

# G Proteins Couple $\alpha$ -Adrenergic and GABA<sub>B</sub> Receptors to Inhibition of Peptide Secretion from Peripheral Sensory Neurons

George G. Holz IV,<sup>1</sup> Richard M. Kream,<sup>2</sup> Allen Spiegel,<sup>3</sup> and Kathleen Dunlap<sup>1</sup>

Departments of <sup>1</sup>Physiology, <sup>2</sup>Anesthesiology and Pharmacology, Tufts University School of Medicine-New England Medical Center, Boston, Massachusetts 02111, and <sup>3</sup>Metabolic Diseases Branch, National Institute of Diabetes, Digestive, and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892

**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  $\alpha$ -adrenergic and GABA<sub>B</sub> 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*) 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  $\alpha$  subunits revealed a minimum of 2 G<sub>i</sub>-like proteins (*M*, 40 and 41 kDa) and a third G<sub>o</sub>-like protein (*M*, 40 kD). Significantly, these findings implicate G<sub>i</sub>- and/or G<sub>o</sub>-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 en-

zymes (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  $\alpha$ -adrenergic (Canfield and Dunlap, 1984) and GABA<sub>B</sub> 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- $\beta$ -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  $\alpha$ -adrenergic and GABA<sub>B</sub> 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  $\alpha$  subunits of G<sub>i</sub> and G<sub>o</sub>. Significantly, these findings implicate G<sub>i</sub>- and/or G<sub>o</sub>-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).

Received May 6, 1988; revised June 28, 1988; accepted July 2, 1988.

This work was supported by grants to K.D. from the NIH (NS 16483), the Klingenstein Fund, and the American Heart Association (with funds contributed, in part, by the Massachusetts affiliate), and to R.M.K. from NIDA (DA 04128) and the New England Medical Center (BRSG 19-807419).

We wish to thank P. Goldsmith and C. Unson for help in preparation of the peptide antisera. R. Vinitsky for help in running the immunoblots, and E. Neer for assistance with the ADP-ribosylation assay.

Correspondence should be addressed to Dr. George G. Holz IV, Department of Physiology, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111.

Copyright © 1989 Society for Neuroscience 0270-6474/89/020657-10\$02.00/0

## 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  $\gamma$ -irradiated to suppress the proliferation of non-neuronal cells and plated on collagen-coated tissue culture dishes at a density of ca.  $1.5 \times 10^5$  neurons/60 mm dish or  $0.5 \times 10^5$  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  $\mu$ g/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  $\geq 95\%$  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<sub>2</sub>, 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<sub>2</sub> and 2 mM CaCl<sub>2</sub>. This solution was chosen because BaCl<sub>2</sub>, 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<sub>2</sub> was omitted from the HBS. For example, in the experiments illustrated in Figures 3 and 6, the HBS contained 3 mM CaCl<sub>2</sub> with no added BaCl<sub>2</sub>.

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<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. 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<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 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  $\gamma$ -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<sub>2</sub>, 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  $\times$  g),

the pellet discarded, and the supernatant recentrifuged (20 min, 50,000  $\times$  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  $\mu$ g of membrane protein were suspended in a final volume of 50  $\mu$ l containing (in mM): 85 Tris (pH 7.8), 10 dithiothreitol, 10 thymidine, 10 isonicotinic acid hydrazide, 6 MgCl<sub>2</sub>, 3 ATP, 0.9 EGTA, and (in  $\mu$ M) 120 EDTA, 100 GTP, 5 NAD, and 0.5–2.0  $\mu$ Ci <sup>32</sup>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  $\mu$ g/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*<sub>r</sub> 40 kDa range. Crude antisera used for immunoblots were raised against synthetic peptides whose sequence was predicted by cDNAs encoding G protein  $\alpha$  subunits. Antisera included AS/7 [directed against the carboxy (C)-terminus decapeptide of transducin  $\alpha$ ], and LE/2 (amino acids 160–169 of G<sub>o2</sub>), whose preparation and characterization was described by Goldsmith et al. (1987). Also tested were GO/1 (C-terminus decapeptide of G<sub>o1</sub>), 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<sub>o1</sub>), 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  $\mu$ M (EC<sub>50</sub> = 1  $\mu$ M) 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  $\mu$ M) 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<sub>1</sub>–S<sub>3</sub>). 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 1A, the control release ratio approximated unity (1.05) when NE was omitted from the HBS during  $S_2$ . In contrast, in the presence of  $50 \mu\text{M}$  NE during  $S_2$ , the release ratio was reduced to 0.40 (Fig. 1B). 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  $50 \mu\text{M}$  NE was  $47 \pm 3\%$  (mean  $\pm$  SEM,  $n = 18$  cultures).

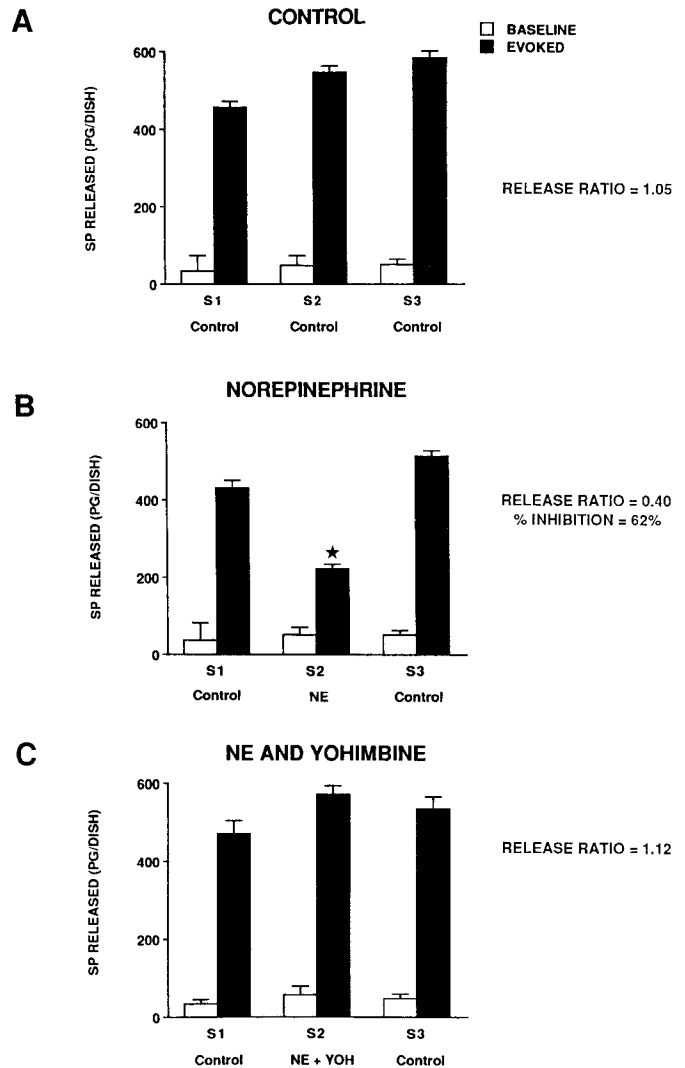
#### Pharmacological properties of NE receptors

In previous electrophysiological studies Canfield and Dunlap (1984) reported that a nonclassical subtype of  $\alpha$ -adrenergic receptor mediates the inhibition of DRG cell calcium channels by NE. This action of NE is blocked by the specific  $\alpha_2$ -receptor antagonist yohimbine ( $IC_{50} = 10 \text{ nM}$ ), but not by micromolar concentrations of the  $\alpha_1$ -receptor antagonist prazosin or the  $\beta$ -receptor antagonist propranolol. Therefore, the antagonist pharmacology of these avian adrenergic receptors matches that previously described for mammalian  $\alpha_2$ -receptors (Langer, 1981). Unexpectedly, however, the  $\alpha_2$ -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 adreno-receptor agonists and antagonists.

Figure 1C illustrates the effect of yohimbine on adrenergic receptor-mediated inhibition of SP release. When cultures were exposed to  $10 \mu\text{M}$  yohimbine and  $50 \mu\text{M}$  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  $10 \mu\text{M}$  prazosin nor  $10 \mu\text{M}$  propranolol blocked the inhibitory action of  $50 \mu\text{M}$  NE. Furthermore, the action of NE was not mimicked by clonidine (the average percent inhibition due to  $50 \mu\text{M}$  clonidine was  $7 \pm 8\%$ ,  $n = 3$  cultures from a single experiment). These findings, summarized in Figure 2, indicate that  $\alpha_2$ -like adrenergic receptors with similar, if not identical, pharmacological properties mediate the inhibitory actions of NE on calcium channels and peptide secretion.

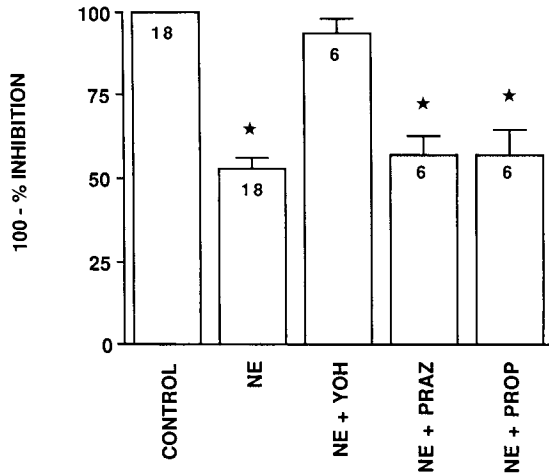
#### Concentration-dependent inhibitory actions of NE

Figure 3 illustrates the concentration dependence of the inhibitory action of NE as determined using a single-phase stimu-



**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  $\text{H}_2\text{O}$ ) in which the drugs were dissolved (A), a second set was exposed to HBS containing  $50 \mu\text{M}$  NE (B), and a third set was exposed to HBS containing  $50 \mu\text{M}$  NE and  $10 \mu\text{M}$  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  $\pm$  SEM ( $n = 3$ ). In these cultures,  $50 \mu\text{M}$  NE decreased the duration of DRG cell action potentials as previously reported (Canfield and Dunlap, 1984).

lation 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.  $0.3$ – $30 \mu\text{M}$  and that  $30 \mu\text{M}$  NE inhibited the release of SP by 68%. The  $IC_{50}$  value for the inhibitory action of NE was ca.  $1 \mu\text{M}$ , a value identical to that previously determined by Canfield



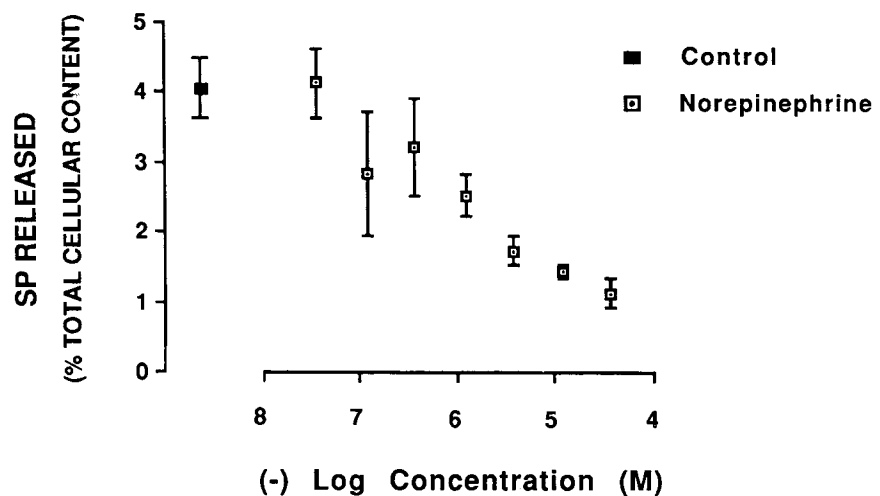
**Figure 2.** Pharmacological properties of norepinephrine receptors. Summary of the findings demonstrating that  $\alpha_2$ -adrenergic-like receptors mediate the inhibitory action of NE on the SP release mechanism. The results of 6 experiments using 54 cultures from 6 different platings are shown. 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  $\pm$  SEM. Note that the action of 50  $\mu$ M NE was antagonized by 10  $\mu$ M yohimbine (YOH, an  $\alpha_2$ -receptor antagonist), but not by 10  $\mu$ M prazosin (PRAZ, an  $\alpha_1$ -receptor antagonist), or 10  $\mu$ M propranolol (PROP, a  $\beta$ -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  $1.03 \pm 0.04$ . 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<sub>A</sub> receptors are activated by relatively high concentrations of GABA ( $EC_{50} = 10 \mu$ M), coupled to chloride ion channels, and mediate the de-

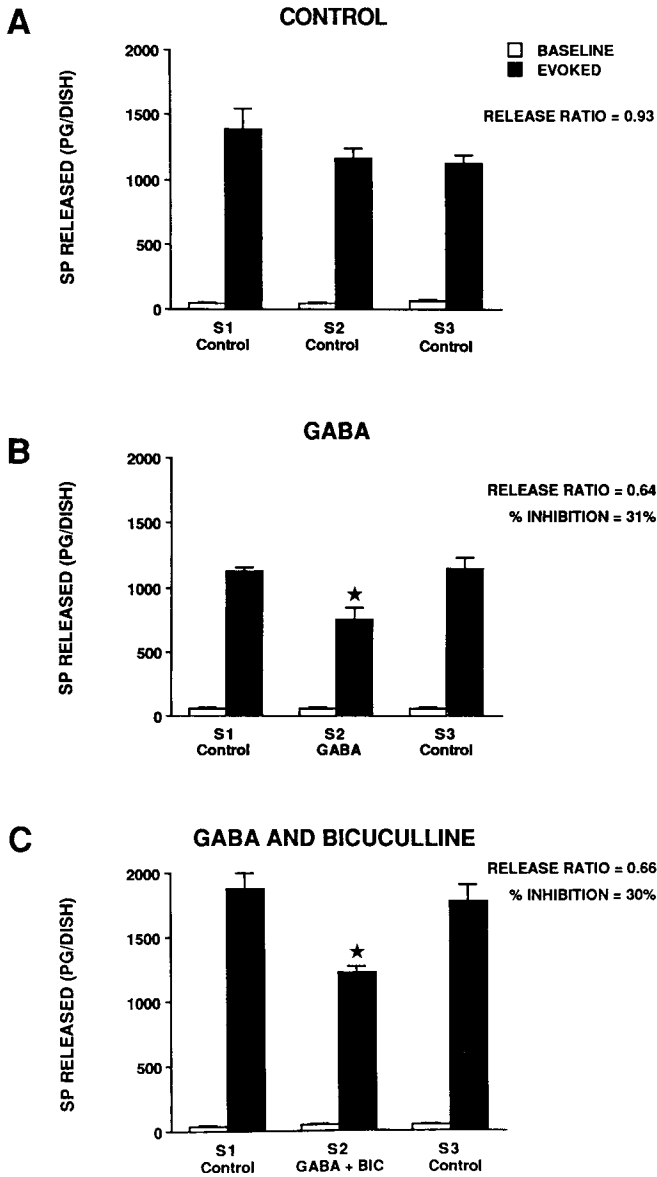
**Figure 3.** Dose–response relationship for norepinephrine. Twenty-four 35 mm cultures from a single plating were divided into 8 sets of 3 [1 control set, and 7 NE-treated sets, each of which was exposed to NE (30 nM–30  $\mu$ M)]. Cultures were stimulated (5 min, 1 Hz) while bathed in HBS containing 3 mM CaCl<sub>2</sub>. 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 ( $IC_{50} 1 \mu$ M). Error bars indicate means  $\pm$  SEM ( $n = 3$ ).



polarizing actions of GABA. In contrast, GABA<sub>B</sub> receptors are activated by lower concentrations of GABA ( $EC_{50} = 1 \mu$ M) and mediate inhibition of voltage-dependent calcium channels. In later-stage cultures, such as those used in the present study, GABA<sub>B</sub> 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 4A, the control release ratio approximated unity (0.93) when GABA was omitted from the HBS during S<sub>2</sub>. In contrast, in the presence of 10  $\mu$ M GABA during S<sub>2</sub>, the release ratio was reduced to 0.64, indicating that GABA inhibited the release of SP by 31% (Fig. 4B). The inhibitory action of GABA was observed in 12 of 12 cultures from 3 different platings. The average percent inhibition due to 10  $\mu$ M GABA was  $31 \pm 4\%$ . Note that as also illustrated in Figure 4C, the inhibitory action of 10  $\mu$ M GABA was not blocked by 50  $\mu$ M (+)bicuculline, a selective antagonist of GABA<sub>A</sub> receptors on chick DRG neurons (Dunlap, 1981; Bowery, 1982).

#### Pharmacological properties of GABA receptors

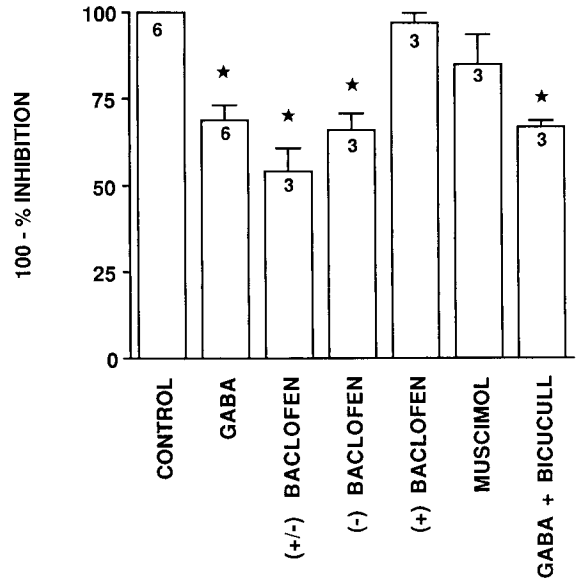
We examined the effects of selective GABA<sub>A</sub> and GABA<sub>B</sub> receptor ligands in order to characterize the pharmacological properties of these GABA receptors. Such an analysis is complicated by the fact that there is, at present, no specific GABA<sub>B</sub> receptor antagonist. Dutar and Nicoll (1988) reported that millimolar concentrations of phaclofen, a phosphonic acid derivative of baclofen, block GABA<sub>B</sub> receptor-mediated responses in hippocampal slice preparations. However, the specificity of this antagonist action remains to be fully characterized. GABA<sub>B</sub> 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  $\mu$ M ( $\pm$ )baclofen (Lioresal), a selective GABA<sub>B</sub> receptor agonist (Bowery, 1982) that mimics the action of GABA on DRG cell calcium channels (Dunlap, 1981). The action of 10  $\mu$ M baclofen was stereoselective. (–)baclofen inhibited the release of SP by 34%, whereas (+)baclofen inhibited release by only 3%. Furthermore, muscimol (10  $\mu$ M), a GABA<sub>A</sub> receptor agonist, was relatively ineffective compared to (–)baclofen. These observations suggest that GABA inhibits the release of SP by activating GABA<sub>B</sub> receptors. The failure of 50  $\mu$ M (+)bicuculline to block the action of 10  $\mu$ M GABA (Fig. 4C) supports this conclusion.



**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<sub>2</sub>, one set of control cultures was bathed in HBS containing only the vehicle (acidified H<sub>2</sub>O) 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<sub>A</sub> receptor antagonist (C). Note that GABA inhibited the release of SP by 31%, and that (+)bicuculline failed to antagonize the response to GABA (\**p* ≤ 0.005, unpaired *t* test). Error bars indicate mean ± SEM (*n* = 3). In this experiment, SP release at S<sub>1</sub> 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.

*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,



**Figure 5.** Pharmacological properties of GABA receptors. Summary of experiments demonstrating that GABA<sub>A</sub> 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<sub>B</sub> receptor agonist but not by 10 μM muscimol, a GABA<sub>A</sub> 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<sub>A</sub> receptor antagonist. Asterisks indicate that the value was significantly different from control (*p* ≤ 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.

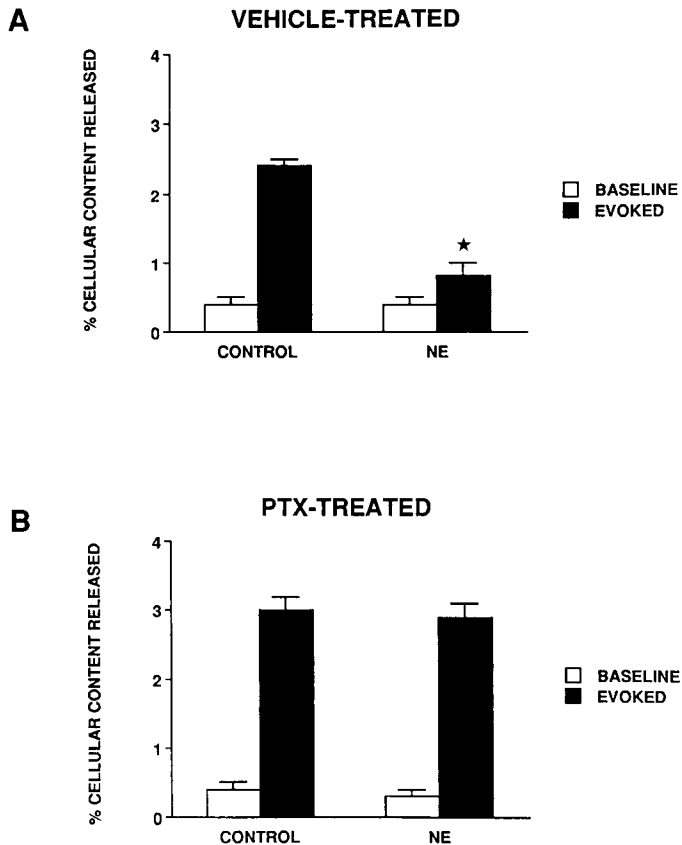
we examined what role G proteins play in this receptor-mediated inhibition of peptide secretion. Specifically, the effects of the transmitters were tested on cultures pretreated with *B. pertussis* toxin, a bacterial exotoxin previously reported to block the G protein-mediated inhibitory actions of NE 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. 6A), whereas in PTX-treated cultures, no significant inhibitory action of the transmitter was observed (Fig. 6B).

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  $(\text{NH}_4)_2\text{SO}_4$  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  $\text{CaCl}_2$ . The amount of SP released from control cultures (columns at *left*) was compared with that released from cultures exposed to 50  $\mu\text{M}$  NE (columns at *right*). Values for baseline and evoked levels of released SP are the means  $\pm$  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  $\alpha$  subunit interacts with cell-surface receptors, binds guanyl nucleotides ( $\alpha$ -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  $\alpha$ -GTP and  $\beta\gamma$  subunits. Activation of the G protein is terminated by hydrolysis of GTP, followed by reassociation of  $\alpha$ -GDP with free  $\beta\gamma$  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  $\alpha$  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.

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

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  $(\text{NH}_4)_2\text{SO}_4$  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 occasions for 90 sec at 1 Hz while bathed in HBS containing 1 mM  $\text{BaCl}_2$  and 2 mM  $\text{CaCl}_2$ . For vehicle-treated cultures, NE (50  $\mu\text{M}$ ) and GABA (10  $\mu\text{M}$ ) 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  $\pm$  SEM.

<sup>a</sup> Significantly different from control ( $p \leq 0.001$ ,  $t$  test).

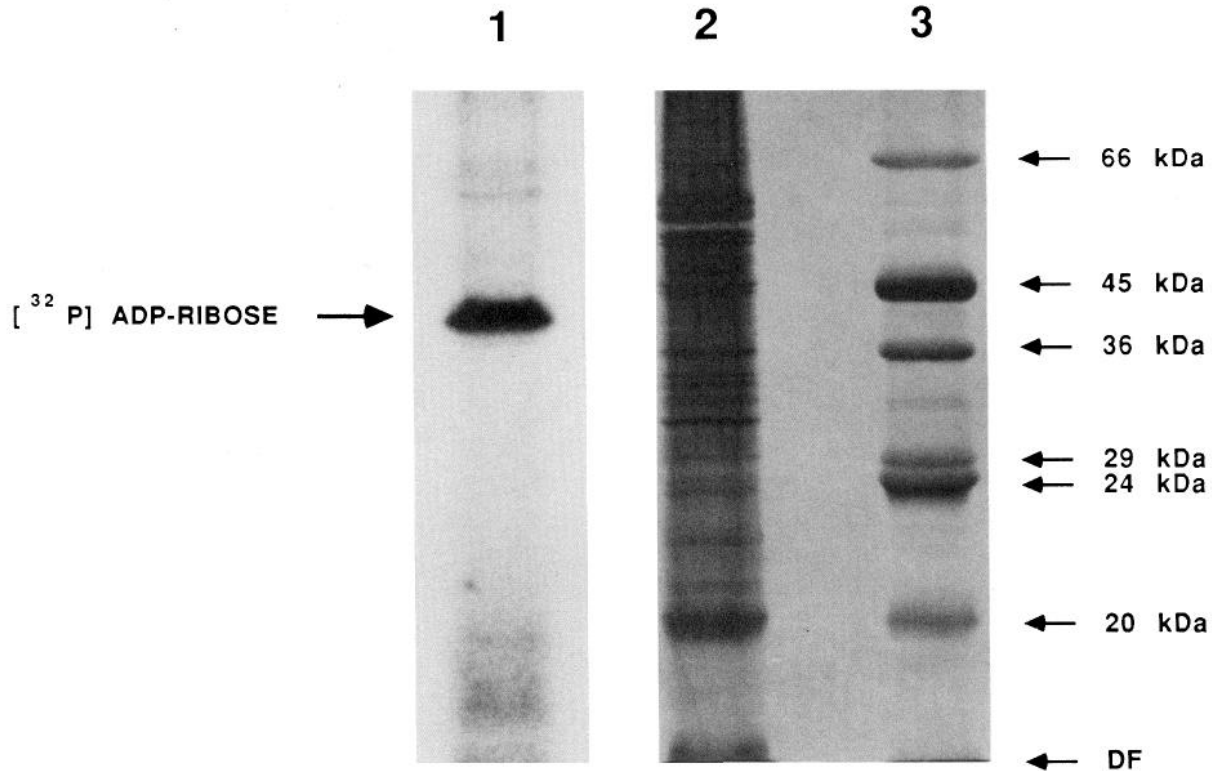
<sup>b</sup> Not significantly different from control ( $p > 0.10$ ).

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  $^{32}\text{P}$ -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  $\alpha$  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  $\alpha$  subunit, each serving as substrate for PTX-catalyzed ADP-ribosylation. These  $\alpha$  subunits include 2 forms of transducin (a G protein found in retinal rods and cones),  $G_{\alpha c}$  (a 39 kDa protein abundant in brain), and at least 3 closely related 40–41 kDa proteins termed  $G_{i1}$ ,  $G_{i2}$ , and  $G_{i3}$  (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  $\alpha$  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  $G_{\alpha c}$  (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  $G_{\alpha c}$  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  $G_{i1}$  and  $G_{i2}$  (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  $\mu$ g total protein) prepared from chick DRG cell cultures were incubated for 45 min at 37°C in a reaction mixture containing 2.4  $\mu$ g/ml PTX and 5  $\mu$ M 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  $G_i$   $\alpha$  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  $G_{i\alpha 1}$  and  $G_{i\alpha 2}$  were tested. As illustrated in Figure 8 (left), in bovine brain, antiserum LD/1, specific for the  $\alpha_1$  subunit (Goldsmith et al., 1988), revealed a 41 kDa protein corresponding to  $G_{i\alpha 1}$ , whereas LE/2, specific for the  $\alpha_2$  subunit (Goldsmith et al., 1987), recognized a 40 kDa protein corresponding to  $G_{i\alpha 2}$ . In contrast, in chick DRG, no specific immunoreactivity was detected with either LD/1 or LE/2 (Fig. 8, right).

## Discussion

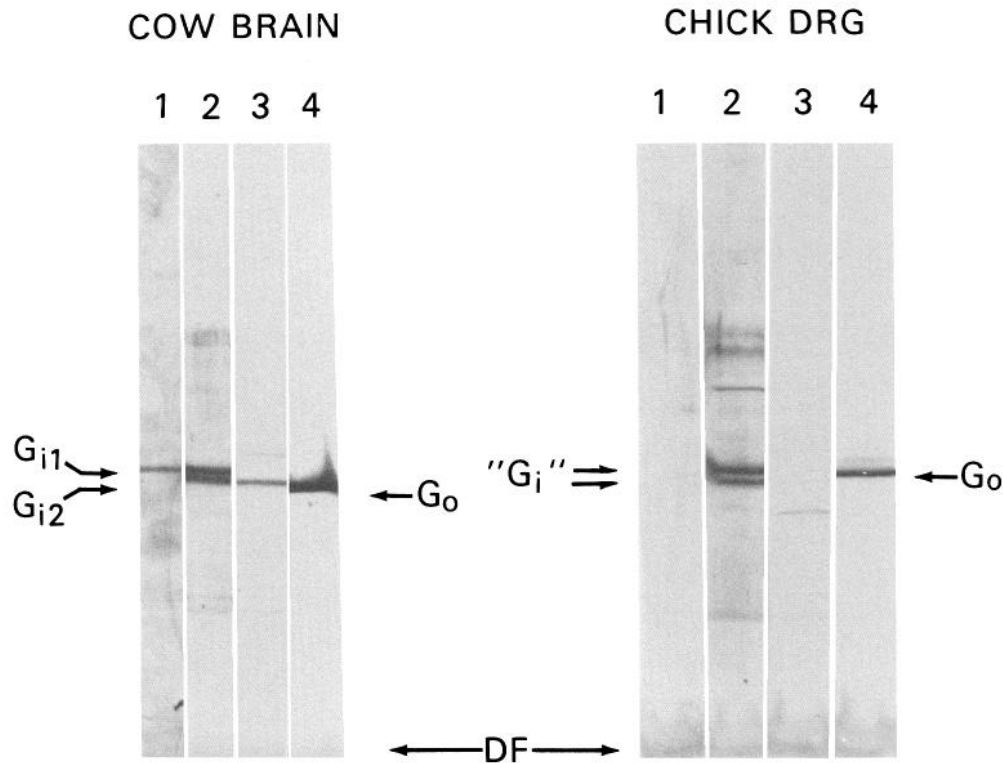
### *G protein-regulated signal transduction in DRG neurons*

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  $\alpha_2$ -adrenergic and GABA<sub>b</sub> 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 pe-

ripheral 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  $\alpha$ -adrenergic and GABA<sub>b</sub> 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  $\alpha$  subunits, each serving as substrates for PTX-catalyzed ADP-ribosylation. These include the 39 and 41 kDa forms of transducin  $\alpha$ , 3 forms of  $G_i$   $\alpha$  with  $M_r$  40 or 41 kDa, and the 39 kDa form of  $G_o$   $\alpha$ . Since



**Figure 8.** Immunoblot of bovine cerebral cortical and chick DRG cell membrane proteins probed with antisera specific for PTX-sensitive G protein  $\alpha$  subunits. A cholate extract of bovine cerebral cortical membranes (150  $\mu$ g protein/lane) and a crude membrane preparation from chick DRG (200  $\mu$ g 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  $\alpha$  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  $G_o$   $\alpha$  subunits. DF indicates the dye front.

transducin  $\alpha$  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  $G_o$   $\alpha$  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  $G_{i\alpha 1}$  and  $G_{i\alpha 2}$ . 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  $G_{i\alpha 1}$ - and  $G_{i\alpha 2}$ -like immunoreactivity. Also unlikely is the possibility that, in general, brain contains  $G_{i\alpha 1}$

and  $G_{i\alpha 2}$ , 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,  $G_o$ -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  $\alpha_2$ -adrenergic and GABA<sub>b</sub> receptors. For example, Cerione et al. (1986) and Asano et al. (1985) reported that purified  $\alpha_2$ -adrenergic and GABA<sub>b</sub> 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  $\alpha$  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  $G_o$  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  $G_o$   $\alpha$  subunits of PTX-sensitive signal transduction pathways that inhibit calcium channel function in neuroblastoma  $\times$  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  $\alpha_2$  and GABA<sub>B</sub> receptors are localized on sensory nerve terminals (Price et al., 1984; Howe et al., 1987) and that  $\alpha_2$  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  $\alpha_2$  and GABA<sub>B</sub> 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  $G_o$ , also recognizes an  $M_r$  40 kDa protein on immunoblots of chick DRG cell membranes, thereby providing additional support for the existence of authentic  $G_o$  in these neurons.

#### References

- Asano, T., M. Ui, and N. Ogasawara (1985) Prevention of the agonist binding to  $\gamma$ -aminobutyric acid B receptors by guanine nucleotides and islet-activating protein, pertussis toxin, in bovine cerebral cortex. *J. Biol. Chem.* 260: 12653–12658.
- Bowery, N. G. (1982) Baclofen: 10 years on. *Trends. Pharm. Sci.* 3: 400–403.
- Bray, P., A. Carter, V. Guo, C. Puckett, J. Kamholz, A. Spiegel, and M. Nirenberg (1987) Human cDNA clones for an  $\alpha$  subunit of  $G_i$  signal-transduction protein. *Proc. Natl. Acad. Sci. USA* 84: 5115–5119.
- Canfield, D. R., and K. Dunlap (1984) Pharmacological characterization of amine receptors on embryonic chick sensory neurons. *Br. J. Pharmacol.* 82: 557–563.
- Cerione, R. A., C. Staniszewski, P. Gierschik, J. Codina, R. L. Somers, L. Birnbaumer, A. M. Spiegel, M. G. Caron, and R. J. Lefkowitz (1986) Functional reconstitution of the  $\alpha_2$ -adrenergic receptor with guanine nucleotide regulatory proteins in phospholipid vesicles. *J. Biol. Chem.* 261: 3901–3909.
- Dunlap, K. (1981) Two types of  $\gamma$ -aminobutyric acid receptor on embryonic sensory neurons. *Br. J. Pharmacol.* 74: 579–585.
- Dunlap, K., and G. D. Fischbach (1978) Neurotransmitters decrease the calcium component of sensory neurone action potentials. *Nature* 276: 837–839.
- Dunlap, K., and G. D. Fischbach (1981) Neurotransmitters decrease the calcium conductance activated by depolarization of embryonic chick sensory neurons. *J. Physiol. (Lond.)* 317: 519–535.
- Dunlap, K., R. M. Kream, and G. G. Holz IV (1986) Alpha-2 adrenergic and GABA-B receptors mediate transmitter inhibition of neuropeptide secretion from dorsal root ganglion cells. *Soc. Neurosci. Abstr.* 12: 1195.
- Dunlap, K., G. G. Holz, and S. G. Rane (1987) G proteins as regulators of ion channel function. *Trends Neurosci.* 10: 241–244.
- Dutar, P., and R. A. Nicoll (1988) A physiological role for GABA<sub>B</sub> receptors in the central nervous system. *Nature* 332: 156–158.
- Ewald, D. A., P. C. Sternweiss, and R. J. Miller (1988) Guanine nucleotide binding protein  $G_o$ -induced coupling of neuropeptide Y receptors to  $Ca^{2+}$  channels in sensory neurons. *Proc. Natl. Acad. Sci. USA* 85: 3633–3637.
- Fischbach, G. D., K. Dunlap, A. Mudge, and S. Leeman (1981) Peptide and amine transmitter effect on embryonic chick sensory neurons *in vitro*. *Adv. Biochem. Psychopharmacol.* 28: 175–188.
- Fox, A. P., M. C. Nowycky, and R. W. Tsien (1987a) Kinetic and pharmacological properties distinguishing three types of calcium currents in chick sensory neurons. *J. Physiol. (Lond.)* 394: 149–172.
- Fox, A. P., M. C. Nowycky, and R. W. Tsien (1987b) Single-channel recordings of three types of calcium channels in chick sensory neurons. *J. Physiol. (Lond.)* 394: 173–200.
- Gierschik, P., G. Milligan, M. Pines, P. Goldsmith, J. Codina, W. Klee, and A. Spiegel (1986a) Use of specific antibodies to quantitate the guanine nucleotide-binding protein  $G_o$  in brain. *Proc. Natl. Acad. Sci. USA* 83: 2258–2262.
- Gierschik, P., J. Falloon, G. Milligan, M. Pines, J. I. Gallin, and A. Spiegel (1986b) Immunochemical evidence for a novel pertussis toxin substrate in human neutrophils. *J. Biol. Chem.* 261: 8058–8062.
- Gilman, A. G. (1987) G proteins: Transducers of receptor-generated signals. *Annu. Rev. Biochem.* 56: 615–649.
- Go, V. L. W., and T. L. Yaksh (1987) Release of substance P from the cat spinal cord. *J. Physiol. (Lond.)* 391: 141–167.
- Goldsmith, P., P. Gierschik, G. Milligan, C. G. Unson, R. Vinitzky, H. L. Malech, and A. M. Spiegel (1987) Antibodies directed against synthetic peptides distinguish between GTP-binding proteins in neutrophil and brain. *J. Biol. Chem.* 262: 14683–14688.

- Goldsmith, P., K. Rossiter, A. Carter, C. G. Unson, R. Vinitzky, and A. M. Spiegel (1988) Identification of the GTP-binding protein encoded for by  $G_{i,3}$  complementary DNA. *J. Biol. Chem.* (in press).
- Harris-Warrick, R. M., C. Hammond, D. Paupardin-Tritsch, V. Homburger, B. Rouot, J. Bockaert, and H. Gerschenfeld (1988) An  $\alpha_{40}$  subunit of GTP-binding protein immunologically related to  $G_o$  mediates a dopamine-induced decrease of  $Ca^{+2}$  current in snail neurons. *Neuron* 1: 27–32.
- Hescheler, J., W. Rosenthal, W. Trautwein, and G. Schultz (1987) The GTP-binding protein,  $G_o$ , regulates neuronal calcium channels. *Nature* 325: 445–447.
- Hill, D. R., N. G. Bowery, and A. L. Hudson (1984) Inhibition of  $GABA_b$  receptor binding by guanyl nucleotides. *J. Neurochem.* 42: 652–657.
- Hoehn, K., A. Reid, and J. Sawynok (1988) Pertussis toxin inhibits antinociception produced by intrathecal injection of morphine, noradrenaline and baclofen. *Eur. J. Pharmacol.* 146: 65–72.
- Holz, G. G. IV, R. M. Kream, and K. Dunlap (1985) Norepinephrine inhibits field stimulation-evoked release of substance P from chick dorsal root ganglion cells in culture. *Soc. Neurosci. Abstr.* 11: 126.
- Holz, G. G. IV, S. G. Rane, and K. Dunlap (1986a) GTP-binding proteins mediate transmitter inhibition of voltage-dependent calcium channels. *Nature* 319: 670–672.
- Holz, G. G. IV, K. Dunlap, and R. M. Kream (1986b) Pertussis toxin-sensitive GTP-binding proteins couple  $\alpha_2$  adrenergic and  $GABA-B$  receptors to inhibition of neurosecretion in dorsal root ganglion cells. *Soc. Neurosci. Abstr.* 12: 1195.
- Holz, G. G. IV, K. Dunlap, and R. M. Kream (1988) Characterization of the electrically-evoked release of substance P from dorsal root ganglion neurons: Methods and dihydropyridine sensitivity. *J. Neurosci.* 8: 463–471.
- Howe, J. R., T. L. Yaksh, and V. L. W. Go (1987) The effects of unilateral dorsal root ganglionectomies or ventral rhizotomies on  $\alpha_2$  adrenoreceptor binding to, and the substance P, enkephalin, and neurotensin content of, the cat lumbar spinal cord. *Neuroscience* 21: 385–394.
- Howell, T. W., S. Cockcroft, and B. D. Gomperts (1987) Essential synergy between  $Ca^{2+}$  and guanine nucleotides in exocytotic secretion from permeabilized rat mast cells. *J. Cell Biol.* 105: 191–197.
- Jones, D. T., and R. R. Reed (1987) Molecular cloning of five GTP-binding protein of cDNA species from rat olfactory neuroepithelium. *J. Biol. Chem.* 262: 14241–14249.
- Katada, T., M. Oinuma, K. Kusakabe, and M. Ui (1987) A new GTP-binding protein in brain tissues serving as the specific substrate of islet-activating protein, pertussis toxin. *FEBS Lett.* 213: 353–358.
- Knight, D. E., and P. F. Baker (1985) Guanine nucleotides and  $Ca$ -dependent exocytosis. *FEBS Lett.* 189: 345–349.
- Kream, R. M., T. A. Schoenfeld, R. Mancuso, A. Clancy, W. El-Bermani, and F. Macrides (1985) Precursor forms of substance P (SP) in nervous tissue: Detection with antisera to SP, SP-Gly, and SP-Gly-Lys. *Proc. Natl. Acad. Sci. USA* 82: 4832–4836.
- Kuraishi, Y., N. Hirota, Y. Sato, S. Kaneko, M. Satoh, and H. Takagi (1986) Noradrenergic inhibition of the release of substance P from the primary afferents in the rabbit spinal dorsal horn. *Brain Res.* 359: 177–182.
- Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685.
- Langer, S. Z. (1981) Presynaptic regulation of the release of catecholamines. *Pharmacol. Rev.* 32: 337–361.
- Lowry, O. H., N. J. Rosebrough, A. L. Fair, and R. J. Randall (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 265–275.
- Mudge, A. W. (1979) Studies on substance P, somatostatin, and enkephalin in cultures of sensory neurons. Ph.D. dissertation, Harvard University, Cambridge, MA.
- Neer, E. J., J. M. Lok, and L. G. Wolf (1984) Purification and properties of the inhibitory guanine nucleotide regulatory unit of brain adenylate cyclase. *J. Biol. Chem.* 259: 14222–14229.
- Nowycky, M. C., A. P. Fox, and R. W. Tsien (1985a) Three types of calcium channel with different calcium agonist sensitivity. *Nature* 316: 440–443.
- Nowycky, M. C., A. P. Fox, and R. W. Tsien (1985b) Long-opening mode of gating of neuronal calcium channels and its promotion by the dihydropyridine calcium agonist Bay K 8644. *Proc. Natl. Acad. Sci. USA* 82: 2178–2182.
- Pang, I. H., and M. R. Vasco (1986) Morphine and norepinephrine but not 5-hydroxytryptamine and  $\gamma$ -aminobutyric acid inhibit the potassium-stimulated release of substance P from rat spinal cord slices. *Brain Res.* 376: 268–279.
- Perney, T. M., L. D. Hirning, and R. J. Miller (1986) Multiple calcium channels mediate neurotransmitter release from peripheral neurons. *Proc. Natl. Acad. Sci. USA* 83: 6656–6659.
- Price, G. W., G. P. Wilkin, M. J. Turnbull, and N. G. Bowery (1984) Are baclofen-sensitive  $GABA_b$  receptors on primary afferent terminals of the spinal cord? *Nature* 307: 71–74.
- Rane, S. G., G. G. Holz IV, and K. Dunlap (1987) Dihydropyridine inhibition of neuronal calcium current and substance P release. *Pflügers. Arch.* 409: 361–366.
- Sabol, S. L., and M. Nirenberg (1979) Regulation of adenylate cyclase of neuroblastoma  $\times$  glioma hybrid cells by  $\alpha$ -adrenergic receptors. *J. Biol. Chem.* 254: 1913–1920.
- Sekura, R. D., F. Falk, C. R. Manclark, B. Meade, and Y. Zhang (1983) Pertussis toxin. Affinity purification of a new ADP-ribosyltransferase. *J. Biol. Chem.* 258: 14647–14651.
- Stryer, L., and H. R. Bourne (1986) G proteins: A family of signal transducers. *Annu. Rev. Cell Biol.* 2: 391–419.
- Ui, M., K. Nogimori, and M. Tamura (1985) Islet-activating protein, pertussis toxin: Subunit structure and mechanism for its multiple biological actions. In *Pertussis Toxin*, R. D. Sekura, J. Moss, and M. Vaughn, eds., pp. 19–43, Academic, New York.
- Vallar, L., T. J. Biden, and C. B. Wollheim (1987) Guanine nucleotides induce  $Ca^{2+}$ -independent insulin secretion from permeabilized RINm5f cells. *J. Biol. Chem.* 262: 5049–5056.
- Worley, P. F., J. M. Baraban, C. Van Dop, E. J. Neer, and S. Snyder (1986)  $G_o$ , a guanine nucleotide-binding protein: Immunohistochemical localization in rat brain resembles distribution of second messenger systems. *Proc. Natl. Acad. Sci. USA* 83: 4561–4565.