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Mechanism of inhibition of calcium channels in rat nucleus tractus solitarius by neurotransmitters

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1 High-threshold Ca^{2+} channel currents were measured every 15 s following a 200 ms voltage step from −80 mV to 0 mV in order to study the coupling mechanism between neurotransmitter receptors and Ca^{2+} channels in neurones acutely isolated from the nucleus tractus solitarius (NTS) of the rat.

2 Application of 30 μM baclofen ($GABA_B$ receptor agonist) caused 38.9±1.2% inhibition of the peak inward Ba^{2+} current ($I_{Ba^{2+}}$) in most NTS cells tested (n=85 of 88). Somatostatin, 300 nM, also reduced $I_{Ba^{2+}}$ by 31.3±1.6% in 53 cells of 82 tested.

3 Activation of μ-opioid-, $GABA_B$- or somatostatin-receptors inhibited both N- and P/Q-type Ca^{2+} channels.

4 The inhibition of Ca^{2+} currents by DAMGO (μ-opioid receptor agonist), baclofen and somatostatin was reduced by treatment with pertussis toxin and partially relieved by application of a 50 ms conditioning prepulse to +80 mV. This suggests that a pertussis toxin-sensitive G-protein was involved in the neurotransmitter-mediated action in the observed inhibition of Ca^{2+} currents.

5 Intracellular loading with an antiserum raised against the amino terminus of $G_{o\alpha}$ (GC/2) markedly attenuated the somatostatin-induced inhibition, but did not block the DAMGO- and baclofen-induced inhibition.

6 These findings suggest at least two different pertussis toxin-sensitive G-protein-mediated pathways are involved in receptor-induced inhibition of Ca^{2+} currents in the NTS.

Keywords: μ-Opioid receptor; $GABA_B$ receptor; somatostatin; pertussis toxin; G-protein; brainstem; autonomic nervous system

Introduction

It is now clear that multiple parallel pathways can regulate voltage-dependent Ca^{2+} channels (see review; Hille, 1994). For example, Beech *et al.* (1992) suggested that there are two rapid routes for Ca^{2+} channel inhibition that differ in their sensitivity to pertussis toxin and in their voltage-dependence. Other examples of pertussis toxin-insensitive pathways inhibiting Ca^{2+} channels are bradykinin-induced inhibition in NG108-15 cells (Taussig *et al.*, 1992; Wilk-Blaszczak *et al.*, 1994), and muscarinic M_1 agonists (Mathie *et al.*, 1992; Shapiro *et al.*, 1994b), angiotensin II (Shapiro *et al.*, 1994a) and substance P (Shapiro & Hille, 1993) inhibiting Ca^{2+} channels in rat superior cervical ganglion neurones. However, the most common form of modulation of Ca^{2+} channels depends on the activation of a pertussis toxin-sensitive G-protein. Indeed, the involvement of G-proteins in this pathway is universally accepted. Intracellular application of a non-hydrolyzable analogue of GTP, GTP-γ-S, mimics the action of transmitters. In many cases, inhibition of Ca^{2+} currents has been postulated to be due to a 'direct action' of the G-protein on the Ca^{2+} channels and not to involve a diffusible cytoplasmic second messenger (Schultz *et al.*, 1990; Hille, 1994). A membrane-delimited signal transduction pathway has been proposed because agonist applied in the bath is unable to depress the Ca^{2+} channels isolated in an on-cell patch. The inhibition of Ca^{2+} channel currents by neurotransmitter application or direct G-protein activation can be relieved by application of a conditioning prepulse (Bean, 1989; Grassi & Lux, 1989; Elmslie *et al.*, 1990). Thus the G-protein is thought to couple to the Ca^{2+} channel in a voltage-dependent manner.

The main exotoxin of *Bordetella pertussis*, pertussis toxin, prevents coupling of activated receptors to some G-proteins (e.g. G_i and G_o) by ADP-ribosylation of G-protein α subunits. Several approaches have been used to investigate which pertussis toxin-sensitive G-proteins transduce the inhibitory signal to the Ca^{2+} channels. In initial studies endogenous G-proteins were inactivated by treating cells with pertussis toxin. Purified G-proteins were then allowed to diffuse from a patch pipette into cells in an attempt to restore neurotransmitter inhibition of Ca^{2+} channels. Hescheler *et al.* (1987) found that intracellular application of purified G_o restored opioid-mediated inhibition of Ca^{2+} currents in NG108-15 cells. In this study, the α-subunit of G_o (with or without βγ in the complex) was 10 times more potent than G_i whereas βγ subunits themselves produced little effect. In contrast, other work has shown that G_i is as effective as G_o in restoring neurotransmitter inhibition of Ca^{2+} currents in submucosal neurones (Surprenant *et al.*, 1990).

Another approach used for investigating G-protein-mediated transduction is to remove a G-protein selectively. This has been done by intranuclear injection of antisense oligonucleotides or with antibodies raised against specific G-proteins. Using antisense oligonucleotides, Kleuss *et al.* (1991) assigned a function to G_o in the receptor-mediated inhibition of voltage-dependent Ca^{2+} channels. They showed that the subtype G_{o1} specifically mediated the action of muscarinic receptors and that G_{o2} transduces the signal from somatostatin receptors in rat pituitary GH_3 cells. Other work, using antisense oligonucleotides, has shown that G_o, rather than G_i, mediates the $GABA_B$-induced inhibition of Ca^{2+} channels in rat DRG neurones (Campbell *et al.*, 1993). Furthermore, intracellular dialysis with $G_{o\alpha}$ antiserum significantly reduced the inhibition of Ca^{2+} currents induced by dopamine in rat pituitary cells (Lledo *et al.*, 1992) and replating cultured DRG cells in the presence of $G_{o\alpha}$ antiserum ('scrape-loading') reduced baclofen inhibition of Ca^{2+} channels (Menon-Johansson *et al.*, 1993).

In our previous studies (Rhim *et al.*, 1993; Rhim & Miller, 1994), we demonstrated that opioids inhibited the high-

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threshold Ca^{2+} currents in the nucleus tractus solitarius (NTS), an area of the brain in which opioids produce many important effects on the activity of the autonomic nervous system (Yeadon & Kitchen, 1989; Van Giersbergen *et al.*, 1992). We have, therefore, examined the coupling mechanism between opioid receptors and Ca^{2+} channels in this area. The results include comparisons with other transmitters, baclofen ($GABA_B$ receptor agonist) and somatostatin, which also modulate synaptic transmission in the NTS and inhibit Ca^{2+} channels in other neurones (see Discussion).

Methods

Acutely dissociated NTS cells

NTS neurones were isolated as described previously (Rhim & Miller, 1994). Briefly, Sprague-Dawley rats (7–21 days old) were anaesthetized with ether, and the whole brain, including the brainstem, was rapidly removed. Transverse slices, 400 μm in thickness, were prepared from the brainstem and placed in a holding chamber filled with 32°C artificial cerebrospinal fluid (aCSF) which contained (in mM): NaCl 126, $NaHCO_3$ 26.2, NaH_2PO_4 1, KCl 3, $MgSO_4$ 1.5, $CaCl_2$ 2.5, glucose 10, gassed with 95% O_2, 5% CO_2. Slices were transferred to a conical tube containing gently bubbled aCSF at 32°C to which 1.8 u ml^{-1} dispace (Grade I, 0.75 ml/slice) was added. After 1 h enzyme treatment, slices were rinsed with enzyme-free aCSF and transferred for storage to a holding chamber with continuously bubbled aCSF at room temperature. When needed, a slice was removed from the chamber and placed on Sylgard-coated petri dish. Under a dissecting microscope, the NTS region was micropunched and placed on a coverslip. The cells were then dissociated by trituration in saline solution containing (mM): NaCl 146, KCl 3, $CaCl_2$ 2, $MgCl_2$ 1, HEPES 10 and glucose 10 (pH 7.4) using progressively smaller diameter pipettes and allowed to settle on a coverslip for 10–30 min.

For pertussis toxin experiments, cells were dissociated in culture medium containing 50% DMEM, 50% F-12 medium, 100 μg ml^{-1} transferrin, 5 μg ml^{-1} insulin, 0.1 mM putrescine, 30 nM sodium selenite, 20 nM progesterone and penicillin/streptomycin (100 u ml^{-1} and 100 μg ml^{-1}, respectively). Cells were treated with pertussis toxin for 18–24 h before recordings were made. Pertussis toxin was stored as a 100 μg ml^{-1} solution in purified water and diluted into cultures of freshly dissociated NTS neurones to a final concentration of 200 ng ml^{-1}. As controls, cells received the same amount of culture medium instead of pertussis toxin.

Electrophysiological recordings

Whole-cell voltage-clamp recordings were used (Hamill *et al.*, 1981). Patch electrodes with resistance of 2.5 to 5 MΩ were filled with the internal solution containing (mM): CsCl 140, $MgCl_2$ 1, BAPTA 10, MgATP 3.6, Tris creatine phosphate 14, GTP-Tris 0.1 and 50 u ml^{-1} creatine phosphokinase, pH adjusted to 7.4 with CsOH. The external solution contained (mM): $BaCl_2$ 10, tetraethylammonium (TEA) chloride 150, $MgCl_2$ 1, HEPES 10, and glucose 10, pH 7.4 with TEAOH. Current recordings were obtained with an Axopatch-1D amplifier (Axon Instruments) and filtered at 2 kHz. Ca^{2+} currents were evoked every 15 s by a 200 ms voltage step from −80 mV to 0 mV. Leak currents were subtracted from the data sweeps by scaling the leak sweep to the data. All data were expressed as the mean ± s.e.mean.

Loading of antiserum

Specific antisera directed against the α subunits of G_o ($G_{o\alpha}$) were obtained from Du Pont/New England Nuclear (Boston, MA, U.S.A.). These are rabbit antisera that were raised against synthetic decapeptide corresponding to the N-terminus of $G_{o\alpha}$, GC/2, and raised against synthetic 15 peptides corresponding to the C-terminus of $G_{o\alpha}$, GO/1. For all recordings in the presence of G-protein antibody, the tip of the recording pipette was dipped into the internal solution without creatine phosphokinase for 10 s and the rest of the pipette was backfilled with the internal solution containing the antibody at a 1:20 dilution. Non-immune rabbit serum (Calbiochem; San Diego, CA, U.S.A.), included in the recording pipette at the same dilution used for antibodies, served as a control.

Loading of cells with antibody by intracellular dialysis was demonstrated using cy3-conjugated IgG (Jackson; West Grove, PA, U.S.A.) which was added to the recording pipette. Under whole-cell voltage clamp, the-time dependent increase in fluorescence in cell soma was monitored at 590 nm while the Ca^{2+} currents of the cell were measured. Fluorescence in the cell soma gradually increased and reached plateau level around 45 min.

When statistical comparisons were made, a paired-sample *t* test was applied to the means of the measurements.

HEK cell preparation

Transfected HEK293 cell line which has stable expression of $\alpha_{1B-1}\alpha_{2b}\beta_{1-2}Ca^{2+}$ channel (A4–A2) was obtained from SIBIA Inc. (La Jolla, CA, U.S.A.). The HEK cells were grown in DMEM containing 5% Defined/Supplemented bovine calf serum, 100 u ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 500 μg ml^{-1} G-418. Cells were prepared for electrophysiology by plating on to poly-L-lysine coated glass coverslips and used within 48 h.

Solutions and reagents

Dispase (Grade I) was purchased from Boehringer Mannheim Co. (Indianapolis, IN, U.S.A.). [D-Ala^2, N-MePhe^4, Gly^5-ol]-enkephalin (DAMGO) and [D-Trp^8]-somatostatin were from Bachem (Torrance, CA, U.S.A.). (−)-Baclofen and pertussis toxin were from RBI (Natick, MA, U.S.A.). ω-Conotoxin-GVIA and ω-agatoxin-IVA were from Peninsula Laboratories Inc. (Belmont, CA, U.S.A.) and Peptides International (Japan), respectively. MgATP, di(Tris)-phosphocreatine, creatine phosphokinase, GTP-Tris, GTP-γ-S, BAPTA, and most other reagents were from Sigma (St. Louis, MO, U.S.A.). Stock solutions of drugs were prepared in purified water and all solutions were applied in the perfusate. Stock solutions of toxins were dissolved in purified water and kept at −20°C until used. Toxins were applied by adding 1 or 2 ml of solution at its final concentration directly to the sample chamber (0.5 ml total volume) and stopping flow for 1.5–2.0 min.

Results

The effect of baclofen on high-threshold Ca^{2+} currents in NTS neurones

Application of the $GABA_B$ receptor agonist, baclofen (30 μM), reversibly reduced the high-threshold Ca^{2+} channel currents in most cells tested from acutely isolated rat NTS (38.9 ± 1.2%, $n = 85$ of 88). Currents were evoked every 15 s by a 200 ms voltage step from −80 mV to 0 mV using Ba^{2+} as the charge carrier. Figure 1a illustrates the effect of baclofen on the Ba^{2+} current ($I_{Ba^{2+}}$) in one acutely isolated NTS neurone. The inhibition by 30 μM baclofen, measured isochronally with the control peak inward $I_{Ba^{2+}}$, was 33.3%. Activation of $GABA_B$ receptors also dramatically slowed the rate of current activation (inset graph), similar to results reported for other G-protein-mediated receptors. In this cell the degree of inhibition by 30 μM baclofen did not change on multiple applications of drug (4 times). In a separate set of experiments, we observed that two applications of baclofen, separated by 5–10 min, produced 38.8 ± 4.6% and 33.0 ± 4.5% inhibition of $I_{Ba^{2+}}$, respectively. Thus, the response to baclofen did not desensitize significantly. Figure 1b shows the dose-response relationship

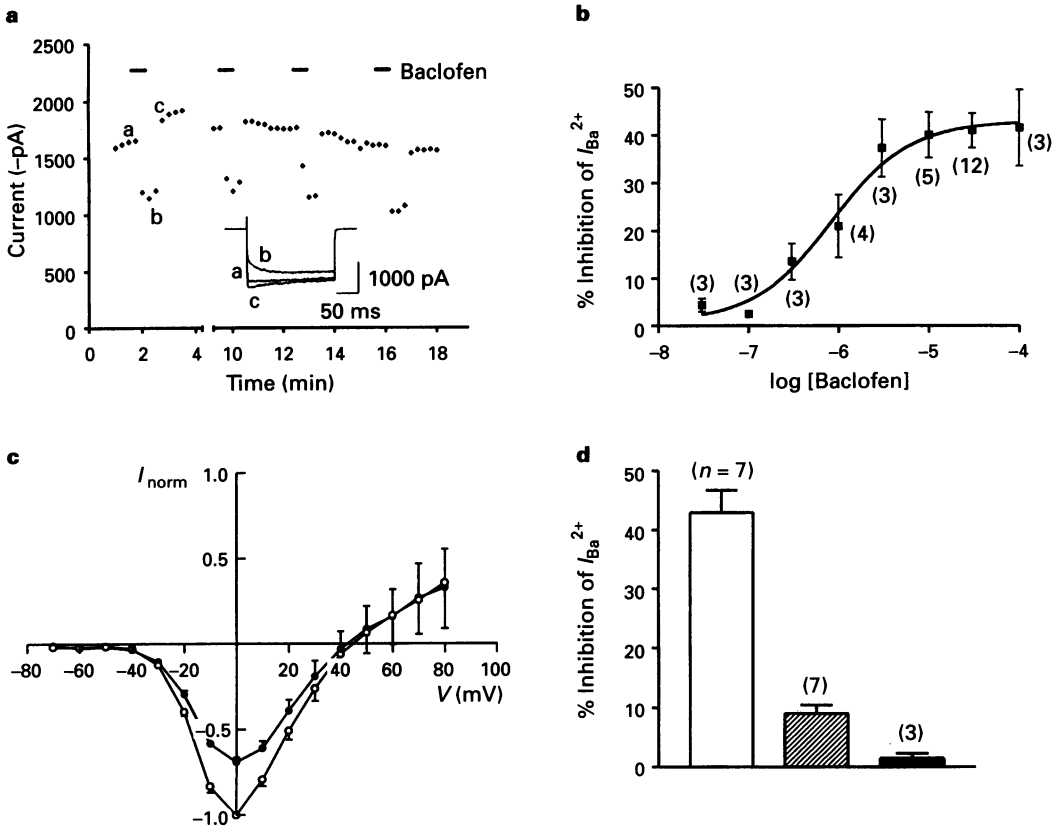

Figure 1 The inhibitory effects of baclofen on high-threshold Ca^{2+} channel currents in NTS neurones. (a) Time course of peak Ba^{2+} current ($I_{Ba^{2+}}$) showing four exposures to baclofen. The inhibition by 30 μM baclofen measured isochronally with the control peak of $I_{Ba^{2+}}$ was 33.3% and did not change with multiple applications of drug (4 times). Inset: Leak-substracted currents of labelled points from the time course of $I_{Ba^{2+}}$. (b) Concentration-response relationship for inhibition of $I_{Ba^{2+}}$ by baclofen. Numbers beside each point indicate the number of cells treated at each concentration. (c) Current-voltage relationship of normalized $I_{Ba^{2+}}$ before (○) and during (●) 30 μM baclofen application from 4 tested cells. Holding potential was −80 mV. (d) Pooled results from Ca^{2+} channel blocker experiments illustrating the mean inhibition of $I_{Ba^{2+}}$ by 30 μM baclofen (control, open column) and following treatment with ω-CgTx-GVIA (hatched column) and co-treatment with ω-CgTx-GVIA and ω-Aga-IVA (solid column). Numbers in parentheses indicate the number of cells treated with each drug.

for baclofen inhibition of $I_{Ba^{2+}}$ was concentration-dependent with an IC_{50} of about 1 μM.

We have previously shown that the μ-opioid agonist, DAMGO, also inhibits high-threshold Ca^{2+} currents in these cells (Rhim & Miller, 1994). DAMGO and baclofen generally produced inhibition of approximately equal magnitude. Since neither DAMGO nor baclofen completely inhibited the current, the additivity of the effects was examined. In this set of experiments a maximal inhibitory concentration of each drug produced 37.0±4.8% inhibition for 3 μM DAMGO and 40.2±1.0% inhibition for 30 μM baclofen. Inhibition of 42.6±4.0% was produced by co-application of DAMGO and baclofen ($n=4$, data not shown). The relationship between the normalized peak current and voltage before and during the application of 30 μM baclofen is shown in Figure 1c ($n=4$). As we previously observed with μ-opioid receptor agonists, baclofen did not affect the voltage-dependence of current activation, nor did it affect the apparent reversal potential. Baclofen inhibited the inward current elicited by small or moderate depolarizations but had almost no effects on the outward current evoked by very large depolarizations.

We attempted to elucidate which types of high-threshold Ca^{2+} channels were inhibited by activation of $GABA_B$ receptors in NTS neurones. The effect of baclofen was examined following application of 1 μM ω-conotoxin-GVIA (ω-CgTx-GVIA). ω-CgTx-GVIA irreversibly inhibited the peak current by 59.9±3.2% ($n=11$). Baclofen, 30 μM, suppressed $I_{Ba^{2+}}$ by 42.9±3.8% before, and 9.0±1.4% after, ω-CgTx-GVIA application in seven cells. The baclofen-mediated inhibition of $I_{Ba^{2+}}$ was completely blocked by pre-treatment with both 1 μM ω-CgTx-GVIA and 100 nM ω-agatoxin-IVA (ω-Aga-IVA; 1.5±0.8% inhibition remaining in 3 cells, Figure 1d). These results suggest that activation of $GABA_B$ receptors can inhibit N- and P/Q-type Ca^{2+} channels in NTS neurones (see Rhim & Miller, 1994).

The effect of somatostatin on high-threshold Ca^{2+} currents in NTS neurones

Somatostatin acts at another member of the family of G-protein-linked receptors that has been shown to affect both Ca^{2+} and K^+ channels. As a comparison with opioid and $GABA_B$ receptors, we examined the action of somatostatin on high-threshold Ca^{2+} currents in acutely isolated NTS neurones. Figure 2a illustrates the effect of somatostatin on $I_{Ba^{2+}}$ through high-threshold Ca^{2+} channels in one acutely isolated NTS neurone. Somatostatin, 300 nM, produced 25.9% inhibition measured isochronally with the control peak inward $I_{Ba^{2+}}$. As with baclofen, this inhibition was accompanied by a characteristic change in the current activation kinetics (inset graph). This effect was reproducible on multiple applications (up to 3 times). In 53 cells of 82 tested, 300 nM somatostatin reduced $I_{Ba^{2+}}$ by 31.3±1.6%. In a separate set of experiments, we observed that two applications of somatostatin, separated by 5–10 min, produced 27.5±2.3% and 27.0±3.6% inhibition of $I_{Ba^{2+}}$, respectively. Therefore, the response to somatostatin did not desensitize significantly. In 5 cells, the effects of somatostatin was examined following 1 μM ω-CgTx-GVIA application. Somatostatin, 300 nM, suppressed $I_{Ba^{2+}}$ by 33.3±8.0% before, and 5.1±1.7% after, ω-CgTx-GVIA ap-

plication. The somatostatin-mediated inhibition of $I_{Ba^{2+}}$ was completely blocked by pretreatment with both 1 μM ω-CgTx-GVIA and 100 nM ω-Aga-IVA (1.9±0.4% inhibition remaining in 3 cells, Figure 2b). These results suggest that activation of somatostatin receptors targeted the same types of Ca^{2+} channels (N and P/Q) that were inhibited by opioids and baclofen in NTS neurones.

Relief of neurotransmitter-mediated inhibition of the Ca^{2+} current by depolarizing prepulses

Inhibition of Ca^{2+} currents by neurotransmitter application or direct G-protein activation has been shown to be relieved by application of a conditioning prepulse before the test step (Bean, 1989; Grassi & Lux, 1989; Elmslie *et al.*, 1990). We therefore examined whether the inhibition of Ca^{2+} channel current by neurotransmitter application in NTS neurones was voltage-dependent.

At the onset, prepulse parameters were varied to examine the conditions necessary for relief of inhibition. Cells were dialyzed with 100 μM GTP-γ-S to mimic the inhibition of $I_{Ba^{2+}}$ produced by DAMGO (see Rhim & Miller, 1994). $I_{Ba^{2+}}$ was elicited by a 25 ms test pulse from −80 to 0 mV. Various prepulses were applied to examine the effects of the prepulse on the current measured during the test pulse. The prepulse voltage-dependence was examined by varying the prepulse potentials between −80 mV and +120 mV for 50 ms. The membrane potential was then returned to −80 mV for 10 ms before the test pulse. The mean normalized $I-V$ curve ($n=4$) is shown in Figure 3a. The normalized currents were obtained from the ratio of test pulse current amplitudes produced by various prepulse potentials to the maximum test pulse current amplitude in each cell. Test pulse currents produced by negative prepulses between −80 to −20 mV were relatively unaltered. With prepulse to potentials, more positive than −20 mV, the test current started to increase. This effect approached a plateau near +80 mV with no further change up to +120 mV. The prepulse duration required to relieve inhibition was examined by varying the duration of prepulses to +80 mV ($n=4$, Figure 3b). Test pulse currents were increased by increasing the duration of the prepulse from 0 to 15 ms and changed little between 15 and 50 ms. These results demonstrate that a 50 ms prepulse to +80 mV provided maximal relief of inhibition.

The prepulse parameters determined above were used to examine the inhibition of $I_{Ba^{2+}}$ by the three neurotransmitters. To compare currents obtained during unconditioned test pulses to those observed during conditioned test pulses, the pulse protocols shown in Figure 4a were used. Figure 4b shows superimposed current traces obtained in the presence of DAMGO. In the absence of transmitter, currents were unaffected by a prepulse in most cells. In some cells, the prepulse induced a

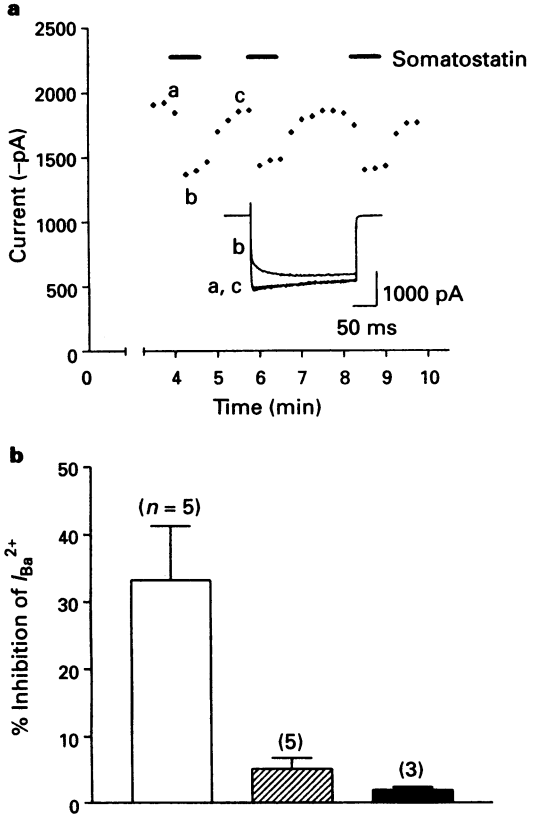

Figure 2 The inhibitory effects of somatostatin on high-threshold Ca^{2+} channel currents in NTS neurones. (a) Time course of peak Ba^{2+} current ($I_{Ba^{2+}}$) showing three exposures to 300 nM somatostatin. The inhibition of $I_{Ba^{2+}}$ was 25.9% following the first and 23.0, 23.9% following the second and third somatostatin applications. Inset: leak-substracted currents of labelled points from the time course of $I_{Ba^{2+}}$. (b) Pooled results from Ca^{2+} channel blocker experiments illustrating the mean inhibition of $I_{Ba^{2+}}$ by 300 nM somatostatin (control, open column) and following treatment with ω-CgTx-GVIA (hatched column) and co-treatment with ω-CgTx-GVIA and ω-Aga-IVA (solid column). Numbers in parentheses indicate the number of cells treated with each drug.

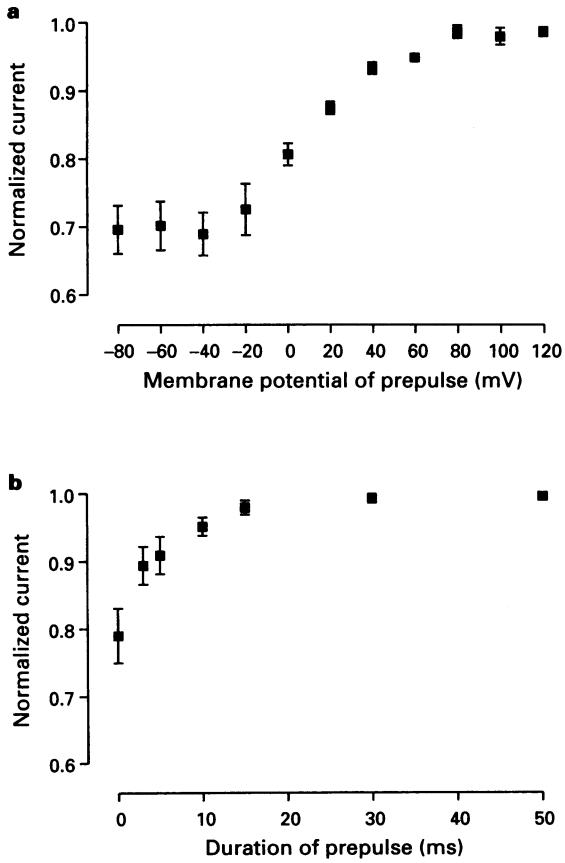

Figure 3 Voltage-dependence and time course of prepulse-induced currents in cells dialyzed with GTP-γ-S. (a) The relationship of mean normalized current and membrane potential of prepulse from 4 tested cells. The prepulse was evoked from holding potential of −80 mV to various prepulse potentials (from −80 mV to +120 mV) for 50 ms. The current amplitude of the test pulse following a prepulse was normalized to the maximum amplitude of test pulse in each cell. (b) The relationship of normalized current and the duration of prepulse from 4 tested cells. The prepulse was evoked from holding potential of −80 to +80 mV and the duration was varied from 0 to 50 ms.

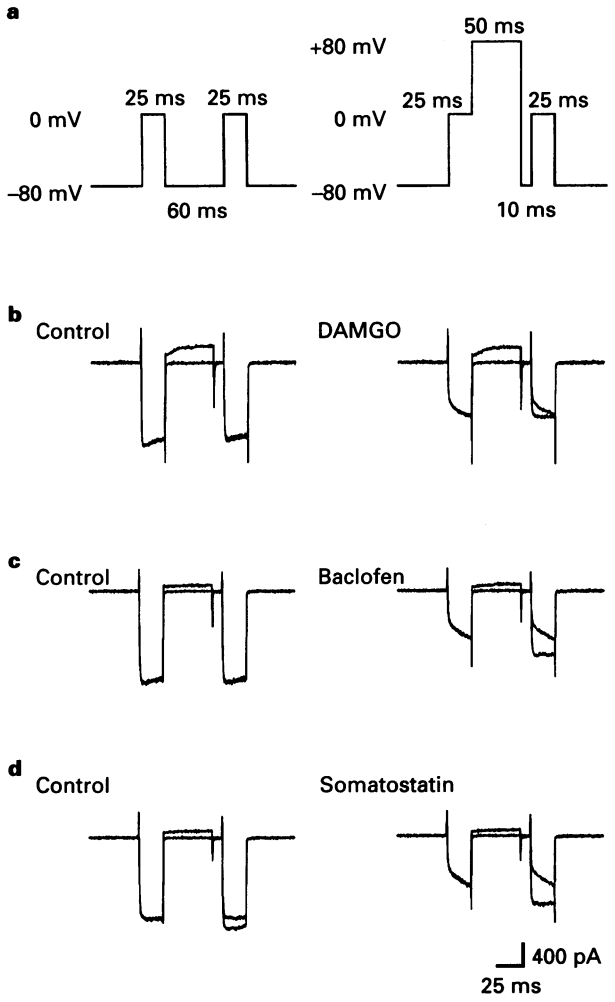

Figure 4 The effect of depolarizing prepulses on neurotransmitter-mediated inhibition of Ca^{2+} currents. (a) The diagrams show the unconditioned and conditioned pulses. The unconditioned pulse (left) was evoked with two identical voltage steps from −80 to 0 mV for 25 ms with 60 ms interval and the conditioned pulse (right) was exactly the same as left one except the second voltage step is preceded by a 50 ms pulse to +80 mV. (b) The overlapped current traces obtained from above two diagrams for control (left) and in the presence of 1 μM DAMGO (right); (c) (for 30 μM baclofen) and (d) (for 300 nM somatostatin) are the same as (b).

small facilitation of the test current in the absence of transmitter as previously reported in rat sympathetic neurones (Figure 4d control; Ikeda, 1991). The mean inhibition of $I_{Ba^{2+}}$ produced by 1 μM DAMGO was 42.3±3.2% during unconditioned test pulses. When test currents were conditioned by a prepulse, the inhibition produced by DAMGO decreased to 23.3±2.5% (4 cells). The same prepulse protocol was used to examine baclofen- or somatostatin-induced inhibition of $I_{Ba^{2+}}$ (Figure 4c and d). The mean inhibition of $I_{Ba^{2+}}$ produced by 30 μM baclofen was 41.2±2.6% in control conditions and this was decreased to 24.8±2.6% by depolarizing a prepulse (10 cells). For somatostatin, the mean inhibition of $I_{Ba^{2+}}$ produced by 300 nM somatostatin was 36.1±5.5% in control conditions and this was decreased to 18.5±3.8% by depolarizing prepulse (6 cells). As discussed earlier, it has been reported that large depolarizing prepulses relieved the rapid ('direct') inhibition of Ca^{2+} currents produced by transmitter-mediated, G-protein activation, although this is incomplete in most cases (Diversé-Pierlussi *et al.*, 1995). The results obtained here agree with these earlier reports and thus provide additional evidence for G-protein involvement in the inhibition of Ca^{2+} currents in NTS neurones.

Pertussis toxin blocks the response to the three neurotransmitters

The results discussed in previous study using the non-hydrolyzable GTP analogue, GTP-γ-S, indicate that a G-protein is involved in the coupling between opioid receptor and Ca^{2+} channel (Rhim & Miller, 1994). However, GTP-γ-S binds to all subtypes of G-proteins, and little can be inferred about the particular class of G-proteins that couples to these receptors in NTS neurones. Pertussis toxin ADP-ribosylates certain G-protein α subunits (G_{α}; e.g. $G_{i\alpha}$ and $G_{o\alpha}$) and prevents receptor activation of the G-protein. We therefore tested the effects of pertussis toxin on the three neurotransmitter-mediated inhibitions of $I_{Ba^{2+}}$ in acutely isolated NTS cells. Experiments were conducted alternately on control cells and on cells treated overnight with pertussis toxin. Inhibition of $I_{Ba^{2+}}$ produced by DAMGO, baclofen or somatostatin was profoundly reduced by treatment with pertussis toxin. In cells incubated with 200 ng ml^{-1} pertussis toxin for 18 to 24 h at 37°C, DAMGO, baclofen, or somatostatin reduced $I_{Ba^{2+}}$ by 2.9±0.8% ($n=14$), 2.2±0.5% ($n=8$), and 2.9±0.7% ($n=10$), respectively. Whereas, in control cells incubated in culture medium without pertussis toxin for the same duration, inhibitions caused by DAMGO, baclofen, or somatostatin were 14.4±2.4% ($n=23$), 24.8±3.7% ($n=12$), and 15.1±2.7% ($n=21$), respectively. These data enabled us to conclude that the DAMGO-, baclofen- and somatostatin-induced decrease in Ca^{2+} current was mediated via activation of a pertussis-sensitive G_i or G_o-type G-protein.

Effect of dialysis of G-protein antisera on responses to the three neurotransmitters

Several approaches might be used to investigate which pertussis toxin-sensitive G-proteins transduce the inhibitory signal to the Ca^{2+} channels. We examined cells dialyzed with specific G-protein antibodies as a means of disrupting the G-protein coupling mechanism. Several advantages of this approach are afforded by the whole-cell recording method. First, there is the ability to introduce large molecular weight molecules into the cell interior. Second, because we tested the effects of agonists during dialysis of neurones with specific G-protein antibody at early and late stage of recording, each neurone could serve as its own control. The paradigm for antibody loading is described in Methods.

Figure 5a shows pairs of $I_{Ba^{2+}}$ recorded in the absence and presence of 1 μM DAMGO, 2.0 min and 30 min after initiation of whole-cell recording. In this experiment, the pipette contained non-immune rabbit serum (NIS) at a 1:20 dilution. NIS was used as a control for examining any non-specific effects that antiserum administration might have on the high-threshold Ca^{2+} channel or the responses to the neurotransmitters. A 30 min infusion of NIS did not affect the inhibitory response to DAMGO. Figure 5b compares $I_{Ba^{2+}}$ recording from another cell after dialysis with GC/2, an antibody raised against the N-terminus of $G_{o\alpha}$. Intracellular loading with GC/2 did not alter the DAMGO-induced inhibition of $I_{Ba^{2+}}$. Figure 5c compares the mean percentage inhibition of $I_{Ba^{2+}}$ produced by DAMGO. The time for drug application varied from cell to cell; first application is 2–6.5 min and second one is 28–32.5 min. On average, responses to DAMGO measured after around 30 min were not significantly different from those measured at early time points after intracellular loading with GO/1, an antibody raised against the C-terminus of $G_{o\alpha}$ ($n=4$), and GC/2 ($n=5$). We also tested DAMGO with the GC/2 antibody around 45 min at which time we observed maximum fluorescence (see Methods), and found its effects were not changed by antibody application in 3 cells.

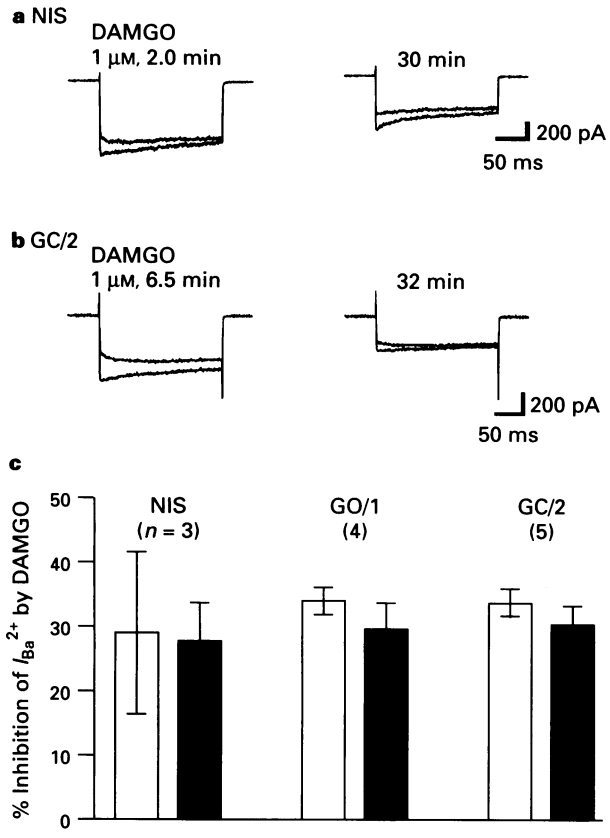

Figure 5 Effect of intracellular dialysis with anti-$G_{o\alpha}$ antiserum on the DAMGO-induced decrease in $I_{Ba^{2+}}$. (a) The superimposed current traces recorded from the cell dialyzed non-immune rabbit serum (NIS) at a 1:20 dilution in the absence and presence of 1 μM DAMGO (indicated by asterisks). After commencing whole-cell recording, DAMGO induced a 18.6% decrease at 2.0 min and 26.6% by the second application at 30 min in $I_{Ba^{2+}}$. (b) Current traces from another cell after dialysis with anti-$G_{o\alpha}$ antiserum, GC/2, raised against the N-terminal of $G_{o\alpha}$ at the same dilution. DAMGO induced a 27.5% decrease at 6.5 min and 23.3% at 32 min in $I_{Ba^{2+}}$. A 32 min infusion of GC/2 slightly decreased the sustained current in this cell, but it did not occur in 4 other tested cells. (c) Pooled results from cells after dialysis with NIS, GO/1 (raised against the C-terminal of $G_{o\alpha}$), and GC/2 illustrating the mean inhibition of $I_{Ba^{2+}}$ by 1 μM DAMGO at first (open columns) and second applications (solid columns). The time for drug application was various from cell to cell; first application is 2–6.5 min and second one is 28–32.5 min. Numbers in parentheses indicate the number of cells dialyzed with each serum.

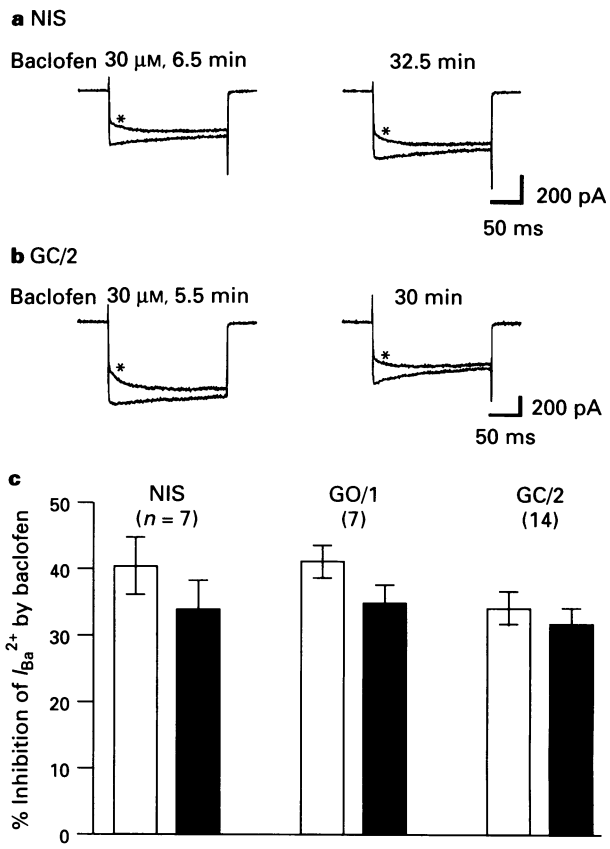

Figure 6 Effect of intracellular dialysis with anti-$G_{o\alpha}$ antiserum on the baclofen-induced decrease in $I_{Ba^{2+}}$. (a) The superimposed current traces recorded from the cell dialyzed NIS at a 1:20 dilution in the absence and presence of 30 μM baclofen (indicated by asterisks). After initiation of whole-cell recording, baclofen induced a 34.8% decrease at 6.5 min and 30.9% by the second application at 32.5 min in $I_{Ba^{2+}}$. (b) Current traces from another cell after dialysis with GC/2 at the same dilution. Baclofen induced a 32.4% decrease at 5.5 min and 34.7% at 30 min in $I_{Ba^{2+}}$. (c) Pooled results from cells after dialysis with NIS, GO/1, and GC/2 illustrating the mean inhibition of $I_{Ba^{2+}}$ by 30 μM baclofen at first (open columns) and second applications (solid columns). Numbers in parentheses indicate the number of cells dialyzed with each serum.

We performed the same kind of experiments for baclofen-mediated inhibition; the results are shown in Figure 6. Intracellular loading of cells with NIS or GC/2 did not change the baclofen-induced inhibition (Figure 6a and b). Figure 6c shows the pooled data for dialysis with NIS ($n=7$), GO/1 ($n=7$) and GC/2 ($n=14$). The responses to baclofen measured after around 30 min were not significantly different from those measured at early time points after intracellular loading of these antibodies.

Figures 7a and 7b show the $I_{Ba^{2+}}$ recorded in the absence and presence of 300 nM somatostatin from a cell dialyzed with NIS and from another cell dialyzed with GC/2. Intracellular loading with GC/2 markedly attenuated the somatostatin-induced inhibition of $I_{Ba^{2+}}$. Figure 7c shows the pooled data after dialysis with NIS, GO/1 and GC/2. The responses to somatostatin measured after 30 min were not significantly different from those measured at early first application after intracellular loading with NIS ($n=6$) or GO/1 ($n=4$). However, the inhibitory effects of somatostatin were significantly reduced after 30 min of intracellular dialysis with GC/2 (from $24.6\pm2.5\%$ to $11.0\pm1.9\%$, $n=11$, $P<0.001$). The loss of somatostatin response cannot be attributed to desensitization, since we have previously shown that the application of 300 nM somatostatin could be repeated without desensitization (Figure 2a). Furthermore, the loss of somatostatin response was not observed in the cells loaded with NIS or GO/1. The attenuation was not due to the prolonged dialysis period, since the use of NIS did not affect the somatostatin-induced decrease in $I_{Ba^{2+}}$. In addition, the decline in response to somatostatin was observed in cells that showed no changed in baclofen-induced inhibition when dialyzed with GC/2 ($n=4$).

We have also examined the effects of somatostatin on Ca^{2+} channels in HEK293 cells which were stably transfected with N-type Ca^{2+} channels (Williams *et al.*, 1992). We have found that N-type Ca^{2+} channels in these cells can be inhibited through activation of an endogenous somatostatin receptor (Toth & Miller, 1994). We found that intracellular loading of the GC/2 antibody attenuated the somatostatin-mediated response (Figure 8a). The inhibitory effect of somatostatin was significantly reduced after 30 min of intracellular administration of GC/2 (from $42.6\pm5.8\%$ to $14.8\pm1.8\%$, $n=5$, $P<0.05$) while the somatostatin responses were not affected after intracellular loading with NIS ($n=5$, Figure 8b). These results support the fact that GC/2 inhibition of the somatostatin effect is a specific effect which is not limited to NTS neurones.

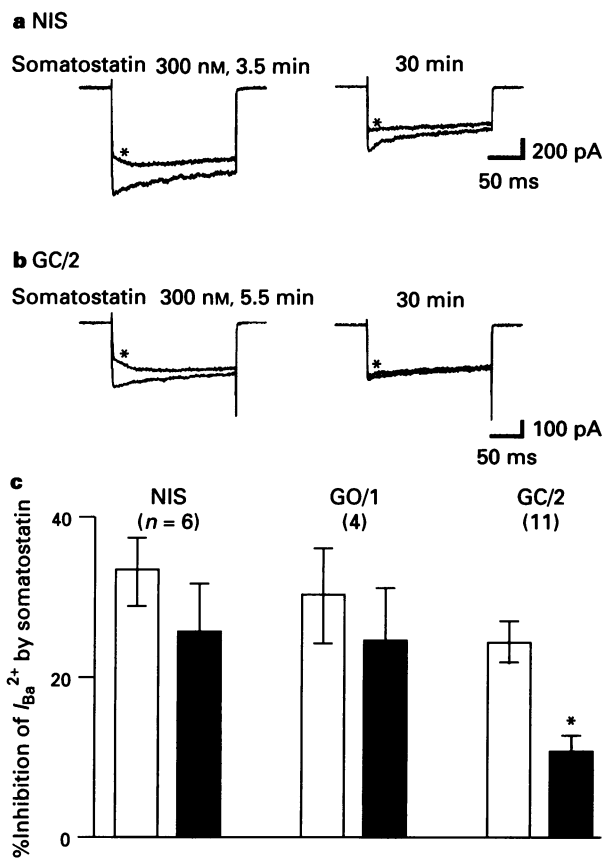

Figure 7 Effect of intracellular dialysis with anti-$G_{o\alpha}$ antiserum on the somatostatin-induced decrease in $I_{Ba^{2+}}$. (a) The superimposed current traces recorded from the cell dialyzed NIS at a 1:20 dilution in the absence and presence of 300 nM somatostatin (indicated by asterisks). After initiation of whole-cell recording, somatostatin induced a 32.0% decrease at 3.5 min and 32.2% by the second application at 30 min in $I_{Ba^{2+}}$. (b) Current traces from another cell after dialysis with GC/2 at the same dilution. Somatostatin induced a 37.9% decrease at 5.5 min and 7.6% at 30 min in $I_{Ba^{2+}}$. A 30 min infusion of GC/2 markedly attenuated the somatostatin-induced decrease in $I_{Ba^{2+}}$. (c) Pooled results from cells after dialysis with NIS, GO/1, and GC/2 illustrating the mean inhibition of $I_{Ba^{2+}}$ by 300 nM somatostatin at first (open columns) and second applications (solid columns). Numbers in parentheses indicate the number of cells dialyzed with each serum. $^*P<0.001$ versus first application at GC/2 ($n=11$).

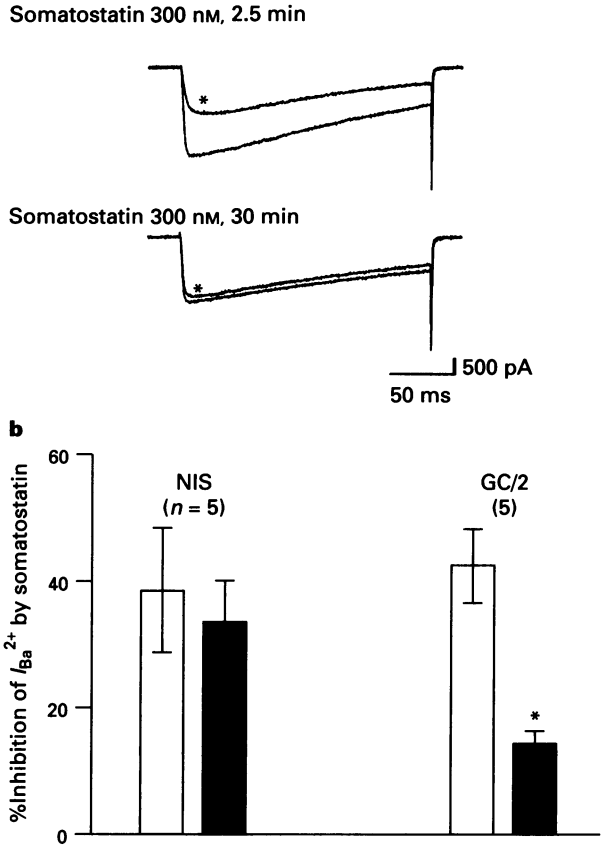

Figure 8 Effect of intracellular dialysis with anti-$G_{o\alpha}$ antiserum on the somatostatin-induced decrease in $I_{Ba^{2+}}$ in HEK cells. (a) The current traces recorded from the cell dialyzed GC/2 at a 1:20 dilution in the absence and presence of 300 nM somatostatin (indicated by asterisks) in a HEK cell. Somatostatin induced a 54.8% decrease at 2.5 min and 11.6% by the second application at 30 min in peak inward $I_{Ba^{2+}}$ after initiation of whole-cell recording. A 30 min infusion of GC/2 markedly attenuated the somatostatin-induced decrease of $I_{Ba^{2+}}$. (c) Pooled results from cells after dialysis with NIS and GC/2 illustrating the mean inhibition of $I_{Ba^{2+}}$ by 300 nM somatostatin at first (open columns) and second applications (solid columns). Numbers in parentheses indicate the number of cells dialyzed with each serum. $^*P<0.05$ versus first application at GC/2 ($n=5$).

Discussion

The results reported in the present study extend our previous studies on the coupling mechanisms between neurotransmitter receptors and Ca^{2+} channels in NTS neurones. We have compared the effects of opioids with those of baclofen or somatostatin, which also inhibited Ca^{2+} channels in a pertussis toxin-sensitive and voltage-dependent manner in these cells.

Physiology of $GABA_B$ and somatostatin receptors in the NTS

Baclofen, a $GABA_B$ receptor agonist, has been known to affect cardiovascular, respiratory and gastrointestinal control through effects in the central nervous system. With regard to the cardiovascular system, application of baclofen to the brainstem causes hypertension, tachycardia and inhibition of the baroreceptor reflex. These effects are mediated through the activation of $GABA_B$ receptors, because they are antagonized by selective $GABA_B$ receptor antagonists (Persson, 1981; Bousquet *et al.*, 1982; Sved & Sved, 1989). Indeed, the results of $GABA_B$ receptor activation in the NTS have been reported using a slice preparation containing the NTS (Brooks *et al.*, 1992). In this preparation, baclofen produced a hyperpolarization of the membrane potential. In addition, baclofen produced inhibition of spontaneous and evoked synaptic transmission at concentrations which have no effect on membrane potential, as we observed for opioids in our previous study (Rhim *et al.*, 1993).

The existence of somatostatin immunoreactive nerve cell bodies and nerve terminals has been demonstrated within the ventrolateral and ventral subnuclei of the NTS of the rat by immunocytochemistry (Kalia *et al.*, 1984b). The involvement of somatostatin in cardiovascular and respiratory control also has been suggested by studies using microinjection of somatostatin into the NTS (Kalia *et al.*, 1984a; Koda *et al.*, 1985). Although few studies examining the action of somatostatin have been performed in NTS neurones, it has been shown that somatostatin induces a hyperpolarization of the membrane potential and the suppression of GABA-mediated synaptic transmission in CA1 neurones of the hippocampus (Xie & Sastry, 1992).

The inhibition of voltage-activated Ca^{2+} channels by baclofen and somatostatin has been studied in various systems.

Baclofen has been shown to inhibit T-type (Deisz & Lux, 1985), L-type (Maguire *et al.*, 1989; Heidelberger & Matthews, 1991) and N-type (Scholz & Miller, 1991; Menon-Johansson *et al.*, 1993) Ca^{2+} channels in various neurones and to inhibit P-type channels in cerebellar Purkinje neurones (Mintz & Bean, 1993). The types of Ca^{2+} channels inhibited by the activation of somatostatin receptors are L-type in GH_3 cells (Kleuss *et al.*, 1991) and N-type in NG108–15 cells (Taussig *et al.*, 1992) and rat sympathetic neurones (Ikeda & Schofield, 1989; Shapiro & Hille, 1993). We have now shown that activation of $GABA_B$ and somatostatin receptors, as with μ-opioid receptors (Rhim & Miller, 1994), can inhibit N- and P/Q-type Ca^{2+} channels in NTS neurones. However, it is less clear what role specific G-proteins play in these modulatory actions. Therefore, we compared the role of specific G-proteins in opioid-, baclofen- and somatostatin-mediated inhibition of Ca^{2+} currents in the NTS.

Types of pertussis toxin-sensitive G-proteins involved in Ca^{2+} channel inhibition

Pertussis toxin prevents coupling of activated receptors to certain G-proteins (e.g. G_i and G_o) by ADP-ribosylation of the G-protein α subunits. With respect to the role of G_i in Ca^{2+} channel inhibition, it has been shown that purified G_i restores receptor-mediated inhibition as effectively as G_o (Surprenant *et al.*, 1990) or at 10 times higher concentration than G_o (Hescheler *et al.*, 1987) in pertussis toxin-treated cells. However, several studies using specific anti-G_i antibodies have shown that G_i is not involved in receptor-mediated Ca^{2+} channel inhibition in many instances (McFadzean *et al.*, 1989; Menon-Johansson *et al.*, 1993; Moises *et al.*, 1994). On the other hand there are reports showing that G_i is involved in receptor-mediated K^+ channel activation (Lledo *et al.*, 1992) or in Ca^{2+} channel inhibition by a mechanism that is voltage-independent (Diversé-Pierlussi *et al.*, 1995).

Accumulated evidence indicates that the α-subunit of G_o ($G_{o\alpha}$) is probably involved in transducing signals from a number of different receptors to Ca^{2+} channels in neurones and secretory cells (Schultz *et al.*, 1990). When an antibody raised against the C-terminus of $G_{o\alpha}$ was microinjected into NG108-15 cells, it significantly reduced the inhibition of Ca^{2+} channels induced by noradrenaline (McFadzean *et al.*, 1989). This antibody also significantly reduced baclofen-mediated Ca^{2+} current inhibition when loaded into DRG cells using scrape loading (Menon-Johansson *et al.*, 1993). A subsequent study from the same group was performed using antisense oligonucleotides complementary to unique sequences of either $G_{o\alpha}$ or $G_{i\alpha}$. Their results strongly suggested that the $GABA_B$ receptor couples to voltage-dependent Ca^{2+} channels via G_o and not G_i in rat DRG neurones (Campbell *et al.*, 1993).

Specific roles for splice variants of G_o (G_{o1} and G_{o2}) in receptor-mediated Ca^{2+} channel inhibition have been reported. Kleuss *et al.* (1991), using antisense oligonucleotides, have shown that G_{o1} specifically mediates the inhibition of Ca^{2+} currents produced by muscarinic receptors and that G_{o2} mediates somatostatin-mediated inhibition in GH_3 cells. In NG108–15 cells, Taussig *et al.* (1992) found that expressing a mutant $G_{o1\alpha}$ subunit that was rendered insensitive to pertussis toxin, rescued the Leu-enkephalin and noradrenaline pathway, but not the somatostatin pathway, when the native pathway had been removed by treatment with pertussis toxin. These studies suggest that specific subtypes of G_o-protein are involved in the receptor-induced inhibition of Ca^{2+} channels.

Which part of the G-protein is coupled to the receptor? The C-terminus of the G_α subunit is thought to be the site that couples to the receptor because the C-terminal domain contains the cysteine residue that is ADP-ribosylated by pertussis toxin (Heideman & Bourne, 1990). Consistent with this, antibodies against the C-terminus of $G_{o\alpha}$ have been successfully used to block receptor-mediated inhibition, as described above. However, it has also been shown that intracellular dialysis with an antiserum raised against the N-terminus of $G_{o\alpha}$ (GC/2) attenuated Ca^{2+} current inhibition by the μ-selective agonist PLO17 in rat DRG neurones (Moises *et al.*, 1994). In addition, it has been reported that antibodies to either the N- or C-terminus of $G_{o\alpha}$ protein (GC/2 or GO/1) can block opioid-mediated inhibition of adenylate cyclase in SH-SY5Y cells (Carter & Medzihradsky, 1993). It is interesting that antibodies to either the N- or C-terminus of $G_{o\alpha}$ protein disrupted the coupling of μ-opioid receptor to adenylate cyclase system. It has been shown that the C-terminus of transducin interacts with rhodopsin (Deretic & Hamm, 1987), while the N-terminus of $G_{o\alpha}$ is important for the interaction with the $\beta\gamma$ subunit (Denker *et al.*, 1992). Thus, the observed effects of the two antisera used above may have been produced by two independent mechanisms: direct interference in receptor-G-protein coupling or blocking formation of the G-protein heterotrimer. However, the role of $\beta\gamma$ subunits in Ca^{2+} channel modulation has not been rigorously investigated at this time. On the other hand, it has been demonstrated that G-protein $\beta\gamma$ subunits act on inwardly rectifying K^+ channels (Logothetis *et al.*, 1987; Rueveny *et al.*, 1994; Takao *et al.*, 1994) and other effectors (Boyer *et al.*, 1994).

Types of G-protein in receptor-mediated Ca^{2+} channel inhibition in the NTS

As with DRG neurones (Moises *et al.*, 1994), we have found that the main opioid receptor coupled to Ca^{2+} channel inhibition in NTS neurones is the μ-type (see Rhim & Miller, 1994). We tested the sensitivity of this effect to an antibody against the N-terminus of $G_{o\alpha}$ (GC/2). Neither responses to μ-opioid nor $GABA_B$ receptors were significantly changed when NTS cells were loaded with GC/2. Furthermore, an antibody against the C-terminus of $G_{o\alpha}$ (GO/1) also did not reduce receptor-mediated Ca^{2+} channel inhibition.

Why did we fail to show that the G_o-protein is involved in μ-opioid- or $GABA_B$-receptor-mediated inhibition in the NTS using specific anti-G-protein antibodies? It is possible that this may be due to the type of neurone involved. G_o involvement in the coupling of μ-opioid and $GABA_B$ receptors to Ca^{2+} channels was reported in studies using cultured or acutely isolated rat DRG neurones, where the content of $G_{o\alpha}$ was reported to be only one fourth of the $G_{o\alpha}$ in whole rat brain (Ewald *et al.*, 1989). Even in DRG neurones, neither use of antibody nor antisense could completely abolish $GABA_B$-mediated inhibition of Ca^{2+} currents, indicating that there may be a large excess of G_o normally present (Campbell *et al.*, 1993; Menon-Johansson *et al.*, 1993). The NTS is the first reported area in the central nervous system where it has been shown that activation of opioid receptors is involved in the modulation of Ca^{2+} channels. Perhaps the content of $G_{o\alpha}$ is extremely high in these cells as compared to DRG neurones and therefore it may be difficult to interfere with its effects. However, it is interesting that we have shown that intracellular loading with GC/2 markedly attenuated the somatostatin-induced inhibition of Ca^{2+} current in the NTS under the same experimental conditions that failed to show an effect of the same antibody on μ-opioid receptor- and $GABA_B$ receptor-mediated actions. It is possible that somatostatin-mediated Ca^{2+} current inhibition is mediated through the G_{o2} type of G_o protein which might be present at a low level compared to that of G_{o1} or through a G_i protein because of the slight cross-reactivity of GC/2 with all subtypes of G_i proteins (Goldsmith *et al.*, 1988). The latter possibility was tested in the HEK293 cell line, which contains somatostatin receptors and in which G_{i1} and G_{i3} appear to be prevalent G-proteins (Lewis *et al.*, 1991; Law *et al.*, 1993). The presence of G_{i2} and G_o could not be demonstrated by Western blotting (Law *et al.*, 1993). However, endogenous G_o expression could be detected using PCR, presumably due to its greater sensitivity (Ma & Miller, unpublished observations). Since the main inhibitory pathway for somatostatin receptors in HEK293 cells may therefore involve G_{i1} or G_{i3}, our finding that intracellular loading of GC/2 attenuated the somatostatin-mediated response in these cells may also be explained by the crossreaction of anti-G_o protein,

GC/2, with G_{i1} or G_{i3}. Indeed, somatostatin block of N-current in these cells can be blocked by treatment with pertussis toxin. Thus, these studies suggest that there are at least two different pertussis-sensitive G-protein-mediated pathways involved in receptor-mediated inhibition of Ca^{2+} currents in rat NTS neurones.

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