taining sequences (Table 1). One possible explanation is that plants have several prolyl hydroxylases that differ in specificity. Although plant prolyl-4-hydroxylases differ from the vertebrate enzyme in specificity (*14*), enzymes that recognize different substrate sequences in a single plant species have not been found.

Alternatively, prolyl hydroxylases from plants might recognize the conformation of the substrate. A common feature of the sequences in Table 1 is the clustering of the 4Hyp residues. Studies of the structure of HRGPs and AGPs performed by spectroscopic methods (*15*) and studies of model peptides performed by x-ray methods (*16*) have shown that sequences that contain clusters of imino acids form poly(L-proline) type II helices. It is this conformation that is recognized by the prolyl-4-hydroxylase of *Vinca rosea* (*17*).

We conclude that CHN-A and CHN-B are examples of a class of HCPs that differ from those described previously in specific ways. (i) CHN-A and CHN-B are vacuolar enzymes (*5*), whereas other HCPs are predominantly secreted, structural proteins (*2, 4*). (ii) The 4Hyp content of chitinases is much lower than in other HCPs, and hydroxylation takes place exclusively at a few unique Pro residues in a short spacer joining two Pro-containing domains. (iii) Unlike other plant HCPs, the 4Hyps in chitinase are not O-glycosylated.

4Hyps in repeated sequences stabilize the polyproline II conformation of collagen, HRGP, and AGP (*1, 15*). The function of the 4Hyps in chitinase is not known. One possibility is suggested by the structural homology of chitinase and bacterial β-1,4-glucanases, which have a lectin domain connected to a catalytic domain by a spacer that contains repeats of the dipeptide Thr-Pro (*18*). Deletion of the lectin domain or of the spacer changes the specificity of this enzyme for different physical forms of cellulose. Modification of the spacer in chitinase by prolyl hydroxylation at specific sites might alter the relative positions of the lectin and catalytic domains and, hence, modulate enzyme activity or specificity. The tobacco chitinases are abundant regulated proteins particularly well suited for the study of the function and specificity of prolyl hydroxylation. The fact that these are intracellular enzymes also raises the possibility that limited prolyl hydroxylation of proteins is a more general phenomenon than previously recognized.

REFERENCES AND NOTES

1. R. A. Berg and D. J. Prockop, *Biochem. Biophys. Res. Commun.* **52**, 115 (1973); S. Sakakibara *et al.*, *Biochim. Biophys. Acta* **303**, 198 (1973).
2. A. M. Showalter and J. E. Varner, *Biochem. Plants* **15**, 485 (1989); A. M. Showalter and D. Rumeau, in *Organization and Assembly of Plant and Animal Extracellular Matrix*, W. S. Adair and R. P. Mecham, Eds. (Academic Press, New York, 1990), pp. 247–281.
3. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; G, Gly; H, His; O, 4Hyp; K, Lys; P, Pro; S, Ser; T, Thr; V, Val; and Y, Tyr.
4. J. Gross, *Harvey Lect.* **68**, 351 (1973).
5. J.-M. Neuhaus, L. Sticher, F. Meins, Jr., T. Boller, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 10362 (1991).
6. T. Boller, *Ox. Surv. Plant Mol. Cell Biol.* **5**, 145 (1988); H. J. M. Linthorst, *Crit. Rev. Plant Sci.* **10**, 123 (1991); F. Meins, Jr., J.-M. Neuhaus, C. Sperisen, J. Ryals, in *Genes Involved in Plant Defense*, T. Boller and F. Meins, Jr., Eds. (Springer-Verlag, Vienna, in press).
7. H. Shinshi, D. Mohnen, F. Meins, Jr., *Proc. Natl. Acad. Sci. U.S.A.* **84**, 89 (1987).
8. H. Shinshi, J.-M. Neuhaus, J. Ryals, F. Meins, Jr., *Plant Mol. Biol.* **14**, 357 (1990); M. van Buuren, J.-M. Neuhaus, H. Shinshi, J. Ryals, F. Meins, Jr., *Mol. Gen. Genet.* **232**, 460 (1992).
9. A mixture of CHN-A and CHN-B was acid-hydrolyzed, and the neutral-sugar fraction was analyzed by thin-layer chromatography [R. G. Spiro, *Methods Enzymol.* **8**, 3 (1966)]. Samples of CHN-A and CHN-B with transferrin as an internal standard were assayed with a DIG glycan detection kit (Boehringer Mannheim).
10. A. S. B. Edge, C. R. Faltynek, L. Hof, L. E. Reichert, Jr., P. Weber, *Anal. Biochem.* **118**, 131 (1990).
11. Means of >21 scans in positive and negative ionization modes [M. Schär, K. O. Börnsen, E. Gassmann, *Rap. Comm. Mass Spec.* **5**, 319 (1991)] yielded 32,168 ± 73 (32,022) daltons for CHN-A and 31,597 ± 47 (31,500) daltons for CHN-B. Values in parentheses were deduced from cDNA clones (*8*).
12. R. L. Jones and D. G. Robinson, *New Phytol.* **111**, 567 (1989).
13. D. T. A. Lamport, *Biochemistry* **8**, 1155 (1969).
14. D. G. Robinson, M. Andreae, P. Blankenstein, in *Organization and Assembly of Plant and Animal Extracellular Matrix*, W. S. Adair and R. P. Mecham, Eds. (Academic Press, New York, 1990), pp. 283–299.
15. R. B. Homer and K. Roberts, *Planta* **46**, 217 (1979); G.-J. van Holst and J. E. Varner, *Plant Physiol.* **74**, 247 (1984); G.-J. van Holst and G. B. Fincher, *ibid.* **75**, 1163 (1984).
16. T. Matsuzaki, *Acta Crystallogr. Sect. B Struct. Sci.* **30**, 1029 (1974).
17. M. Tanaka, K. Sato, T. Uchida, *J. Biol. Chem.* **256**, 11397 (1981).
18. H. Shen *et al.*, *ibid.* **266**, 11335 (1991).
19. J. Hofsteenge, S. R. Stone, A. Donella-Deana, L. A. Pinna, *Eur. J. Biochem.* **188**, 55 (1990).
20. R. Knecht and J.-Y. Chang, *Anal. Chem.* **58**, 2375 (1986).
21. R. M. Hewick, M. W. Hunkapiller, L. E. Hood, W. J. Dreyer, *J. Biol. Chem.* **256**, 7990 (1981).
22. J. H. Waite, *ibid.* **258**, 2911 (1983).

23. We thank O. Börnsen, Ciba-Geigy Ltd., Basel, for mass analyses, R. Matthies for technical assistance, and E. Shaw, T. Boller, and P. Caroni for comments.

13 April 1992; accepted 16 June 1992

Calcium-Dependent Transmitter Secretion Reconstituted in *Xenopus* Oocytes: Requirement for Synaptophysin

Janet Alder, Bai Lu, Flavia Valtorta, Paul Greengard, Mu-ming Poo*

Calcium-dependent glutamate secretion was reconstituted in *Xenopus* oocytes by injecting the oocyte with total rat cerebellar messenger RNA (mRNA). Co-injection of total mRNA with antisense oligonucleotides to synaptophysin message decreased the expression of synaptophysin in the oocyte and reduced the calcium-dependent secretion. A similar effect on secretion was observed for oocytes injected with total mRNA together with an antibody to rat synaptophysin. These results indicate that synaptophysin is necessary for transmitter secretion and that the oocyte expression system may be useful for dissecting the molecular events associated with the secretory process.

Synaptic transmission between nerve cells depends on impulse-triggered, Ca^{2+}-dependent transmitter secretion from the presynaptic nerve terminal (*1*), a process poorly understood at the molecular level. Physiological studies in a variety of systems have provided important clues to the process of synaptic vesicle exocytosis and its regulation. The application of protein purification as well as molecular cloning techniques to the study of synaptic vesicle proteins has led to the identification and characterization of the major components of these organelles. However, attempts to determine the precise function of the synaptic vesicle proteins have been hampered by the inaccessibility of the small nerve terminal to experimental manipulations. We have now examined the role of synaptophysin, a major integral membrane protein of synaptic vesicles (*2*), in transmitter secretion by reconstituting Ca^{2+}-dependent transmitter release in *Xenopus* oocytes. The use of *Xenopus* oocytes for expressing neuronal properties in a millimeter-size cell (*3*) offers an opportunity for studying secretion mechanisms in vitro. Recently, Ca^{2+}-dependent

J. Alder and M-m. Poo, Department of Biological Sciences, Columbia University, New York, NY 10027.
B. Lu and P. Greengard, Laboratory of Molecular and Cellular Neuroscience, Rockefeller University, New York, NY 10021.
F. Valtorta, B. Ceccarelli Center, Department of Pharmacology, National Research Council, Center of Cytopharmacology, University of Milan, Milan, Italy 20129.

*To whom correspondence should be addressed.

secretion of acetylcholine (ACh) was shown to be expressed in *Xenopus* oocytes that were injected with total mRNA from electric lobe tissue of *Torpedo* (*4*). In the present report, we show that *Xenopus* oocytes injected with rat cerebellar mRNA are capable of secreting the excitatory transmitter glutamate in a Ca^{2+}-dependent manner and that this property depends on the expression of synaptophysin.

For reconstitution of the secretion mechanism in *Xenopus* oocytes, we started with total mRNA from nervous tissue, because the entire complement of the molecules involved in neurosecretion is unknown. Cerebellum was chosen as the source of mRNA for its richness in glutamatergic granule cells (*5*). Total mRNA was isolated from rat cerebellum by the guanidinium-thiocyanate-phenol-chloroform method followed by selection of polyadenylated RNA. Fifty nanograms of isolated total mRNA dissolved in 50 nl of water were injected into individual *Xenopus* oocytes (stage VI), and experiments were performed 2 days later (*6*). Neurons can synthesize a Ca^{2+}-dependent, releasable pool of glutamate from the precursor glutamine in synaptosomes, in tissue slices, and in vivo (*7*), and *Xenopus* oocytes express amino acid transport mechanisms at higher than endogenous levels (*8*) in their plasma membranes when injected with total mRNA from various nervous tissues (*9*). Figure 1A shows a time course of [^{3}H]-labeled glutamine uptake into oocytes 2 days after injection with cerebellar mRNA. Time-dependent glutamine uptake, which saturated by 2 hours of incubation, was seen in mRNA-injected oocytes, but not in control, water-injected ones (*10*). This glutamine transport system expressed in the oocyte membrane was used to load radioactive glutamine into the oocytes, where it could be converted to glutamate and incorporated into the transmitter pool.

We assayed the secretion of glutamate by measuring the release of radioactivity into the medium in response to extracellular application of the Ca^{2+} ionophore A23187, in the presence of various concentrations of external Ca^{2+} (*11*). As shown in Fig. 1B, there was a clear Ca^{2+} dependence in the release of radioactivity from mRNA-injected oocytes but not from control oocytes. The bar graph in Fig. 1E depicts normalized data from 42 release assays performed on injected oocytes. The radioactivity released was dependent on external Ca^{2+} ($[Ca^{2+}]_o$); at 0 mM $[Ca^{2+}]_o$ the release was significantly lower than that at 5 and 10 mM. High $[Ca^{2+}]_o$ by itself did not induce secretion, because at 10 mM $[Ca^{2+}]_o$ the release was significantly lower in the absence of ionophore than in its presence. The residual secretion observed at 0 mM $[Ca^{2+}]_o$ appears to be caused by ionophore-induced release of Ca^{2+} from internal stores; at 0 mM $[Ca^{2+}]_o$ without ionophore, residual secretion was significantly lower. Furthermore, after preincubation of the oocytes in 1 mM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraceticacid,acetoxymethyl ester (BAPTA-AM), which is known to reduce cytosolic Ca^{2+} to a low level (*12*), the release at 0 mM $[Ca^{2+}]_o$ remained low even in the presence of ionophore. Taken together, these results indicate that the released radioactivity from the mRNA-injected oocytes was predominantly Ca^{2+}-dependent.

As a test for the specificity of the secretion mechanism, parallel experiments were carried out with total rat liver mRNA (*6*). Oocytes injected with liver mRNA also expressed the glutamine uptake system (Fig. 1C). However, these oocytes did not show more or Ca^{2+}-dependent release of radioactivity as compared to uninjected, control ones (Fig. 1D).

We examined the synthesis and distribution of synaptophysin in oocytes injected with cerebellar mRNA by immunocytochemistry. Synaptophysin is a vesicle membrane protein specifically localized to neurons and neuroendocrine cells (*2*). Its presence on the outer surface of the axolemma after nerve stimulation provides support for the vesicle hypothesis of transmitter release (*13*). Immunofluorescence microscopy of oocyte sections revealed the presence of a punctate pattern of synaptophysin staining dispersed throughout the cytoplasm (*14*). The localization of synaptophysin in oocytes was further studied by immunoelectron microscopy with colloidal gold (*15*). Most gold particles were associated with small (40 to 80 nm) clear vesicles that were

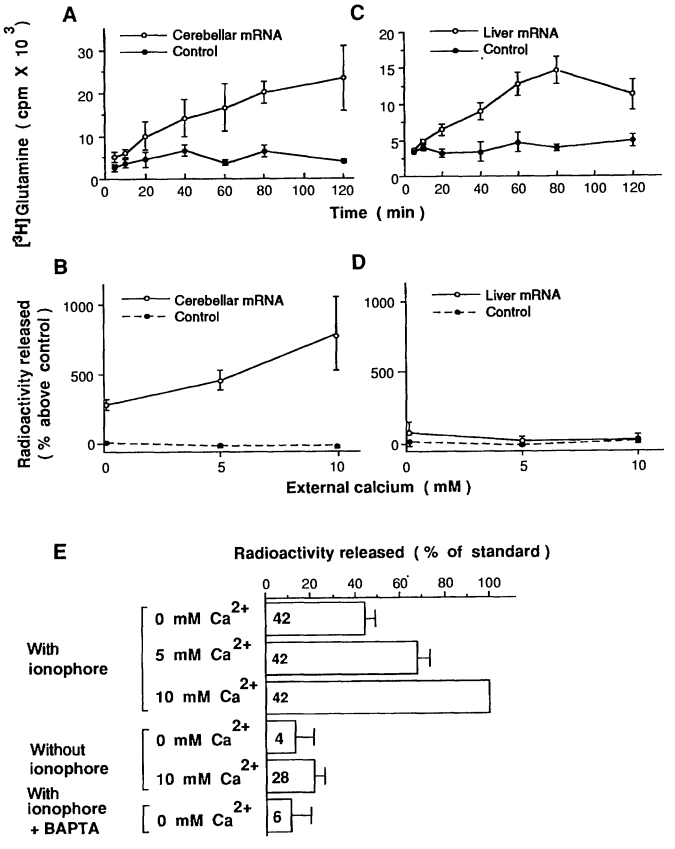

Fig. 1. Transmitter uptake and release mechanisms reconstituted in *Xenopus* oocytes. The oocytes were injected with total cerebellar polyadenylated [poly(A)$^+$] mRNA 2 days before the experiments. (**A**) Time course of the uptake of [^{3}H]glutamine. Oocytes were incubated in 1 μM [^{3}H]glutamine, and uptake was assayed as described (*10*). Uptake was calculated for groups of three oocytes, and mean values ± SEM for three separate experiments were presented. (**B**) Ca^{2+}-dependent release of radioactivity from oocytes loaded with [^{3}H]glutamine. Oocytes were incubated with [^{3}H]glutamine overnight. In each experiment, groups of five oocytes were treated for 10 min in 10 μM Ca^{2+} ionophore (A23187) in ORII solution containing various $[Ca^{2+}]_o$, and the supernatant was collected for scintillation spectrophotometry (*11*). For each concentration of $[Ca^{2+}]_o$, the mean value of release from control oocytes was subtracted from that of mRNA-injected oocytes at that concentration. For each experiment, the release above the mean control value was normalized by setting the mean control value as 100%. Data represent the average ± SEM from four experiments. The difference between values at 0 and 5 or 10 mM $[Ca^{2+}]_o$ is statistically significant ($P < 0.05$, one-tail *t* test). (**C**) Uptake of glutamine by oocytes injected with liver mRNA ($n = 3$). Same protocol as in (A). Error bars represent SD. (**D**) Release of radioactivity from liver mRNA-injected oocytes loaded with [^{3}H]glutamine ($n = 4$). Same protocol as in (B). (**E**) Calcium- and ionophore-dependent release of radioactivity from oocytes injected with cerebellar mRNA. The data were obtained by subtracting the radioactivity released from water-injected, control oocytes, and normalized by the value at 10 mM $[Ca^{2+}]_o$. The bars represent the mean value ± SEM, and the number of experiments is shown inside the bars. The differences among the values at 0, 5, and 10 mM $[Ca^{2+}]_o$ with ionophore were significant [$P < 0.001$, analysis of variance (ANOVA)].

concentrated in regions of the cytoplasm from which other organelles were excluded (Fig. 2). Mitochondria and yolk granules were virtually devoid of labeling. The synaptophysin-positive vesicles resembled in size and appearance the synaptic-like microvesicles previously described in neuroendocrine cells (*16*). The expression of synaptophysin does not induce the formation of these microvesicles, because a population of microvesicles virtually identical to those to which synaptophysin is targeted was also present in control, uninjected oocytes. However, in the latter case the vesicles were not labeled by synaptophysin antibodies. These results are consistent with previous findings indicating that nonneuronal cells, when transfected with synaptophysin cDNA or microinjected with its mRNA, correctly incorporate the protein into membranes of preexisting vesicles that structurally resemble synaptic vesicles (*17*).

The primary sequence of rat synaptophysin has been determined (*18*); it has four transmembrane regions and a cytoplasmic COOH-terminal domain that contains the major antigenic site (*19*). Although the biochemical properties and cellular localization of synaptophysin have been studied extensively, little is known about its biological function. Using the reconstituted secretion system in *Xenopus* oocytes, we examined directly whether the presence of synaptophysin is obligatory for Ca^{2+}-dependent secretion.

The expression of synaptophysin was blocked by co-injection of antisense oligonucleotides to synaptophysin with total cerebellar mRNA. Antisense oligonucleotides injected into *Xenopus* oocytes hybridize to their complementary mRNA and activate RNase H, which in turn specifically digests oligonucleotide-bound endogenous and foreign messages (*20*). Sequences corresponding to the first 19 nucleotides of rat synaptophysin in either the sense or antisense configuration (*21*) were co-injected with the same amount of total mRNA (6), and the expression of synaptophysin was determined by Western blot (*22*) (Fig. 3A). Cerebellar mRNA-injected oocytes expressed synaptophysin, whereas the uninjected ones did not. The antisense oligonucleotide co-injection consistently diminished synaptophysin expression as compared to oocytes co-injected with sense oligonucleotides. The expression of synaptobrevin (*23*) was also induced by cerebellar mRNA injection (Fig. 3B), thus suggesting that other neuronal proteins may be present in the injected oocyte. However, the expression of synaptobrevin was not affected by antisense oligonucleotides to synaptophysin, indicating the specificity of the antisense oligonucleotides. The molecular weights of the single protein bands observed were slightly higher than those of purified rat synaptophysin and synaptobrevin, presumably due to post-translational modification of the rat gene product in *Xenopus* oocytes. Indeed, amphibian synaptophysin has a higher apparent molecular weight than mammalian synaptophysin (*13*).

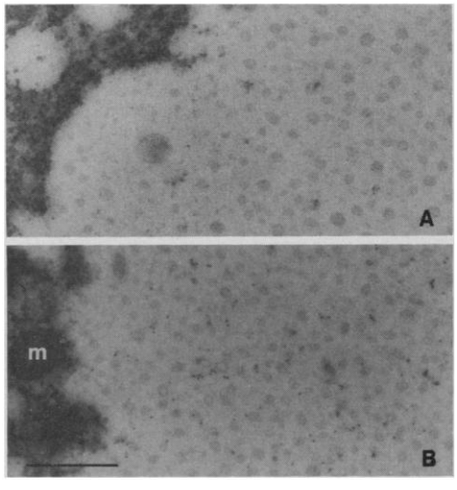

Fig. 2. Distribution of synaptophysin immunoreactivity in control (**A**) and mRNA-injected (**B**) oocytes. Ultrathin frozen section were prepared (*15*) and stained with immunoglobulins (IgGs) prepared from a rabbit antiserum raised against frog synaptophysin, followed by antibody to rabbit IgGs conjugated with 4-nm colloidal gold particles. A population of microvesicles is specifically labeled in the mRNA-injected oocytes. Mitochondrion, m. Calibration bar, 0.5 μm.

When the radioactivity release assays were performed on oocytes co-injected with total rat cerebellar mRNA and antisense oligonucleotides to synaptophysin, we found that at 10 mM $[Ca^{2+}]_o$ the ionophore-induced release was reduced to an average of 37.6 ± 8.8% SEM (n = 38) of that observed for oocytes injected with mRNA alone (Fig. 4A). The inhibition was sequence-specific because sense oligonucleotides were ineffective in inhibiting secretion [86.7 ± 10.8% (SEM)] (n = 23). When 2.5 ng of synaptophysin antibody (SY38) (6) was co-injected with total mRNA, we again found marked reduction in secretion [13.7 ± 6.0% of control (SEM)] (n = 29). The antibody effect was specific because co-injection of control antibody to glial fibrillary acidic protein (GFAP) (6) was much less effective [73.7 ± 8.5% of control (SEM)] (n = 20). This marked blockade of Ca^{2+}-dependent secretion by antisense oligonucleotides and synaptophysin antibody was further tested on another reconstituted secretion system: the ACh release mechanism in oocytes injected with *Torpedo* electric lobe mRNA (6). Oocytes injected with electric lobe mRNA were loaded with radioactive acetate, and

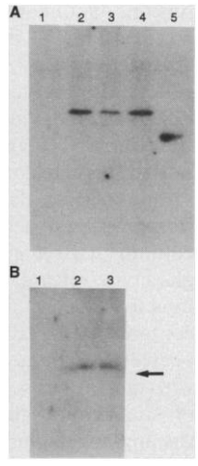

Fig. 3. Co-injection of antisense synaptophysin oligonucleotides with total cerebellar mRNA reduced the expression of synaptophysin but not of synaptobrevin. (**A**) Western blot of mRNA-injected oocytes stained for synaptophysin. Individual mRNA-injected oocytes were sonicated in homogenization buffer and centrifuged, and total protein was separated by SDS-PAGE. Monoclonal rat synaptophysin antibody followed by ^{125}I-labeled protein A was used for detection (*22*). Lane 1, uninjected oocyte; lane 2, cerebellar mRNA–injected oocyte; lane 3, cerebellar mRNA plus antisense oligonucleotides; lane 4, cerebellar mRNA plus sense oligonucleotides; and lane 5, rat synaptophysin standard (10 μg). (**B**) Western blot of cerebellar mRNA–injected oocytes stained with rat synaptobrevin antiserum. Arrow indicates position of rat brain synaptobrevin. Lanes 1 to 3 are the same as in (A).

release assays were performed; Ca^{2+}-dependent secretion of ACh was observed as previously reported (*4*). Using antisense oligonucleotides corresponding to the first 19 bases of *Torpedo* synaptophysin (*21*) and a polyclonal frog anti-synaptophysin antibody (*24*), we observed similar inhibition of release as seen in the glutamate system (Fig. 4B).

Finally, as an independent monitor of glutamate secretion from mRNA-injected oocytes, a fluorometric assay was used (*25*). We examined the time course of glutamate release by incubating oocytes in a solution containing glutamate dehydrogenase and nicotinamide adenine dinucleotide phosphate ($NADP^+$). An increase in fluorescence occurs when glutamate is released and NADPH is formed. When mRNA-injected oocytes were stimulated with Ca^{2+} ionophore, the fluorescence showed detectable increase after a lag of about 30 min and reached an maximum after 80 min (Fig. 5A). Secretion was inhibited by the absence of external Ca^{2+} or the co-injection with antisense oligonucleotides to synaptophysin. Although the basis for the lag in secretion on stimulation is not known, these results clearly confirm the conclusion that a large amount of endogenous glutamate is recruited for release in the mRNA-injected oocytes. Figure 5B depicts the final glutamate content in the supernatant of oocytes stimulated in a petri dish for 80 min as determined by fluorescence intensity. Water-injected oocytes did not release sig-

nificant glutamate. The release of glutamate was Ca^{2+}-dependent, since in the absence of $[Ca^{2+}]_o$, fourfold less fluorescence was observed. In addition, antisense oligonucleotides to synaptophysin resulted in decreased glutamate release, whereas sense oligonucleotides had no effect on release. These data support the findings from the radioactivity release assay and confirm that synaptophysin is necessary for glutamate release. We found that mRNA-injected oocytes have a total glutamate content similar to that of water-injected oocytes (~20 nmol), even after preloading with glutamine, suggesting that the amount of endogenous glutamate greatly exceeds the amount of glutamate incorporated from exogenous glutamine.

Our experiments suggest that synaptophysin is required for Ca^{2+}-dependent secretion of neurotransmitter to occur. Because we have shown that in mRNA-injected oocytes synaptophysin is targeted to a population of microvesicles of unidentified function, it is conceivable that, on expression of synaptophysin (and possibly of other synaptic vesicle proteins), these vesicles acquire the ability to release neurotransmitter in a Ca^{2+}-dependent fashion. Previous biochemical studies have shown that several synaptophysin subunits are linked to each other to form a homooligomer (*26*) associated with an as yet unidentified low molecular weight protein (*27*). Such a homooligomer of synaptophysin could bind to the plasma membrane and facilitate fusion of the synaptic vesicle with the plasma membrane (*28*). Furthermore, synaptophysin may be responsible for the formation of fusion pores during vesicular exocytosis, since purified synaptophysin has been reported to form gap junctionlike channels in black lipid membranes (*29*).

In conclusion, our study provides the first indication of a functional role for synaptophysin in transmitter secretion and demonstrates that *Xenopus* oocytes can be used as an accessible in vitro system for dissecting the role of specific molecules in a complex cellular process, such as that involved in Ca^{2+}-dependent neurosecretion.

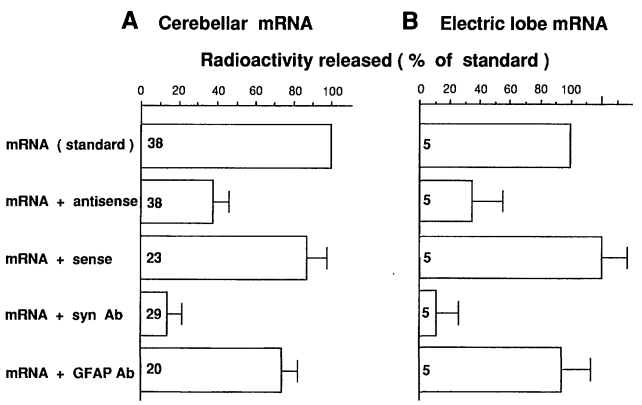

Fig. 4. Inhibition of transmitter release by antisense oligonucleotides to synaptophysin and by antibodies raised against synaptophysin. (**A**) Release assays from cerebellar mRNA-injected oocytes were performed in 10 mM $[Ca^{2+}]_o$ and in the presence of 10 μM Ca^{2+} ionophore (A23187), as described in (*11*). The release of radioactivity was greatly inhibited in oocytes co-injected with cerebellar mRNA and either antisense oligonucleotides (25 ng) or antibodies (2.5 ng), but unaffected by co-injection with similar concentrations of sense oligonucleotides or control antibodies (anti-GFAP). Data were normalized and averaged in the same manner as in Fig. 1E. (**B**) Inhibition of ACh release from oocytes injected with *Torpedo* lobe mRNA. Methods for the assay of ACh secretion followed those previously described (*4, 11*). Data are presented as in (A). In both (A) and (B), the difference of values between antisense and standard was significant ($P < 0.01$, t test), whereas that between sense and standard was not ($P > 0.05$).

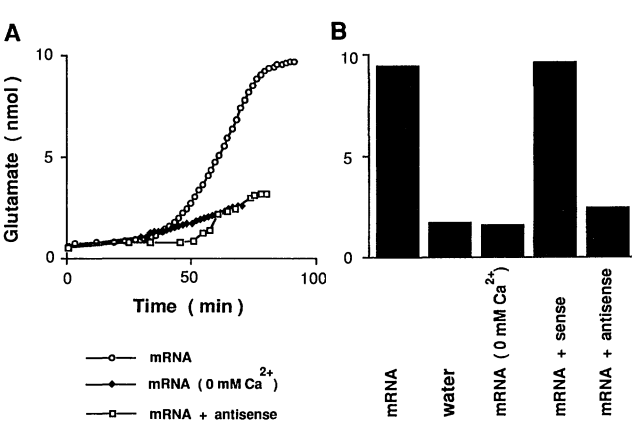

Fig. 5. Fluorimetric measurements of glutamate secretion from oocytes. Release of glutamate was assayed by an enzyme-linked fluorescence assay in the presence of Ca^{2+} ionophore (ionomycin), as described (*25*). All experiments were performed in 10 mM $[Ca^{2+}]_o$, except as indicated. (**A**) Time course of glutamate release from mRNA-injected oocytes. Each curve represents measurements obtained for a group of five oocytes. (**B**) Final glutamate content of the supernatant obtained after prolonged ionophore-stimulation of oocytes. Each bar represents the average value of glutamate secretion obtained from two separate experiments, consisting of five oocytes each.

REFERENCES AND NOTES

1. P. De Camilli and R. Jahn, *Annu. Rev. Physiol.* **52**, 625 (1990).
2. B. Wiedenmann and W. W. Franke, *Cell* **41**, 1017 (1985); R. Jahn *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4137 (1985).
3. L. Kushner *et al.*, *Methods Neurosci.* **1**, 3 (1989).
4. A. Cavelli *et al.*, *EMBO J.* **10**, 1671 (1991).
5. A. B. Young *et al.*, *Brain Res.* **73**, 1 (1974).
6. Cerebella from six or seven Sprague-Dawley rats and 1 g of rat liver tissue or *Torpedo* electric lobe were frozen in liquid nitrogen immediately after dissection. Total RNA was isolated by the guanidinium-thiocyanate-phenol-chloroform method as described [P. Chomczynski and N. Sacchi, *Anal. Biochem.* **162**, 156 (1987)]. All precipitations were done in the presence of glycogen. Poly(A)$^+$ RNA was selected by binding to oligo(dT) cellulose (Sigma, St. Louis, MO) with a series of centrifugation steps [*Promega Protocols and Applications* (Promega, Madison, WI, 1990), p. 116]. We confirmed the integrity of the mRNA with an in vitro reticulocyte lysate translation assay (NEN-DuPont, Boston, MA) containing [^{35}S]-labeled methionine, followed by SDS–polyacrylamide gel electrophoresis (PAGE), and autoradiography. The mRNA was dissolved in ribonuclease (RNase)-free H_2O at a concentration of 1 or 2 μg per microliter and stored at −70°C. Stage VI *Xenopus* oocytes were defolliculated by collagenase treatment (Sigma type 1A, 2 mg/ml) in a Ca^{2+}-free medium for 2 hours and were allowed to recover for at least 4 hours at 19°C in Barth's solution [88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.32 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 75 mM tris (pH 7.5), penicillin-streptomycin (100 U/m)]. Then, 50 nl of the mRNA solution was microinjected into the vegetal hemisphere of oocytes, which were incubated in Barth's solution for 2 days before use in experiments. Antisense and sense oligonucleotides (1 μg/μl), as well as antibodies to synaptophysin and GFAP (from Boehringer-Mannheim, Germany, used at 0.1 μg/μl), were individually diluted 1:1 with mRNA (2 μg/μl), and 50 nl were co-injected into the oocytes.
7. H. F. Bradford, H. K. Ward, A. J. Thomas, *J. Neurochem.* **30**, 1453 (1978); A. C. Hamberger *et al.*, *Brain Res.* **168**, 513 (1979); C. M. Thanki *et al.*, *J. Neurochem.* **41**, 611 (1983); H. K. Ward, C. M. Thanki, H. F. Bradford, *ibid.* **40**, 855 (1983); J. C. Szerb and P. A. O'Reagan, *ibid.* **44**, 1724 (1985); H. T. McMahon and D. G. Nicholls, *ibid.* **54**, 373 (1990).
8. P. M. Taylor, H. S. Hundel, M. J. Rennie, *J. Membr. Biol.* **112**, 149 (1989).
9. R. D. Blakely, M. B. Robinson, S. G. Amara, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 9846 (1988).
10. Time-dependent [^{3}H]glutamine uptake was measured 48 hours after mRNA injection as described [R. D. Blakely *et al.*, *J. Neurochem.* **56**, 860 (1991)]. Oocytes were individually transferred to microfuge tubes containing 500 μl of 1 μM L-[^{3}H]-glutamine (47 Ci/mmol; Amersham, Arlington Heights, IL) at 19°C. Uptake was terminated by three washes in ice-cold saline, and the oocytes were solubilized in 500 μl of 1% SDS for 2 hours. Scintillation fluid (10 ml) (New England Nuclear) was added, and scintillation spectrophotometry was performed (Beckman, Carlsbad, CA). Uptake rates from three separate experiments usually consisting of three to five oocytes per condition were averaged. Data represent mean values ± SEM. The uptake after 15 hours of incubations was similar to that observed for 2 hours, indicating

that the uptake had reached its maximum at 2 hours.
11. Oocytes were incubated in 0.5 μM [^{3}H]glutamine overnight at 19°C. In experiments where ACh secretion from oocytes injected with *Torpedo* mRNA was assayed, 1 μM [^{14}C]acetate was used in the overnight incubation. After three washes in Ca^{2+}- and Mg^{2+}-free ORII medium [82.5 mM NaCl, 2.5 mM KCl, 5 mM Hepes (pH 7.5)], groups of five oocytes were transferred to wells containing 0.5 ml of ORII medium supplemented with 10 mM, 5 mM, or 0 mM Ca^{2+} in the presence or absence of 10 μM A23187 Ca^{2+} ionophore (Calbiochem, La Jolla, CA) (final dimethyl sulfoxide concentration 0.1%). After 10 min, the oocytes were transferred to a well containing 1 ml of Ca^{2+}- and Mg^{2+}-free ORII to stop the release of transmitter. The two supernatants were combined in a scintillation vial with 15 ml of scintillation fluid and the radioactivity was counted. We have tried high concentrations of extracellular K^+ (50 mM) to depolarize the oocyte membrane and found no depolarization-dependent release of glutamate. Veratridine does cause release, although to a lesser extent than the Ca^{2+} ionophore. The failure of the oocyte to respond to depolarization was likely due to a deficiency in the expression of voltage-dependent Ca^{2+} channels in these oocytes.
12. E. M. Adler *et al.*, *J. Neurosci.* 11, 1496 (1991). For experiments involving BAPTA-AM, oocytes were preincubated for 15 min with Ca^{2+}-free ORII containing 1 mM BAPTA, and 1 mM BAPTA was present during the ionophore application. The basal level of release from water-injected oocytes was not affected by BAPTA treatment.
13. F. Valtorta *et al.*, *J. Cell Biol.* **107**, 2717 (1988).
14. F. Valtorta, unpublished observations.
15. *Xenopus* oocytes were fixed for 2 hours at room temperature in 2% formaldehyde plus 0.2% glutaraldehyde plus 100 mM sodium phosphate buffer (pH 7.0). Small wedges of oocytes were dissected, washed for 1 hour in 100 mM sodium phosphate buffer (pH 7.0), infiltrated in 2.3 M sucrose, and frozen in Freon 22 cooled with liquid nitrogen. Ultrathin frozen sections were prepared with an Ultracut microtome equipped with an FC4 attachment (Reichert Jung, Austria), were collected onto Formvar-coated nickel grids, and were processed for immunogold labeling as described [F. Torri-Tarelli *et al.*, *J. Cell Biol.* **110**, 449 (1990)]. Rabbit antisera against frog synaptophysin and immunoglobulin G, fractions were prepared as described (*13*).
16. P. De Camilli *et al.*, *EMBO J.* **10**, 1275 (1991).
17. R. E. Leube, B. Weidenmann, W. W. Franke, *Cell* **59**, 433 (1989); P. A. Johnston *et al.*, *EMBO J.* **8**, 2863 (1989); A. D. Linstedt and R. B. Kelly, *Neuron* **7**, 309 (1991).
18. R. E. Leube *et al.*, *EMBO J.* **6**, 3261 (1987); K. M. Buckley, E. Floor, R. B. Kelly, *J. Cell Biol.* **105**, 2447 (1987); T. C. Südhof *et al.*, *Science* **238**, 1142 (1987).
19. P. A. Johnson, R. Jahn, T. C. Südhof, *J. Biol. Chem.* **264**, 1258 (1989); P. Knaus and H. Betz, *FEBS Lett.* **261**, 358 (1990).
20. C. Cazenave *et al.*, *Nucleic Acids Res.* **15**, 10507 (1987); P. Dash *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7896 (1987); J. Shuttleworth *et al.*, *Gene* **72**, 267 (1988).
21. Oligonucleotides were prepared on a Pharmacia DNA synthesizer and deprotected in ammonium hydroxide, which was then evaporated in a Speed-Vac. The oligonucleotides were desalted by reprecipitation with NaCl and ethyl alcohol in the presence of glycogen. Sense rat cerebellar synaptophysin oligonucleotide: ATGGACGTGGTGAATCAGC. Antisense rat synaptophysin oligonucleotide: GCTGATTCACCACGTCCAT. Antisense *Torpedo* synaptophysin oligonucleotide: TCTGGTTGACGATCTCCAT [D. Cowan, M. Linial, R. H. Scheller, *Brain Res.* **509**, 1 (1990)]. The rat sense oligonucleotides were also used in the *Torpedo* experiments as a control.
22. Oocytes were sonicated in homogenization buffer [20 mM tris (pH 7.6), 50 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM iodoacetamide, aprotinin (200 U/ml)]. The homogenates were subjected to low-speed centrifugation (10,000*g*, 15 min) to separate yolk granules. Total protein from one oocyte was loaded onto each lane, was separated by SDS-PAGE electrophoresis, and was blotted onto nitrocellulose membrane. The blot was incubated with a monoclonal antibody specific to rat synaptophysin or an antiserum to synaptobrevin followed by ^{125}I-labeled protein A and autoradiography.
23. M. Baumert *et al.*, *EMBO J.* **8**, 379 (1989).
24. This frog polyclonal anti-synaptophysin antibody is known to have diverse cross-reactivity, including goldfish and rat (F. Valtorta, unpublished observations).
25. D. G. Nicholls, T. S. Sihra, J. Sanchez-Prieto, *J. Neurochem.* **49**, 50 (1987); D. G. Nicholls and T. S. Sihra, *Nature* **321**, 772 (1986). Oocytes were placed in a cuvette with 2 ml of ORII solution either with 10 mM or 0 mM $[Ca^{2+}]_o$. $NADP^+$ (1 mM) and 50 units of L-glutamate dehydrogenase were added and, after the initial exogenous glutamate was assayed, 10 μM Ca^{2+} ionophore (ionomycin) was added. Ionomycin was used instead of A23187 because the intrinsic fluorescence of the latter interfered with the assay. A Perkin-Elmer spectrofluorimeter (U 50) and a fluorescence data manager program with excitation at 330 nm and emission at 460 nm were used to monitor the increase of fluorescence over time. For final glutamate release values, oocytes were stimulated with the ionophore in a small petri dish, the supernatant (2 ml) was transferred to a cuvette where $NADP^+$ and glutamate dehydrogenase were added, and the fluorescence was measured. Total glutamate content was determined after solubilization of the oocytes in 0.4% Triton X-100.
26. H. Rehm, B. Wiedenmann, H. Betz, *EMBO J.* **5**, 535 (1986).
27. P. A. Johnston and T. C. Südhof, *J. Biol. Chem.* **264**, 7849 (1990).
28. L. Thomas and H. Betz, *J. Cell Biol.* 11, 2041 (1990); T. C. Südhof and R. Jahn, *Neuron* **6**, 665 (1991).
29. L. Thomas *et al.*, *Science* **292**, 1050 (1988).
30. This work was supported by grants from USPHS to M-m.P. (NS 22764) and to P.G. (MH 39327), and from NSF to M-m.P. (BNS 13306 and BNS 12398). J.A. was supported by a NSF Predoctoral Fellowship. We thank A. Cavelli, Y. Dunant, D. Weilner, R. Blakely, and S. Omara for valuable communications; N. Iezzi for performing the electron microscopy experiments; and R. Jahn and J. Rubenstein for the gift of antibodies.

13 April 1992; accepted 16 June 1992

Functional Modulation of $GABA_A$ Receptors by cAMP-Dependent Protein Phosphorylation

Stephen J. Moss, Trevor G. Smart, Craig D. Blackstone, Richard L. Huganir*

γ-Aminobutyric $acid_A$ ($GABA_A$) receptors are ligand-gated ion channels that mediate inhibitory synaptic transmission in the central nervous system. The role of protein phosphorylation in the modulation of $GABA_A$ receptor function was examined with cells transiently transfected with $GABA_A$ receptor subunits. $GABA_A$ receptors consisting of the α_1 and β_1 or the α_1, β_1, and γ_2 subunits were directly phosphorylated on the β_1 subunit by adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase (PKA). The phosphorylation decreased the amplitude of the GABA response of both receptor types and the extent of rapid desensitization of the $GABA_A$ receptor that consisted of the α_1 and β_1 subunits. Site-specific mutagenesis of the serine residue phosphorylated by PKA completely eliminated the PKA phosphorylation and modulation of the $GABA_A$ receptor. In primary embryonic rat neuronal cell cultures, a similar regulation of $GABA_A$ receptors by PKA was observed. These results demonstrate that the $GABA_A$ receptor is directly modulated by protein phosphorylation and suggest that neurotransmitters or neuropeptides that regulate intracellular cAMP levels may modulate the responses of neurons to GABA and consequently have profound effects on synaptic excitability.

Molecular cloning of $GABA_A$ receptor subunits has revealed a large number of diverse subunits that can be divided into five classes—α, β, γ, δ, and ρ—on the basis of sequence similarity (*1–4*). Expression of different combinations of these subunits in heterologous systems produces $GABA_A$ receptors that vary in their pharmacology and ion channel properties (*1*). The major intracellular domains of many of these receptor subunits contain consensus sites for protein phosphorylation by PKA, protein kinase C, and protein tyrosine kinases (*5*).

To examine directly the phosphorylation of $GABA_A$ receptors, we expressed the α_1 and β_1 or the α_1, β_1, and γ_2 subunits in human embryonic kidney 293 (HEK293) cells (*6–9*). These cells were labeled with [^{35}S]methionine, and the $GABA_A$ receptors were isolated by immunoprecipitation with antibodies to a bacterial fusion protein containing the major intracellular domain of the β_1 subunit (anti-β_1) (Fig. 1A) (*9*). The α_1 subunit migrated as a 52-kD protein, whereas the β_1 subunit migrated as a 58-kD protein with a proteolytic break-

S. J. Moss, C. D. Blackstone, R. L. Huganir, Department of Neuroscience, Howard Hughes Medical Institute, The Johns Hopkins University School of Medicine, Baltimore, MD 21205.
T. G. Smart, The School of Pharmacy, Department of Pharmacology, London, United Kingdom WCIN 1AX.
*To whom correspondence should be addressed.