terminal and COOH-terminal SH3 regions alter the activity of the SEM-5 protein from *Caenorhabditis elegans* (*22*). GAP (275 to 351) may thus in itself constitute a domain allowing binding to one or several downstream molecules. These data are consistent with evidence suggesting that the SH2-SH3 domains of GAP are sufficient to inhibit muscarinic atrial K^+ channel currents (*5*).

Our results provide evidence that SH3 domains can regulate cellular signal transduction. Ras might be regulated both downstream and upstream by SH2-SH3–containing proteins. GAP (275 to 351) should prove to be useful for the identification of downstream components that participate in Ras-GAP signaling. Recently a complementary DNA was isolated that encoded a protein (3BP-1) similar to proteins with GAP activity that bound the SH3 domain of Abl (*23*). The Ras-GAP–associated protein p190 (*10*) has sequence similarities with 3BP-1 and may participate in Ras signaling by binding to the SH3 domain of Ras-GAP. Direct links have yet to be made between Ras and the growth factor receptors that increase the GTP-bound active form of Ras and between Ras and activation of the mitogen-actived protein kinases. The SH3 domain of GAP may constitute a direct link in the control of Ras signaling, and within this SH3 domain the GAP (317 to 326) peptide may represent a binding site for an effector protein.

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19. C. Pernelle (Rhone Poulenc Rorer), unpublished data. Overlapping decapeptides derived from GAP (273 to 351) were synthesized according to the technique reported by Cambridge Research Biochemicals. Peptides were reacted with MAb200 (10 nM), and the enzyme-linked immunosorbent assay was developed with the 2,2′-azino-di-(3-ethyl benzthiazoline sulfonate)-hydrogen peroxide substrate.
20. M. N. Thang and M. Duchesne, unpublished data. MAb 200, when added to the GAP assay solution, did not modify the rate of GTP hydrolysis, even after immunoprecipitation. The effect of an increase in the amount of GAP (275 to 351) or GAP (317 to 326) on the rate of p100-GAP–stimulated GTPase activity was measured according to (*9*).
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Modulation of Anxiety and Neuropeptide Y-Y1 Receptors by Antisense Oligodeoxynucleotides

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The function of neuropeptide Y, one of the most abundant peptide transmitters of the mammalian brain, remains unclear because of a lack of specific receptor antagonists. An antisense oligodeoxynucleotide corresponding to the NH_2-terminus of the rat Y1 receptor was constructed and added to cultures of rat cortical neurons. This treatment resulted in a reduced density of Y1 (but not Y2) receptors and diminished the decrease in adenosine 3′,5′-monophosphate (cAMP) usually seen after Y1 receptor activation. Repeated injection of the same oligodeoxynucleotide into the lateral cerebral ventricle of rats was followed by a similar reduction of cortical Y1 (but not Y2) receptors. Such antisense-treated animals displayed behavioral signs of anxiety. Thus, specific inhibition of neurotransmitter receptor expression can be accomplished in the living brain and demonstrates that altered central neuropeptide Y transmission produces an anxiety-like state.

Neuropeptide Y (NPY) (*1*) is present in high concentrations in the hypothalamus, the limbic system, and the cortex of mammals (*2*). Because NPY is abundantly expressed, exhibits bioactivity in numerous systems at nanomolar concentrations, and shows a remarkable degree of conservation throughout phylogenesis (*3*, *4*), it is likely to be an important brain transmitter. On the basis of NPY administration to the central nervous system, the peptide has been proposed to participate in the central regulation of endocrine and autonomic function, circadian rhythms, and food intake. In addition, disturbed NPY transmission may contribute to clinical symptoms of anxiety and depression (*5*). In rats, central administration of NPY produces effects indistinguishable from clinically effective anxiolytics (*6*). A direct demonstration of the role played by endogenous NPY, however, has been hampered by the lack of specific NPY receptor antagonists.

Two subclasses of receptors for NPY have been described both in the periphery and in the brain (*7*–*12*). NPY-Y1 receptors require the intact sequence of amino acids 1 to 36 of NPY for activation, whereas Y2 receptors can also be activated by shorter COOH-terminal fragments such as $NPY_{13–36}$ (*7*). The anxiolytic action of NPY in animal models of anxiety has been linked to the Y1 subclass of NPY receptors (*6*). The rat NPY-Y1 receptor and the highly homologous human NPY-Y1 receptor have been cloned (*8*), and their nucleotide sequences indicate that the Y1 receptor belongs to the superfamily of G protein–coupled receptors. On the basis of the sequence of the rat Y1 receptor, we designed and synthesized an antisense 18-base oligodeoxynucleotide (D-oligo) that corresponded to the receptor NH_2-terminus immediately downstream from the initiation codon; the corresponding sense and missense D-oligos were used as controls.

Rat cortical neurons obtained at embryonic day 14 were cultured for 7 days. RNA was then isolated, and the presence of Y1 receptor mRNA was determined by reverse transcriptase–polymerase chain reaction (RT-PCR) with the use of the sense D-oligo and a reverse primer (Fig. 1B) (*13*). Intact cultured cortical neurons displayed specific binding sites for ^{125}I-labeled peptide YY (PYY), which binds to multiple NPY receptors (*7*–*12*). Heterogeneity among the ^{125}I-

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labeled PYY binding sites was determined by a masking approach (*7, 12, 13*), which indicated that approximately 55% of the sites were of the Y1 subclass, with the remaining sites being Y2 (Fig. 1A).

We examined whether treating cultured neurons with Y1 receptor antisense D-oligo would affect the density of Y1 binding sites and the associated second messenger coupling. Neurons were treated with 0.2 μM antisense or sense D-oligo added cumulatively—that is, once daily for 5 days—without a change of medium under serum-free conditions. Antisense treatment reduced Y1-type binding by approximately 60%. Y2-type binding was unaffected. The sense D-oligo was without effect on either Y1- or Y2-type binding (Fig. 1A). In a separate experiment, Y1 binding in cultures treated in an identical fashion with a missense D-oligo (that is, a random sequence of the 18 bases that make up the antisense D-oligo) also did not differ from binding in untreated control cultures (n = 6; 14.2 ± 0.9 binding sites per neuron versus 13.5 ± 0.7 binding sites per neuron, mean ± SEM × 10^2). The neuronal cultures were monitored for viability by lactate dehydrogenase release, Trypan blue exclusion, staining for neuron-specific enolase, and cell counting and assessment of total protein content. Cultures treated with D-oligos (in concentrations up to 1 μM), and control cultures were equally viable by these methods.

The ability of NPY to reduce forskolin (3 μM)-stimulated adenosine 3′,5′-monophosphate (cAMP) accumulation, an action frequently associated with Y1 receptors (*4, 7, 10*), was studied in the cortical cultures. [Pro^{34}]NPY (100 nM), a selective Y1 receptor agonist, reduced cAMP accumulation by 43% ± 5% in untreated cultures but only by 18% ± 3% in antisense-treated neuronal cultures (n = 6; mean ± SEM; $P < 0.001$) (*13*). The threefold smaller effect of 200 nM NPY_{13-36}, a Y2 receptor agonist, on cAMP accumulation was not affected by antisense treatment. This suggests that the reduction of Y1-type binding sites produced by antisense treatment reflected a loss of functional, G protein–coupled receptors.

Next, we examined whether the Y1 receptor antisense D-oligo could decrease the density of cerebral Y1 receptors in vivo in a manner similar to that seen in culture and whether such a decrease would be associated with functional effects opposite those seen when the Y1 receptor is activated by central administration of NPY. Rats equipped with chronic intracerebroventricular (i.c.v.) guide cannulas were injected four times over 2 days with 50 μg of antisense or sense D-oligo or saline.

Twelve hours after the last i.c.v. injection, the animals were tested in a pharmacologically validated animal model of anxiety, the elevated plus maze. The plus maze consists of two open and two enclosed compartments and is based on an ethologically defined conflict between an animal's need to explore its environment and its fear of open spaces, where it may fall prey to predators (*14*). Prototypical anxiolytics, such as the benzodiazepines, increase the proportion of exploratory time spent in, and the number of entries made into, open compartments with a high degree of specificity. Anxiogenic drugs result in the opposite effect (*15, 16*). In antisense-treated animals, the ratios of time spent in, and number of entries made into, open arms were decreased by more than 60%, which indicates a marked anxiogenic-like effect. Results in animals injected with control sense D-oligo did not differ from those in saline-injected control animals (Fig. 2). There was no difference in overall locomotor activity. Because NPY has been implicated in the central control of feeding, food intake was measured for 4 hours after plus maze testing, and no effect on food intake was observed (*17*).

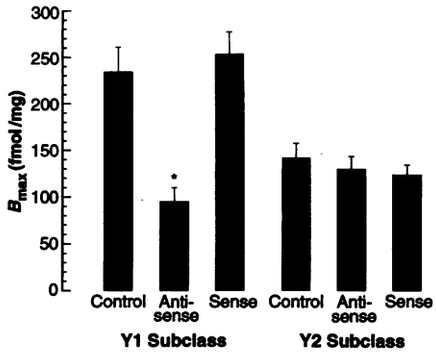

Fig. 3. Down-regulation of NPY-Y1 receptors in rat cerebral cortex induced by in vivo treatment with an antisense oligodeoxynucleotide (D-oligo). Three groups of ten rats received i.c.v. injections of 50 μg of antisense or sense D-oligo or saline (control) twice daily for 2 days; these rats were the same as those subjected to behavioral testing. Mean B_{max} values of cortical NPY-Y1 and NPY-Y2 receptors in antisense-, sense-, or saline-treated rats were obtained by nonlinear regression analysis (*18*). Cortical Y1-type sites were selectively reduced in antisense-treated rats [means ± SEM, n = 10; ANOVA for treatment effect: $F(27,2) = 11.9$; $P <$ 0.001 is indicated by an asterisk; Tukey's HSD test was used for individual comparisons].

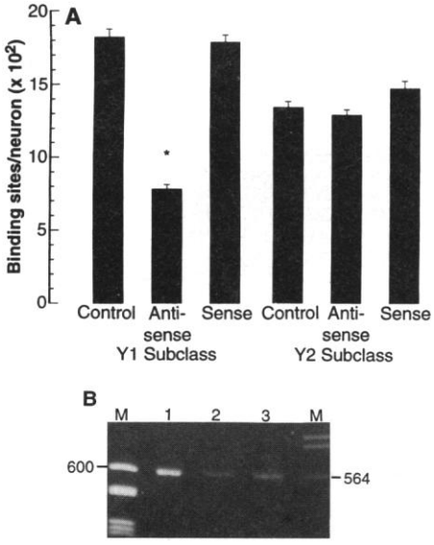

Fig. 1. (**A**) Down-regulation of NPY-Y1 binding site density by antisense treatment in rat cultured cortical neurons. The other cortical NPY receptor subtype, Y2, was not affected [means ± SEM, n = 9 to 10; analysis of variance (ANOVA) for treatment effect: $F(27,2) = 18.6$; $P <$ 0.001 is indicated by an asterisk. Tukey's honestly significant difference (HSD) test was used for individual comparisons]. (**B**) PCR products of the expected size [582 bases, indicated by marker (M) DNA] for the rat Y1 receptor, with the use of rat cloned Y1 receptor (lane 1), rat cDNA from whole brain (lane 2), or rat cultured cortical neurons (lane 3) (used in the binding experiments) as a template. The same sense D-oligo used as control served as the forward primer in the PCR. Numbers indicate length in number of bases.

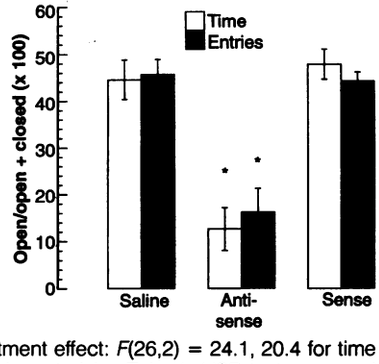

Fig. 2. Anxiogenic-like action of antisense treatment in the elevated plus maze. The ratio of time spent in, and entries made into, the open arms of a plus maze (open) to the time spent in, and entries into, both the open and enclosed arms (open + closed) is shown during 5-min sessions. The two measures are correlated, and increase or decrease when anxiolytic drugs or anxiogenic drugs, respectively, are administered. The time measure is most sensitive (*13, 14*). Administration of NPY (i.c.v.) produces marked anxiolytic-like effects in this model (*6*). Both the time and the entry ratio were markedly lower in animals treated with antisense D-oligos compared to both saline- and sense-treated controls, which in turn did not differ from each other [means ± SEM, n = 9 to 10; ANOVA for treatment effect: $F(26,2) = 24.1$, 20.4 for time and entries, respectively; $P < 0.001$ for both is indicated by asterisks. Tukey's HSD test was used for the individual comparisons]. No group differences were seen in the total number of entries, a measure of overall locomotor activity.

After testing, the rats were killed, and cortical tissue, containing both Y1 and Y2 receptors, and striatum, harboring Y2 receptors only (*12*), were obtained. Binding was performed as described (*10*) with [^{3}H]-propyonyl-NPY as a ligand (*18*). Because this ligand labels both Y1 and Y2 receptors, masking was used as described (*9, 13*) to determine Y1 and Y2 sites (*12, 18*). In both control groups, approximately 70% of cortical [^{3}H]NPY binding sites were of the Y1 type. These Y1-type binding sites were decreased by almost 60% in antisense-treated rats. Y2-type sites were unaffected by antisense treatment (Fig. 3). No differences between groups were detected when [^{3}H]NPY binding was performed on striatal membranes, and there was no change in the affinity of cortical Y1 receptors [dissociation constant (K_d) 0.9 to 2 nM for [^{3}H]NPY] (*18*).

The ability of unmodified (D-) as well as phosphorothioate-substituted (S-) oligonucleotides to enter cells depends on receptor-mediated endocytosis (*19, 20*). Antisense oligonucleotides can interfere with protein expression by several mechanisms (*21, 22*). For D-oligos, the inhibition of translation (translation arrest) is thought to result from ribonuclease H–mediated degradation of mRNA, interference with ribosomal activity (blockade of message read-through), or both. In vitro exposure of cells to antisense oligonucleotides inhibits the expression of receptors for luteinizing hormone (*23*), interleukin-1 (*24*), and interleukin-6 (*25*), as well as the T cell receptor (*26*). In all these studies, decreased receptor density was associated with attenuated functional responses to receptor activation. The in vitro part of our study is consistent with these results and shows that a similar approach can be successfully applied to neurons and a neurotransmitter receptor. The unexpected effectiveness of our D-oligo in vivo may be a result of the relative stability of nucleic acids in the brain (*27*).

We were able to circumvent the lack of specific NPY receptor antagonists and to examine the hypothesis that NPY may be involved in mechanisms of anxiety. Central administration of NPY produces marked anxiolytic-like effects in animal models of anxiety (6). Our results support the hypothesis that endogenous NPY participates in regulating anxiety and that a disturbed NPY transmission may produce anxiety symptoms in psychiatric patients. This is in agreement with reports that concentrations of NPY-like immunoreactivity in the cerebrospinal fluid of depressed patients correlate negatively with clinical anxiety scores (*5*). Exogenous NPY, at doses higher than those required for anxiolytic-like action, produces sedation by acting on Y1 receptors (*28*). In our study, however, locomotor activity was unaffected. Sedation thus may be a pharmacological rather than a physiological effect of NPY.

Finally, NPY is a potent stimulant of food intake (*29, 30*), and this seems to represent a physiological action of the peptide (*4, 31, 32*). This action of NPY, however, is localized to the hypothalamus (*31, 32*), whereas the anxiolytic-like effects are produced in telencephalic structures (*33*). The cloned Y1 receptor does not seem to be expressed in hypothalamic areas implicated in food intake regulation (*4*); also, the receptor mediating food intake effects of NPY seems to differ pharmacologically from typical Y1 receptors (*34*). Thus, NPY receptors mediating food intake actions of the peptide and those responsible for its anxiolytic-like effects may represent different gene products or may be differentially regulated.

The function of many other bioactive peptides remains unclear because of a lack of selective receptor antagonists. Initial hypotheses regarding the functional role of neuropeptide transmitters can be postulated on the basis of effects observed after exogenous administration of the compounds. Because of the differences in spatial and temporal patterns of availability, however, establishing a physiological role for a peptide transmitter must ultimately rely on a demonstration of deficits produced by antagonizing the action of endogenous transmission. Our antisense approach may be a rational alternative to the laborious or fortuitous development of traditional competitive receptor antagonists. Moreover, the selectivity of an antisense D-oligo is likely to match or exceed that of classical drugs.

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13. Cortical neurons were plated on 20-mm dishes (1.5×10^6 neurons per dish) coated with polyornithine and grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum (Irvine Scientific, Santa Ana, CA), 20% glucose, 2.0 mM L-glutamine, and penicillin-streptomycin. After 40 hours of culture, fetal bovine serum was omitted. At this time, 0.2 μM NPY-Y1 receptor antisense (5′-GGAGAACAGAGTTGAATT-3′), sense (5′-AATTCAACTCTGTTCTCC-3′), or missense (5′-GTTAAAGGTCAGGAATAG-3′) D-oligos were added once daily for 5 days; controls did not receive D-oligo. For binding, cells were rinsed three times with binding buffer and incubated for 60 min at 37°C. The binding site density was estimated at a saturating concentration of iodinated PYY. This methodology adequately estimates the density of binding sites in SK-N-MC, a neuroblastoma cell line that expresses Y1 receptors (*7, 9, 10*), and in COS-1 or CHO-K1 cells transfected with the human Y1 receptor [(*8*); C. Wahlestedt, unpublished data] as well as in brain homogenates (*10, 11*). The specific activity of the ligand used, porcine (p) [^{125}I] PYY (New England Nuclear, Boston, MA), was reduced to a 1:10 ratio with unlabeled pPYY (Bachem California, Torrance, CA), and the ligand was used as described (*11*) at a total concentration of 2 nM. Specific binding was defined as that displaced by excess (1 μM) pNPY. Because [^{125}I] PYY labels both NPY-Y1 and NPY-Y2 sites, masking was used as described (*10*) to determine total Y1- and Y2-type binding. Total [^{125}I]PYY binding, representing a sum of Y1 and Y2 sites, was obtained in the absence of any masking agent. To determine Y1 sites, we performed [^{125}I] PYY binding in the presence of 0.2 μM unlabeled Y2 ligand $pNPY_{13-36}$. To determine Y2 binding, we used 0.1 μM unlabeled Y1 ligand, p[Pro^{34}]NPY. We stopped incubation by washing the cells four times in ice-cold buffer and exposing them to lysis buffer [3 M acetic acid with 8 M urea and 2% (v/v) Nonidet P-40]. The remaining radioactivity was determined and converted into binding sites per cell. cAMP accumulation was determined by radioimmunoassay (Advanced Magnetics, Cambridge, MA) as described (*7, 9*). Some control cultures, as well as whole rat brain, were used for RNA extraction and cDNA synthesis by reverse transcriptase. These cDNAs, in addition to the cloned rat Y1 receptor cDNA, were used for PCR performed as described (*8*) except that the annealing temperature was 45°C. The sense D-oligo was the forward primer, and a 40-mer nucleotide (5′-ATACTTGTCCTTGAACGCCGCAAGTGATACATTTTGGAAG-3′, corresponding to the second extracellular loop) was the reverse primer. The PCR products were analyzed by agarose gel electrophoresis and exhibited the expected size, 582 base pairs, when compared to marker DNA.
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17. Male Wistar rats (250 g) were housed three per cage under reversed light cycle conditions (on at 10:00 p.m., off at 10:00 a.m.) with free access to tap water and standard laboratory chow. Under halothane anesthesia, the animals were stereotactically implanted with chronic 23-G guide cannulas (Plastic One, Roanoke, VA) aimed at the lateral cerebral ventricle and secured to the skull with stainless steel screws and dental cement; placement was controlled by the insertion of a 28-G injector and by the injection of 5 μl of saline by gravity. After a 10-day recovery period during which the animals were handled and sham-injected, they were housed one per cage, and injections were initiated. Sense or antisense oligonucleotide (50 μg in 5 μl of 0.9% saline) or vehicle alone was injected from 10:00 to 11:30 a.m. and from 10:00 to 11:30 p.m. on days 1 and 2. On day 2, food was removed at noon. On day 3, plus maze testing was started at 10:00 a.m. Scoring was performed as described (*6*) by an observer blind to treatment given. Immediately after the plus maze session, sawdust was removed from the cage, an amount of preweighed food was given, and the remaining food (including spill) was measured after 30 min, 1 hour, 2 hours, and 4 hours. Finally, the animals were decapitated,

their brains were removed, and cortical as well as striatal tissue was dissected, weighed, and frozen at −70°C until assayed.
18. Cortical tissue from individual rats was used for preparation of the P2 synaptosomal membrane fraction, which was used in radioreceptor binding studies at 0.2 mg of protein per milliliter of incubation solution. Porcine (p) [^{3}H]-propyonyl-NPY (Amersham) was used in seven concentrations ranging from 0.25 to 25 nM. Specific binding was defined as that displaced by excess (1 μM) unlabeled NPY. Incubations were for 180 min at room temperature in a final volume of 250 μl. All conditions were tested in triplicate. Total [^{3}H]NPY binding, representing a sum of Y1- and Y2-type receptors, was obtained with no masking agent present. Determination of Y1 sites and Y2 binding was done as for [^{125}I]PYY (*13*). Saturation curves, constructed separately from each rat with the use of computerized nonlinear least squares regression (Accufit and Accucomp, Lundon Software, Chagrin Falls, OH), yielded individual estimates of binding site numbers (B_{max}) and binding affinities (K_d). Group averages of these parameters were compared.
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GABA-Activated Chloride Channels in Secretory Nerve Endings

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Neurotransmitters acting on presynaptic terminals regulate synaptic transmission and plasticity. Because of the difficulty of direct electrophysiological recording from small presynaptic terminals, little is known about the ion channels that mediate these actions or about the mechanisms by which transmitter secretion is altered. The patch-clamp technique is used to show that the predominant inhibitory presynaptic neurotransmitter, γ-aminobutyric acid (GABA), activates a $GABA_A$ receptor and gates a chloride channel in the membranes of peptidergic nerve terminals of the posterior pituitary. The opening of a chloride channel by GABA weakly depolarizes the nerve terminal membrane and blocks action potentials. In this way, GABA limits secretion by retarding the spread of excitation into the terminal arborization.

The neurotransmitter GABA inhibits the release of transmitters from nerve terminals throughout the nervous system. This action has been demonstrated in preparations as diverse as the arthropod neuromuscular junction, fish retina, and mammalian spinal cord and hippocampus (*1–5*). In contrast to its actions at postsynaptic targets, the actions of GABA at presynaptic targets are poorly understood; the nature of the channels gated by GABA at presynaptic sites is not known, and the evaluation of the pharmacological properties of presynaptic GABA receptors relies heavily on extrapolations from studies of postsynaptic receptors. The situation is the same for virtually all substances that act at presynaptic targets. Little is known of the ionic mechanisms by which neurotransmitters alter the release of neurotransmitters from nerve endings.

The posterior pituitary is very rich in secretory nerve terminals, some of which are larger than 10 μm in diameter. Patch-clamp recordings from posterior pituitary neurosecretosomes (*6, 7*) and slices (*8, 9*) have revealed some of the basic membrane mechanisms that govern the excitability of nerve terminals. Using thin slices of the posterior pituitary, we studied the membrane mechanisms that underly the responses of nerve endings to chemical signals (*10*). Under whole cell voltage clamp, the application of GABA elicited a membrane current in 85 of 95 recordings (89%) (Fig. 1A). With Cl^- concentration nearly equal on both sides of the membrane and a holding potential of −70 mV, the mean GABA-activated current was 92 ± 8 pA (mean ± SEM; $n = 30$). The current varied linearly with voltage, reversing at 0.1 ± 1.4 mV ($n = 11$) (Fig. 1B). The reversal potential was close to the Cl^- Nernst potential of 0.9 mV (Fig. 1C). With fixed extracellular Cl^- concentration, the reversal potential varied linearly with the logarithm of the intracellular Cl^- concentration with a slope of 63.7 mV per tenfold change. These data indicate that GABA gates a Cl^--selective ion channel in the membranes of posterior pituitary nerve endings.

GABA application to outside-out patches produced single-channel currents (Fig. 2A). Individual patches had as many as three channels; about one-third of the patches had none. At a holding potential of −70 mV, the average single-channel current was 1.8 pA. Amplitude distributions revealed only one open conductance level (Fig. 2B, inset) and no subconductance states. In contrast, GABA-activated channels in nerve cell bodies exhibit abundant subconductance state activity (*11*), reflecting a subtle difference in channel properties. Single-channel current varied linearly with voltage (Fig. 2B) and reversed at 0.0 ± 1.1 mV ($n = 4$), a value again near the Nernst potential for Cl^-. The slope from the best fitting line furnished an average single-channel conductance of 26.3 ± 0.9 pS ($n = 4$). This conductance is similar to the conductance of GABA-activated channels in nerve cell bodies (*11, 12*), endocrine secretory cells (*13*), and glia (*14*).

A $GABA_A$ receptor mediated these responses. The responses were blocked more than 90% by the $GABA_A$ receptor antagonists bicuculline and picrotoxin in each of four nerve terminals tested with each drug (Fig. 3, A and B). Muscimol, a $GABA_A$ receptor agonist, produced responses that were similar in shape but were 28 ± 8% larger ($n = 4$; $P < 0.005$ of zero difference by z statistic) than the responses of the same nerve endings to GABA (Fig. 3C). The benzodiazepine chlordiazepoxide enhanced GABA responses 27 ± 8% ($n = 8$; $P < 0.005$) (Fig. 3D). These drug actions resemble those at $GABA_A$ receptors of cell bodies. The $GABA_B$ receptor agonist baclofen (100 μM), on the other hand, did not change the holding current when applied to five different nerve endings (*15*). In these experiments, voltage was varied in 20-mV increments from −70 to 30 mV. Because the K^+ Nernst potential was −90 mV, these experiments would have detected $GABA_B$ receptors if they were coupled to a K^+ channel (*16*). Furthermore, 200 μM baclofen had no effect on the maximum amplitude and the voltage depen-

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