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THE PEPTIDE CGRP INCREASES A HIGH-THRESHOLD Ca^{2+} CURRENT IN RAT NODOSE NEURONES VIA A PERTUSSIS TOXIN-SENSITIVE PATHWAY

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SUMMARY

1. The whole-cell variation of the patch clamp technique was used to study the effect of calcitonin gene-related peptide (CGRP) on voltage-gated calcium currents in acutely dissociated rat nodose ganglion neurones and to determine if its effects were mediated via a guanine nucleotide binding (G) protein.

2. Both low- and high-threshold calcium current components were present in nodose ganglion neurones. CGRP had no effect on the isolated low-threshold current component. However, CGRP (1–1000 nM, ED_{50} = 50 nM) caused a concentration-dependent increase in high-threshold calcium currents. CGRP (1 μM) increased the peak of these calcium currents $21 \pm 4\%$ over controls.

3. CGRP enhanced a transient high-threshold calcium current evoked from a holding potential of -80 mV but did not affect the slowly inactivating high-threshold current evoked from -40 mV. Multiple high-threshold calcium currents have been reported in sensory neurones. We cannot state unequivocally which high-threshold calcium current component was enhanced by CGRP. However, based on the observation that CGRP increased a transient but not the slowly inactivating high-threshold calcium current, we believe the peptide enhanced primarily the N-type calcium current component.

4. CGRP increased the maximal peak current and caused a modest negative shift of $\leqslant 10$ mV in the peak of the current–voltage (I–V) relation in three of six neurones. In the remaining three neurones the peptide increased the maximal peak current without a detectable shift in the peak of the I–V relation.

5. To determine if the CGRP-induced enhancement in calcium current was associated with an increase in calcium conductance, we studied the effect of the peptide on the instantaneous current–voltage (I–V) relation when currents were evoked at a clamp potential (V_c) of $+30$ mV, positive to the observed maximal current ($V_c = 0$ to $+10$ mV). CGRP increased the maximal conductance $23 \pm 4\%$.

6. The enhancement of calcium current by CGRP was not due to a shift in the voltage dependency of steady-state inactivation of the calcium channels. The

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stimulatory effect of CGRP on calcium current was evaluated by evoking currents from different holding potentials (V_h) at the same V_c (+10 mV). CGRP-induced increases in calcium currents were similar over the range of (V_h) from −60 to −110 mV, suggesting that the peptide did not alter voltage-dependent steady-state inactivation.

7. CGRP-induced enhancement of calcium current was antagonized by GDP-β-S (0·1 mM), a non-hydrolyzable analogue of GTP that prevents activation of G proteins. The effect of CGRP was also abolished after neurones were treated with 100 ng/ml pertussis toxin (PTX) for 12–24 h.

8. In separate studies we examined the effect of CGRP on [^{3}H]acetylcholine release from isolated intact nodose ganglia. CGRP (0·01 and 1 μM) produced a concentration-dependent increase in acetylcholine release of 12±3 and 22±5%, respectively, over basal release levels. The effect of CGRP was absent in the presence of calcium-free media or tetrodotoxin and reduced after treatment with PTX (250 ng/ml for 3 h).

9. We conclude that CGRP enhanced primarily a transient high-threshold calcium current component and stimulated acetylcholine release in nodose ganglion neurones via a pertussis toxin-sensitive mechanism.

INTRODUCTION

Calcitonin gene-related peptide (CGRP) is a thirty-seven amino acid peptide encoded in the calcitonin gene that is present in a variety of central and peripheral neurones, including hypothalamic, primary afferent, and intrinsic neurones of the gastrointestinal tract (Ishida-Yamamoto & Tohyama, 1989). CGRP increased calcium current in rat dorsal root ganglion (DRG) neurones and enhanced excitatory synaptic transmission in the spinal cord (Ryu, Gerber, Murase & Randic, 1988). In addition, CGRP had an excitatory action on guinea-pig myenteric neurones and coeliac ganglion neurones and stimulated acetylcholine release from myenteric ganglia (Palmer, Schemann, Tamura & Wood, 1986; Dun & Mo, 1989; Mullholland & Jaffer, 1990). The mechanism by which CGRP altered calcium channel activity is not known.

It is unknown, for example, whether enhancement of calcium currents by CGRP was mediated by a guanine nucleotide binding (G) protein. Several inhibitory neuropeptides including the κ-receptor selective opioid peptide dynorphin A, neuropeptide Y (NPY) and somatostatin decreased calcium currents (Gross & Macdonald, 1987; Ewald, Sternweiss & Miller, 1988; Lewis, Weight & Luini, 1986) via a pertussis toxin (PTX)-sensitive mechanism, suggesting that inhibitory (G_i/G_o-type) G protein(s) mediated this action (Lewis *et al.* 1986; Ewald *et al.* 1988; Gross, Moises, Uhler & Macdonald, 1990). In addition, the α-subunit of G_o 'reconstituted' the effect of NPY to reduce calcium current in PTX-treated dorsal root ganglion (DRG) neurones (Ewald *et al.* 1988). Previous studies have shown that pertussis toxin blocked angiotensin II-induced stimulation of voltage-dependent calcium currents in an adrenal cortical cell line and angiotensin II-induced calcium influx, but not inositol trisphosphate production, in adrenal glomerulosa cells (Kojima, Shibata & Ogata, 1986; Hescheler, Rosenthal, Hinsch, Wulfern, Trautwein &

Schultz, 1988). Taken together, these studies suggest that PTX-sensitive G proteins may mediate stimulatory as well as inhibitory effects of peptides on calcium currents.

Multiple low- and high-threshold voltage-dependent calcium current components have been recorded from primary sensory neurone cell bodies. The transient low-threshold (T-type), relatively transient high-threshold (N-type) and slowly inactivating high-threshold (L-type) currents differed in their electrophysiological properties and responses to application of pharmacological agents (Nowycky, Fox, & Tsien, 1985; Wanke, Ferroni, Malgaroli, Ambrosini, Pozzan & Meldolesi, 1987; Fox, Nowycky & Tsien, 1987*a*, *b*; Gross & Macdonald, 1987). For example, the snail toxin ω-conotoxin GVIA reduced neuronal N-type currents (McCleskey, Fox, Feldman, Cruz, Olivera, Tsien & Yoshikami, 1987), whereas dihydropyridines selectively inhibited the L-type current (Fox *et al.* 1987*a*). Additional high-threshold calcium currents have been recorded from a variety of neurones that exhibited distinctive electrophysiological properties and sensitivity to the spider venom toxin ω-Aga-111A but insensitivity to ω-conotoxin GVIA and dihydropyridines (Plummer, Logothetis & Hess, 1989; Plummer & Hess, 1991; Regan, Sah & Bean, 1991; Mintz, Venema, Adams & Bean, 1991). It is unknown if CGRP receptors couple to one or more of these channel subtypes. Dynorphin A and NPY, for example, primarily decreased transient high-threshold calcium currents in primary sensory neurones (Gross & Macdonald, 1987; Wiley, Gross, Lu & Macdonald, 1990). Differential coupling of neurotransmitter receptors to calcium channels is of physiological interest in light of recent studies showing that different calcium channel subtypes may be primarily responsible for modulating neurotransmitter release (Perney, Hirning, Leeman & Miller, 1986; Hirning, Fox, McClesky, Olivera, Thayer, Miller, Tsien, 1988). In a previous study we demonstrated that reduction of a transient high-threshold calcium current by NPY correlated with a reduction in evoked acetylcholine release (Wiley *et al.* 1990). Such correlations have not been reported for stimulatory peptides such as CGRP.

The goals of this study were, therefore, to determine the effect of CGRP on calcium current components in rat vagal sensory (nodose ganglion) neurones, to determine whether the coupling of CGRP receptors to calcium channels was G protein dependent and to evaluate the effect of CGRP on the release of the neurotransmitter acetylcholine in the isolated nodose ganglion and assess whether G protein(s) mediated this action.

METHODS

Preparation of acutely dissociated nodose ganglion neurones

Nodose ganglion neurones were prepared using methods described previously (Gross, Wiley, Ryan-Jastrow & Macdonald, 1990). Briefly, rats were decapitated after ether anaesthesia; nodose ganglia were removed and treated with 1 mg/ml type Ia collagenase (Sigma Chemical Company, St Louis, MO, USA) in minimal essential medium (GIBCO Laboratories, Grand Island, NY) buffered with 16·5 mM-$NaHCO_3$ for 25 min at 37 °C. The tissue was then transferred to a tube containing Dulbecco's minimum essential medium (GIBCO) and 5% fetal calf serum (GIBCO) to inhibit the enzyme, triturated gently to disperse the cells and then centrifuged and resuspended in minimum essential medium with Earle's salts supplemented with $NaHCO_3$ (16·5 mM), glucose (28·2 mM), nerve growth factor (10 ng/ml; Boehringer Mannheim, Indianapolis, IN, USA), penicillin (50 U/ml), streptomycin (50 mg/ml), $CaCl_2$ (3·6 mM) and 10% fetal calf serum (GIBCO). The suspension (approximately 0·5 ml/ganglion) was plated on 35 mm polystyrene Petri dishes

(Corning Glass Works, Corning, NY), without a substrate, 0·3 ml/dish and incubated at 37 °C in a 93% air–7% CO_2 atmosphere. An additional 1 ml of medium was added after 30 min, and the cells were used for recordings within 1–24 h.

Whole-cell patch clamp recordings

Whole-cell voltage clamp recordings were obtained using the whole-cell variation of the patch clamp technique. Cells were bathed in a solution containing (mM): 67 choline chloride, 100 tetraethylammonium, 5·6 glucose, 5·3 KCl, 5·0 $CaCl_2$, 10 HEPES (pH 7·3–7·4) 310–330 mosM; all reagents from Sigma. Glass recording patch pipettes (Fisherbrand microhaematocrit tubes, Pittsburgh, PA, USA) with resistances of 1·0–2·0 MW were filled with a recording solution containing (mM): 140 CsCl, 10 HEPES, 10 EGTA, 5 ATP-Mg^{2+} and either 0·1 GTP or GDP-β-S, guanosine, 5′-*O*-(2-thiodisphosphate) (all reagents from Sigma). The pH (7·3–7·4) was adjusted with 1 M-CsOH after the addition of ATP. The osmolarity was 10–15% below that of the bath solution, 280–300 mosM.

Recordings were made at room temperature using the Axopatch 1-C patch clamp amplifier (Axon Instruments, Burlingame, CA, USA). Pipette and whole-cell capacitance and series resistance were corrected by compensation circuitry on the patch amplifier. Typical initial input resistances were 500 MW to 1GW. Series resistance was estimated by cancellation of the capacitance-charging current transient after patch rupture; typical values for the series resistance were 1·5–4 MΩ. Generally, series resistance compensation of 80–90% was possible without significant noise or oscillation. Voltage step commands were generated, and currents were digitized (5 kHz), stored and analysed by a microcomputer (IBM AT or equivalent) using the program pCLAMP (Axon Instruments). The current traces were filtered with a Bessel filter at 10 kHz (−3 dB).

Analysis of current components

Leak current was estimated either as the inverse of the current evoked using hyperpolarizing voltage commands of equal magnitude to the depolarizing commands used to evoked the inward currents, or as the current evoked with depolarizing commands in the presence of cadmium chloride (200 μM). Leak current was digitally subtracted from the relevant inward current to obtain the calcium current. Leak currents were not affected by CGRP, GDP-β-S or PTX.

At least three calcium current components were present in nodose ganglion neurones (Gross *et al.* 1990), similar to the transient low-threshold (T-type), relatively high-threshold (N-type) and slowly inactivating high-threshold (L-type) currents reported in dorsal root ganglion neurones (Nowycky *et al.* 1985; Gross & Macdonald, 1987). The presence of additional high-threshold calcium currents similar to the ω-conotoxin- and dihydropyridine-insensitive currents reported in other peripheral neurones has not been examined in nodose neurones (Plummer *et al.* 1989; Regan *et al.* 1991; Mintz *et al.* 1991). The transient low-threshold current component was isolated by evoking currents from holding potentials (V_h) = −80 or −90 mV at clamp potentials (V_c) = −50 to −20 mV. At V_c positive to −20 mV more slowly inactivating high-threshold current components were evoked. To distinguish between transient and slowly inactivating high-threshold current components, we evoked currents at 1 min intervals after patch rupture, alternating between V_h of −80 and −40 mV. The current evoked from V_h = −40 mV at V_c = +10 to +20 mV was used as an estimate of the slowly inactivating high-threshold current component, and the current evoked from V_h = −80 mV as an estimate of the transient high-threshold component. Results are presented in terms of the evoked currents unless we refer to a specific current component. We use the nomenclature T-type, N-type and L-type calcium current components to describe the transient low-threshold, transient high-threshold and slowly inactivating high-threshold current components, respectively with the understanding that the whole-cell technique does not allow unequivocal identification of currents carried by channel subtypes. Statistical comparisons were made by using Student's two-tailed *t* test.

Drug preparation and application

Rat CGRP (Pennisula Laboratories, Belmont, CA), was dissolved in external recording solution supplemented with 0·1% bovine serum albumin (BSA, Sigma). Compounds were applied by pressure ejection (1·7–6·9 kPa) for 2 s from micropipettes (tip diameters 10–15 μm) that were positioned 20–30 μm from the cell body. Application of diluent had no effect on evoked calcium

currents or holding currents. After application, micropipettes were removed from the bath. Currents were evoked 5 s after completion of CGRP application.

For some experiments neurones were pre-incubated with PTX (Sigma). Four microlitres of 50 μg/ml in PTX in 0·1 % BSA was added to neuronal cultures containing 2 ml of culture medium resulting in a concentration of 100 ng/ml PTX. Control Petri dishes received 4 μl of 1% BSA. Cultures were incubated for 12–24 h. Prior to recording, culture medium was exchanged for external recording solution containing either 100 ng/ml PTX (treated cultures) or diluent (control cultures). Comparisons were made between treated and untreated cultures of the same plating.

[^{3}H]Acetylcholine (ACh) release studies

Postnatal rats (1–3 weeks old) were fasted for 12 h, anaesthetized by carbon dioxide inhalation and decapitated. The nodose ganglia from nine animals were removed and cleaned of surrounding connective tissue. The release of [^{3}H]ACh from nodose ganglia was examined using a method similar to that described previously (Wiley *et al.* 1990). Briefly, the isolated intact ganglia were incubated for 1 h with [^{3}H]choline (0·2 μM, specific activity 80 Ci/mmol) in 2 ml of oxygenated Krebs–Ringer buffer. The baskets were serially transferred to scintillation vials containing fresh buffer every 10 min. At 40–50 and 80–90 min during the release period, baskets were placed into vials containing 0·01 or 1·0 μM-CGRP. Some experiments were performed in calcium-free media containing 2 mM-EGTA or in Krebs–Ringer buffer containing 1 μM tetrodotoxin (TTX).

In experiments evaluating the effect of PTX, ganglia were pretreated of 3 h with PTX (250 ng/ml) prior to the [^{3}H]ACh release period. The concentration of PTX and duration of pretreatment were derived from previous studies (Wiley *et al.* 1990). In each experiment ganglia not exposed to PTX served as controls. In all experiments, total ^{3}H was determined and used to indicate transmitter release. Column chromatography was used to verify that the ^{3}H released was ACh as described previously (Wu, Kisslinger & Gaginella, 1982).

Fractional release was calculated by expressing the radioactivity in each vial as a percentage of the total radioactivity in the tissue at the time of collection as described previously (Wiley & Owyang, 1987). All results were expressed as means ± S.E.M. The number of experimental trials was taken as the sample size.

RESULTS

CGRP increased the transient high-threshold calcium current component in nodose neurones

We studied the effect of CGRP on the calcium current components of nodose ganglion neurones. Calcium current components were similar to those described previously (Gross *et al.* 1990; Wiley *et al.* 1990). Briefly, the low-threshold currents generally remained stable during a typical 30 min recording period (not shown, see Gross *et al.* 1990), whereas high-threshold currents decreased slowly to approximately 60% of their maximal values. Application of CGRP had not effect on either the low-threshold (Fig. 1*A*, traces 1 and 2) or slowly inactivating high-threshold currents (Fig. 1*B*, traces 4 and 5). However, the peptide reversibly increased high-threshold calcium currents containing the transient and slowly inactivating components in thirty-two out of fifty-five neurones (Fig. 1*B*, traces 1, 2 and 3). The effect of CGRP was concentration dependent (1–1000 nM, ED_{50} = 50 nM). CGRP (1 μM) produced a mean increase of the peak current (I_p), evoked from $V_h = -80$ mV at $V_c = +10$ to +20 mV, of 21 ± 4% with a range 12 ± 3 to 38 ± 5% (mean ± S.E.M.; $P < 0.05$). CGRP-mediated enhancement of calcium current was observed within 5 s of application of the peptide. Calcium currents returned to control values within 1–3 min of CGRP application.

We examined whether CGRP affected the voltage dependence of calcium channel activity, as has been suggested for neurotransmitters that decrease calcium current

(Bean, 1989). A series of currents were evoked from $V_h = -80$ mV at V_c ranging from -110 to $+80$ mV, and the peak current values were plotted as a function of V_c (Fig. 1*C*). The maximum current typically occurred between 0 and $+10$ mV (Fig. 1*C*). CGRP increased the maximal peak current and caused a modest negative shift of

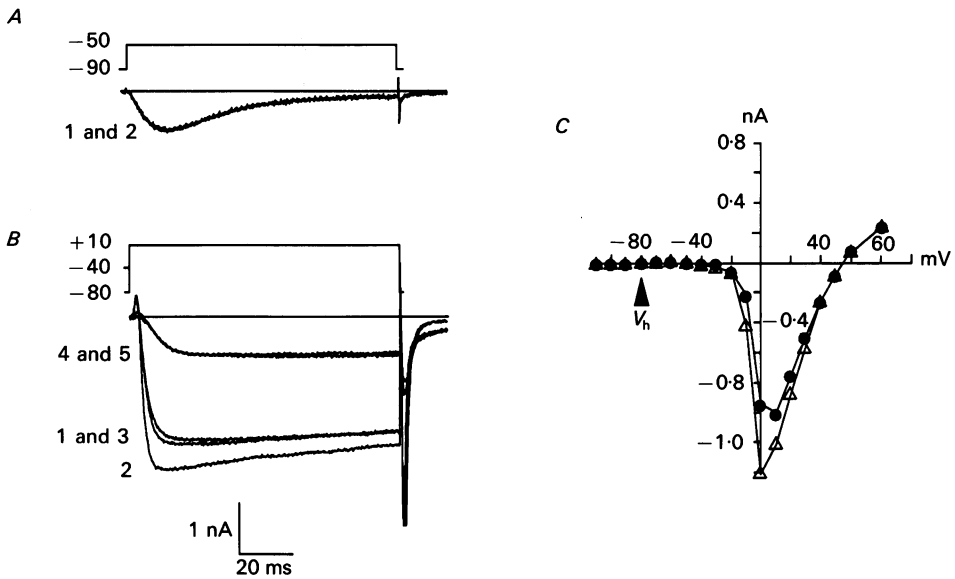

Fig. 1. CGRP selectively increased calcium currents in rat nodose ganglion neurones. Currents are leak substracted and inward currents are downward. In this and subsequent figures, representative voltage command protocols (mV) are shown above the calcium current traces. The holding potential (V_h) and clamp potential (V_c) are depicted. Data depicted in *A–C* were recorded from the same neurone using the whole-cell voltage clamp technique. *A*, transient low-threshold calcium currents. V_h was -90 mV; the 100 ms step command was to $V_c = -50$ mV. *B*, high-threshold calcium currents. Slowly inactivating high-threshold currents were evoked from $V_h = -40$ mV; the 100 ms command was to $V_c = +10$ mV. High-threshold currents consisting of both transient and slowly inactivating components were evoked from $V_h = -80$ mV at $V_c = +10$ mV. Superimposed traces demonstrate currents before (traces 1 and 4) and 5 to 10 s after (traces 2 and 5) a 2 s application of CGRP (1 μM). Trace 3 shows recovery at 3 min after application of the peptide. *C*, effect of CGRP on current–voltage relation. The current–voltage plot was constructed from peak currents recorded before (control, ●) and after (△) application of CGRP (1 μM). V_h was -80 mV and currents were evoked using 100 ms hyperpolarizing and depolarizing steps ranging from -110 mV to $+80$ mV in 10 mV increments.

$\leqslant 10$ mV in the peak of the *I–V* relation in three out of six neurones. In the remaining three neurones the peptide increased the maximal peak current without a detectable shift in the peak of the *I–V* relation.

To determine whether CGRP increased the maximal calcium conductance we studied the effect of peptide on the instantaneous *I–V* relation at a V_c positive to that at which maximal calcium currents were evoked. During a large depolarizing command from $V_h = -80$ mV to $V_c = +30$ mV, a series of 5 and 10 mV depolarizing and hyperpolarizing commands were performed to generate an instantaneous *I–V* relation. Representative current tracings, shown in Fig. 2*Aa* and *b*, reveal that

CGRP increased the current required to attain a given voltage (Fig. 2*B*) consistent with an increase in maximum calcium conductance (23±4%, $n = 4$; mean±S.E.M.).

We evaluated whether CGRP affected voltage-dependent steady-state inactivation of calcium current by evoking calcium currents at the same V_c from two

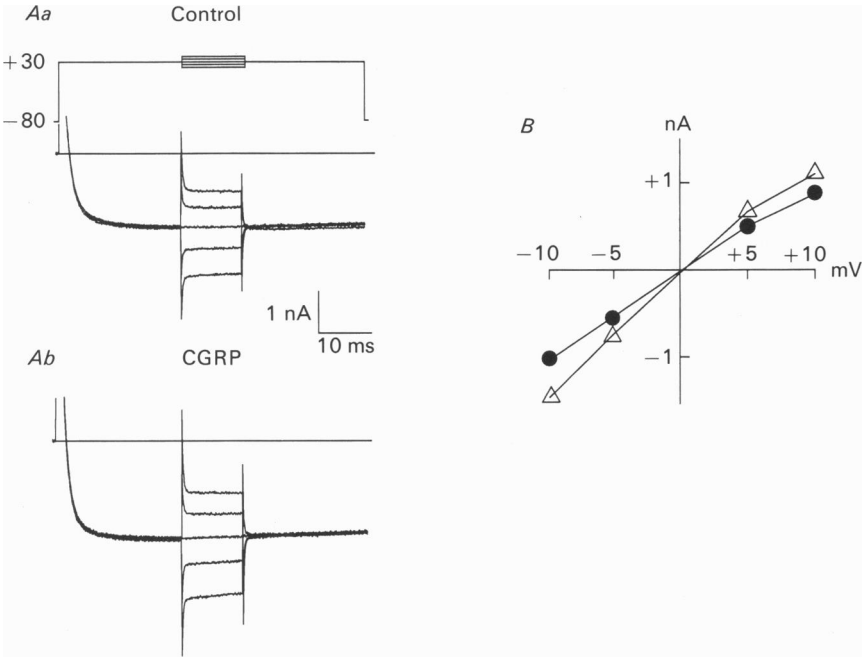

Fig. 2. CGRP increased the instantaneous current–voltage relation. Currents were evoked from $V_h = -80$ mV at $V_c = +30$ mV. During the large depolarizing command to V_c, sequential 5 and 10 mV hyperpolarizing and depolarizing voltage steps were performed. Currents before (control, *A a*) and after (*A b*) application of CGRP (1 μM) are depicted. *B*, instantaneous current–voltage relation demonstrating the current recorded at the end of each hyperpolarizing and depolarizing voltage step from V_c. Control (●) and after application of CGRP (△).

different V_h in the presence and absence of the peptide. If CGRP were enhancing calcium current by reducing voltage-dependent steady-state inactivation, we would anticipate observing a larger increase in calcium currents evoked from a more positive V_h. Instead, we observed that the percentage increase in the peak calcium current produced by CGRP was similar, if not somewhat greater, when currents were evoked from, $V_h = -110$ mV (28±4%) compared to -60 mV (18±3%) ($n = 5$, $P < 0{\cdot}05$; Fig. 3).

GDP-β-S and PTX prevented the CGRP-induced increase in calcium current

Experiments were designed to determine whether the pathway(s) by which CGRP receptors were coupled to calcium channels involved G proteins. First we examined the effect of substituting GDP-β-S which prevents activation of G proteins (Casey & Gilman, 1988) for GTP in the recording solution. CGRP was applied at 5 and 20 min to control cells and to cells from the same preparation in which GDP-β-S was

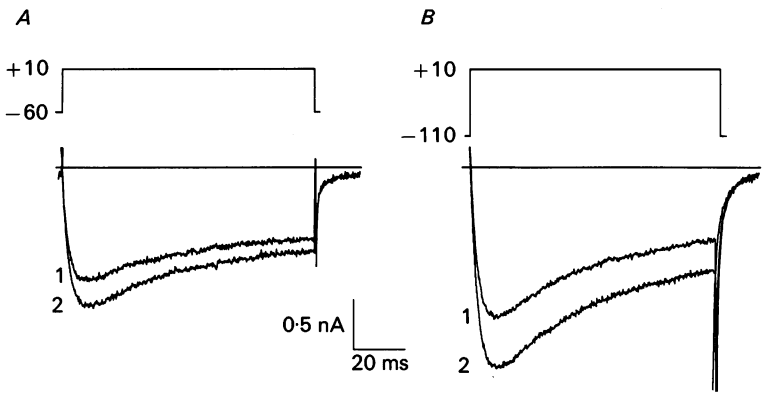

Fig. 3. CGRP increased calcium current independently of a shift in the voltage dependence of steady-state inactivation. The currents shown in *A* and *B* were recorded from the same neurone before (trace 1) and after (trace 2) a 2 s application of CGRP (1 μM). The currents were evoked from $V_h = -60$ mV (*A*) or $V_h = -110$ mV (*B*) at $V_c = +10$ mV for 100 ms.

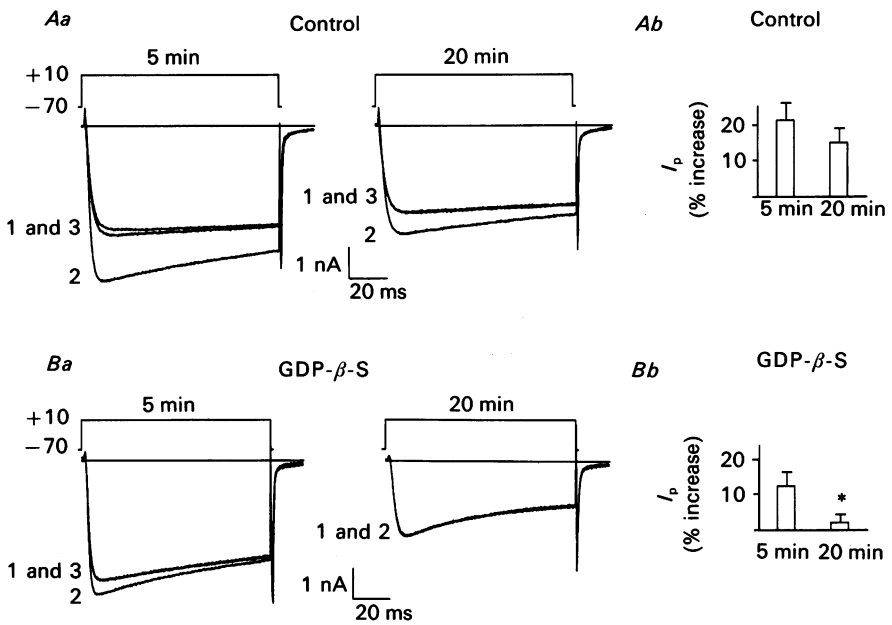

Fig. 4. CGRP-induced increase in calcium current was antagonized by GDP-β-S. *A* and *B* show the effect of CGRP at 5 and 15 min after patch rupture in a typical control cell 'dialyzed' with GTP (0·1 mM). *C* and *D* show the effect of substituting GDP-β-S (0·1 mM) for GTP in a representative CGRP-responsive cell. The percentage increase in the peak calcium currents (I_p) (mean ± S.E.M) are shown to the right of the current traces. Calcium currents before (control, trace 1) and after CGRP (1 μM) application (trace 2) were evoked from $V_h = -80$ mV at $V_c = +10$ mV for 100 ms.

substituted for GTP in the recording pipette solution (Fig. 4). In twelve of twenty-one control cells CGRP increased the peak calcium current 20±4% at 5 min and 14±3% at 20 min in twelve out of twenty-two cells ($P < 0{\cdot}05$) (Fig. 4*Aa* and *b*). When GDP-β-S was substituted for GTP in the recording pipette solution, CGRP

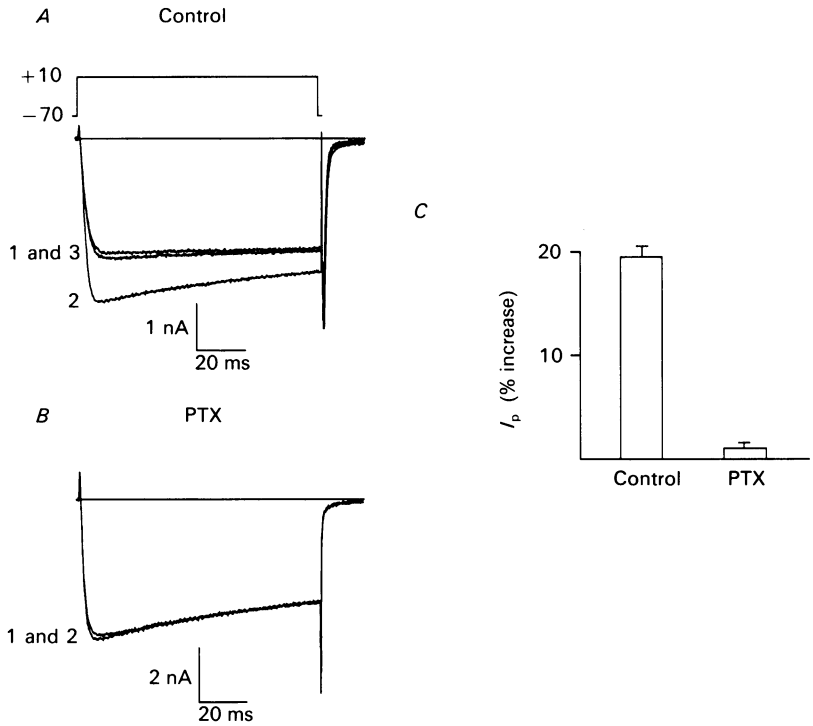

Fig. 5. Pertussis toxin (PTX) abolished CGRP-induced increase in calcium current. *A*, CGRP increased the calcium current in seven of twelve untreated neurones. Calcium currents are shown for a representative untreated cell before (control, trace 1), after CGRP (1 μM) application (trace 2) and recovery (trace 3). *B*, after pretreatment with PTX (100 ng/ml for 12–24 h) none of twelve neurones responded to application of CGRP. Cells tested were from the same plating. Calcium currents before (trace 1) and after (trace 2) CGRP application were evoked from $V_h = -70$ mV at $V_c = +10$ mV for 100 ms. *C*, histogram shows response to application of CGRP for untreated (control) cells and treated (PTX) cells. Results are presented as a percentage increase in the peak (I_p) of the calcium current after application of CGRP compared to control currents.

increased the peak calcium current 12±3% at 5 min and 3±2% at 20 min of six of twelve cells (Fig. 4*Ba* and *b*). The negligible increase in the peak calcium current produced by CGRP at 20 min in cells 'dialysed' with GDP-β-S was not significantly greater than control currents.

We studied the effect of CGRP in the presence of pertussis toxin (PTX) by comparing the effect of CGRP on cells exposed to PTX for 12–24 h after plating to time-matched control (vehicle-treated) cells from the same preparation. CGRP significantly increased the peak calcium current evoked at $V_c = +10$ mV from $V_h = -70$ mV in seven out of twelve control cells (Fig. 5*A*) but had no effect on twelve cells pretreated with PTX (Fig. 5*B*). The results are summarized in Fig. 5*C*.

CGRP stimulated acetylcholine release in the nodose ganglia via a PTX-sensitive mechanism

We examined the effect of CGRP on acetylcholine (ACh) release in excised intact nodose ganglia to correlate our observations on calcium currents with the release of

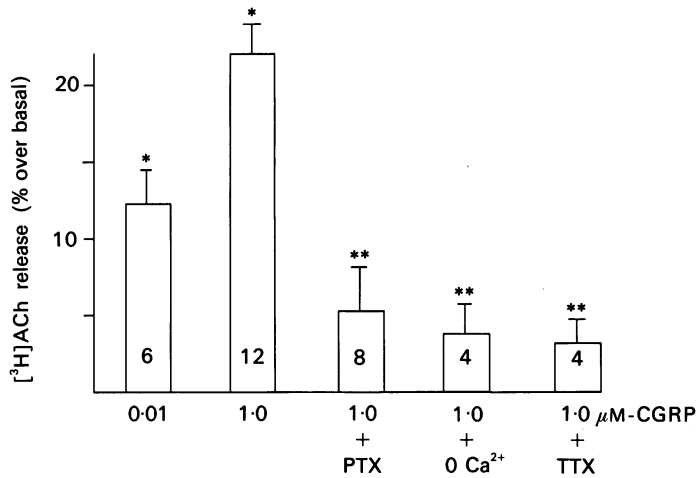

Fig. 6. CGRP-stimulated acetylcholine (ACh) release in nodose ganglia was reduced by pertussis toxin (PTX). CGRP (0·01 and 1·0 μM)-induced release of [^{3}H]ACh from excised intact nodose ganglia was tested after pretreatment with PTX (250 ng/ml for 3 h) in the presence of calcium-free media containing EGTA (2 mM) or after treatment with tetrodotoxin (1 μM). Number of results in each experiment is given within columns.

a classical neurotransmitter known to be present in this preparation (Cooper, 1984; Ternaux, Falemopin, Palouzier, Chamoin & Portalier, 1989). Specifically, we evaluated: (i) whether CGRP induced calcium-dependent release of ACh and (ii) whether CGRP-evoked release of ACh was PTX sensitive. CGRP (0·01 and 1·0 μM) produced a concentration-dependent increase of 12 ± 3 and 22 ± 5%, respectively, in the release of acetylcholine over basal levels (mean $\pm$ S.E.M., $P < 0{\cdot}05$) (Fig. 6). CGRP-evoked release of acetylcholine was blocked in the presence of calcium-free medium containing EGTA (2 mM; $n = 4$) and by tetrodotoxin (1 μM; $n = 4$). Pretreatment with PTX significantly reduced the stimulatory effect of CGRP on acetylcholine release by 73% ($n = 8$, $P < 0{\cdot}05$).

DISCUSSION

This study demonstrated that CGRP selectively increased a voltage-activated calcium current and acetylcholine release in rat nodose neurones via a pertussis toxin-sensitive mechanism. Ryu *et al.* (1988) reported that CGRP enhanced calcium current and synaptic transmission in rat dorsal root ganglion neurones. Our results confirm and extend their observations by showing that CGRP preferentially enhanced a transient high-threshold calcium current component and acetylcholine

release in vagal sensory neurones via a pertussis toxin-sensitive pathway. Our data suggest that CGRP increased the N-type current component. However, additional single-channel and pharmacological studies will be needed to determine unequivocally if CGRP acts on one or more subtypes of transient high-threshold calcium currents (Plummer *et al.* 1989; Plummer & Hess, 1991; Regan *et al.* 1991; Mintz *et al.* 1991).

Previous studies have shown that pertussis toxin blocked angiotensin II-induced stimulation of voltage dependent calcium currents in an adrenal cortical cell line and angiotensin II-induced calcium influx, but not inositol trisphosphate production, in adrenal glomerulosa cells (Kojima *et al.* 1986; Hescheler *et al.* 1988). Taken together these studies suggest that PTX-sensitive G protein(s) can mediate the effects not only of transmitters that reduce calcium currents but also the effects of stimulatory transmitters.

Potential mechanisms that could explain the enhancement of calcium currents produced by CGRP include: (i) increasing the number of open channels at a given clamp potential, (ii) shifting the voltage dependency of calcium channel activation in the negative direction which would have the effect of opening channels at more negative clamp potentials but the maximum number of open channels would remain the same, (iii) increasing the single-channel amplitude and/or (iv) increasing the channel open time. Our data suggest that the enhancement in calcium currents by CGRP in rat nodose ganglion neurones was associated with an increase in maximal calcium conductance, as shown by the instantaneous current–voltage relation. However, in three neurones CGRP produced a modest increase ($\sim 12\%$) in calcium current and a negative shift (~ 5 mV) in the peak of the current–voltage relation, but without a detectable increase in conductance. The data from these two cells suggest, by contrast, that the increase in calcium current may be due to a transmitter-induced change in the voltage dependence of channel activation. It has been proposed, for example, that neurotransmitters that reduce calcium current act to alter the voltage dependence of channel activation (Bean, 1989). We do not feel, however, that such a modest shift in the I–V relation should be taken as unequivocal evidence of a transmitter-induced change in the voltage-dependent properties of calcium channels. For the typical 20% increase in peak current produced by CGRP (1 μM), a negative shift of 10–20 mV in the peak of the I–V relation would be expected if the peptide was acting solely by altering the voltage dependence of channel activation. We did not observe shifts in the peak of the I–V relation of this magnitude. In separate studies, we examined whether CGRP increased calcium current by altering the voltage dependence of steady-state inactivation. Our studies indicated that the percentage increase in the peak calcium current produced by CGRP was similar, if not somewhat greater, when currents were evoked at $V_c = +10$ mV from $V_h = -110$ mV compared to $V_h = -60$ mV, suggesting that the peptide's effect occurred independently of a shift in the voltage dependence of steady-state inactivation over this range. Although our data do not allow a definite statement regarding the mechanism(s) by which CGRP increased calcium currents, we favour the view that more calcium channels are opened in the presence of the peptide, since the results do not strongly support the hypothesis that the peptide altered voltage-dependent properties.

The signal transduction pathway(s) underlying the G protein-dependent CGRP-mediated increase in calcium current remains to be fully elucidated, however. It is unclear, for example, whether the specific pathway involved direct coupling of the G protein to the calcium channel or indirect coupling via second messenger pathway(s) such as a cyclic AMP-dependent pathway. Since CGRP increased adenylate cyclase activity in other preparations, it is possible that the peptide acted via G_s to directly or indirectly activate calcium channels. Direct coupling of G_s to activation of calcium channels has been reported in cardiac myocytes (Yatani, Codina, Imoto, Reeves, Birnbaumer & Brown, 1987). Indirect activation of calcium current in cardiac myocytes and hippocampal neurones via cyclic AMP-dependent phosphorylation occurred with application of β-adrenoceptor agonists (Reuter, 1983; Kameyama, Hofmann & Trautwin, 1985; Kameyama, Hescheler, Hofmann & Trautwein, 1986; Gray & Johnston, 1987). In addition, the cyclic AMP-dependent protein kinase catalytic subunit selectively increased calcium current components in rat nodose neurones (Gross, Uhler & Macdonald, 1990). While these observations suggest that enhancement of calcium current can occur either via G_s or through cyclic AMP-dependent phosphorylation of calcium channels (or associated proteins), they do not readily explain our observation that the effect of CGRP was antagonized by PTX, which blocks the actions of G_i/G_o-type G proteins. These proteins are more commonly thought of as mediating the inhibition of adenylate cyclase or the reduction of calcium currents by inhibitory neurotransmitters. It is possible, for example, that G_i/G_o-type G proteins subserve tonic inhibition of the adenylate cyclase–cyclic AMP system and that PTX, by inactivating G_i/G_o produced a full activation of this pathway. If the effect of CGRP on I_{Ca} was the result of increased intracellular levels of cyclic AMP, this would result in a PTX-sensitive 'blockade' of its stimulatory action, because the pathway would be maximally activated in the presence of PTX. This hypothesis is supported by the observation that cardiac myocytes preincubated with pertussis toxin exhibited a twofold increase in the L calcium current component, suggesting that a pertussis toxin substrate significantly inhibited I_{Ca} in the basal state (Keung & Karliner, 1990). Thus, the present results support the idea that the coupling of CGRP receptors to calcium channels is G protein dependent, but further studies will be necessary to determine the exact nature of the biochemical pathway(s) involved.

We also examined the effect of CGRP on the release of the neurotransmitter acetylcholine in excised intact nodose ganglia to determine if CGRP-induced release of this neurotransmitter was calcium-dependent and pertussis toxin-sensitive. The nodose ganglion is a useful preparation to study the regulation of acetylcholine release because the neurotransmitter is present and dispersed nodose neurones are known to form nicotinic-type synapses in culture (Cooper, 1984; Ternaux *et al.* 1989). CGRP induced calcium-dependent release of acetylcholine that was significantly reduced after treatment with PTX and was blocked by tetrodotoxin. Thus, both CGRP-induced enhancement of calcium current and stimulation of acetylcholine release were PTX sensitive. The cellular mechanism(s) underlying CGRP-induced release of acetylcholine in nodose ganglion neurones remain to be clarified. Our neurotransmitter release studies were performed in Krebs–Ringer buffer; therefore, we cannot rule out that CGRP may also act on other ion channels, in addition to

calcium channels, to stimulate acetylcholine release. For example, CGRP evoked three types of excitatory responses in guinea-pig coeliac ganglion cells: a fast, a slow and a biphasic depolarization supporting involvement of different ion conductances (Dun & Mo, 1989). In myenteric neurones the primary mechanism underlying CGRP-induced slow depolarization appeared to involve inactivation of a calcium-activated potassium conductance (Palmer, Schemann, Tamura & Wood, 1986). CGRP-mediated release of acetylcholine may also involve activation of second messenger pathways. For example, studies involving myenteric neurones indicated that CGRP stimulated acetylcholine release via a cyclic AMP-dependent pathway (Mullholland & Jaffer, 1990). Therefore, it is possible that CGRP-induced release of acetylcholine in nodose neurones may utilize a cyclic AMP-dependent pathway (see above). Alternatively, CGRP receptors could be coupled via a PTX-sensitive form of the G protein G_p to activation of phospholipase C, mobilization of intracellular calcium and the subsequent release of acetylcholine. Our data suggest, however, that activation of calcium channels is responsible, at least in part, for the CGRP-induced increase in acetylcholine release.

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REFERENCES

BEAN, B. P. (1989). Neurotransmitter inhibition of neuronal calcium currents by changes in channel voltage dependence. *Nature* **340**, 153–156.

CASEY, P. J. & GILMAN, A. G. (1988). G protein involvement in receptor–effector coupling. *Journal of Biological Chemistry* **263**, 2577–2580.

COOPER, E. (1984). Synapse formation among developing sensory neurones from rat nodose ganglia grown in tissue culture. *Journal of Physiology* **351**, 263–274.

DUN, M. J. & MO, N. (1989). Calcitonin gene-related peptide evokes distinct types of excitatory response in guinea pig coeliac ganglion cells. *Brain Research* **476**, 256–264.

EWALD, D. A., STERNWEISS, P. C. & MILLER, R. J. (1988). Guanine nucleotide-binding protein G_o-included coupling of neuropeptide Y receptors to Ca^{2+} channels in sensory neurons. *Proceedings of the National Academy of Sciences of the USA* **85**, 3633–3637.

FOX, A. P., NOWYCKY, M. C. & TSIEN, R. W. (1987*a*). Kinetic and pharmacological properties distinguishing three types of calcium currents in chick sensory neurones. *Journal of Physiology* **394**, 149–172.

FOX, A. P., NOWYCKY, M. C. & TSIEN, R. W. (1987*b*). Single-channel recordings of three types of calcium channels in chick sensory neurones. *Journal of Physiology* **394**, 173–200.

GRAY, R. & JOHNSTON, D. (1987). Noradrenaline and b-adrenoceptor agonists increase activity of voltage-dependent calcium channels in hippocampal neurones. *Nature* **327**, 620–622.

GROSS, R. A. & MACDONALD, R. L. (1987). Dynorphin A selectively reduces a large transient (N-type) calcium current of mouse dorsal root ganglion neurons in cell culture. *Proceedings of the National Academy of Sciences of the USA* **84**, 5469–5473.

GROSS, R. A., MOISES, H. C., UHLER, M. D. & MACDONALD, R. L. (1990). Dynorphin A and cAMP-dependent protein kinase independently regulate neuronal calcium currents. *Proceedings of the National Academy of Sciences of the USA* **87**, 7025–7029.

GROSS, R. A., UHLER, M. D. & MACDONALD, R. L. (1990). The cyclic AMP-dependent protein kinase catalytic subunit selectively enhances calcium currents in rat nodose neurones. *Journal of Physiology* **429**, 483–496.

GROSS, R. A., WILEY, J. W., RYAN-JASTROW, T. & MACDONALD, R. L. (1990). Regulation by GTP and it stable thiol derivatives of calcium current components in rat nodose ganglion neurons. *Journal of Molecular Pharmacology* **37**, 546–553.

HESCHELER, J., ROSENTHAL, W., HINSCH, K. D., WULFERN, M., TRAUTWEIN, W. & SCHULTZ, G. (1988). Angiotensin II-induced stimulation of voltage-dependent Ca^{2+} currents in an adrenal cortical cell line. *EMBO Journal* **7**, 619–624.

HIRNING, L. D., FOX, A. P., MCCLESKY, E. W., OLIVERA, B. M., THAYER, S. A., MILLER, R. J. & TSIEN, R. W. (1988). Dominant role of N-type calcium channels in evoked release of norepinephrine from rat sympathetic neurons. *Science* **239**, 57–61.

HIRNING, L. D., FOX, A. P. & MILLER, R. J. (1990). Inhibition of calcium currents in cultured myenteric neurons by neuropeptide Y: evidence for direct receptor/channel coupling. *Brain Research* **532**, 120–130.

ISHIDA-YAMAMOTO, A. & TOHYAMA, M. (1989). Calcitonin gene-related peptide in the nervous tissue. *Progress in Neurobiology* **33**, 335–386.

KAMEYAMA, M., HESCHELER, J., HOFMANN, F. & TRAUTWEIN, W. (1986). Modulation of Ca current during the phosphorylation cycle in the guinea pig heart. *Pflügers Archiv* **407**, 123–128.

KAMEYAMA, M., HOFMANN, F. & TRAUTWEIN, W. (1985). On the mechanism of beta-adrenergic regulation of the Ca channels in the guinea pig heart. *Pflügers Archiv* **405**, 285–293.

KEUNG, E. C. & KARLINER, J. S. (1990). Complex regulation of calcium current in cardiac cells. *Journal of Clinical Investigation* **85**, 950–954.

KOJIMA, I., SHIBATA, H. & OGATA, E. (1986). Pertussis toxin blocks angiotensin II-induced calcium influx but not inositol trisphosphate production in adrenal glomerulosa cells. *FEBS Letters* **204**, 347–351.

LEWIS, D. L., WEIGHT, F. F. & LUINI, A. (1986). A guanine nucleotide-binding protein mediates the inhibition of voltage-dependent calcium current by somatostatin in a pituitary cell line. *Proceedings of the National Academy of Sciences of the USA* **83**, 9035–9039.

MCCLESKEY, E. W., FOX, A. P., FELDMAN, D. H., CRUZ, L. J., OLIVERA, B. M., TSIEN, R. W. & YOSHIKAMI, D. (1987). ω-Conotoxin: direct and persistent blockade of specific types of calcium channels in neurons but not muscle. *Proceedings of the National Academy of Sciences of the USA* **84**, 4327–4331.

MINTZ, I. M., VENEMA, V. J., ADAMS, M. E. & BEAN, B. P. (1991). Inhibition of N- and L-type Ca^{2+} channels by the spider venom toxin ω-Aga-111A. *Proceedings of the National Academy of Sciences of the USA* **88**, 6628–6631.

MULLHOLLAND, M. W. & JAFFER, S. (1990). Stimulation of acetylcholine release in myenteric plexus by calcitonin gene-related peptide. *American Journal of Physiology* **259**, G934–939.

NOWYCKY, M., FOX, A. P. & TSIEN, R. W. (1985). Three types of neuronal calcium channel with different calcium agonist sensitivity. *Nature* **316**, 440–443.

PALMER, J. M., SCHEMANN, M., TAMURA, K. & WOOD, J. D. (1986). Calcitonin gene-related peptide excites myenteric neurons. *European Journal of Pharmacology* **132**, 163–170.

PERNEY, T. M., HIRNING, L. D., LEEMAN, S. E. & MILLER, R. J. (1986). Multiple calcium channels mediated neurotransmitter release from peripheral neurons. *Proceedings of the National Academy of Sciences of the USA* **83**, 6656–6659.

PLUMMER, M. R., LOGOTHETIS, D. E. & HESS, P. (1989). Elementary properties and pharmacological sensitivities of calcium channels in peripheral neurons. *Neuron* **2**, 1453–1463.

PLUMMER, M. R. & HESS, P. (1991). Reversible uncoupling of inactivation in N-type calcium channels. *Nature* **351**, 657–659.

REGAN, L. J., SAH, D. W. Y. & BEAN, B. P. (1991). Ca^{2+} channels in rat central and peripheral neurons: high-threshold current resistant to dihydropyridine blockers and ω-conotoxin. *Neuron* **6**, 269–280.

REUTER, H. (1983). Calcium channel modulation by neurotransmitters, enzymes and drugs. *Nature* **301**, 569–574.

RYU, D P. D., GERBER, G., MURASE, K. & RANDIC, M. (1988). Calcitonin gene-related peptide enhances calcium current of rat dorsal root ganglion neurons and spinal excitatory synaptic transmission. *Neuroscience Letters* **89**, 305–312.

TERNAUX, J. P., FALEMPIN, M., PALOUZIER, B., CHAMOIN, M. C. & PORTALIER, P. (1989). Presence of cholinergic neurons in the vagal afferent system: Biochemical and immunohistochemical approaches. *Journal of the Autonomic Nervous System* **28**, 233–242.

WALKER, M. W., EWALD, D. A., PERNEY, T. M. & MILLER, R. J. (1988). Neuropeptide Y modulates neurotransmitter release and Ca^{2+} currents in rat sensory neurons. *Journal of Neuroscience* **8**, 2438–2446.

WANKE, E., FERRONI, A., MALGAROLI, A., AMBROSINI, A., POZZAN, T. & MELDOLESI, J. (1987). Activation of a muscarinic receptor selectively inhibits a rapidly inactivated Ca^{2+} current in rat sympathetic neurons. *Proceedings of the National Academy of Sciences of the USA* **84**, 4313–4317.

WILEY, J. W., GROSS, R. A., LU, Y. & MACDONALD, R. L. (1990). Neuropeptide Y reduces calcium current and inhibits acetylcholine release in nodose neurons via a pertussis toxin-sensitive mechanism. *Journal of Neurophysiology* **63**, 1499–1507.

WILEY, J. W. & OWYANG, C. (1987). Somatostatin inhibits cyclic AMP mediated cholinergic transmission in the myenteric plexus. *American Journal of Physiology* **253**, G607–612.

WU, T., KISSLINGER, S. D. & GAGINELLA, T. S. (1982). Functional evidence for the presence of cholinergic nerve endings in the colonic mucosa of the rat. *Journal of Pharmacology and Experimental Therapeutics* **221**, 664–669.

YATANI, A., CODINA, J., IMOTO, Y., REEVES, J. P., BIRNBAUMER, L. & BROWN, A. M. (1987). A G protein directly regulates mammalian cardiac calcium channels. *Science* **238**, 1288–1292.

YAWO, H. (1990). Voltage activated calcium currents in presynaptic nerve terminals of the chicken ciliary ganglion. *Journal of Physiology* **428**, 199–213.