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G-Protein Types Involved in Calcium Channel Inhibition at a Presynaptic Nerve Terminal

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The inhibition of presynaptic calcium channels via G-protein-dependent second messenger pathways is a key mechanism of transmitter release modulation. We used the calyx-type nerve terminal of the chick ciliary ganglion to examine which G-proteins are involved in the voltage-sensitive inhibition of presynaptic N-type calcium channels. Adenosine caused a prominent inhibition of the calcium current that was totally blocked by pretreatment with pertussis toxin (PTX), consistent with an exclusive involvement of G_o/G_i in the G-protein pathway. Immunocytochemistry was used to localize these G-protein types to the nerve terminal and its transmitter release face. We used two approaches to test for modulation by other G-protein types. First, we treated the terminals with ligands for a variety of G-protein-linked neurotransmitter receptor types that have been associated with different G-protein families. Although small inhibitory effects were observed, these could all be eliminated by PTX, indicating that in this terminal the G_i family is the sole transmitter-induced G-protein inhibitory pathway. Second, we examined the kinetics of calcium channel inhibition by uncaging the nonselective and irreversible G-protein activator GTPγS, bypassing the receptors. A large fraction of the rapid GTPγS-induced inhibition persisted, consistent with a G_o/G_i-independent pathway. Immunocytochemistry identified G_q, G_{11}, G_{12}, and G_{13} as potential PTX-insensitive second messengers at this terminal. Thus, our results suggest that whereas neurotransmitter-mediated calcium channel inhibition is mainly, and possibly exclusively, via G_o/G_i, other rapid PTX-insensitive G-protein pathways exist that may involve novel, and perhaps transmitter-independent, activating mechanisms.

Key words: nerve terminal; G-protein; G-protein type; calcium channel; presynaptic; calcium channel modulation; calcium channel inhibition; transmitter release; synaptic strength; chick; calyx synapse; chick ciliary ganglion

The inhibition of presynaptic calcium channels via trimeric G-protein second messenger pathways is a key mechanism whereby transmitter release, and hence, synaptic strength can be modulated (Hille, 1994). However, relatively little is known about which specific G-proteins are involved in this pathway in intact nerve terminals. We have used the large presynaptic nerve terminal of the chick ciliary ganglion to examine the diversity of G-protein types involved in calcium channel regulation.

The chick calyx nerve terminal preparation (Stanley and Goping, 1991) has several key advantages for an analysis of G-protein action. First, it is sufficiently large to allow direct recording of whole-cell calcium currents. Second, the channels are almost exclusively N-type (Stanley, 1991; Yawo and Momiyama, 1993) and located in the presynaptic region (Stanley, 1993; Haydon et al., 1994). Third, the large size allows protein components to be localized to the surface membrane by immunocytochemistry and, by costaining for vesicle clusters, to the transmitter release site regions (Stanley and Mirotznik, 1997).

The N-type calcium channel is known to be sensitive to transmitter-mediated, G-protein-dependent, inhibition (for review, see Dolphin, 1998; Ikeda and Dunlap, 1999). A major element of this inhibition is via a voltage-sensitive mechanism (Bean, 1989) that can be relieved, and hence assayed, by a strong preceding depolarizing pulse (Grassi and Lux, 1989; Elmslie, 1990).

There is considerable diversity in G-protein pathways leading to N-type calcium channel inhibition. A wide range of metabotropic receptor types may be involved, and a number of different G-proteins are capable of acting as second messengers, indicating a high level of heterogeneity. The pertussis toxin (PTX)-sensitive G-proteins G_o and G_i are the most commonly identified types in both primary neurons (Diversé-Pierluissi and Dunlap, 1993; Hille, 1994; Filippov et al., 1998; Park and Dunlap, 1998) and cell lines (Toth et al., 1996; Morikawa et al., 1998). However, PTX-insensitive G-proteins can also modulate these channels. Thus, G_s can modulate N-type channels in rat sympathetic neurons (Zhu and Ikeda, 1994), G_{13} does so in the NG 108–15 cell line (Wilk-Blaszczak et al., 1994), and G_z will substitute for PTX-sensitive G-proteins when overexpressed in superior cervical ganglia neurons (Jeong and Ikeda, 1998).

The presynaptic calcium channels in the chick calyx are inhibited via a G-protein-dependent pathway. Adenosine, a potent modulator of transmitter release at a variety of synapses, inhibits N-type calcium channels (Yawo and Chuhma, 1993). G-protein-dependent, voltage-sensitive inhibition of the calcium channels has been shown directly using the nonhydrolyzable (and irreversible) GTP analog GTPγS (Stanley and Mirotznik, 1997).

The goal of this study was to determine which G-protein type or types modulate presynaptic N-type calcium channels at the chick calyx synapse. Our results indicate that extracellular transmitters, including adenosine, all appear to act via G_o/G_i. However, we also demonstrate that other classes of G-protein are present, and we present evidence that one or more of these can also inhibit presynaptic calcium channel activity.

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Table 1. Antibodies used in this study

Antibody	Source	Immunostain dilution	Western blot dilution
Poly anti-$G\alpha_{o/i}$	J. K. Northup	1:200	1:1000
Poly anti-$G\alpha_{i1-3}$	Santa Cruz (sc262)	1:100	1:1000
Poly anti-$G\alpha_{o+i3}$	DuPont (NEI-803)	1:100	1:1000
Poly anti-$G\alpha_{i3}$	Calbiochem (371729)	1:100	1:1000
Mono anti-$G\alpha_o$ (Ab-1)	Lab Vision	1:200	1:1000
Mono anti-$G\alpha_o$ (Ab-2)	Lab Vision	1:200	1:1000
Mono anti-$G\alpha_{i1}$	Lab Vision	1:100	1:1000
Mono anti-$G\alpha_{i2}$	Lab Vision	1:100	1:1000
Poly anti-$G\alpha_{q/11}$	DuPont	1:100	1:1000
Poly anti-$G\alpha_{q/11}$	Calbiochem	1:100	1:1000
Poly anti-$G\alpha_{q/11}$	Santa Cruz	1:100	1:1000
Poly anti-$G\alpha_{12,13}$	Gutkind	1:100	1:1000
Poly anti-$G\alpha_s$	DuPont	1:100	1:1000
Poly anti-$G\alpha_z$	Calbiochem	1:100	1:500
Mono SV2	DSHB	1:0–1:1	
Poly SV2A	StressGen	1:200	
Mono neurofilament	DSHB	1:1	
Mono tubulin α	Lab Vision	1:100	
Mono tubulin β	Lab Vision	1:100	

MATERIALS AND METHODS

Electrophysiology

Calyx nerve terminal preparation. Ciliary ganglia were removed from embryonic day 15 (E15) chicks and were enzymatically dissociated in minimal Eagle's medium (MEM), as previously described (Stanley and Goping, 1991; Haydon et al., 1994). The dissociated preparation was transferred to a coverslip recording chamber containing the external solution, and calyx nerve terminals were identified visually under high-power magnification with oil-immersion lenses (40–60×; 1.35–1.4 numerical aperture). The cells were washed three times in MEM before use.

Patch-clamp recording. Currents were recorded using the whole-cell variant of the patch-clamp technique. Patch electrodes (1.5 mm outside diameter, thin wall microcapillary glass; World Precision Instruments), were fire-polished and had resistances in the range of 3.5–4.5 MΩ when filled with the internal solution described below. Currents were amplified, and cell membrane capacitance and series resistance were electronically compensated (Axopatch 200A; Axon Instruments, Foster City, CA). Voltage protocol generation and data acquisition were performed using pClamp 7.0 software. Current traces were generally low-pass filtered at 5 kHz, and leak currents were subtracted with a standard P/6 protocol, using a positive polarity leak subtraction pulse. All recordings were performed at room temperature.

The external (bath) medium was (in mM): NaCl 160, $CaCl_2$ 5, $MgCl_2$ 1, D-glucose 5, 4-aminopyridine 2, tetrodotoxin 0.001, HEPES-Na 10, and the patch electrode internal solution was: Cs-gluconate 120, CsCl 10, EGTA-Cs 10, $MgCl_2$ 1, HEPES-Cs 10, tetraethylamonium-Cl 20, MgATP 1, with GTP 0.1 (except where specified). GTPγS (0.1 mM) was included in the internal solution as described. PTX treatment was performed by incubating the preparation at 20°C (8% CO_2) in MEM overnight with or without 0.5–10 μg/ml PTX (Research Biochemicals, Natick, MA). Overnight incubation reduced the number of available calyx terminals for recording, consistent with the degeneration of the nerve stump after nerve section (Stanley and Drachman, 1980), and greatly increased the difficulty of these experiments. For flash photolysis, release of intracellular GTPγS, *S*-DMNPE-caged GTPγS (400 μM; Molecular Probes, Eugene, OR) was added to the internal solution, and a single 200 msec flash of unfiltered light from a mercury bulb (Uniblitz shutter; 40×; 1.35 NA quartz objective) was used to liberate the free nucleotide.

Voltage protocol and data analysis. A double trial protocol was used to measure voltage-sensitive G-protein inhibition. The cells were held at −80 mV. In the first trial a single 80 msec test pulse to 0 mV activated a calcium current. In the second trial, delivered 5 sec later, the test pulse was preceded by a 60 msec depolarization to +80 mV, a conditioning pulse that maximally relieves prepulse-sensitive G-protein inhibition in this nerve terminal (Stanley and Mirotznik, 1997). Data were acquired at 5–10 sec intervals. Current recruitment was measured as the maximal difference between the amplitude of the current induced by the test pulse with and without the conditioning pulse, at ~10 msec after the onset of the test pulse. Data were analyzed using ClampFit 6.0 (pClamp suite) and are presented as mean ± SEM. Student's *t* test was used to determine statistical significance.

Drug treatments. Drugs were dissolved in external solution and were applied either by addition to the bath or by pulse-triggered pressure ejection (Medical Systems) from a puff pipette (~5 μm diameter) at a distance of ~10 μm. The chamber was not perfused because only one calyx was treated in each dish, and the recordings were typically of short duration and with few individual drug applications. The following G-protein receptor agonists were used: substance P, bradykinin, somatostatin, neuropeptide Y (Peninsula), BRL52537 (Tocris), VIP, serotonin, ATP (Sigma, St. Louis, MO), adenosine, and noradrenalin (Research Biochemicals). The drugs were diluted in external solution and were applied by a pneumatic pressure ejection from a closely positioned micropipette or by addition to the bath.

Immunocytochemistry

Antibody characterization. Primary G-protein antibodies (Table 1) were characterized by standard Western blot (10% SDS gel) techniques. After electrophoresis, the proteins were transferred onto nitrocellulose membrane and probed with the indicated G-protein antibodies. Western blots were visualized with enhanced chemiluminescence.

G-protein antibodies used in this study are listed in Table 1. All primary antibodies were first tested for cross-reactivity against chick G-proteins by Western blot against chick brain protein (10 μg/lane; Fig. 1*A*). We only relied on antibodies that gave bands restricted to the appropriate molecular weight for the Gα subunit (~40 kDa).

The specificity of each antibody for its particular G-protein subtype was tested against the following individual recombinant Gα subunits (Calbiochem, La Jolla, CA): G_o, G_s, G_q, G_{11}, G_{i1}, G_{i2}, and G_{i3} (Fig. 1*B, panels 1–12*). Anti-$G\alpha_z$ was tested against all of these plus a mix of G-proteins (Calbiochem) reported to contain $G\alpha_{i1-3}$, $G\alpha_o$, $G\alpha_s$, $G\alpha_z$, $G\alpha_\beta$s, and $G\alpha_\gamma$ (Fig. 1*B, panel 12*). The $G_{12,13}$ antibody was tested against the above single recombinant G-proteins plus recombinant G_{13} (Calbiochem) (Fig. 1*B, panel 10*). Recombinant $G\alpha_{12}$ was unavailable.

On the whole, the antibodies distinguished far better between G-protein families (G_o/G_i, G_s, $G_{q/11}$, and $G_{12/13}$) than between members of the same family (Fig. 1*B, panels 1–12*). We made considerable effort to differentiate between specific PTX-sensitive members of the G_o/G_i family (G_o, G_{i1}, G_{i2}, and G_{i3}). Monoclonal antibodies against $G\alpha_o$ (Fig. 1*B, lanes 1,2*) and $G\alpha_i$ (data not shown) were particularly selective, but of these only the anti-$G\alpha_o$ antibodies were of use for immunocytochemistry. Commercially available polyclonal anti-G-protein antibodies for particular members of the G_o/G_i group typically exhibited little specificity and cross-reacted with all members (Fig. 1*B, panels 4–6*). Although we were unable to obtain recombinant $G\alpha_z$ to test the anti-$G\alpha_z$ antibody, our evidence suggests that this antibody was specific because a band of the appropriate molecular weight was observed against the $G\alpha_z$-containing mixture of G-proteins, whereas none was observed with any of the other recombinant G-proteins alone (Fig. 1*B, panel 12*).

Immunostaining of calyx nerve terminals. Ganglia from E15 chicks were dissociated and plated on coverslips, as described above. The preparation was fixed in 2% paraformaldehyde for 45 min and then permeabilized in 0.5% polyoxyethylene-20-cetyl ether with 0.5% paraformaldehyde for 10 min. Cells were stained by exposure to primary antibodies overnight. To identify the nerve terminal and the transmitter release zones, all preparations were double-labeled with the appropriate complimentary monoclonal or polyclonal antibody against the synaptic vesicle protein SV2 (Table 1).

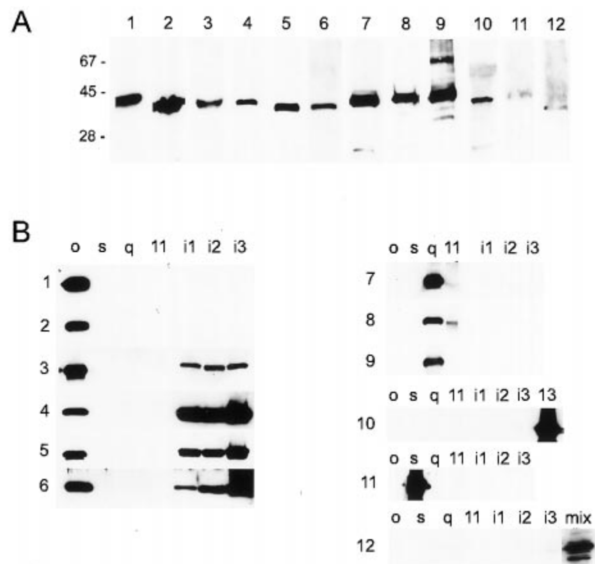

Figure 1. Characterization of G-protein antibodies. *A,* All antibodies gave an appropriate ~40 kDa band for chick brain protein (10 μg/lane). *Lane 1,* Monoclonal anti-G_o (Ab-1); *2,* monoclonal anti-G_o (Ab-2); *3,* polyclonal anti-G_o (J. K. Northup); *4,* polyclonal anti-G_{i3} (Calbiochem); *5,* polyclonal anti-G_{i1-3} (Santa Cruz Biotechnology); *6,* polyclonal anti-G_{o+i3} (DuPont); *7,* polyclonal anti-$G_{q/11}$ (DuPont); *8,* polyclonal anti-$G_{q/11}$ (Santa Cruz Biotechnology); *9,* polyclonal anti-$G_{q/11}$ (Calbiochem); *10,* polyclonal anti-$G_{12,13}$ (Gutkind); *11,* polyclonal anti-G_s (DuPont); *12,* polyclonal anti-G_z (Calbiochem). *B,* Blot of antibodies against the recombinant Gα subunits: G_o, G_s, G_q, G_{11}, G_{i1}, G_{i2}, G_{i3}, and G_{13}, as well as a G-protein mixture that includes G_z.

FITC and LRSC-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) were applied at 1:50 dilution for 1 hr. Definitive localization of staining to the transmitter release face or the external, Schwann cell face of the nerve terminal was only possible in the dissociated preparations when the calyces remained attached to the postsynaptic ciliary neuron.

Immunostaining was also performed on ciliary ganglia slices prepared by cryostat section, without previous treatment with dissociation enzymes. Ganglia were fixed in 4% paraformaldehyde in 15% picric acid for 1–1½ hr, infiltrated with 15% sucrose for 1 hr and 30% sucrose overnight, and were then sliced on a cryostat into 12 μM sections. Staining was performed as above and, except where noted, the staining patterns between dissociated cells and cryostat cells were consistent.

Dissociated cells and cryostat slices were visualized under fluorescent illumination on a Zeiss Axiophot with a 63 or 100×, 1.4 NA lens. Images were acquired and analyzed using a Scanalytics Cellscan deconvolution system as described (Juhaszova et al., 2000). This system uses Exhaustive Photon Reassignment to yield confocal-like images of slices through the sample. At least 30 calyces were examined for each stain combination.

RESULTS

Detection of G-protein-dependent inhibition of presynaptic calcium channels

The object of this study was to determine which G-protein families are involved in the modulation of calcium currents in the presynaptic terminal of the chick ciliary ganglion. G-protein-dependent inhibition was monitored as the percentage of current increase after a strong depolarizing prepulse (Fig. 2) and is termed here the "prepulse recruitment." There was no evidence of calcium channel inhibition in the absence of intracellular GTP (Fig. 2, *top left panel*).

Adenosine modulates presynaptic calcium channels via a PTX-sensitive G-protein pathway

Transmitter-induced inhibition of calcium channels can involve many different G-protein species, the most common of which appear to be G_o and G_i. Adenosine, which inhibits N-type calcium channels in many neuronal cells, is believed to act solely via G_o/G_i, and this agent inhibits calcium influx at the calyx nerve terminal (Yawo and Chuhma, 1993). Thus, our first objective was to demonstrate that this inhibition involved a characteristic voltage-sensitive inhibition mechanism. We then tested if the adenosine inhibition pathway was via G_o/G_i by blocking these G-proteins with the selective toxin PTX.

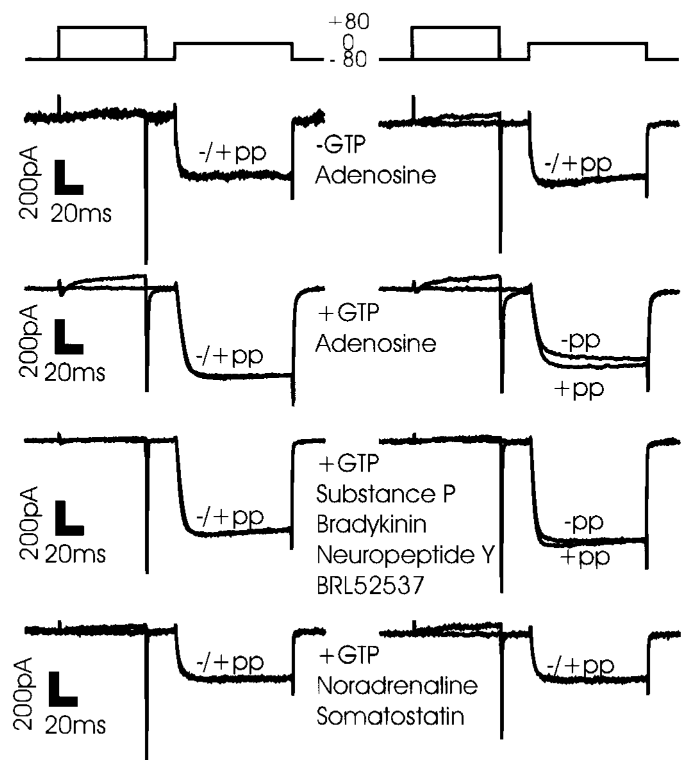

Figure 2. Modulation of chick ciliary ganglion presynaptic calcium current by neurotransmitter. The degree of current recruitment was determined using a double-trial protocol (see Materials and Methods) before, during, and after a puff of transmitter onto the terminal. Each panel shows before (*left column*) and during (*right column*) transmitter application in a single calyx nerve terminal. GTP (0.1 mM) was included in the internal solution in all experiments except in panel 1. *Panel 1,* Adenosine (10 μM) in the absence of internal GTP. *Panel 2,* Adenosine (10 μM) treatment. *Panel 3,* Treatment with a mix of substance P (0.5 μM), bradykinin (1 μM), neuropeptide Y (0.1 μM), and BRL52537 (1 μM). *Panel 4,* Treatment with a mix of noradrenaline (100 μM) and somatostatin (10 μM). *−pp,* Without prepulse; *+pp,* with prepulse.

A step voltage depolarization of the calyx nerve terminal triggered a calcium current with characteristic properties of rapid activation with little inactivation during the current pulse, terminated by a rapid, monotonic tail current on return to the resting potential (Fig. 2, *left column*). In the absence of drug treatment with or without intracellular GTP (Fig. 2, *left column*) or in the presence of adenosine but while omitting intracellular GTP (Fig. 2, *top right panel*), little or no prepulse recruitment was observed. However, when adenosine (10 μM) was puff-applied in the presence of intracellular GTP (Fig. 2, *second panel*) a brief puff application caused a significant calcium current inhibition and a prepulse recruitment of 16.1 ± 2.7%, (n = 9), consistent with voltage-sensitive inhibition via the G-protein pathway. Inhibition was maintained only during the 5 sec puff application but persisted beyond the puff with longer treatment durations that can be attributed to extracellular accumulation. Repeated applications did not show significant desensitization (Fig. 3*A*).

We next tested for G_o/G_i involvement in the adenosine pathway by pretreatment with PTX. Block with PTX typically requires hours of exposure necessitating the development of a method for long-term maintenance of the dissociated calyces. After ~15 hr incubation (in MEM at 20°C, 8% CO_2) nerve terminals survived but were less common and were more fragile. Control calyces were incubated in the same conditions in the absence of PTX. In these a prominent inward calcium current was still present, and adenosine-dependent prepulse recruitment was similar to calyces before incubation at 13.6 ± 2.4% (n = 6; $p > 0.05$). PTX was tested

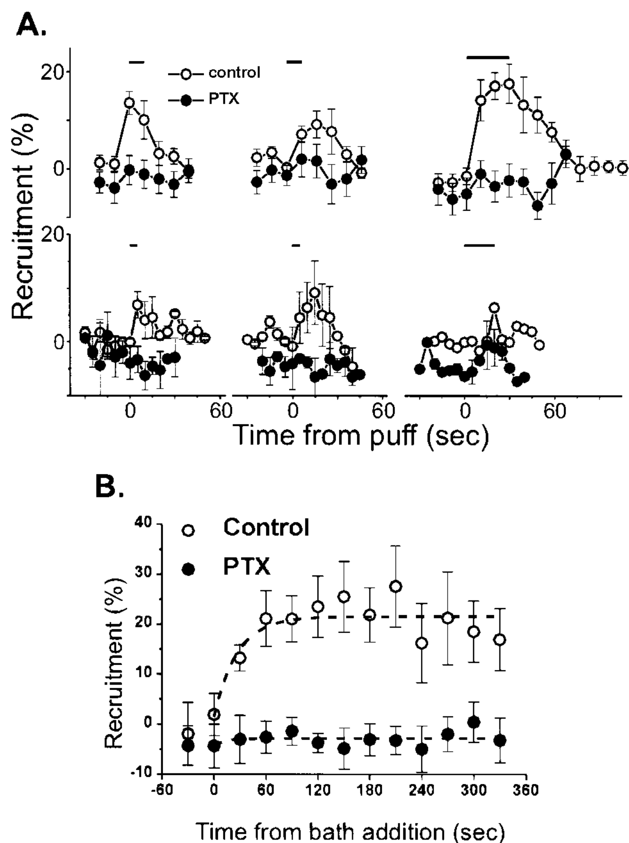

Figure 3. Effect of PTX pretreatment on adenosine-induced calcium current inhibition. *A,* Time course of calcium channel inhibition with puff application of transmitter (*horizontal bar*) with or without overnight PTX treatment. The effects of three consecutive trials given ~1 min apart to each group of calyces are shown. *Top series,* Adenosine (10 μM) treatment (control, $n = 6$; PTX, $n = 14$). *Bottom series*, Treatment with a cocktail of substance P (0.5 μM), bradykinin (1 μM), neuropeptide Y (0.1 μM), and BRL52537 (1 μM; control, $n = 4$; PTX, $n = 3$). *B,* Bath application of adenosine. Current inhibition was monitored after the addition ($t = 0$) of adenosine (0.2 mM) to control (*open symbols*, $n = 6$) or PTX-treated (*filled symbols*, $n = 6$) calyces. In both *A* and *B* current inhibition is monitored by the percentage of prepulse recruitment.

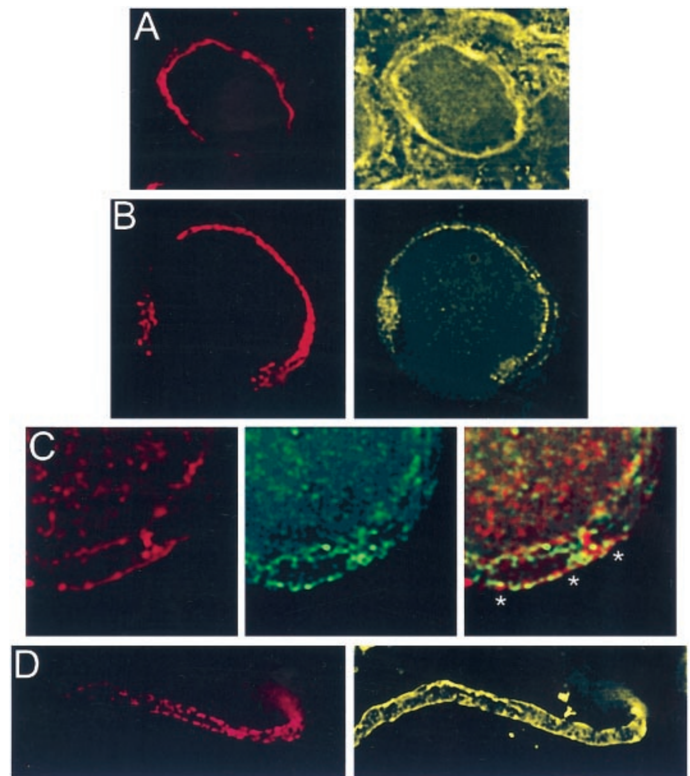

Figure 4. Localization of PTX-sensitive G-proteins. $G\alpha_o$ and $G\alpha_i$ localized to the membrane of the calyx presynaptic terminal. Calyx terminals are identified by SV2 (*red*) in the *left panel* of each pair except in *C. A,* Monoclonal antibodies (Ab-1 + Ab-2, *yellow*) localized G_o to the membrane of calyx terminals in cryostat sections of whole ciliary ganglia. *B,* In dissociated preparations, monoclonal anti-$G\alpha_o$ (Ab-2) stained the membranes of presynaptic calyces with especially bright, patchy staining at the synaptic interface. *C,* G_i was localized by a multistaining approach. G_o plus G_i were stained in *red* with polyclonal anti-$G\alpha_o$ (Northup; *red; left panel*) whereas G_o was stained in *green* with monoclonal anti-$G\alpha_o$ (Ab-1 plus Ab-2; *middle panel*). Both labeled the calyx membrane. Superimposing the two stains (*right panel*) localizes Go by the costained regions (*yellow*). The distinct regions of red staining (e.g., *asterisks*) identify membrane regions with G_i but not G_o. *D,* Fully isolated nerve terminals exhibited staining of the surface membrane with polyclonal anti-$G\alpha_o$ (Northup), confirming a presynaptic localization of G_o/G_i.

at concentrations ranging from 0.5 to 10 μg/ml but was fully effective at 1 μg/ml. Pretreatment with PTX reduced adenosine-induced prepulse recruitment to undetectable levels at 1.6 ± 2.2% ($n = 14$; Fig. 3*A*).

In an attempt to saturate the effect of adenosine, we bath-applied the transmitter at a high concentration (0.2 mM). In the absence of PTX, adenosine treatment for ~2 min resulted in 23.5 ± 6.1% ($n = 8$) prepulse recruitment, which was maintained for up to 6 min without significant desensitization (Fig. 3*B*). PTX pretreatment eliminated prepulse recruitment (−2.6 ± 3.2%; $n = 6$; $p < 0.01$; Fig. 3*B*). Thus, adenosine inhibits the calcium current via a $G_{o/i}$ pathway, in agreement with findings from other preparations.

G_o/G_i are located in the presynaptic nerve terminal

We used high-resolution immunocytochemistry to determine whether G_o/G_i proteins were present in the calyx. The G_i protein family comprises G_{o1}, G_{o2}, G_{i1}, G_{i2}, G_{i3}, G_z, G_{t1}, G_{t2}, and G_{gust}. G_o, G_i and G_z have been implicated in the modulation of N-type calcium channels. With the exception of G_z, all are blocked by PTX. The synaptic vesicle marker SV2 was used to positively identify calyx nerve terminals, and the bright patches of staining for this protein mark the clusters of vesicles at the transmitter release sites.

We examined calyces that remained attached to a postsynaptic neuron and also those that had become fully detached. The former provides a view of the target protein locations at an intact synapse, whereas the latter can be used to demonstrate unambiguously that the G-protein is located in the presynaptic terminal and that we are not observing staining in the synaptic space or on the surface of the postsynaptic ciliary neuron. We also examined staining in calyces from whole fixed cryostat-sectioned ciliary ganglion (Fig. 4*A*). With this technique we were able to reproduce the main features of our findings in the absence of enzymatic dissociation.

G_o was localized with two different monoclonal antibodies (Ab-1 and Ab-2). These antibodies gave essentially the same staining pattern in cryostat sections (Fig. 4*A*) and in dissociated ganglia (Fig. 4*B*). Staining was almost exclusively on the surface membrane of both the back (Schwann cell) and release-face aspects of the terminal, with particularly bright, patchy staining at the synaptic interface. We could not find an antibody suitable for selective immunostaining of chick G_i. However, a polyclonal antibody, anti-$G\alpha_{o/i}$ (Table 1), recognized G_o and all three G_i α subunits (Fig. 1*B, panel 3*). If we assume that the monoclonal antibodies against G_o localize all of this G-protein, then the distribution of G_i (or, more accurately, the G_i that is not colocalized with G_o) can be deduced as the regions stained with anti-$G\alpha_{o/i}$ but not with either of the monoclonal antibodies. Thus, in Figure 4*C*, G_o/G_i is stained in red (*left panel*), G_o in green (*middle panel*), and costained regions are yellow in the superimposed images (*right panel*). While much of the G_o/G_i and G_o staining is colocalized and indistinguishable from G_o alone, some distinct regions of red staining at the calyx mem-

brane were evident (*asterisks*). These spots were seen near the vesicle clusters and are consistent with the presence of G_i at the transmitter release face of the calyx nerve terminal.

In fully isolated nerve terminals anti-$G\alpha_{o/i}$ staining was noted primarily on the surface membrane (Fig. 4*D*). Staining partially corresponded with SV2 staining, indicating the presence of G_o/G_i at the transmitter release sites, but with a distribution that also extended to nonterminal regions. Other polyclonal antibodies against G_o/G_i gave similar results (data not shown).

Calcium channel inhibition by metabotropic neurotransmitter receptors

Whereas the inhibition of N-type calcium channels via the adenosine pathway involves primarily the PTX-sensitive members of the G_i family, other metabotropic receptor types are known to use G-protein from different families (for review, see Hille et al., 1995). As a first attempt to identify inhibitory pathways that involved other G-protein types, we screened a number of different receptor agonists for voltage-dependent inhibition of the calcium channels. Ligands were puff-applied, and we tested for the presence of prepulse-dependent calcium current recruitment.

A mix (combined to speed the screening process) of substance P (0.5 μM), neuropeptide Y (0.1 μM), bradykinin (1 μM), and BRL52537 (1 μM) resulted in a weak, but significant, current recruitment (7.0 ± 2.4%, $n = 4$, $p < 0.05$; Figs. 2, third panel, 3*A*). Current inhibition by this cocktail was, however, also blocked by PTX pretreatment (Fig. 3*A*) and, hence, was also consistent with the involvement a G_o/G_i pathway. Other receptor ligands including noradrenaline (100 μM) and somatostatin (3 μM; Fig. 2, *bottom panel*), VIP, ATP, or serotonin (both 10 μM; data not shown) did not inhibit the calcium current. Thus, this approach failed to demonstrate the involvement of other G-protein types. In fact, our findings suggest that the PTX-sensitive G_o/G_i may mediate all neurotransmitter-induced, voltage-sensitive calcium channel inhibition via G-proteins at this nerve terminal.

Calcium channel inhibition by GTPγS

We used GTPγS to test if there was any evidence for calyx calcium channel inhibition by non-G_o/G_i G-proteins. GTPγS is a nonhydrolyzable analog of GTP that irreversibly activates all trimeric G-proteins and has been demonstrated to strongly inhibit the calcium channels at this nerve terminal (Stanley and Mirotznik, 1997). Our strategy was to first compare adenosine and GTPγS-dependent calcium channel inhibition and then to test for persisting GTPγS inhibitory effects after G_o/G_i block with PTX.

Infusion of GTPγS (0.1 mM) into the untreated calyx terminals resulted in a robust and maintained prepulse-sensitive calcium channel inhibition (Fig. 5*A*; Stanley and Mirotznik, 1997). The degree of current inhibition was double that observed with bath application of adenosine (GTPγS: 47.1 ± 12.6%, $n = 5$; adenosine: 23.5 ± 6.1%, $n = 8$ as above, $p < 0.01$), suggesting that GTPγS activates a larger pool of inhibitory trimeric G-proteins than adenosine. This conclusion was supported by the finding that, in contrast to the results with adenosine, almost half of the GTPγS-induced current inhibition persisted after PTX pretreatment of the nerve terminals (Fig. 3*A*; 18.2 ± 4.1%, $n = 6$).

The above results suggest, but do not prove, that the more pronounced calcium channel inhibition observed with GTPγS than adenosine is attributable to non-G_o/G_i pathways. Although it is known that GTPγS can still activate PTX-inhibited G-protein, published evidence suggests that its action is markedly slowed (see Discussion). We therefore compared the kinetics of GTPγS-dependent inhibition with or without previous PTX treatment using flash photolysis of caged GTPγS (Dolphin et al., 1988). No prepulse-sensitive calcium channel inhibition was detected before flash treatment (Fig. 5*B*). In control terminals flash photolysis resulted in significant prepulse-dependent calcium current recruitment (23.8 ± 6%, $n = 7$). Maximum inhibition was detected within 5 sec, the interval between trials. The effect of flash photolysis after PTX was essentially indistinguishable, with a prepulse-dependent

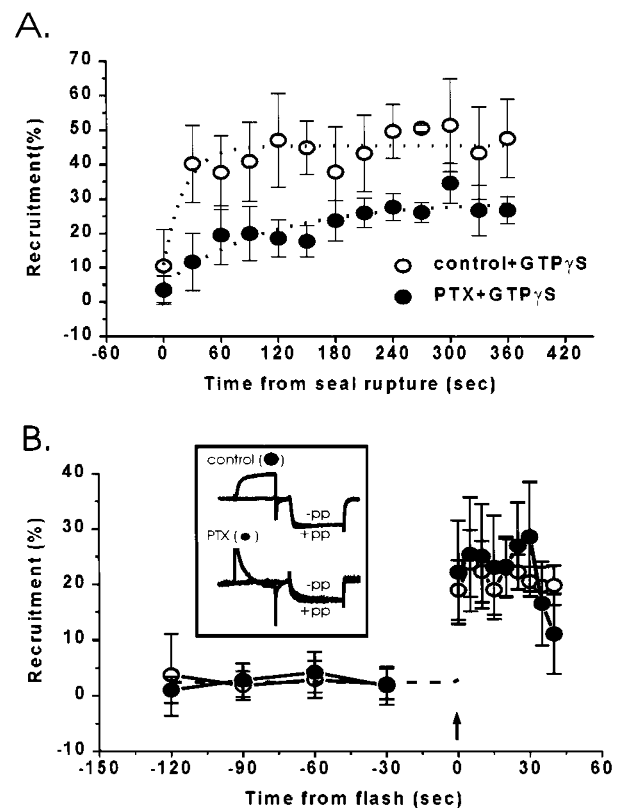

Figure 5. Effect of PTX pretreatment on GTPγS-induced calcium channel inhibition. *A,* Intracellular infusion of GTPγS. Calcium currents were recorded every 10 sec after membrane rupture in each experiment, and the percentage of recruitment was averaged for 30 sec periods. Control, $n = 5$ experiments (*open symbols*); PTX, $n = 6$ (*closed symbols*). *B,* Flash photolysis of caged GTPγS with or without PTX pretreatment. Caged GTPγS was included in the internal solution, and ~2 min after membrane seal rupture the caged nucleotide was released by a 200 msec flash (*arrow*). Immediately after, current trials were initiated (control, $n = 7$; PTX, $n = 5$). *Insert,* Representative current traces recorded 5–10 sec after the flash in a control and a PTX-treated terminal. −*pp*, Without prepulse; +*pp,* with prepulse. Symbols as in *A*.

recruitment of 25.5 ± 4% ($n = 5$) and an abrupt onset within the first 5 sec. An anomaly in these results was that the amplitude of inhibition with flash photolysis was significantly less than that observed when (free) GTPγS was introduced directly into the nerve terminal. We do not know the reason for this disparity but it may reflect, in part, the liberation of a lower concentration of intracellular GTPγS from the caged compound or perhaps a component that is inhibited with a much slower time constant (possibly via recruitment of PTX-inhibited G_o/G_i) than could not be reliably detected within the limitations of the uncaging technique. Because of the technical difficulty of these experiments (see Materials and Methods) these possibilities were not examined further. The important point was, however, that rapid inhibition could still be detected, even after PTX treatment, in stark contrast to the findings with adenosine.

PTX-insensitive G-proteins localized at the presynaptic nerve terminal

We used immunocytochemistry to test for the presence and distribution in the calyx nerve terminal of PTX-insensitive G-proteins in the G_q, G_{12}, and G_s families and also for G_z, a PTX-insensitive member of the G_i family.

Members of the G_q subfamily were examined with three polyclonal antibodies that cross-reacted with $G\alpha_q$ and $G\alpha_{11}$ (Table 1, Fig. 1), and all three gave similar staining patterns at the calyx nerve terminal. The results obtained with the DuPont (Billerica, MA) antibody are presented. Attached calyces had staining throughout the nerve terminal that often colocalized with SV2

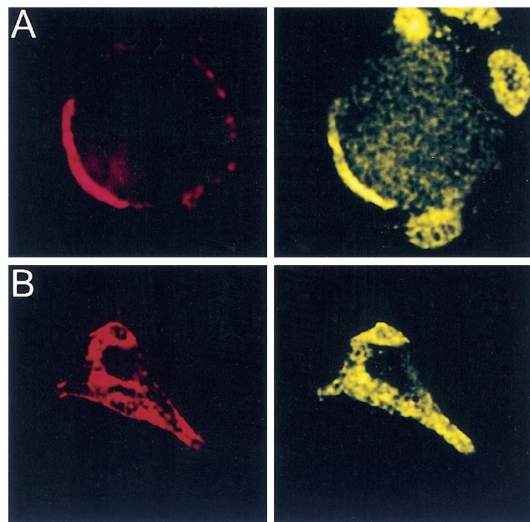

Figure 6. Localization of PTX-insensitive G-proteins: $G_{q/11}$. $G_{\alpha q/11}$ (DuPont antibody) localized to the membrane and cytoplasm of the presynaptic terminal. Calyx terminals were identified by SV2 (*red*) in the *left panel*. Attached (*A*) and isolated (*B*) calyces had bright staining throughout the terminal that frequently colocalized with SV2.

(Fig. 6*A*) and was particularly bright at the synaptic interface. Fully isolated calyx nerve terminals also exhibited spotty staining that localized to both the membrane and the cytoplasm (Fig. 6*B*).

The G_{12} subfamily of G-proteins contains two members, G_{12} and G_{13}, and our antibody recognized both (Fig. 1). With most attached calyces, the staining of the presynaptic terminal was comparable in intensity to the postsynaptic soma and thus difficult to distinguish. However, some somata were less brightly stained, and the calyx could then be seen to exhibit clear spotty staining throughout the terminal (Fig. 7*A*). However, unlike G_o or $G_{q/11}$, prominent staining of the synaptic interface was not observed. Fully isolated calyces stained both at the terminal region, as identified by the SV2 staining, and further up the length of the axon (Fig. 7*B*).

The G_s family contains G_{olf} and G_s. We only investigated G_s staining because the former is expressed solely in the olfactory system. An antibody that recognized only $G\alpha_s$ (Fig. 1*B, panel 11*) produced bright staining of the soma that rivaled that of the calyx (Fig. 8*A*). The staining was, if anything, reduced along the synaptic interface. This impression was supported by the observation that in fully isolated calyx nerve terminals, punctate G_s staining was

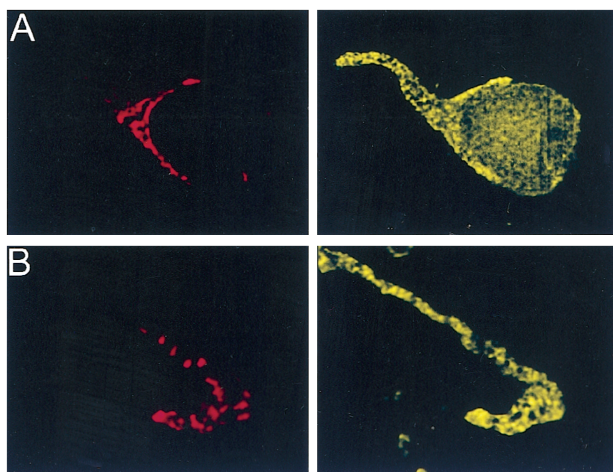

Figure 7. Localization of PTX-insensitive G-proteins: $G_{12,13}$. $G_{\alpha 12,13}$ localized to both the membrane and cytoplasm of the calyx terminal. Calyx terminals are identified by SV2 (*red*) in the *left panel*. *A,* Attached calyces had spotty staining throughout the membrane and the cytoplasm. *B,* The staining remained in isolated calyces.

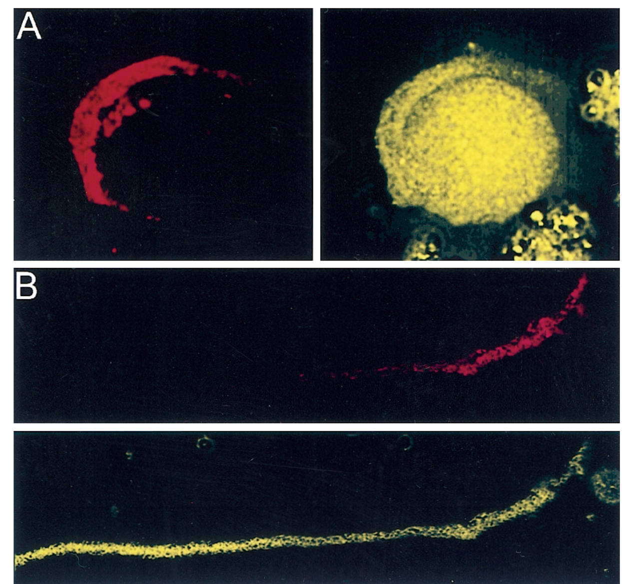

Figure 8. Localization of PTX-insensitive G-proteins: G_s. $G_{\alpha s}$ predominantly localized to the calyx cytoplasm. Calyx terminals are identified by SV2 (*red*) in each panel pair. *A,* Attached calyces were as stained as the postsynaptic neuron with reduced staining at the interface. *B,* Isolated calyces showed bright $G_{\alpha S}$ staining along the axon that decreased at the terminal region.

negatively correlated with SV2 staining and, thus, with the presynaptic region (Fig. 8*B*).

Anti-$G\alpha_z$ gave a novel staining pattern, not seen for any other G-protein. Staining was limited to a bright fibrous band that coursed through the cytoplasm of the calyx axon and into the terminal (Fig. 9*A,B*). Staining for G_z and SV2 were mutually exclusive, and no clear G_z staining was associated with the surface membrane in the nerve terminal. In the postsynaptic soma, the stained fibrous pattern formed a faint web-like pattern just beneath the membrane that came together to course out of the soma and stream down the axon (data not shown). Because this pattern of staining was strongly suggestive of the cytoskeleton, we colabeled G_z with cytoskeletal proteins. G_z did not colocalize with tubulin α or β (Fig. 9*C* shows tubulin α) but exhibited near perfect colocalization with the phosphorylated 200 kDa subunit of neurofilament protein (Fig. 9*D*). This G_z staining pattern was not attributable to cross-reaction of the antibody with chick neurofilament protein because Western blots for the antibody did not show a 200 kDa band (data not shown) or, for that matter, any band other than the G-protein itself (Fig. 1).

DISCUSSION

We have examined the diversity of G-proteins involved in the voltage-dependent modulation of N-type calcium channels at an identified presynaptic nerve terminal. Our main findings are first, numerous G-proteins from several families exist at this nerve terminal but that only certain types are closely associated with the transmitter release face. Second, PTX-sensitive members of the G_i family are involved in the adenosine-dependent, and possibly all, neurotransmitter-induced calcium channel inhibition. Third, we present evidence that PTX-independent G-proteins can also modulate the presynaptic calcium channels, although their role in nerve terminal function remains to be established.

The isolated chick calyx synapse preparation exhibits several methodological advantages for the study of presynaptic calcium channels and their modulation. These include excellent visualization of the whole nerve terminal and the ability to achieve an effective voltage clamp of membrane currents. Furthermore, immunofluorescent staining can be performed on cryostat sections, dissociated calyx synapses free from surrounding cells, and even

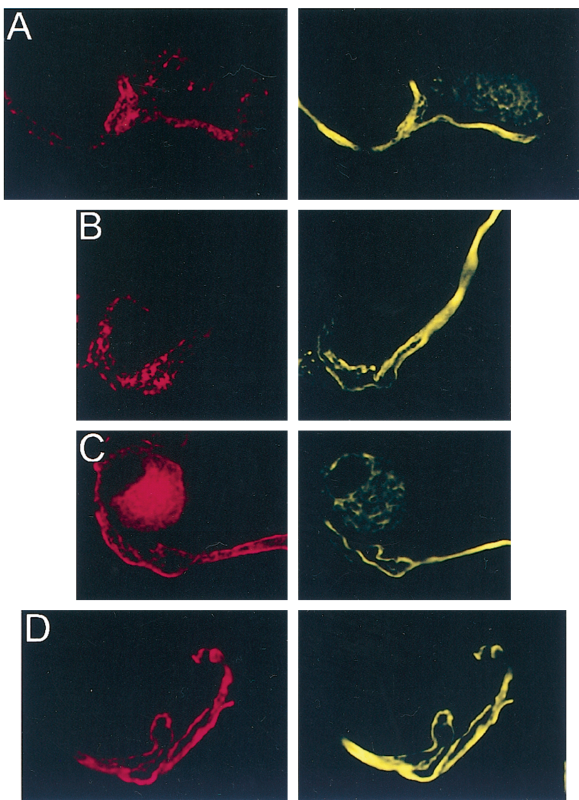

Figure 9. Localization of PTX-insensitive G-proteins: G_z. Attached (*A*) and isolated (*B*) calyces had bright fibrous staining for G_z that coursed through the axon and partially into the terminal and was inversely correlated with SV2 (*red, left panel*). This staining pattern was suggestive of colocalization with the cytoskeleton. *C,* Tubulin α (*red, left panel*) stained predominantly near the calyx membrane and, hence, did not colocalize with the predominantly intracellular staining of G_z (*yellow; right panel*). *D,* G_z showed almost perfect colocalization (*yellow, right panel*) with the phosphorylated 200 kDa subunit of neurofilament protein (*red, left panel*).

fully isolated nerve terminals, ensuring unambiguous localization of staining to the nerve terminal.

Adenosine-induced calcium current inhibition was demonstrated to occur via a PTX-sensitive pathway. G_o was localized to the membrane of the calyx nerve terminal (Fig. 4*A,B*), and evidence was presented for similar localization of G_i (Fig. 4*C*). Thus, we conclude that one or both of these G-protein species mediate the adenosine inhibitory pathway at the calyx presynaptic nerve terminal.

The involvement of PTX-insensitive G-proteins in calcium channel inhibition was initially tested by treating the nerve terminal with a variety of neurotransmitters that have been associated with non-G_o/G_i inhibitory pathways. Ligands for noradrenaline, somatostatin, $P2_Y$, muscarinic, VIP, serotonin, substance P, NPY, bradykinin, and κ-opiate G-protein receptors were tested. However, minimal inhibition of the calcium channels was observed, and even this was blocked by PTX. Thus, our results suggest that at this nerve terminal, neurotransmitter-dependent inhibition of presynaptic calcium channels occurs exclusively via the PTX-sensitive G-proteins G_o and G_i.

A more general test for G-proteins involved in calcium channel inhibition was based on the nonselective G-protein activator GTPγS. Intracellular treatment with this agent caused a far greater calcium current inhibition (~40%) than that observed with a saturating dose of adenosine (~20%). This finding in itself suggests the recruitment of an additional pool of G-proteins. However, it does indicate whether the increased inhibition is attributable to the activation of additional, perhaps reserve, G_o/G_i or the recruitment of distinct G-protein types.

To test for channel inhibition via non-G_o/G_i G-proteins, we tested GTPγS after PTX treatment. Although the degree of GTPγS-dependent calcium current inhibition was reduced, a significant level persisted. However, an alternative interpretation for this PTX-insensitive fraction is that GTPγS overcomes the action of the toxin on G_o/G_i and that calcium channel inhibition still involves these G-proteins. Early studies by Gilman (Katada et al., 1984) on purified G-proteins *in vitro* concluded that PTX treatment markedly impedes the activation of G_i by GTPγS. However, a later report by Huff and Neer (1986) contradicted this finding, reporting that GTPγS can overcome the inhibitory action of PTX. The anomaly in these two reports is readily attributable to differences in the assay conditions: the former study exposed the G-proteins to GTPγS for only 2 min at 30°C and, hence, tested only for short-term effects. However, the study by Huff and Neer (1986) incubated GTPγS with the G-proteins for 30 min at 30°C followed by overnight at 4°C, testing for completion of its action. Although no subsequent study has attempted to reconcile these findings directly, they can be explained if GTPγS can relieve PTX block but its latency or kinetics are greatly slowed. Indeed, such a slowing was demonstrated directly in an earlier study (Jakobs et al., 1984) in which activation of G-protein by GTPγS was assayed by the inhibition of adenylate cyclase. This study found that the onset of G-protein activation was markedly slowed after PTX, from ≤1 min in control cell fractions (the minimum time tested) to ~5 min after toxin treatment. Thus, all previous reports are consistent with the conclusion that PTX slows activation of G-protein by GTPγS by several minutes. Thus, the inhibition of the presynaptic calcium current by GTPγS after PTX block within 5–10 msec, as noted here, strongly suggests the involvement of a G_o/G_i-independent G-protein pathway.

Few studies have examined the spectrum of G-protein types in presynaptic nerve terminals and which of these might be involved in calcium channel modulation. We have examined the diversity and location of G-proteins at the chick calyx nerve terminal by immunocytochemistry and high-resolution imaging. All primary antibodies used in this study were characterized in detail, testing each against recombinant G-proteins to confirm specificity as well as against rat and chick neural tissue to confirm cross-reactivity. These studies indicated that G_o, G_i (Fig. 4), G_q or G_{11} (Fig. 6), and G_{12} or G_{13} (Fig. 7) were located in the nerve terminal and at the transmitter release site regions. G_s was located primarily outside the nerve terminal and synaptic cleft and exhibited little staining on the surface membrane (Fig. 8), suggesting that it is less likely to act as a primary modulator of the calcium channels. G_z was not observed on the surface membrane but instead colocalized with the neurofilaments (Fig. 9). The functional significance of this localization is unclear, but it makes it unlikely that this G-protein modulates the calcium channels at the transmitter release site. Thus, our study identifies G_q, G_{11}, G_{12}, and G_{13} as possible PTX-insensitive G-proteins capable of presynaptic calcium channel modulation at this terminal.

If, as suggested by our results, G_o or G_i are the only G-proteins mediating calcium channel inhibition via neurotransmitter receptors at this nerve terminal, what then is the role of the PTX-insensitive pathway? An obvious possibility is that these G-proteins are linked to an as yet untested membrane receptor pathway that we have not explored. Alternatively, the potent action of GTPγS induces inhibition by G-proteins that are not normally involved in calcium channel modulation. However, if this was the case one might expect a much slower inhibition via the PTX-insensitive G-proteins, whereas a very rapid effect, more consistent with a membrane-delimited pathway, was noted (Fig. 5*B*). One exciting possibility is that mechanisms other than that initiated by metabotropic receptors can modulate the release site-associated calcium channels. Possibilities might include inhibition associated with the transmitter release steps, such as the loading or unloading of vesicles into the transmitter release site, and interactions between the presynaptic and postsynaptic cells mediated by extracellular matrix proteins.

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