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G Proteins Couple α-Adrenergic and $GABA_b$ Receptors to Inhibition of Peptide Secretion from Peripheral Sensory Neurons

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Regulation of neuronal calcium channels by GTP-binding proteins (G proteins) is likely to be an important mechanism by which inhibitory transmitters influence excitation-secretion coupling in presynaptic nerve endings. Here, we report that in peripheral sensory neurons from embryonic chick dorsal root ganglia (DRG), the G protein-mediated inhibition of voltage-dependent calcium channels may best explain how norepinephrine (NE) and GABA inhibit the electrically evoked, calcium-dependent release of substance P (SP). As is the case for the previously reported inhibitory actions of these transmitters on DRG cell calcium channels, we demonstrate that NE and GABA inhibit peptide secretion through activation of α-adrenergic and $GABA_b$ receptors that are functionally coupled to pertussis toxin (PTX)-sensitive G proteins. Pretreatment of DRG cell cultures with PTX blocked the ability of NE and GABA to inhibit the release of SP, an action correlated with PTX-catalyzed ADP-ribosylation of membrane proteins with apparent molecular weight (M_r) of 40–41 kDa. Western immunoblot analysis of chick DRG cell membrane proteins using antisera directed against synthetic peptides corresponding to amino acid sequences predicted from cDNAs for PTX-sensitive G protein α subunits revealed a *minimum* of 2 G_i-like proteins (M_r 40 and 41 kDa) and a third G_o-like protein (M_r 40 kD). Significantly, these findings implicate G_i- and/or G_o-like GTP-binding proteins as mediators of presynaptic inhibition in peripheral sensory neurons.

GTP-binding proteins (G proteins) comprise a family of structurally homologous, yet functionally distinct, regulatory proteins serving as intermediaries in transmembrane signal transduction (reviewed by Stryer and Bourne, 1986; Gilman, 1987). G proteins couple cell-surface receptors to plasma membrane effector molecules, including second-messenger-generating enzymes (e.g., adenylate cyclase, polyphosphoinositide phosphodiesterase; see reviews cited above) and ion channels (reviewed by Dunlap et al., 1987). Recently, we have begun to examine what role G proteins play in the regulation of neuronal excitability and excitation–secretion coupling. To this end, we have focused on the mechanism by which norepinephrine (NE) and GABA inhibit voltage-dependent calcium channels in, and peptide secretion from, dorsal root ganglion (DRG) sensory neurons, and have sought to identify the G protein-regulated signal transduction pathway(s) responsible for such modulatory influences.

In earlier studies it was reported that NE and GABA inhibit calcium currents in DRG neurons (Dunlap and Fischbach, 1978, 1981) through stimulation of α-adrenergic (Canfield and Dunlap, 1984) and $GABA_b$ receptors (Dunlap, 1981). These receptors are functionally coupled to a G protein-regulated signal transduction mechanism. The responses to NE and GABA are blocked by intracellular application of GDP-β-S (an analog of GDP that competitively inhibits the binding of GTP to, and activation of, G proteins) or by prior exposure of DRG neurons to *Bordetella pertussis* toxin (PTX), a bacterial exotoxin catalyzing ADP-ribosylation and inactivation of G proteins (Holz et al., 1986a). On the basis of these findings it was proposed that the G protein-mediated inhibition of neuronal calcium channels might be one mechanism by which inhibitory transmitters suppress excitation–secretion coupling in presynaptic nerve endings.

To test this hypothesis we have characterized the electrically evoked release of substance P (SP) from DRG cell cultures and have confirmed the previous report of Mudge and coworkers that NE and GABA inhibit peptide secretion from these sensory neurons (Mudge, 1979; Fischbach et al., 1981; Holz et al., 1985). Here we report that, as is the case for the previously reported inhibitory actions of NE and GABA on DRG cell calcium channels, the transmitters inhibit peptide secretion through activation of α-adrenergic and $GABA_b$ receptors and that these receptors are functionally coupled to PTX-sensitive G proteins. Furthermore, we report the initial characterization of DRG cell G proteins on the basis of molecular weight, susceptibility to PTX-catalyzed ADP-ribosylation, and immunological profile as assessed by Western blot analysis using antisera that distinguish between α subunits of G_i and G_o. Significantly, these findings implicate G_i- and/or G_o-like proteins as mediators of presynaptic inhibition in peripheral sensory neurons. For preliminary reports of these findings, see Holz et al. (1985, 1986b) and Dunlap et al. (1986).

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Materials and Methods

Preparation of DRG cell cultures. Primary cultures of embryonic chick DRG neurons were prepared as previously described (Holz et al., 1988). Briefly, freshly dissected DRG from 10- to 12-d-old embryos were mechanically dissociated by trituration to yield a single-cell suspension. The cells were γ-irradiated to suppress the proliferation of non-neuronal cells and plated on collagen-coated tissue culture dishes at a density of ca. 1.5×10^5 neurons/60 mm dish or 0.5×10^5 neurons/35 mm dish, as determined by cell counts 6 d postplating. Cultures were fed Dulbecco's Modified Eagle's Medium (DMEM, supplemented with 7S nerve growth factor, 5% chick embryo extract, 10% horse serum, 1 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin). The peak phase of non-neuronal cell death was observed on days 3–5 postplating. Cell counts performed on day 6 demonstrated that ≥95% of the remaining cells could be classified as sensory neurons on the basis of morphological criteria.

Preparation of solutions. DRG cell cultures were bathed in saline buffered with 25 mM HEPES (pH 7.4). The HEPES-buffered saline (HBS) contained (in mM): 132 NaCl, 2.5 KCl, 0.8 $MgCl_2$, and 0.04% BSA. In the experiments summarized in Figures 1, 2, 4, and 5 and in Table 1, the HBS also contained 1 mM $BaCl_2$ and 2 mM $CaCl_2$. This solution was chosen because $BaCl_2$, although not a secretagogue, facilitates the electrically evoked release of SP (by prolonging the duration of DRG cell action potentials), thereby allowing reduced assay sensitivity and increased assay accuracy (Holz et al., 1988). It is important to note, however, that in the present study inhibitory responses to NE and GABA were routinely observed when $BaCl_2$ was omitted from the HBS. For example, in the experiments illustrated in Figures 3 and 6, the HBS contained 3 mM $CaCl_2$ with no added $BaCl_2$.

NE (*d,l*-arterenol), yohimbine, propranolol, GABA, muscimol, and (+)bicuculline (free base) were obtained from Sigma. Lioresal, (+)baclofen and (−)baclofen were obtained from CIBA-GEIGY, prazosin from Pfizer, and clonidine from Boehringer-Ingelheim, Ltd. Drugs were prepared as concentrated stock solutions immediately prior to each experiment. Stock solutions were diluted in HBS to obtain the final desired concentrations. Control HBS solutions (i.e., no added drugs) were prepared with appropriate vehicle solutions added.

Stimulation protocol for SP release experiments. Cultures were stimulated under sterile conditions at room temperature using bipolar platinum stimulating electrodes (square-wave direct-current pulses, 3 msec duration, 110 V, 1 Hz). As previously reported (Holz et al., 1988), intracellular recordings from DRG cell bodies demonstrated that this innocuous form of stimulation generates action potentials in all neurons tested, thereby inducing the calcium-dependent release of SP.

Radioimmunoassay for released SP. SP was measured by direct radioimmunoassay (RIA) of the solution bathing the cells (Holz et al., 1988) using an antiserum specific for SP and its sulfoxide derivative (Kream et al., 1985). Standard curves were generated by assaying serial dilutions of synthetic SP standard (Sigma) that was diluted in the appropriate HBS test solution. None of the drugs tested interfered with binding of the tracer to the antibody. For determination of the cellular content of SP, cultures were extracted in 2 ml of 2 N acetic acid containing 0.5% BSA. Aliquots of this solution were lyophilized and resuspended in assay buffer, and SP content was determined by RIA. The RIA intra-assay coefficient of variation was <10%.

PTX treatment of DRG cell cultures. PTX was a generous gift of Dr. Ronald Sekura, National Institutes of Health (Sekura et al., 1983). PTX was stored at 4°C as a stock suspension (1.4 mg/ml) in saturated $(NH_4)_2SO_4$. The stock suspension was diluted 1:1000 in 10 mM sodium phosphate buffer (pH 7.2) containing 50 mM NaCl and 0.04% protease-inactivated BSA. Freshly diluted PTX was then diluted an additional 10-fold in DMEM containing 0.1% glutamine to yield a final dilution factor of 1:10,000 containing 140 ng/ml PTX. Control (i.e., vehicle-treated) cultures were incubated in DMEM containing 0.1% glutamine and saturated $(NH_4)_2SO_4$ diluted 1:10,000.

Preparation of DRG cell and cerebral cortical membranes. Chick DRG and cerebral cortices were dissected from 12-d-old embryos. DRG cells were γ-irradiated, and plated on tissue culture dishes, and the neurons were harvested 6 d postplating. The DRG neurons and cerebral cortices were homogenized while suspended in ice-cold buffer containing (in mM): 100 Tris (pH 7.8), 1.2 $MgCl_2$, 0.2 EDTA, 1.5 EGTA, 0.2 dithiothreitol, and (in mg/ml) 5.0 dextrose, 0.1 leupeptin, and 0.1 soybean trypsin inhibitor. The homogenate was centrifuged (20 min, 200 × *g*), the pellet discarded, and the supernatant recentrifuged (20 min, 50,000 × *g*) to obtain a membrane fraction. Membranes were resuspended in buffer (protein concentration, 6–9 mg/ml, as determined by the method of Lowry et al., 1951). Bovine cerebral cortical membranes, and cholate extracts thereof, were prepared as described by Gierschik et al. (1986a).

ADP-ribosylation of DRG cell membrane proteins. ADP-ribosylation was examined by a modification of the method of Neer et al. (1984). For the ribosylation reaction, 50–200 μg of membrane protein were suspended in a final volume of 50 μl containing (in mM): 85 Tris (pH 7.8), 10 dithiothreitol, 10 thymidine, 10 isonicotinic acid hydrazide, 6 $MgCl_2$, 3 ATP, 0.9 EGTA, and (in μM) 120 EDTA, 100 GTP, 5 NAD, and 0.5–2.0 μCi ^{32}P-NAD (New England Nuclear NEG-023; final specific activity, 3–12 Ci/mmol). PTX (List Biochemicals) was preactivated at room temperature in a solution containing (in mM) 100 Tris (pH 7.8), 25 dithiothreitol, and 1 ATP, and stored at 4°C in 50% glycerol. To each assay, PTX was added such that its final concentration was 2.4 μg/ml. The ribosylation reaction was allowed to proceed for 45 min at 37°C and terminated by addition of SDS sample buffer. Proteins were solubilized by boiling for 10 min and applied to 13% SDS-polyacrylamide gels, and electrophoresis was performed as described by Laemmli (1970). Gels were silver-stained, dried, and used to expose Kodak X AR50 film for 1–4 d at −70°C.

Immunoblot analysis of DRG and cerebral cortical membranes. SDS-PAGE and immunoblotting were performed as described previously (Gierschik et al., 1986a; Goldsmith et al., 1987), except that the 10% gels contained half (0.13 gm/100 ml) the usual concentration of bisacrylamide. This permitted greater resolution of proteins in the M_r 40 kDa range. Crude antisera used for immunoblots were raised against synthetic peptides whose sequence was predicted by cDNAs encoding G protein α subunits. Antisera included AS/7 [directed against the carboxy (C)-terminus decapeptide of transducin α], and LE/2 (amino acids 160–169 of $G_{i\alpha 2}$), whose preparation and characterization was described by Goldsmith et al. (1987). Also tested were GO/1 (C-terminus decapeptide of $G_{o\alpha}$), whose preparation and characterization will be described elsewhere (P. Goldsmith, C. G. Unson, and A. Spiegel, unpublished observations), and LD/1 (amino acids 159–168 of $G_{i\alpha 1}$), whose preparation and characterization has been reported by Goldsmith et al. (1988).

Results

Electrically evoked release of SP

Release experiments were performed using DRG cell cultures 9–16 d postplating, at which time the average cellular content of SP was 10–15 ng/60 mm dish. As previously reported, electrical stimulation of these cultures induces calcium-dependent release of SP-like immunoreactivity into the solution bathing the cells (Holz et al., 1988). High-pressure liquid chromatography demonstrated that the SP-like immunoreactivity synthesized by DRG neurons is authentic SP (Holz et al., 1988). Therefore, we shall refer to this immunoreactivity as SP. In the experiments described below, the effects of NE and GABA on the electrically evoked release of SP were assessed.

NE inhibits peptide secretion

Dunlap and Fischbach (1981) and Canfield and Dunlap (1984) reported that NE in concentrations ranging from 0.1 to 100 μM (EC_{50} = 1 μM) inhibits voltage-dependent calcium currents and calcium-dependent action potentials recorded from chick DRG cell bodies. On average, saturating concentrations of NE (and GABA, see below) inhibit the calcium current by ca. 35–40%. Figure 1 illustrates that a saturating concentration of NE (50 μM) also inhibits, but does not completely block, the electrically evoked release of SP. To examine modulation of the release mechanism by NE, cultures were stimulated on 3 successive occasions (stimulation phases S_1–S_3). Each phase of stimulation was separated by a 1.5 hr interval, during which the cultures were returned to the incubator for reequilibration in culture

medium. This insured that reproducible amounts of SP would be released during all 3 phases of stimulation. Phases S_1 and S_3 served as controls (i.e., the HBS contained only the vehicle in which the drugs were dissolved), whereas stimulation phase S_2 served as a test (i.e., the HBS contained the drug to be tested). For each phase of stimulation, the evoked release of SP was determined by subtracting baseline values of SP from the total amount released. By calculating the *release ratio,*

$$S_2/[(S_1 + S_3)/2],$$

we compared how different drugs affect the SP release mechanism.

As shown in Figure 1*A*, the control release ratio approximated unity (1.05) when NE was omitted from the HBS during S_2. In contrast, in the presence of 50 μM NE during S_2, the release ratio was reduced to 0.40 (Fig. 1*B*). The percent inhibition due to NE was calculated as

$$[1 - (\text{Test release ratio}/\text{Control release ratio})] \times 100\%.$$

Using this analysis, NE inhibited SP release by 62%. The inhibitory action of NE was observed in 6 of 6 release experiments using 54 cultures from 6 different platings. The average percent inhibition due to 50 μM NE was 47 ± 3% (mean ± SEM, n = 18 cultures).

Pharmacological properties of NE receptors

In previous electrophysiological studies Canfield and Dunlap (1984) reported that a nonclassical subtype of α-adrenergic receptor mediates the inhibition of DRG cell calcium channels by NE. This action of NE is blocked by the specific α_2-receptor antagonist yohimbine (IC_{50} = 10 nM), but not by micromolar concentrations of the α_1-receptor antagonist prazosin or the β-receptor antagonist propranolol. Therefore, the antagonist pharmacology of these avian adrenergic receptors matches that previously described for mammalian α_2-receptors (Langer, 1981). Unexpectedly, however, the α_2-receptor agonists clonidine and xylazine were ineffective when tested for their ability to inhibit calcium channel function. To determine if this unusual pharmacological profile is also characteristic of DRG cell receptors that mediate inhibition of peptide secretion, we compared the relative potency of equimolar concentrations of these adrenoreceptor agonists and antagonists.

Figure 1*C* illustrates the effect of yohimbine on adrenergic receptor-mediated inhibition of SP release. When cultures were exposed to 10 μM yohimbine and 50 μM NE during stimulation phase S_2, the release ratio approximated unity (1.12), a value not significantly different ($p > 0.20$, t test) from control (cf. Fig. 1, *A*, *C*). Yohimbine, itself, did not affect the SP release mechanism. In contrast, neither 10 μM prazosin nor 10 μM propranolol blocked the inhibitory action of 50 μM NE. Furthermore, the action of NE was not mimicked by clonidine (the average percent inhibition due to 50 μM clonidine was 7 ± 8%, n = 3 cultures from a single experiment). These findings, summarized in Figure 2, indicate that α_2-like adrenergic receptors with similar, if not identical, pharmacological properties mediate the inhibitory actions of NE on calcium channels and peptide secretion.

Concentration-dependent inhibitory actions of NE

Figure 3 illustrates the concentration dependence of the inhibitory action of NE as determined using a single-phase stimulation protocol (5 min, 1 Hz). In this experiment, the amount of SP released was expressed as a percentage of the total cellular content of SP prior to stimulation. Note that NE inhibited the release of SP in a graded fashion over a concentration range of ca. 0.3–30 μM and that 30 μM NE inhibited the release of SP by 68%. The IC_{50} value for the inhibitory action of NE was ca. 1 μM, a value identical to that previously determined by Canfield

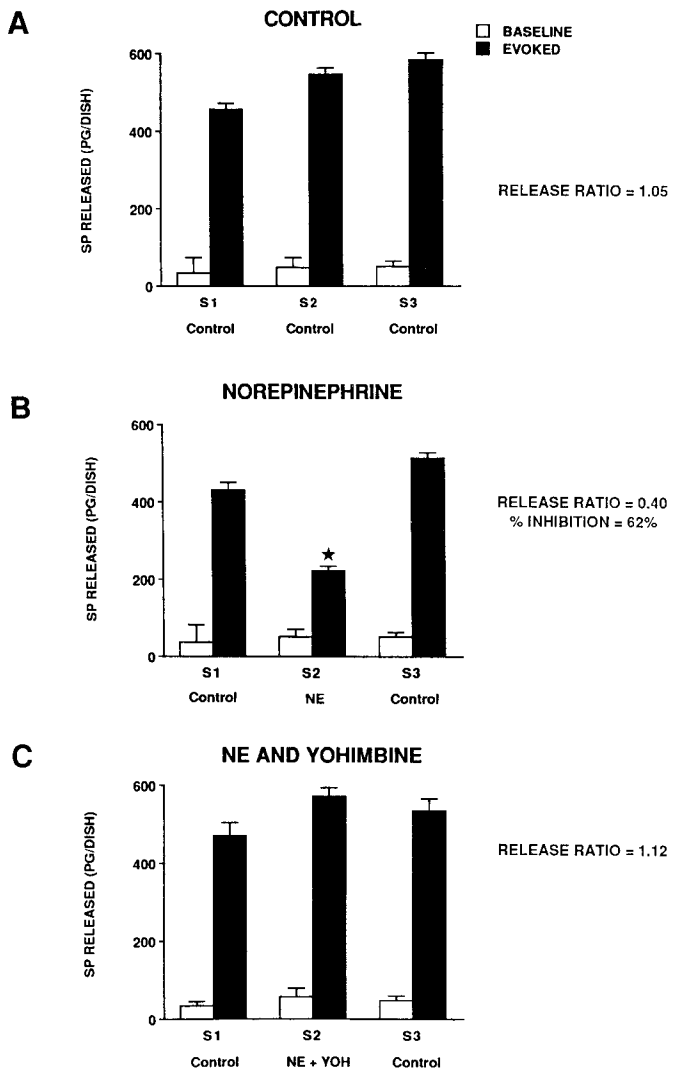

Figure 1. Norepinephrine inhibits the release of SP. Nine 60 mm cultures from a single plating were divided into 3 sets of 3—a control set, a norepinephrine (NE)-treated set, and a set treated with both NE and yohimbine (YOH). Each culture was stimulated for 90 sec at 1 Hz while bathed in HEPES-buffered saline (HBS). Baseline and evoked levels of SP immunoreactivity were determined by direct RIA of the HBS for 3 successive phases of stimulation (S_1–S_3). Each phase of stimulation was separated by a 1.5 hr interval during which the cultures were returned to the incubator while bathed in culture medium. During S_2 one set of cultures was exposed to HBS containing only the vehicle (acidified H_2O) in which the drugs were dissolved (*A*), a second set was exposed to HBS containing 50 μM NE (*B*), and a third set was exposed to HBS containing 50 μM NE and 10 μM YOH (*C*). Here, and in all subsequent figures, the drugs tested did not significantly affect baseline levels of released SP. The release ratio and percent inhibition were calculated as described in Results. Note that NE inhibited the release of SP by 62% (* $p \leq 0.001$, unpaired t test), and that the action of NE was antagonized by YOH. Error bars indicate the mean ± SEM (n = 3). In these cultures, 50 μM NE decreased the duration of DRG cell action potentials as previously reported (Canfield and Dunlap, 1984).

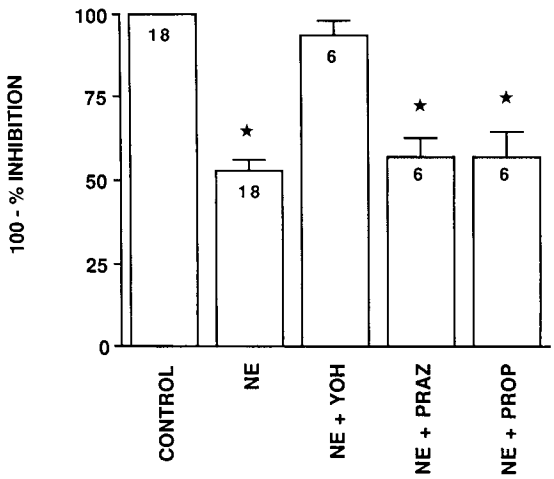

Figure 2. Pharmacological properties of norepinephrine receptors. Summary of the findings demonstrating that α_2-adrenergic-like receptors mediate the inhibitory action of NE on the SP release mechanism. The results of 6 experiments using 54 cultures from 6 different platings are shown. The experimental protocol was identical to that illustrated in Figure 1. For each experiment the mean release ratio and percent inhibition of SP release were calculated as described in Results. Values for all experiments were then averaged, and a statistical comparison was made between control and test values by unpaired *t* tests. Error bars indicate mean ± SEM. Note that the action of 50 μM NE was antagonized by 10 μM yohimbine (*YOH*, an α_2-receptor antagonist), but not by 10 μM prazosin (*PRAZ*, an α_1-receptor antagonist), or 10 μM propranolol (*PROP*, a β-receptor antagonist). *Asterisks* indicate that values were significantly different from control ($p \leq 0.001$); the absence of an asterisk indicates that the value was not significantly different from control ($p > 0.05$). In this series of experiments, the control release ratio was 1.03 ± 0.04. Values within each histogram indicate the number of cultures tested.

and Dunlap (1984) for the inhibitory action of NE on DRG cell calcium channels.

GABA inhibits the release of SP

Embryonic chick DRG neurons express 2 types of GABA receptor on their cell bodies (Dunlap, 1981). $GABA_a$ receptors are activated by relatively high concentrations of GABA (EC_{50} = 10 μM), coupled to chloride ion channels, and mediate the depolarizing actions of GABA. In contrast, $GABA_b$ receptors are activated by lower concentrations of GABA (EC_{50} = 1 μM) and mediate inhibition of voltage-dependent calcium channels. In later-stage cultures, such as those used in the present study, $GABA_b$ receptors predominate (Dunlap, 1981). To examine inhibition of peptide secretion by GABA, experiments were conducted using the 3-phase stimulation protocol. As illustrated in Figure 4*A*, the control release ratio approximated unity (0.93) when GABA was omitted from the HBS during S_2. In contrast, in the presence of 10 μM GABA during S_2, the release ratio was reduced to 0.64, indicating that GABA inhibited the release of SP by 31% (Fig. 4*B*). The inhibitory action of GABA was observed in 12 of 12 cultures from 3 different platings. The average percent inhibition due to 10 μM GABA was 31 ± 4%. Note that as also illustrated in Figure 4*C*, the inhibitory action of 10 μM GABA was not blocked by 50 μM (+)bicuculline, a selective antagonist of $GABA_a$ receptors on chick DRG neurons (Dunlap, 1981; Bowery, 1982).

Pharmacological properties of GABA receptors

We examined the effects of selective $GABA_a$ and $GABA_b$ receptor ligands in order to characterize the pharmacological properties of these GABA receptors. Such an analysis is complicated by the fact that there is, at present, no specific $GABA_b$ receptor antagonist. Dutar and Nicoll (1988) reported that millimolar concentrations of phaclofen, a phosphonic acid derivative of baclofen, block $GABA_b$ receptor-mediated responses in hippocampal slice preparations. However, the specificity of this antagonist action remains to be fully characterized. $GABA_b$ receptors must therefore be characterized on the basis of agonist selectivity. As summarized in Figure 5, the inhibitory action of GABA was mimicked by 10 μM (±)baclofen (Lioresal), a selective $GABA_b$ receptor agonist (Bowery, 1982) that mimics the action of GABA on DRG cell calcium channels (Dunlap, 1981). The action of 10 μM baclofen was stereoselective. (−)baclofen inhibited the release of SP by 34%, whereas (+)baclofen inhibited release by only 3%. Furthermore, muscimol (10 μM), a $GABA_a$ receptor agonist, was relatively ineffective compared to (−)baclofen. These observations suggest that GABA inhibits the release of SP by activating $GABA_b$ receptors. The failure of 50 μM (+)bicuculline to block the action of 10 μM GABA (Fig. 4*C*) supports this conclusion.

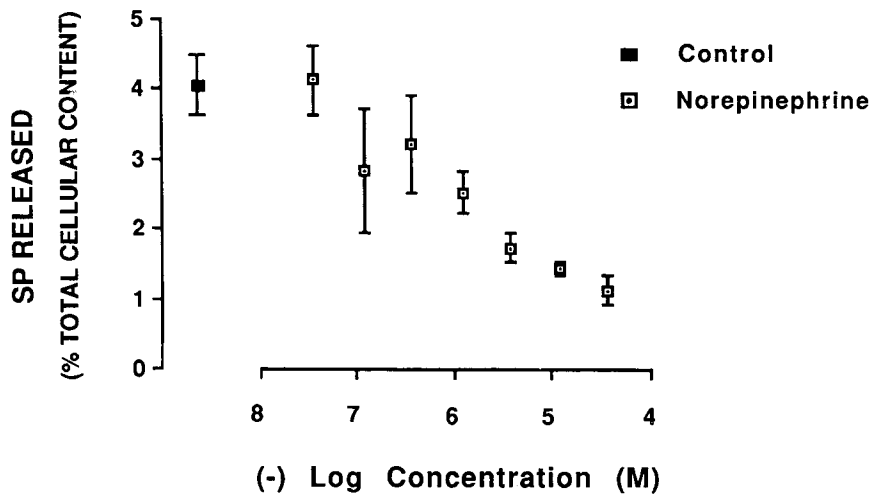

Figure 3. Dose–response relationship for norepinephrine. Twenty-four 35 mm cultures from a single plating were divided into 8 sets of 3 [1 control set, and 7 NE-treated sets, each of which was exposed to NE (30 nM–30 μM)]. Cultures were stimulated (5 min, 1 Hz) while bathed in HBS containing 3 mM $CaCl_2$. The amount of SP released is expressed as a percentage of the total cellular content of SP prior to stimulation, as determined after extraction of the cultures in acetic acid. The average cellular content of SP was 1.7 ng/dish (n = 24). Note that NE inhibited the release of SP in a concentration-dependent fashion (IC_{50} 1 μM). Error bars indicate means ± SEM (n = 3).

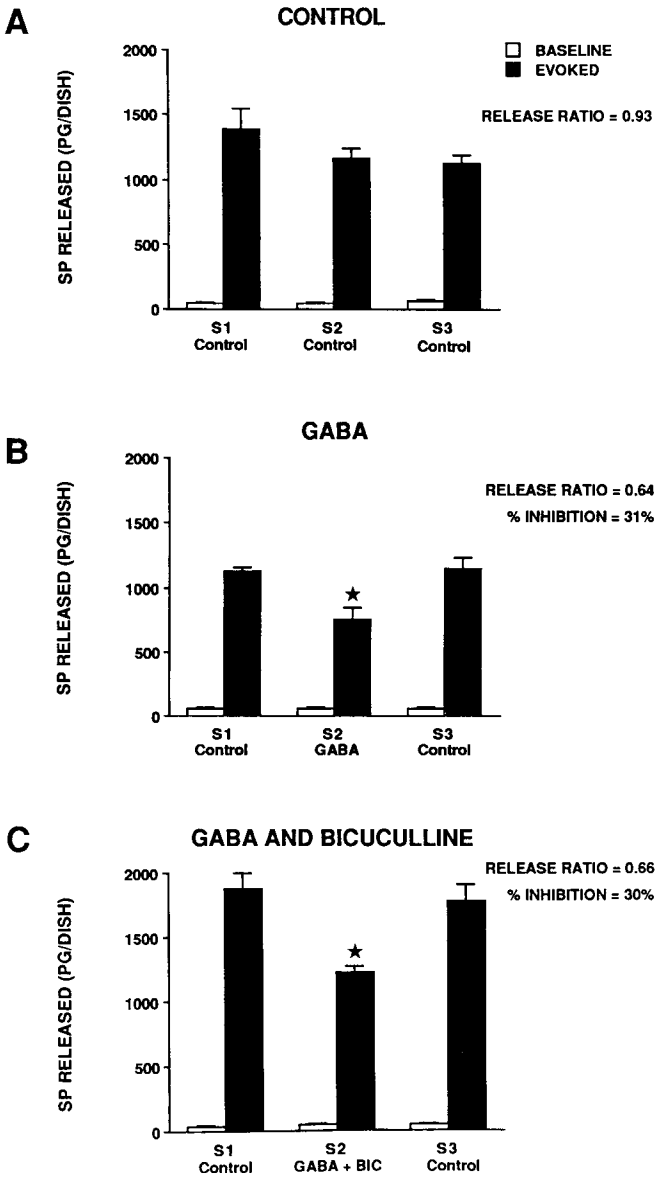

Figure 4. Inhibition of peptide secretion by GABA. Nine 60 mm cultures from a single plating were divided into 3 sets of 3. The stimulation protocol and composition of the bathing solution was identical to that described in Figure 1. During stimulation phase S_2, one set of control cultures was bathed in HBS containing only the vehicle (acidified H_2O) in which the drugs were dissolved (*A*), a second set was exposed to HBS containing 10 μM GABA (*B*), and a third set was exposed to HBS containing 10 μM GABA and 50 μM (+)bicuculline, a $GABA_a$ receptor antagonist (*C*). Note that GABA inhibited the release of SP by 31%, and that (+)bicuculline failed to antagonize the response to GABA (*$p \leq 0.005$, unpaired *t* test). Error bars indicate mean ± SEM ($n = 3$). In this experiment, SP release at S_1 ranged from 1.4–1.9 ng/dish depending on which set of cultures was tested. This variability simply reflects differences in the average cellular content of SP per dish within each of the sets.

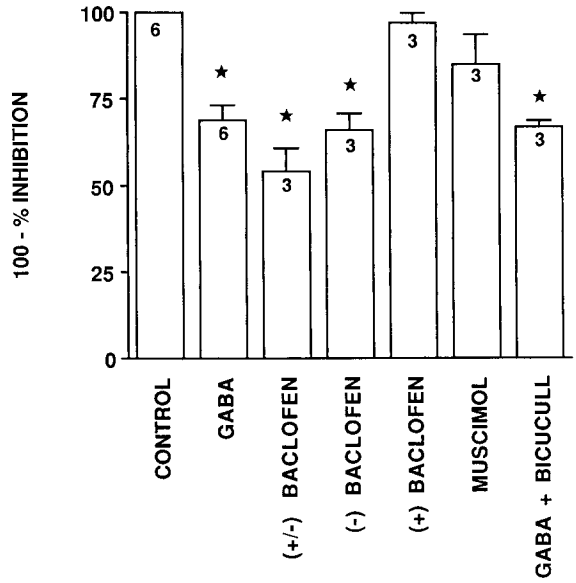

Figure 5. Pharmacological properties of GABA receptors. Summary of experiments demonstrating that $GABA_b$ receptors mediate the inhibitory action of GABA on the SP release mechanism. The results of 2 experiments using 27 cultures from 2 different platings are shown. The experimental protocol was similar to that described in Figure 4. Note that the inhibitory action of 10 μM GABA was mimicked by 10 μM (±)baclofen, a $GABA_b$ receptor agonist but not by 10 μM muscimol, a $GABA_a$ receptor agonist. Note also that the action of baclofen was stereoselective: 10 μM (−)baclofen inhibited the release of SP, whereas 10 μM (+)baclofen was without significant effect. Furthermore, the action of 10 μM GABA was not antagonized by 50 μM (+)bicuculline, a $GABA_a$ receptor antagonist. *Asterisks* indicate that the value was significantly different from control ($p \leq 0.005$, unpaired *t* test); the absence of an asterisk indicates that the value was not significantly different from control ($p > 0.05$). In this series of experiments the control release ratio was 0.99 ± 0.03. Error bars indicate means ± SEM. Values within each histogram indicate the number of cultures tested.

Role for G proteins in the regulation of peptide secretion

On the basis of the pharmacological analysis presented above, we suggest that NE and GABA inhibit the release of SP by suppressing the influx of calcium ions through voltage-dependent calcium channels. As an additional test of this hypothesis, we examined what role G proteins play in this receptor-mediated inhibition of peptide secretion. Specifically, the effects of the transmitters were tested on cultures pretreated with *B. pertussis* toxin, a bacterial exotoxin previously reported to block the G protein-mediated inhibitory actions of NE and GABA on DRG cell calcium currents (Holz et al., 1986a).

Cultures were pretreated with PTX (140 ng/ml, 16 hr, 37°C, conditions that effectively block the inhibitory actions of the transmitters on calcium channels), and experiments were performed using a single-phase stimulation protocol (5 min, 1 Hz). In cultures not treated with PTX, NE (50 μM) inhibited the release of SP by 82% (Fig. 6*A*), whereas in PTX-treated cultures, no significant inhibitory action of the transmitter was observed (Fig. 6*B*).

Table 1 summarizes an additional experiment in which the action of PTX was examined using a 3-phase stimulation protocol. In cultures not pretreated with PTX, NE (50 μM) and GABA (10 μM) inhibited the release of SP by an average of 60 and 52%, respectively, whereas in PTX-treated cultures, the transmitters were without significant effect. This action of the toxin was observed in 3 of 3 identical release experiments using 54 cultures from 3 different platings.

PTX-catalyzed ADP-ribosylation of DRG cell proteins

G proteins are membrane-associated heterotrimers consisting of α (*MW* 39–52 kDa), β (35 and 36 kDa), and γ (8–11 kDa)

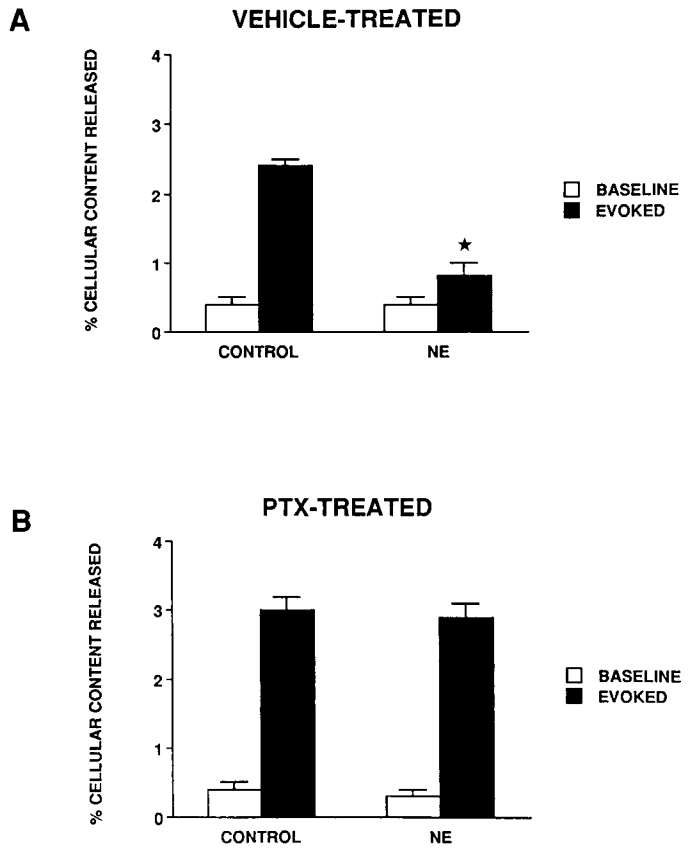

Figure 6. PTX blocks the inhibitory action of NE, as demonstrated using a single-phase stimulation protocol. Twelve 35 mm cultures from a single plating were divided into 4 sets of 3. Two sets of cultures (*A*) were incubated for 16 hr at 37°C in MEM containing no PTX, but to which the $(NH_4)_2SO_4$ vehicle solution was added. The remaining 2 sets of cultures were incubated for 16 hr at 37°C in 140 ng/ml PTX (*B*). Each set of cultures was then electrically stimulated (5 min, 1 Hz) while bathed in HBS containing 3 mM $CaCl_2$. The amount of SP released from control cultures (columns at *left*) was compared with that released from cultures exposed to 50 μM NE (columns at *right*). Values for baseline and evoked levels of released SP are the means ± SEM for 3 cultures of a single set, expressed as a percentage of the total cellular content of SP per culture prior to stimulation. The average cellular content of SP was 2.8 ng/dish ($n = 12$). NE inhibited the release of SP from vehicle-treated cultures by 82%, whereas no significant inhibitory action of NE was observed in cultures treated with PTX. *Asterisks* indicate that the value was significantly different from control ($p \leq 0.001$, unpaired t test). Note, also, that PTX, by itself, facilitated the evoked release of SP by ca. 30% but had no effect on basal levels of SP immunoreactivity.

subunits. The α subunit interacts with cell-surface receptors, binds guanyl nucleotides (α-GDP, when inactive), and has intrinsic GTP-ase activity. Binding of agonist to these receptors catalyzes the exchange of GDP for GTP and consequent dissociation of the heterotrimer into fully active α-GTP and βγ subunits. Activation of the G protein is terminated by hydrolysis of GTP, followed by reassociation of α-GDP with free βγ to reform the inactive heterotrimer. According to one presently accepted model, PTX blocks G protein-regulated signal transduction by catalyzing ADP-ribosylation of G protein α subunits, thereby preventing agonist-induced dissociation of the heterotrimeric G protein complex into its active subunits (Ui et al., 1985). To ascertain whether the effects of PTX on DRG neurons are consistent with this proposed mechanism of action, we tested for PTX-catalyzed ADP-ribosylation of DRG cell membrane proteins.

Table 1. PTX blocks the responses to NE and GABA, as demonstrated using a 3-phase stimulation protocol.

	Vehicle-treated ($n = 9$)		PTX-treated ($n = 9$)	
Group	Release ratio	Percent inhibition	Release ratio	Percent inhibition
Control	1.04 ± 0.07	—	0.87 ± 0.03	—
NE	0.42 ± 0.04	60 ± 4[a]	0.78 ± 0.08	n.s.[b]
GABA	0.50 ± 0.06	52 ± 6[a]	0.92 ± 0.07	n.s.[b]

Eighteen 60 mm cultures from a single plating were divided into 6 sets of 3. Nine cultures were incubated for 16 hr at 37°C in MEM containing no PTX but to which the $(NH_4)_2SO_4$ vehicle solution was added. The remaining 9 cultures were incubated for 16 hr at 37°C in MEM containing 140 ng/ml PTX. Each set of cultures was then stimulated on 3 successive occassions for 90 sec at 1 Hz while bathed in HBS continaing 1 mM $BaCl_2$ and 2 mM $CaCl_2$. For vehicle-treated cultures, NE (50 μM) and GABA (10 μM) inhibited the release of SP by 60 and 52%, respectively. In contrast, neither NE nor GABA significantly inhibited the release of SP from PTX-treated cultures. Note, also, that in PTX-treated cultures the control *release ratio* was reduced to 0.87, whereas in vehicle-treated cultures it approximated unity. This phenomenon was observed in all 3 release experiments using the 3-phase stimulation protocol and is attributable to a small decrement in SP release observed between phases S_1 and S_2. This phenomenon did not, however, obscure the ability of the toxin to block the actions of the transmitters. The release ratio and percent inhibition were calculated as described in Results. All values are means ± SEM.

[a] Significantly different from control ($p \leq 0.001$, t test).

[b] Not significantly different from control ($p > 0.10$).

Figure 7 illustrates that DRG cell membranes do, in fact, contain substrates for PTX-catalyzed ADP-ribosylation, as ascertained by SDS-PAGE and autoradiography. Note that under conditions in which ^{32}P-NAD served as the donor source of ADP-ribose, PTX catalyzed the ribosylation of membrane proteins with M_r of approximately 40–41 kDa.

Immunological characterization of DRG cell G proteins

Immunochemical studies (Gierschik et al., 1986b), sequencing of cloned DNAs (cDNAs) encoding G protein α subunits (Bray et al., 1987; Jones and Reed, 1987), and purification of GTP-binding proteins (Katada et al., 1987) indicate that there are several distinct forms of G protein α subunit, each serving as substrate for PTX-catalyzed ADP-ribosylation. These α subunits include 2 forms of transducin (a G protein found in retinal rods and cones), $G_{o\alpha}$ (a 39 kDa protein abundant in brain), and at least 3 closely related 40–41 kDa proteins termed $G_{i\alpha1}$, $G_{i\alpha2}$, and $G_{i\alpha3}$ (arbitrarily designated in order of cDNA cloning by Jones and Reed, 1987, without implying which, if any, is responsible for inhibition of adenylate cyclase). To determine which PTX substrates are present in chick DRG cell membranes, we performed immunoblots using antisera raised against synthetic peptides corresponding to sequences predicted by cDNAs encoding G protein α subunits.

Immunoblots of chick DRG cell membranes were probed with 4 distinct peptide antisera, and the pattern of immunoreactivity was compared with that observed using bovine cerebral cortical membranes. As illustrated in Figure 8, an antiserum specific for the C-terminus decapeptide amino acid sequence of $G_{o\alpha}$ (GO/1, Goldsmith et al., unpublished observations) revealed a M_r 39 kDa protein in bovine brain and a protein of slightly slower mobility in chick DRG. The significance of this small difference in migration of bovine and chick immunoreactive $G_{o\alpha}$ is unclear, but it could reflect minor species differences in primary sequence. Antiserum AS/7, which recognizes the C-terminus decapeptide amino acid sequence of multiple forms of $G_{i\alpha}$, including $G_{i\alpha1}$ and $G_{i\alpha2}$ (Goldsmith et al., 1987), revealed a doublet (M_r 40 and 41 kDa) in cow brain and

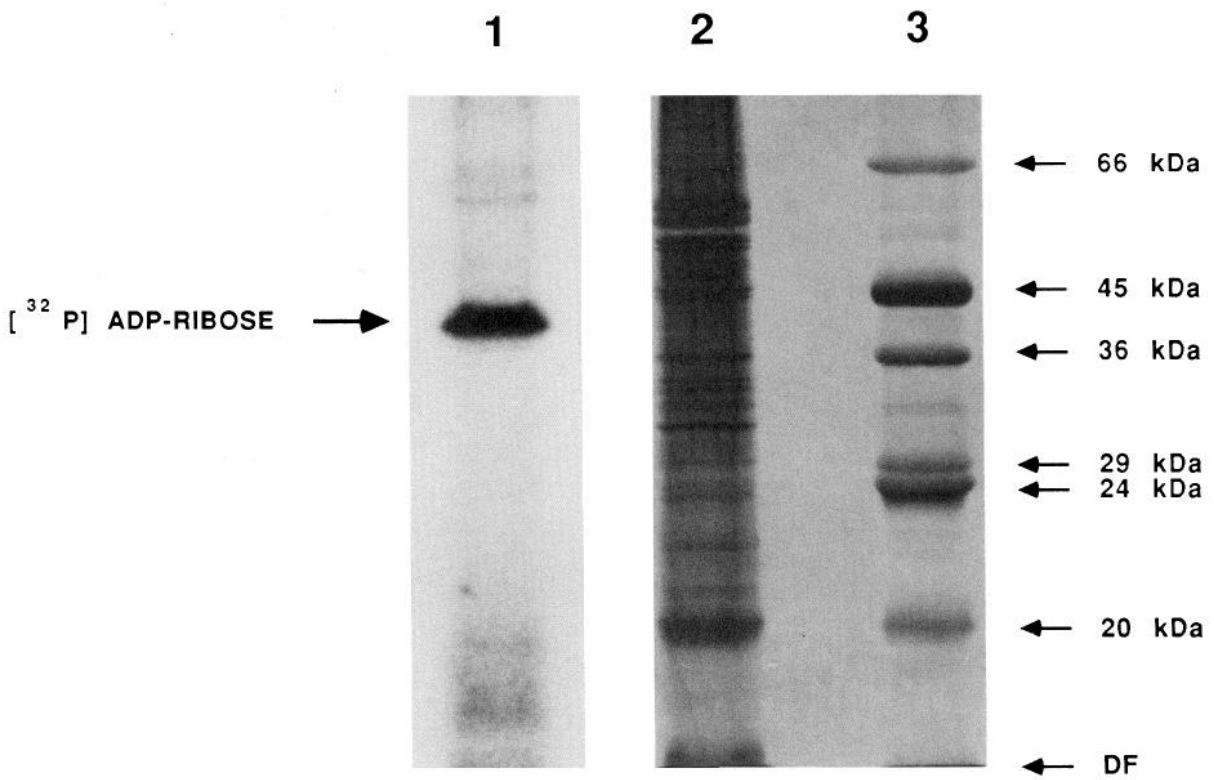

Figure 7. PTX-catalyzed ADP-ribosylation of DRG cell membrane proteins. Membranes (70 μg total protein) prepared from chick DRG cell cultures were incubated for 45 min at 37°C in a reaction mixture containing 2.4 μg/ml PTX and 5 μM nicotinamide adenine dinucleotide (3 Ci/mmol ^{32}P-NAD). Proteins were then solubilized in SDS sample buffer (containing 5% 2-mercaptoethanol) and subjected to SDS-PAGE on 13% polyacrylamide gels (20 mA/gel, 4 hr). The gels were then silver-stained and dried. Illustrated in column *1* is an autoradiogram prepared from the representative gel shown in columns *2* and *3*. Note that the predominant substrates for PTX-catalyzed ADP-ribosylation are proteins of M_r 40–41 kDa, as assessed by the pattern of ^{32}P incorporation. Failure to include PTX in the ribosylation assay resulted in the complete absence of ^{32}P incorporation at this M_r. Molecular-weight standards (Sigma, MW-SDS-70L) included BSA (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), carbonic anhydrase (29 kDa), trypsinogen (24 kDa), and soybean trypsin inhibitor (20.1 kDa). *DF* indicates the dye front.

in chick DRG, indicating that both tissues contain at least 2 forms of G_i α subunits. AS/7 does not cross-react with $G_{o\alpha}$ (Goldsmith et al., 1987); thus, neither of the bands corresponds to G_o. In an attempt to determine the subtypes of G_i recognized by AS/7, antisera specific for internal decapeptide sequences of $G_{i\alpha1}$ and $G_{i\alpha2}$ were tested. As illustrated in Figure 8 (left), in bovine brain, antiserum LD/1, specific for the α_1 subunit (Goldsmith et al., 1988), revealed a 41 kDa protein corresponding to $G_{i\alpha1}$, whereas LE/2, specific for the α_2 subunit (Goldsmith et al., 1987), recognized a 40 kDa protein corresponding to $G_{i\alpha2}$. In contrast, in chick DRG, no specific immunoreactivity was detected with either LD/1 or LE/2 (Fig. 8, right).

Discussion

G protein-regulated signal transduction in DRG neurons

Using RIA for the electrically evoked release of SP, we have demonstrated that in DRG neurons, PTX-sensitive G proteins couple NE and GABA receptors to the inhibition of peptide secretion. Furthermore, we report that the pharmacological properties of these receptors most closely resemble those of α_2-adrenergic and $GABA_b$ receptors, 2 receptor subtypes whose affinity is known to be regulated by guanyl nucleotides (see, for example, Sabol and Nirenberg, 1979; Hill et al., 1984). On the basis of these findings we propose that the G protein-mediated inhibition of voltage-dependent calcium channels may best explain how NE and GABA inhibit peptide secretion from peripheral sensory neurons. Support for this proposed mechanism of action is based on a comparison of the pharmacological properties of the cell-surface receptors and G proteins responsible for inhibition of macroscopic calcium currents and transmitter release. Both inhibitory processes are mediated by α-adrenergic and $GABA_b$ receptors, and these receptors are functionally coupled to PTX-sensitive G proteins. These findings do not, however, rule out additional G protein-mediated inhibitory actions of NE or GABA to block secretion at a step subsequent to the entry of calcium ions across the plasma membrane (see Knight and Baker, 1985; Howell et al., 1987; Vallar et al., 1987, for studies indicating a *direct* action of G proteins on excitation–secretion coupling in non-neuronal cells).

Characteristics of DRG cell G proteins

To identify which G proteins mediate the inhibitory actions of NE and GABA we characterized DRG cell G proteins on the basis of molecular weight, susceptibility to PTX-catalyzed ADP-ribosylation, and immunological profile as assessed by Western blot analysis. SDS-PAGE of membrane proteins revealed PTX substrates of M_r 40–41 kDa, an observation consistent with the findings of cloning studies documenting the predicted molecular weights of several distinct forms of G protein α subunits, each serving as substrates for PTX-catalyzed ADP-ribosylation. These include the 39 and 41 kDa forms of transducin α, 3 forms of G_i α with M_r 40 or 41 kDa, and the 39 kDa form of G_o α. Since

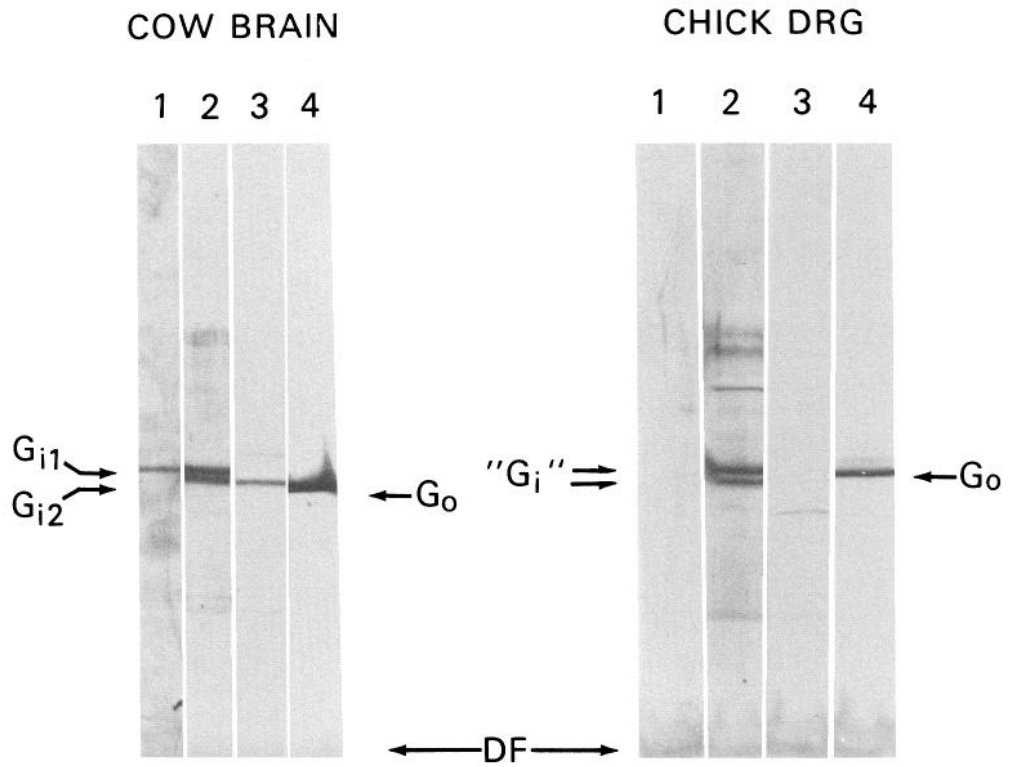

Figure 8. Immunoblot of bovine cerebral cortical and chick DRG cell membrane proteins probed with antisera specific for PTX-sensitive G protein α subunits. A cholate extract of bovine cerebral cortical membranes (150 μg protein/lane) and a crude membrane preparation from chick DRG (200 μg protein/lane) were treated with sample buffer for SDS-PAGE and loaded onto a 10% polyacrylamide gel as described in Materials and Methods. The separated proteins were transferred to nitrocellulose paper and immunoblotting performed with antisera: *1*, LD/1 (1:100 dilution); *2*, AS/7 (1:250); *3*, LE/2 (1:100); *4*, GO/1 (1:250). Molecular weights were estimated by comparison with either prestained molecular-weight markers (Bethesda Research Labs) or purified G protein α subunits run in parallel with the membrane samples. The entire nitrocellulose strip is shown, and *arrows* denote the positions of specific immunoreactive bands corresponding to G_i and G_o α subunits. *DF* indicates the dye front.

transducin α is found only in retinal rods and cones, we focused on the possible role of G_i or G_o as signal transducers in DRG neurons.

Immunoblots of DRG cell membranes, performed using antisera raised against synthetic peptides corresponding to predicted cDNAs of PTX-sensitive G proteins, revealed a *minimum* of 2 G_i-like proteins (M_r 40 and 41 kDa) and a third G_o-like protein (M_r 40 kDa). These antisera (AS/7 and GO/1) recognize the C-terminus decapeptide sequence of G_i and G_o α subunits, sequences containing the cysteine residue ADP-ribosylated by PTX. Therefore, the 3 G_i-and G_o-like immunoreactivities recognized by AS/7 and GO/1 appear to correspond to the M_r 40–41 kDa PTX substrates migrating as a single broad band on autoradiograms of the gels. (Two-dimensional gel electrophoresis and immunoblotting will be required to demonstrate that this band contains the 3 predicted PTX substrates recognized by AS/7 and GO/1.)

We were unable to determine which subtypes of G_i are present in chick DRG cell membranes, despite testing antisera LD/1 and LE/2 which recognize internal decapeptide sequences characteristic of mammalian $G_{i\alpha1}$ and $G_{i\alpha2}$. Several factors could account for this observation. For example, these proteins may be present in DRG but at concentrations below the detection limit of the antisera. This seems unlikely since the G_i-like immunoreactivity detected by AS/7 in DRG stained with approximately the same intensity as that detected in bovine brain, a tissue with significant $G_{i\alpha1}$- and $G_{i\alpha2}$-like immunoreactivity. Also unlikely is the possibility that, in general, brain contains $G_{i\alpha1}$ and $G_{i\alpha2}$, whereas DRG does not, since we observed essentially identical patterns of G_i-like immunoreactivity in chick brain and chick DRG when testing AS/7, but failed to detect immunoreactivity in either tissue when testing LD/1 and LD/2 (data not shown). Therefore, the most likely explanation is that the failure of LD/1 and LE/2 to detect immunoreactivity in chick DRG reflects species-specific changes in primary sequence in the region (residues 159–169) probed with these antisera. Species-specific differences in primary sequence may also explain our finding that in chick DRG, $G_{o\alpha}$-like immunoreactivity recognized by GO/1 is of M_r 40 kDa, whereas in bovine brain it is 39 kDa. Alternatively, we cannot rule out the possibility that chick DRG may contain G proteins not previously characterized in mammalian tissues.

G protein structure–function relationships

Attention has recently focused on the biochemical characterization of G proteins that mediate signal transduction via α_2-adrenergic and $GABA_b$ receptors. For example, Cerione et al. (1986) and Asano et al. (1985) reported that purified α_2-adrenergic and $GABA_b$ receptors interact with resolved heterotrimers of G_i and G_o using *in vitro* reconstitution assays. However, it remains to be determined exactly which of these 2 classes of G protein mediate the inhibitory actions of NE and GABA in the DRG cell system. Such an assessment requires pharmacological probes capable of discriminating, on a functional basis, between individual G protein α subunits. PTX fails in this respect since all 3 G_i- and G_o-like proteins found in DRG neurons appear to

be substrates for PTX-catalyzed ADP-ribosylation. In contrast, our immunological characterization of DRG cell G proteins suggests that it may be possible to establish a functional role for G_i or G_o by testing whether G protein-specific antisera such as AS/7 or GO/1 block transmitter responses in intact neurons. This strategy has proven successful in a previous study examining the ability of antisera directed against mammalian $G_{o\alpha}$ to block receptor-mediated inhibition of calcium channels in identified molluscan neurons (Harris-Warrick et al., 1988). A primary role for G_o as a regulator of voltage-dependent calcium channels is also suggested by previous studies reporting the functional reconstitution by G_o α subunits of PTX-sensitive signal transduction pathways that inhibit calcium channel function in neuroblastoma × glioma cells (Hescheler et al., 1987) and rodent DRG neurons (Ewald et al., 1988).

Calcium channel heterogeneity in DRG sensory neurons

It has been reported that in chick DRG neurons at least 3 distinct types of voltage-dependent calcium current may be recorded from the cell soma using whole-cell and single-channel patch-clamp analysis (Nowycky et al., 1985a, b; Fox et al., 1987a, b). These currents, designated types L, N, and T by Tsien and coworkers, are distinguishable on the basis of biophysical and pharmacological criteria, as expected if depolarization-induced calcium influx occurs through 3 functionally distinct classes of channel. In DRG neurons dihydropyridine calcium channel antagonists inhibit peptide secretion, as expected if transmitter release results, at least in part, from calcium influx through the L-type channels (Perney et al., 1986; Rane et al., 1987; Holz et al., 1988). This observation is consonant with previous macroscopic current analysis demonstrating that the predominant calcium current recorded from embryonic chick DRG cell somata exhibits a high threshold for activation, slow inactivation, and dihydropyridine sensitivity (Rane et al., 1987), qualities characteristic of L-type calcium currents. Therefore, the G protein-mediated inhibitory actions of NE or GABA described here may result from a suppression of L-type calcium channel function, a conclusion that remains to be directly tested using single-channel recording.

Functional implications of G protein-regulated signal transduction in peripheral sensory neurons

The G protein-mediated inhibition of peptide secretion, as reported here, may be one mechanism by which inhibitory neurotransmitters regulate excitation–secretion coupling at the spinal terminations of peripheral sensory neurons *in vivo*. Support for this concept is provided by previous studies demonstrating that in the spinal cord, a subpopulation of α_2 and $GABA_b$ receptors are localized on sensory nerve terminals (Price et al., 1984; Howe et al., 1987) and that α_2 receptor agonists inhibit the release of SP (Kuraishi et al., 1986; Pang and Vasco, 1986; Go and Yaksh, 1987). SP is implicated as a transmitter, or modulator, released from finely myelinated and unmyelinated sensory neurons, many of which subserve nociceptive sensory modalities. Therefore, especially intriguing are reports that in the spinal cord, the antinociceptive actions of α_2 and $GABA_b$ receptor agonists are blocked by pretreatment with PTX (Hoehn et al., 1988), and that G_o-like immunoreactivity is concentrated in the substantia gelatinosa (Worley et al., 1986), a region in which nociceptive sensory neurons synapse with spinal interneurons. In light of these, and our own findings in the DRG cell system, we would like to propose that G_i- and/or G_o-like proteins may play an important role as mediators of presynaptic inhibition at the spinal terminations of peripheral sensory neurons.

Note added in proof. Antiserum IM/1 (kindly provided by Dr. Graeme Milligan, University of Glasgow), specific for amino acid residues 22–36 of bovine $G_{o\alpha}$, also recognizes an M_r 40 kDa protein on immunoblots of chick DRG cell membranes, thereby providing additional support for the existence of authentic $G_{o\alpha}$ in these neurons.

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