experiments of Figs. 1 and 2 is that in the presence of GTP[γS], substance P produces an almost irreversible suppression of the inward rectification channels.

Pertussis Toxin. Certain kinds of G proteins (G_i, G_o, and transducin) are ADP-ribosylated by pertussis toxin and lose their ability to become activated (20). We pretreated our cultures with pertussis toxin (500 ng/ml) for 15–22 hr. As shown in Fig. 3, neurons pretreated with pertussis toxin (*B*) can still respond to substance P. Table 2 summarizes results from three different cultures. Substance P-sensitive conductance does not differ significantly between control and pertussis toxin-treated neurons. The maximal inward current (inwardly directed shift of base-line current) produced by substance P was 94 pA in control and 62 pA in the toxin-treated neurons. The difference is not significant at the 95% level but is significant at the 90% level. There were three pertussis toxin-treated neurons (out of 26) that showed an unusual response; a very large inward current (>200 pA) was produced by substance P not coincident with the time of maximal conductance decrease. Because inward currents and the decrease in membrane conductance occurred almost coincidentally in other substance P-applied cases, the above-mentioned result suggests that these inward currents were not caused by decrease in the inward rectification channels. If such unusual cells are omitted, we find no significant difference (even at the 90% level) between the control and the toxin-treated group in magnitude of the inward current.

The virtual ineffectiveness of pertussis toxin on substance P-induced responses raises the question of whether procedures used for pertussis toxin treatment were effective. However, using an identical procedure for pertussis toxin treatment, we found that somatostatin-induced hyperpolarization was totally abolished in locus coeruleus neurons (10). To verify the effectiveness of pertussis toxin treatment in the present experiments, we conducted pertussis toxin experiments on cultured noradrenergic neurons from the locus coeruleus, neurons known to respond to both substance P and somatostatin (21).

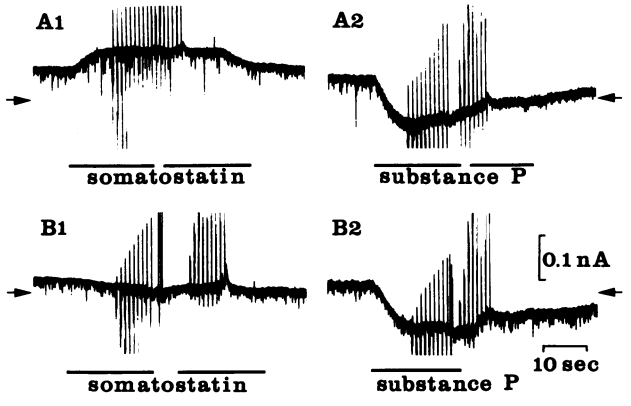

FIG. 4. Substance P effects were preserved, but somatostatin effects were abolished by pertussis toxin treatment. Locus coeruleus neurons cultured for 35 days from 3-day-old postnatal rats were used. Whole-cell recording was used. Substance P (300 nM) or somatostatin (100 nM) was pressure ejected during the time indicated by horizontal bars. Arrows indicate zero current levels. (*A 1* and *2*) Control neuron. The neuron responded to somatostatin with an outward current (*A1*) and to substance P with an inward current (*A2*). Holding potential, −80 mV. (*B 1* and *2*) Pretreated with pertussis toxin (500 ng/ml) for 19 hr. The effect of somatostatin was abolished (*B1*), but the effect of substance P remained intact (*B2*). Holding potential, −80 mV. The spike-like current pulses are artifacts from injecting voltage pulses.

Fig. 4 shows experiments in which somatostatin and substance P were applied to single noradrenergic neurons from the locus coeruleus. Under voltage-clamp conditions, application of somatostatin produced an outward current, and application of substance P generated an inward current in a control neuron (*A1* and *A2*). On the other hand, in a pertussis toxin-treated neuron (*B1* and *B2*), the response to somatostatin was almost totally abolished, whereas the same neuron responded with an inward current of a normal magnitude (103 pA) to substance P. We conducted eight such experiments on locus coeruleus neurons (five pertussis toxin-treated neurons and three control neurons) with similar results. We conclude that the substance P-induced response is not significantly suppressed by pertussis toxin.

Experiments with cAMP and Adenylate Cyclase. There is a possibility that the so-called stimulatory G protein (G_s) mediates the effect of substance P. G_s is known to stimulate the activity of adenylate cyclase, thereby enhancing the production of cAMP. We examined the effect of substance P while the intracellular concentration of cAMP was greatly increased. This was done by including 100 μM cAMP and 1 mM iBuMeXan in the patch pipette (22). iBuMeXan inhibits phosphodiesterase and would therefore prevent the breakdown of cAMP. We found that under this condition of increased cAMP concentration, the substance P-induced response was normal (Fig. 5); the average substance P-sensitive conductance in five cells was 6.4 ± 1.8 nS (mean ± SEM), a value that is not significantly different from the control value (Table 1). We also applied SQ 22,536 intracellularly. This chemical is known to suppress the activity of adenylate cyclase in mammalian platelets or sympathetic ganglia (23, 24). The substance P-sensitive conductance in SQ

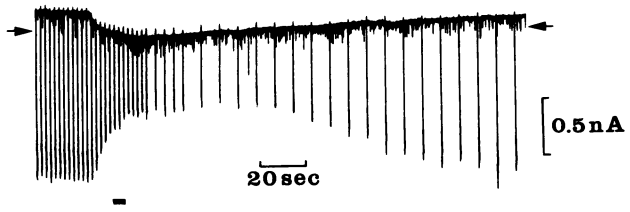

FIG. 5. Effects of increasing intracellular concentration of cAMP in a nucleus basalis neuron. The patch pipette contained 100 μM cAMP and 1 mM iBuMeXan. Records were obtained 5 min after breaking the patch. Hyperpolarizing rectangular-wave pulses (50 mV, 100 msec) were intermittently imposed. See Fig. 1 legend for the general protocol. Substance P (300 nM), pressure ejected during the time indicated by the horizontal bar, produced an inward current and a decrease in conductance. Holding potential, −74 mV.

22,536-treated neurons was 9.9 ± 2.4 nS (mean ± SEM, n = 4), compared with that of the control neurons from the same series of culture, which was 10.5 ± 2.6 nS (mean ± SEM, n = 6). These results indicate that adenylate cyclase or cAMP is probably not involved in the substance P-induced inhibition of K^+ currents.

DISCUSSION

Substance P enhances neuronal excitability partly by inhibiting the inwardly rectifying K^+ channels (3). The present investigation has shown that a G protein couples the substance P signal to the inward rectification channels. This G protein is insensitive to pertussis toxin. Thus, we can rule out the possibility that G_i, G_o, G_k, or other recently discovered pertussis toxin-sensitive G proteins (25–27) mediate the substance P effect. One possible candidate is G_s (stimulatory G protein), which is known to be insensitive to pertussis toxin. However, the only function of G_s so far known is to enhance the activity of adenylate cyclase, resulting in an increase in cAMP, which in turn modulates certain types of K^+ or Ca^{2+} channels in some cells (28, 29). In the present work we could not find any evidence that cAMP is an integral part of the signal transduction system for substance P effects on the K^+ channels. Nevertheless, our data do not exclude the possibility that G_s has other functions, such as acting directly on the K^+ channels.

Possibly we are dealing with an unidentified G protein. A G protein has been recently reported to couple substance P receptors to phospholipase C in the rat parotid gland (30). G proteins that couple receptors to phospholipase C or inositol phospholipid metabolism are called G_ps (31). Some of these G_ps are sensitive to pertussis toxin (32), whereas some are not (32–35). Wakelam *et al.* (36) reported that normal N-*ras*-encoded p21 may correspond to the putative G_p. One of these G_ps could well mediate the substance P action on K^+ channels.

Recently inhibitory actions of several transmitter substances, such as acetylcholine, γ-aminobutyric acid (GABA), or somatostatin, have been shown to be mediated through a pertussis toxin-sensitive G protein (G_i or G_k) (6–11). In these cases, the G protein (or its α subunit) most likely directly enhances the K^+ channel activity (11). On the other hand, a family of neurotransmitters, including substance P, produces an excitatory action by suppressing the resting K^+ conductance; however, little is known about the role of G proteins on this excitatory action. The present report demonstrates that this excitatory action, which is brought about by suppressing the inward rectification channels, is also mediated by a G protein. Although superficially there appears to be a mirror image between the inhibitory and excitatory actions on the K^+ channels mediated by the two different G proteins, the mechanism of action could be totally different. The unidentified G protein quite possibly does not act directly on the K^+ channels (unlike the inhibitory G protein), but acts on the K^+ channels through a third messenger, such as the polyphosphoinositide system.

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