of the amplified product was subjected to a second round of amplification using a third internal C_α primer (5′-GTGAACAGGCAGAGGGTGCTG-3′) and the same anchor primer. The amplified products were ligated to the Hph I–digested pCR1000 plasmid vector (Invitrogen) and transformed into the INVαF′ bacteria. The resultant kanamycin colonies were screened successively with C_α and V_α3.1 probes (same probes as described for Northern analysis).

13. TCRα surface expression and heterogeneity were assessed by subjecting immunoprecipitates from surface ^{125}I-labeled lymphocytes to two-dimensional electrophoresis employing pH-dependent separation in the horizontal dimension and then SDS–polyacrylamide gel electrophoresis in the vertical dimension [P. A. O'Farrell, H. M. Goodman, P. H. O'Farrell, *Cell* **12**, 1133 (1977)]. Although the basic β chain components of control NOD mice exhibited considerable microheterogeneity in terms of both charge and molecular size, there was little structural heterogeneity detectable in the acidic α chain components. These findings, which are similar to the reports of others [B. W. McIntyre and J. P. Allison, *Cell* **34**, 739 (1983); E. L. Reinherz *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **80**, 4104 (1983)], precluded analysis of the α chain repertoire by this approach.
14. We generated T cell hybridomas by fusing, according to the fusion procedure of L. Glimcher and E. M. Shevach [*J. Exp. Med.* **156**, 640 (1982)], nylon-wool–purified splenic T cells (1×10^7 to 2×10^7) from a 6-month-old transgenic and a nontransgenic littermate with the TCRαβ$^-$ thymoma [J. White *et al.*, *J. Immunol.* **143**, 1822 (1989)]. T cell hybridomas were cloned by limiting dilution. Total mRNA from 1×10^6 to 2×10^6 hybridoma T cells were prepared from each clone, and were transferred onto nitrocellulose by the slot-blot method (Schleicher & Schuell, Keene, NH). The same blots were sequentially hybridized with ^{32}P-labeled V_α3.1, V_β8.2, and C_α probes and stripped between hybridizations.
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A Functional Role for GTP-Binding Proteins in Synaptic Vesicle Cycling

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The squid giant synapse was used to test the hypothesis that guanosine-5′-triphosphate (GTP)–binding proteins regulate the local distribution of synaptic vesicles within nerve terminals. Presynaptic injection of the nonhydrolyzable GTP analog GTPγS irreversibly inhibited neurotransmitter release without changing either the size of the calcium signals produced by presynaptic action potentials or the number of synaptic vesicles docked at presynaptic active zones. Neurotransmitter release was also inhibited by injection of the nonhydrolyzable guanosine diphosphate (GDP) analog GDPβS but not by injection of AlF_4^-. These results suggest that a small molecular weight GTP-binding protein directs the docking of synaptic vesicles that occurs before calcium-dependent neurotransmitter release. Depletion of undocked synaptic vesicles by GTPγS indicates that additional GTP-binding proteins function in the terminal at other steps responsible for synaptic vesicle replenishment.

Rapid transmission of signals between neurons is achieved by secretion of neurotransmitters at synapses. Neurotransmitters are stored in synaptic terminals in membrane-bound organelles, the synaptic vesicles, and are secreted by the process of exocytosis. During repeated bouts of synaptic transmission, neurotransmitters are newly synthesized while synaptic vesicles are retrieved and refilled through a local cycling pathway (*1*, *2*). The molecular mechanisms that mediate the cycling of synaptic vesicles are not known. Because the trafficking of other intracellular organelles involves proteins that bind GTP (*3*) and such proteins are also associated with synaptic vesicles (*4*–*6*), GTP-binding proteins may regulate synaptic vesicle traffic (*6*). To test this proposal, we used the squid giant synapse to examine how the activation of GTP-binding proteins affects two consequences of vesicle trafficking: the release of neurotransmitters and the distribution of synaptic vesicles within the terminal.

Transmission across the giant synapse was assayed by measurement of postsynaptic currents (PSCs) or potentials (PSPs) that were evoked by presynaptic action potentials (*7*). Both iontophoretic and pressure microinjections of guanosine-5′-O-(3-thio-triphosphate) (GTPγS), a nonhydrolyzable analog of GTP, into the giant presynaptic terminal produced a slow and irreversible depression of synaptic transmission (Fig. 1A). Because the nucleotide was injected only into the presynaptic terminal, this inhibition reflects a decrease in neurotransmitter release from the terminal. This effect contrasts with the case of mast cells, where GTPγS stimulates secretion (*8*). In this experiment (Fig. 1A), release diminished to ~15% of its preinjection value when transmission was assayed 30 min after the start of the injection (at $t = 54$ min), and there was no reversal of the effect. Similarly, irreversible depression was ob-

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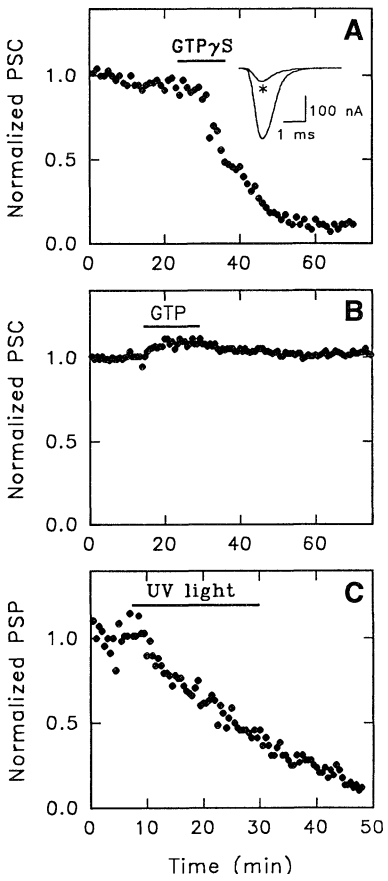

Fig. 1. Effects of presynaptic injection of guanine nucleotides on transmission at the squid giant synapse. (**A**) Time course of reduction in PSC amplitude caused by iontophoretic injection of GTPγS (36 μC) during the period indicated by the horizontal bar. The inset shows superimposed PSCs recorded before and 20 min after GTPγS injection (asterisk). (**B**) Lack of inhibition of PSC amplitude after injection of GTP. The transient increase in PSC is due to hyperpolarization of the terminal by injection current. (**C**) Time course of reduction in PSP amplitude caused by illumination of caged GTPγS with UV light. Synaptic responses here and in subsequent figures have been normalized by division by the mean signal amplitude measured before injection.

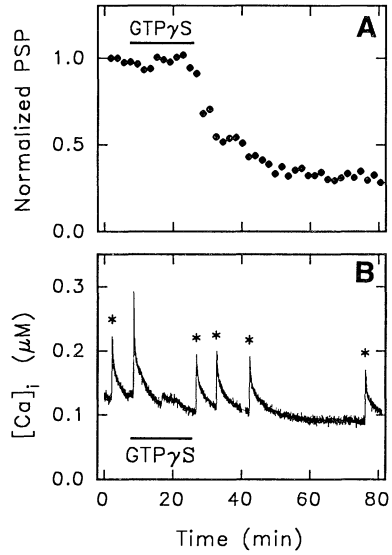

Fig. 2. Absence of changes in Ca^{2+} signaling during GTPγS-induced inhibition of synaptic transmitter release. (**A**) Time course of inhibition of transmitter release caused by intraterminal injection of GTPγS. The ordinate shows the normalized maximal rate of PSP rise that was evoked by single presynaptic action potentials that were triggered every minute. (**B**) Simultaneous measurement of presynaptic $[Ca^{2+}]_i$ at rest and during trains of presynaptic action potentials (asterisks). The transient rise in $[Ca^{2+}]_i$ at 13 min was produced by a longer stimulus train.

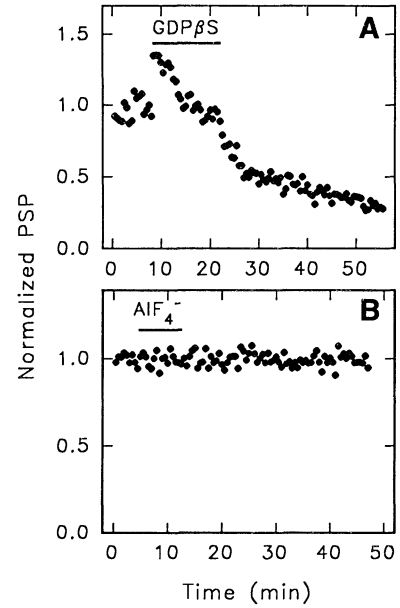

Fig. 3. G proteins are not involved in inhibition of transmitter release. (**A**) Time course of inhibition of synaptic transmission that was produced by iontophoretic injection of GDPβS (90 μC). (**B**) Pressure injection of AlF_4^- does not inhibit synaptic transmission. In this experiment 15 pulses of 2.5 bar and 170 ms in duration were applied to the injection pipette.

served in more than 20 experiments. In a subset of five injections of GTPγS of identical amplitude and duration (100-nA injection current for 10 min), the amplitude of PSCs elicited by presynaptic action potentials decreased to 34.2 ± 0.1% (mean ± SEM) of the preinjection value when it was assessed 35 min after the start of the injection.

In control experiments, injection of GTP caused only slight changes in transmission over the same period (Fig. 1B). Analysis of ten experiments in which the same injection protocols were used to inject either GTP or GTPγS indicated that transmission in synapses that were injected with GTPγS was significantly lower than in those receiving GTP 20 min after the start of injections (t = 2.23 min, $P < 0.05$, and 8 df), and the difference later increased. To exclude possible inhibitory effects due to thiophosphorylation (*9*), we injected adenosine-5′-O-(3-thiotriphosphate) (ATPγS) presynaptically using similar protocols. This nucleotide analog did not inhibit transmission (n = 2). Furthermore, injection of a different type of nonhydrolyzable GTP analog, guanylyl-imidodiphosphate (GMP-PNP), mimicked the inhibitory effect of GTPγS (n = 3). These results indicate that the observed inhibition results from the action of GTPγS on GTP-binding proteins.

To test whether the slow time course of inhibition by GTPγS was due to slow diffusion of the nucleotide through the terminal (*10*), we used the light-induced release of GTPγS from caged GTPγS. After injecting this compound into the presynaptic terminal and allowing it to diffuse, we illuminated the terminal with ultraviolet (UV) light to convert caged GTPγS to free GTPγS (*11*). Illumination of the caged GTPγS caused an irreversible inhibition of synaptic transmission that continued after illumination ended (Fig. 1C). Similar effects were observed in three experiments and were not seen in control experiments in which terminals that were not injected with caged GTPγS were illuminated with more intense UV light. Thus, the rate of inhibition is not limited by diffusion of GTPγS but by the response of the nerve terminal to this nucleotide analog.

Transmitter release is highly sensitive to the waveform of the presynaptic action potential, which regulates both the amount of Ca^{2+} influx into the terminal (*12*, *13*) and the magnitude of the resultant rise in the intraterminal Ca^{2+} concentration, $[Ca^{2+}]_i$. The change in $[Ca^{2+}]_i$ determines the rate of transmitter release that is evoked by presynaptic action potentials (*14*). Several results suggest that none of these signaling steps is affected by GTPγS. Neither presynaptic resting potentials nor action potentials were affected by GTPγS injections. The resting potential remained stable, within 1 to 2 mV, after the injection, and action potentials did not change their shape. Preliminary voltage-clamp experiments suggest that GTPγS does not affect the presynaptic Ca^{2+} current, a measure of Ca^{2+} influx into the terminal. This possibility was tested further with spectrofluorometric measurements of $[Ca^{2+}]_i$ in terminals that were preinjected with the Ca^{2+} indicator Fura 2 (*15*). The increase in $[Ca^{2+}]_i$ that was induced by a train of action potentials was not changed significantly by injection of GTPγS (Fig. 2B), whereas transmitter release from the same terminals was inhibited dramatically (Fig. 2A). In six experiments, the peak amplitude of the Ca^{2+} signals that were measured 13 to 18 min after the GTPγS injections was 93 ± 5% (mean ± SEM; range, 73 to 106%) of the preinjection value. The known relations among action potential–induced Ca^{2+} influx,

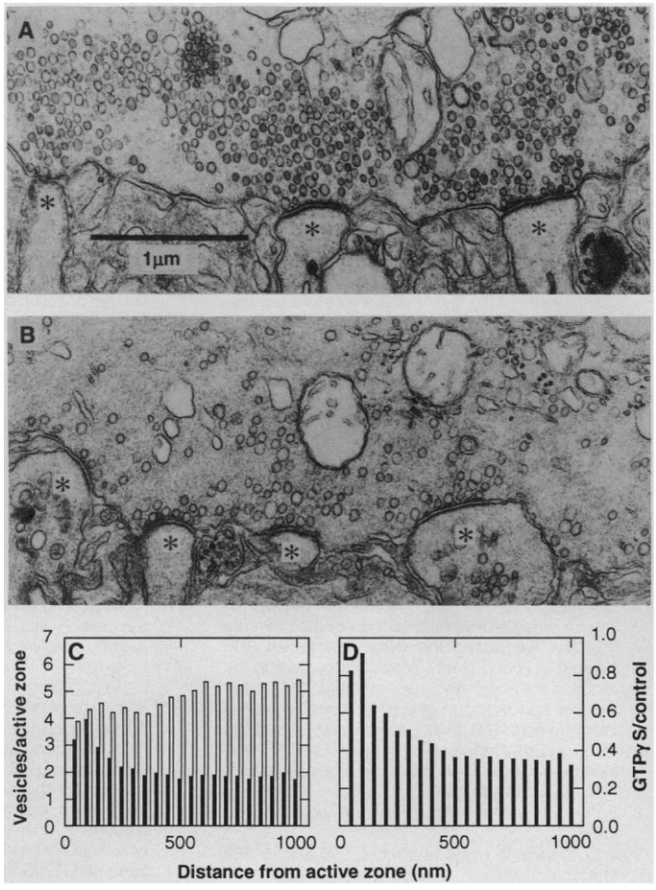

Fig. 4. Changes in synaptic vesicle distribution induced by GTPγS. Electron micrographs of active zones from (**A**) a control terminal and (**B**) a terminal injected with GTPγS. Asterisks mark postsynaptic spines that are opposite the presynaptic active zones. (**C**) Spatial distribution of synaptic vesicles around (open bars) 170 active zones in three control terminals and (solid bars) 155 active zones in two terminals that were injected with GTPγS. The abscissa indicates distance between adjacent vesicles and the electron-dense region of the presynaptic membrane. (**D**) Spatial gradient of the GTPγS effect. For each spatial compartment, the number of synaptic vesicles found in GTPγS-injected terminals [solid bars in (C)] is divided by the number of synaptic vesicles that were found in control terminals [open bars in (C)].

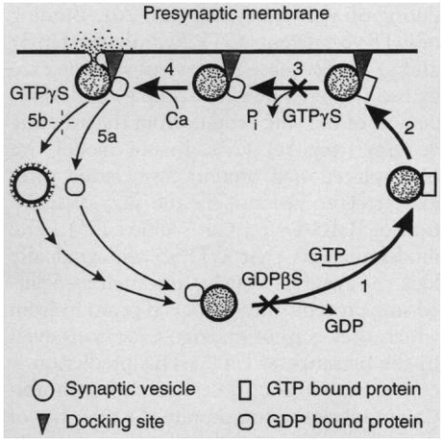

Fig. 5. The hypothetical presynaptic sites of action of nonhydrolyzable guanine nucleotides. We propose that the GTP-bound form of a GTP-binding protein is responsible for docking synaptic vesicles (step 2) and that GTPγS inhibits transmitter release (step 3) by preventing the hydrolysis of GTP that normally allows docked vesicles to undergo exocytosis (step 4). GDPβS inhibits release by preventing GDP-GTP exchange by the smg protein (step 1) and thus inhibits docking (step 2). Depletion of synaptic vesicles results from the effect of GTPγS on other GTP-binding proteins that are involved at other steps in the vesicle life cycle, such as endocytosis (step 5b). P_i, inorganic phosphorus.

$[Ca^{2+}]_i$ increases, and transmitter release (*13, 16*) require that the measured Ca^{2+} signal decrease by more than 70% to produce the degree of inhibition that we observed. In addition, there was no consistent change in the resting $[Ca^{2+}]_i$ of the terminal. These results indicate that GTPγS acts by altering the amount of transmitter release that is evoked by the rise in $[Ca^{2+}]_i$ rather than by regulating action potential–induced Ca^{2+} entry into, or movement within, the terminal.

Synaptic transmission could be regulated by two classes of GTP-binding proteins. Heterotrimeric G proteins (*17*) can regulate release at presynaptic terminals by altering Ca^{2+} signaling (*18*) or by acting on other steps (*19*). Alternatively, secretion could be regulated by monomeric GTP-binding proteins with a smaller molecular weight (smg's) (*3*). Current views (*17, 20*) hold that G proteins act catalytically. Nonhydrolyzable GTP or GDP analogs affect them in opposite ways; that is, if GTPγS activates a process that is mediated by a G protein (which inhibits release), then GDPβS will block this process and yield no inhibition of transmission. In contrast, the smg proteins are thought to act cyclically; both nucleotide analogs inhibit the action of these proteins (*20*). Thus the relative effects of GTPγS and GDPβS provide a means to distinguish between responses mediated by G proteins and those mediated by smg proteins. We tested this criterion by injecting GDPβS into the presynaptic terminal, which inhibited transmitter release ($n = 8$) (Fig. 3A). As was the case for GTPγS, GDPβS inhibition was irreversible and was not due to changes in presynaptic membrane potential or $[Ca^{2+}]_i$ signals. This effect suggests that smg's mediate inhibition at the giant synapse of squid.

Another way to discriminate between the two classes of GTP-binding proteins is by the use of AlF_4^-, which potently activates heterotrimeric G proteins but does not activate smg's (*21*). In contrast to the effects of the nonhydrolyzable guanine nucleotides, injection of AlF_4^- [either ionophoretically ($n = 2$) or by pressure ($n = 3$)] did not affect synaptic transmission (Fig. 3B) (*22*). Thus the similar inhibitory effects of GTPγS and GDPβS as well as the inability of AlF_4^- to inhibit transmission implicate smg proteins in the inhibition of release.

To learn more about the cellular mechanisms responsible for this inhibition of release, we used electron microscopy (*23, 24*) to examine the spatial distribution of synaptic vesicles near active zones of three control presynaptic terminals (two injected with GTP and one uninjected) (Fig. 4A) and of two terminals injected with GTPγS (Fig. 4B). The average numbers of synaptic vesicles in concentric shells 50 nm wide that surrounded each active zone were used to construct vesicle distribution histograms (Fig. 4C). The GTPγS-injected terminals had a reduced number of synaptic vesicles, less than half of those in the control terminals at distances between 500 and 1000 nm from the active zones (Fig. 4D). However, the terminals injected with GTPγS had almost normal numbers of docked vesicles, which are defined as vesicles within 50 nm of the active zone (Fig. 4D). Docked vesicles are thought to be the only ones that undergo Ca^{2+}-dependent release (*2, 6*). Release from these same terminals was reduced approximately 90% by GTPγS, which means that the inhibition of release occurred without a concomitant reduction in the number of vesicles that were available for release. Thus GTPγS must interfere with GTP hydrolysis at a step that follows vesicle docking but precedes release.

To explain our results, we propose a model (Fig. 5) that combines previous schemes for synaptic vesicle trafficking (*1, 6*) and smg protein cycling (*20*). According to this model, GTPγS substitutes for GTP in binding to an smg protein (step 1) that is responsible for docking synaptic vesicles (step 2) at the active zone. This docking protein may be Rab3A, which is associated with synaptic vesicles (*4, 5*) but dissociates

during or after exocytosis (*25*, *26*). Binding of GTPγS prevents GTP hydrolysis (step 3) and so blocks subsequent steps such as exocytosis triggered by Ca^{2+} (step 4) and dissociation of the smg protein from the synaptic vesicles (step 5a) (*27*). In our model, we have placed smg protein dissociation after exocytosis to account for the slow dissociation of Rab3A after Ca^{2+} influx (*25*). The model predicts that GTPγS will gradually lock the synaptic vesicles and their associated smg proteins into a docked position from which they cannot undergo exocytosis even in the presence of Ca^{2+}. This prediction is consistent with our observation that inhibition of release occurs despite the presence of docked synaptic vesicles. Further, because rates of exocytosis are low under our experimental conditions, this locking model could also account for the slow time course of inhibition of release.

Our model cannot account for the depletion of undocked synaptic vesicles by GTPγS because locking should disrupt the cycling of synaptic vesicles (*1*, *2*) at a point that would result in an accumulation of undocked synaptic vesicles in the active zone. However, because we observed depletion of these vesicles, GTPγS must affect one or more additional GTP-binding proteins that are involved in other steps of the cycle. One such step may be endocytosis (step 5b), which may be regulated by dynamin, another GTP-binding protein (*28*). If GTPγS also inhibits endocytosis, the observed loss of synaptic vesicles would then be due to an accumulation of their membranes in the presynaptic plasma membrane (*1*). GTP-binding proteins—either smg's (*3*) or heterotrimeric G proteins (*29*)—regulate every step in the trafficking of other intracellular organelles. If we extend the parallels between the trafficking of synaptic vesicles and that of these other organelles, it seems possible that GTP-binding proteins regulate every step in the transit of a synaptic vesicle through its cycle.

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15. Presynaptic terminals were injected with the Ca^{2+}-sensitive dye Fura 2 by iontophoresis (200 to 300 μC) from a microelectrode that contained 1 mM Fura 2, 0.25 M KCl, and 100 mM Hepes-NaOH, pH 7.2. About 1 hour was allowed afterward for diffusional equilibration of the dye within the terminal Dual-wavelength excitation of Fura 2 (at 360 and 390 nm) and measurement of fluorescence emission (at 510 nm) were performed with a Deltascan 4000 microspectrofluorometer (Photon Technology International) on a Microphot 5A microscope (Nikon). Fluorescence ratios were converted to presynaptic Ca concentrations as described by G Grynkiewicz, M. Poenie, and R. Y Tsien [*J. Biol Chem.* **260**, 3440 (1985)] with calibration parameters that were determined as in D. Swandulla, M Hans, K. Zipser, G. J. Augustine, *Neuron* **7**, 915 (1991). Rises in presynaptic $[Ca^{2+}]_i$ were elicited by trains of action potentials (50 Hz, 5 s) because $[Ca^{2+}]_i$ signals that were produced by single action potentials were slightly beyond the system's detection limit. Previous work has shown that $[Ca^{2+}]_i$ responses to trains of action potentials are similar in time course to responses that are produced by single action potentials [M. P Charlton, S. J. Smith, R. S. Zucker, *J Physiol.* **323**, 173 (1982)] and that the amplitude of responses to trains is directly proportional to the number of action potentials in the train [K. Zipser, G. J Augustine, J. Deitmer, *Biophys J* **59**, 594a (1991)]. Thus, no change in the $[Ca^{2+}]_i$ signal that was produced by trains of action potentials means that there was no change in the $[Ca^{2+}]_i$ response to a single action potential
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