Tonic Activation of Presynaptic \textit{GABA}\textsubscript{B} Receptors in the Opener Neuromuscular Junction of Crayfish

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\textsuperscript{1}The Otto Loewi Minerva Center for Cellular and Molecular Neurobiology, Department of Neurobiology, The Hebrew University, Jerusalem, Israel; and \textsuperscript{2}Department of Anaesthesia and Intensive Care, The University of Adelaide, Adelaide, South Australia 5005, Australia

Parnas, Itzchak, Grigory Rashkovan, Jennifer Ong, and David I. B. Kerr. Tonic activation of presynaptic \textit{GABA}\textsubscript{B} receptors in the opener neuromuscular junction of crayfish. \textit{J. Neurophysiol.} 81: 1184–1191, 1999. 1184–1191 Release of excitatory transmitter from boutons on crayfish nerve terminals was inhibited by (R,S)-baclofen, an agonist at \textit{GABA}\textsubscript{B} receptors. Baclofen had no postsynaptic actions on crayfish nerve terminals was inhibited by (R,S)-baclofen, an agonist at \textit{GABA}\textsubscript{B} receptors. Baclofen had no postsynaptic actions as it reduced quantal content without affecting quantal amplitude. The effect of baclofen increased with concentration producing 18% inhibition at 10 \textmu M; EC\textsubscript{50}, 50% inhibition at 30 \textmu M; maximal inhibition, 85% at 100 \textmu M and higher. There was no desensitization, even with 200 or 320 \textmu M baclofen. Phaclofen, an antagonist at \textit{GABA}\textsubscript{A} receptors, competitively antagonized the inhibitory action of baclofen (\textit{K}_D = 50 \textmu M, equivalent to a pA$_2$ = 4.3 ± 0.1). Phaclofen on its own at concentrations below 200 \textmu M had no effect on release, whereas at 200 \textmu M phaclofen itself increased the control level of release by 60%, as did 2-hydroxy-saclofen (200 \textmu M), another antagonist at \textit{GABA}\textsubscript{B} receptors. This increase was evidently due to antagonism of a persistent level of \textit{GABA} in the synaptic cleft, since the effect was abolished by destruction of the presynaptic inhibitory fiber, using intraneuronal pronase. We conclude that presynaptic \textit{GABA}\textsubscript{B} receptors, with a pharmacological profile similar to that of mammalian \textit{GABA}\textsubscript{B} receptors, are involved in the control of transmitter release at the crayfish neuromuscular junction.

\textbf{INTRODUCTION}

Receptors for the inhibitory transmitter \textit{GABA} (\textit{\gamma}-amino butyric acid) can be divided into ionotropic and metabotropic subtypes. The ionotropic \textit{GABA}$_A$ and \textit{GABA}$_C$ receptors, with an integral picrotoxin-sensitive chloride channel, are members of the ligand-gated ion channel super family of receptors that includes glycine, nicotinic, and serotonin (5-HT$_3$) receptors. The metabotropic \textit{GABA}\textsubscript{B} receptors belong to the larger super family of the ligand-gated ion channel super family of receptors that includes glycine, nicotinic, and serotonin (5-HT$_3$) receptors. The metabotropic \textit{GABA}\textsubscript{B} receptors belong to the larger super family of heptahelical transmembrane receptors that are G-protein–linked to a variety of cellular effectors, including calcium and potassium channels, which they regulate (Kerr and Ong 1995). \textit{GABA}\textsubscript{A} receptors are picrotoxin insensitive but respond selectively to baclofen as an agonist and are antagonized by phaclofen, the phosphonic analogue of baclofen, as well as by the related sulfonic derivative 2-hydroxy-saclofen (Kerr et al. 1987, 1988). Baclofen has no actions at either \textit{GABA}\textsubscript{A} or \textit{GABA}\textsubscript{C} receptors.

There is now increasing evidence for both invertebrate and vertebrate presynaptic \textit{GABA}\textsubscript{B} receptors. Using baclofen as an agonist, Miwa et al. (1990) found a pertussis toxin–sensitive potassium-dependent hyperpolarization of the excitatory axon at the lobster neuromuscular junction. Blundon and Bittner (1992) showed that baclofen depresses the amplitude of the action potentials in crayfish excitatory axons. These actions suggest that \textit{GABA}\textsubscript{B} receptors are located on the excitatory axon itself and may be involved in presynaptic inhibition by affecting the amplitude of the excitatory action potential. However, using a macropatch electrode, to give focal depolarization of individual release boutons, Fischer and Parnas (1996a,b) demonstrated the presence of both \textit{GABA}\textsubscript{A} and \textit{GABA}\textsubscript{B} receptors on one and the same release bouton at the crayfish opener muscle. When using such localized depolarization, activation of \textit{GABA}\textsubscript{B} receptors affects the release machinery by a mechanism other than reduction of the amplitude of the action potential in the excitatory axon.

In the present study we further characterize the \textit{GABA}\textsubscript{B} receptors in the crayfish neuromuscular junction. We found considerable resemblance in the pharmacological properties of the mammalian and crayfish receptors. In addition, the simpler anatomic organization of the crayfish preparation enabled us to unravel a presynaptic tonic inhibitory effect on release, exerted through \textit{GABA}\textsubscript{A} receptors by the small concentration of \textit{GABA} normally present in the synaptic cleft.

\textbf{METHODS}

\textbf{Preparation}

The opener neuromuscular system of the crayfish \textit{Procambarus clarkii} was used (Fischer and Parnas 1996a,b). Animals were purchased from Atachafalaya Biological Supply (Raceland) and kept in aquaria with circulating filtered fresh water. The crayfish were fed with fish fillets twice a week. The first walking leg was removed by autotomy and the opener muscle exposed as described previously (Dudel and Kuffler 1961; Fischer and Parnas 1996a). The preparation was held by small springs in a chamber (3 \times 2 cm) with shallow walls (4 mm). The chamber was placed on the stage of a Zeiss upright microscope (Axioskop FS). Modified Van-Harreveld solution in the chamber was circulated through a cooling device using a peristaltic pump (Gilson Minipuls 3), to keep the temperature at 12°C, and contained the following (in mM): 220 NaCl, 5.4 KCl, 13.5 CaCl$_2$, 2.5 MgCl$_2$, and 10 tris (hydroxymethyl) aminomethane-maleate; pH was adjusted to 7.4 by adding NaOH. TTX (2 \times 10$^{-5}$M) was added to block sodium excitability.

\textbf{Stimulation and recording}

To visualize single release boutons, an objective (Acroplan \times 40/0.75 W) with 1.8-mm working distance was used, requiring horizontal positioning of the macropatch electrode (Ravin et al. 1997). Macro-
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In the presence of 30 μM baclofen, C: after washing. Traces were selected to show a failure in release, a single quantum, or 2 or 3 quanta. The onset of each quantal event is marked by an asterisk. D: averages of 200 single quanta events in control, and in the presence of 30 μM baclofen.

The tip (8 μm) was slightly bent to allow positioning of the macropatch electrode over a single release bouton in the small space between the objective and the preparation. In the different experiments, the seal resistance varied between 200 and 250 kΩ, but it was constant throughout each of the experiments. The bouton was depolarized by constant negative current pulses (0.7 ms, −0.7 μA at a rate of 2 Hz) (Dudel 1981, 1983). At the temperature used (12°C), quanta appeared after the stimulus artifact (Fig. 1), and single quanta events could be detected and counted. Traces were digitized using a neurodata (Neuro-Recorder DR-484) A/D converter at 50 kHz, and stored on video cassettes. In parallel, the data were transferred to a Pentium computer (Philips 90 MHz) using the Labview (AT-MIO-16F-5, NI-DAQ 4.9.0 driver software) interface. The number of quantal events was counted for a given number of pulses (usually 120, 1 min). Dividing this number by the number of pulses gave the quantal content.

**Experimental procedure**

First, we established the control quantal content by counting the number of quanta for several sets of stimuli. When the quantal content stabilized, we changed the circulation fluid to one containing either an agonist or an antagonist of GABA<sub>B</sub> receptors, or both. The time required for a complete change of solution in the chamber was estimated to be 1.1 min, using a calibrated test solution containing Coomassie Blue. Because the crayfish presynaptic GABA<sub>B</sub> receptors did not show desensitization to agonists, this period required for a drug to reach its final concentration had no effect on the final steady level of the quantal content.

Concentration-response curves for the agonist baclofen were constructed in the presence and absence of the antagonist phaclofen. As a measure of agonist potency, the half-maximally effective concentration (EC<sub>50</sub>) was the concentration of the agonist required to produce 50% depression of release, estimated from the concentration-response curve. Potency of the antagonist phaclofen was obtained from the rightward shift of the baclofen concentration-response curve in its presence, using the Gaddum-Schild relationship CR = 1 + [B]/[K<sub>I</sub>], rearranging, to convert to logs, and averaging, gives an estimate of antagonist potency (pA<sub>2</sub> = log (CR − 1) − log [B]), the concentration ratio (CR) being derived from the shift of the concentration-response curve in the presence of each concentration of the antagonist [B] (see Barlow et al. 1997). By definition, the pA<sub>2</sub> of an antagonist is the concentration causing a two-fold rightward shift of the agonist concentration-response curve. The pA<sub>2</sub> for phaclofen was also estimated from the inhibition curve for increasing concentrations of phaclofen against the response to a fixed concentration of baclofen (30 μM, close to the EC<sub>50</sub>), based on the method described by Lazareno and Birdsall (1993).

**Intracellular injection of the proteolytic enzyme pronase**

In some preparations, it was desired to have an opener muscle innervated only by the excitatory axon. This was achieved by intra-axonal injection of the proteolytic enzyme pronase, as described by Parnas and Bowling (1977) for leech neurons, and by Dudel and Parnas (1987) for lobster axons. The pronase diffuses into the very small terminals to dissolve them without any overt damage to nearby neurons (Bowling et al. 1978). The injection solution contained 0.4% sulforhodamine B (RBI) 0.5% protease type 14 (Sigma) 200 mM KCl, and the pH was adjusted to 7.4 by adding NaOH. Beveled electrodes were inserted into one of the main secondary branches of the inhibitor fiber, and the protease solution was injected by pressure using a Pico-Injector (Medical System, PLI-100). The preparation was then incubated for at least 2 h after which the action of the agonist or antagonist was tested on quantal content.

**Electron microscopy**

To ensure that the inhibitory axon was dissolved, we studied the ultrastructure of the neuromuscular junction as described by Atwood and Morin (1970) and found that incubation with intra-axonal pronase for 2 h was sufficient to destroy the inhibitory terminals (not shown).

**Statistical evaluation**

In each experiment the quantal content was established for a series of groups of pulses (usually 120 pulses). The range of fluctuation of the quantal content in the controls or after treatment is given together with the average and the standard deviation (SD). Significance was estimated using the paired two-tailed t-test. For comparison of results between groups of experiments, we used the unpaired two-tailed t-test. The level of significance has been set at P = 0.01. Theoretical curves were fitted to the experimental data points (Figs. 2A, 4, and 5) using the Prism computer program: sigmoidal dose response equation (variable slope).

**Drugs**

(R,S)-Baclofen (RBI) was used as a GABA<sub>B</sub> receptor agonist. The GABA<sub>B</sub> receptor antagonists, phaclofen and 2-hydroxy-saclofen (Kerr et al. 1987, 1988) were synthesized by Professor R. H. Prager and his colleagues (The Flinders University of South Australia, Australia). 3-Aminopropylphosphonic acid (3-APPA) was purchased from Sigma.

**RESULTS**

**Lack of effect of baclofen on quantum size**

Baclofen had no effect on quantum size. Figure 1 shows samples of recordings in a control (A), in the presence of 30...
μM baclofen (B), and after washing (C). Traces were selected to show a failure of release, a single quantum, and two or three quanta (the onset of quanta is marked by asterisks). Note that even though quanta varied in amplitude, the smaller quanta could be easily distinguished from the noise level. The average amplitude of 200 consecutive single quanta (without any selection) was the same in controls as in the presence of 30 μM baclofen, which reduced the quantal content by 50% (Fig. 1D).

**Baclofen reduced release in a concentration-dependent manner**

Application of baclofen led to a reduction in the evoked quantal content. The lowest detectable threshold for this effect was 7–10 μM (14–18% reduction); effects of baclofen at lower concentrations could not be detected, because of small fluctuations in quantal content seen in controls. Upon switching from the control solution to baclofen-containing medium, it took some minutes for the full depressant effect on transmitter release to appear (Figs. 2 and 3), lower concentrations (<50 μM) requiring at least an additional 5 min after complete exchange of the medium in the chamber. The action of baclofen on release was concentration dependent, with an EC50 of 30 μM and a maximum reduction of 87% achieved at 100–120 μM (B100 μM; Fig. 2A). This same level of reduction was seen at 200 μM baclofen (B200 μM; Fig. 2B), and higher concentrations of baclofen, even up to 320 μM, never completely abolished release. There was no evidence of desensitization with prolonged application of baclofen (>20 min, Fig. 2B; see also Fig. 3), which enabled cumulative concentration-response curves to be constructed.

Partial agonist/antagonist properties have been described for the congener 3-aminopropylphosphonic acid (3-APPA), from which phaclofen is derived (Chiefari et al. 1987; Kerr et al. 1989). Such partial agonist properties were also found here, at the crayfish opener muscle (Fig. 2C). In four preparations, application of 3-APPA (300 μM) reduced transmitter release by an average of 35 ± 8% (mean ± SD), whereas 100 μM or
200 μM 3-APPA had no effect on release. In combination with 30 μM baclofen, which reduced release by 76%, the effect of 300 μM 3-APPA did not add. Instead, in its continued presence, 3-APPA significantly reduced the response to baclofen by an average of 35%, in keeping with a partial agonist/antagonist action of 3-APPA at this receptor. After a 15-min wash out, 30 μM baclofen was again fully effective in reducing release (Fig. 2C).

**Antagonism of baclofen by phaclofen, a GABA B receptor antagonist**

Phaclofen is a competitive, surmountable, and reversible antagonist of baclofen (Kerr et al. 1987). At the crayfish presynaptic GABA B receptor, it was also an effective antagonist as seen in Fig. 3. The control average quantal content was 0.15 ± 0.01; in the presence of baclofen (50 μM), this was reduced to 0.02 ± 0.01, recovering to 0.12 ± 0.01 after wash out. Phaclofen (100 μM) did not affect basal release (0.14 ± 0.01), but in its continued presence, the response to 50 μM baclofen was virtually blocked (release 0.13 ± 0.01). Upon wash out, the original basal level of stimulated release was restored, whereupon another application of 50 μM baclofen again depressed release to the same extent as previously (0.02 ± 0.01), indicating reversibility of the antagonism. In a similar experiment, phaclofen (100 μM) antagonized the action of baclofen (100 μM) by 25%. The average quantal content in the control was 0.36 ± 0.03. In the presence of 100 μM baclofen, this declined to 0.09 ± 0.02 (75% inhibition), whereas in the combined presence of phaclofen and baclofen, the quantal content was 0.17 ± 0.01, a 21% change from the level with baclofen alone (highly significant P < 0.01).

To establish the properties of phaclofen as an antagonist at GABA B receptors in this preparation, we measured the effects of different concentrations of phaclofen on the concentration-response curve for the presynaptic action of baclofen. Figure 4 shows that the typical concentration-response curve for baclofen was shifted to the right, in a parallel manner, in the presence of 60 μM phaclofen, indicative of surmountable, competitive antagonism. Interestingly, as can be seen, the concentration-response curve for baclofen, over the lower concentration range 10–30 μM, was altered in the presence of phaclofen (60 μM, Fig. 4). From the control concentration-response curve, the threshold response to baclofen was found to be a minimal 18% depression of release at 10 μM. However, in the presence of phaclofen (60 μM), which shifted the curve to the right, the minimum detectable response induced by 10 μM baclofen became a 5% depression. After wash out of phaclofen, the control concentration-response curve to baclofen was reestablished, although the response to 10 μM baclofen remained near 10%, rather than the original 18% depression. Using the rightward shifts of the baclofen concentration-response curve due to various concentrations of phaclofen and applying the Gaddum-Schild relationship (see Methods), a mean value of pA2 = 4.3 ± 0.1 was found for its antagonist action at the crayfish GABA B receptor.

In a further six experiments, we examined the concentration-dependent displacement of baclofen from the GABA B receptor by the antagonist. Three different concentrations of phaclofen (15, 30, and 60 μM) were used against the response to 30 μM baclofen, which is close to the EC50 for reduction in release; Fig. 5 shows the resultant concentration-dependent reduction of the baclofen response (taking the control response to 30 μM baclofen alone as 100%). Phaclofen at 15 μM had no effect on the baclofen response, but at 30 and 60 μM, phaclofen reduced the effect of baclofen by 24 ± 12.1% and 82 ± 9.75%, respectively. Using these results, an EC50 value of 45 μM was calculated for phaclofen to reduce the response to 30 μM baclofen by half; applying the method of Lazareno and Birdsall...
(1993), these results again yielded an estimated $pA_2$ of 4.3 $\pm$ 0.1 for phaclofen as an antagonist at the crayfish GABA$_B$ receptor. Each of these estimates was based on steady-state analysis with prolonged exposure to the agents, which overcomes any problem of limitation of drug penetration at the macropatch electrode.

**Over antagonism at high antagonist concentrations**

Although 100 $\mu$M phaclofen had no effect on the resting level of evoked release, phaclofen at 200 $\mu$M increased the level of evoked release, even in the presence of 100 $\mu$M baclofen. Such an increase of release above the previous basal control indicates that 200 $\mu$M phaclofen had removed an underlying tonic inhibitory action of GABA at the synapse ("over-antagonism"). In a typical experiment, 100 $\mu$M baclofen reduced the control level of release (0.15 $\pm$ 0.014) by 69%, after which phaclofen (200 $\mu$M) was added in the continued presence of baclofen without washing. Even though baclofen (100 $\mu$M) was still present, the quantal content recovered to above its original control level, to an average of 0.23 $\pm$ 0.01, an increase of 153% (significant, $P < 0.01$). After washing, the quantal content declined to its initial level (0.15 $\pm$ 0.014), and a further application of 100 $\mu$M baclofen again inhibited release, as in the beginning of the experiment (not shown). This effect was not seen when using lower concentrations of phaclofen.

In Fig. 6, different concentrations of phaclofen (50, 100, and 200 $\mu$M) were added after 100 $\mu$M baclofen (B 100 $\mu$M) had produced its maximal effect (76% depression of release). With baclofen (100 $\mu$M) still present, addition of the lowest concentration of phaclofen (P 50 $\mu$M) had only a very slight effect, whereas phaclofen at 100 $\mu$M (P 100 $\mu$M) produced partial recovery, to 50% of control release. However, in the presence of 200 $\mu$M phaclofen (P 200 $\mu$M), the quantal content rose to a peak level of 78% over and above the initial control level; washing then restored release to the basal level, whereupon adding 100 $\mu$M baclofen once again effectively reduced release. In all experiments ($n = 5$) where 100 $\mu$M phaclofen was used, there was no increase in the basal level of evoked release, whereas in all experiments with 200 $\mu$M phaclofen ($n = 4$), evoked release increased above the basal level, on average by 62 $\pm$ 8%.

When phaclofen (200 $\mu$M) was added alone in the absence of baclofen, there was again an increase in the basal level of evoked release. In four experiments, 200 $\mu$M phaclofen significantly increased release, to an average of 167 $\pm$ 24% (Fig. 7A, $P = 0.01$) of the control. Also, we tested for any similar effect of high concentrations of the GABA$_B$ receptor antagonist 2-OH-saclofen (Fig. 7A); at 100 $\mu$M 2-OH-saclofen had no effect on the basal level of evoked release (average 99.3 $\pm$ 10.2$\%$).
3.7%; \( n = 4 \); nonsignificant, \( P = 0.7 \)), whereas 200 \( \mu M \)
2-OH-saclofen significantly increased the basal level of evoked
release to 144 \( \pm 20.3\% \) (\( n = 4 \), \( P = 0.01 \)).

Effect of phaclofen and 2-OH-saclofen on preparations
without an inhibitory axon, following intra-axonal pronase
treatment

An increase in the basal level of release after addition of
phaclofen or 2-OH-saclofen could have resulted from one or
both of the following mechanisms: phaclofen or 2-OH-saclofen
may, at high concentrations, affect release directly, or they may
act by the removal of a tonic inhibitory effect of GABA present
in the synaptic cleft, as found for example for the postsynaptic
membrane of the neuromuscular junction of the crab (Parnas et
al. 1975). One way to distinguish between these two mechan-
isms is to test for effects of phaclofen or 2-OH-saclofen, on
the opener neuromuscular system, after removal of the inhib-
itory axon by intracellular injection of the proteolytic enzyme
pronase (Bowling et al. 1978; Parnas and Bowling 1977).
Removal of the inhibitory axon presumably leaves the presyn-
aptic release bouton without inhibitory innervation, and there-
fore without an inhibitory synaptic cleft containing GABA. If
the effect of the antagonists is to increase release directly, we
would expect high antagonist concentrations to still increase
the release by an action at the excitatory boutons. If, on the
other hand, the effect is indirect, because of removal of tonic
inhibition, then we expect the effect to disappear. Comparison
of Fig. 7A with Fig. 7B shows that the latter indeed was the
case; in preparations with the inhibitory axon removed, 200
\( \mu M \) phaclofen or 200 \( \mu M \) 2-OH-saclofen no longer had any
effect on the basal level of release, in contrast to preparations
with intact inhibitory input. The average percentage of the
quantal content in comparison with the control was 101 \( \pm 10.8\% \) SD (4 experiments, insignificant change, \( P = 0.2 \)).
If there was any leakage of pronase from the damaged inhibi-
tory terminals into the synaptic cleft, it had no effect on the GABA
receptors of the excitatory terminal because baclofen at 100
\( \mu M \) was still effective in reducing release (Fig. 7B), as in
normal noninjected preparations.

**DISCUSSION**

Although Kaupmann et al. (1997) could not detect any
invertebrate GABA \(_B\) receptor protein in an insect (*Drosophila*)
or a nematode (*Hemonchus*), nevertheless the present results
have demonstrated inhibitory GABA \(_B\) receptors on presynaptic
terminals in a crustacean (*Procambarus*). From their agonist
and antagonist profiles, using known agents selective for mam-
malian GABA \(_B\) receptors, these presynaptic receptors at the
crayfish opener muscle are typical GABA \(_B\) receptors, their
pharmacology being very similar to that found in the mamma-
lan CNS. The crayfish GABA \(_B\) receptors are activated by
GABA itself, as well as by the agonist baclofen, and both these
agonists are blocked by 2-OH-saclofen. Moreover, complete
block of the inhibitory actions of GABA requires the combined
application of picrotoxin and 2-OH-saclofen, which are antag-
onists at GABA \(_A\) and GABA \(_B\) receptors, respectively (Fischer
and Parnas 1996a,b), so that each of these receptor types must be present in this preparation.

In the present study, activation of the presynaptic GABA<sub>B</sub> receptors with baclofen gave a depression of excitatory transmitter release at the crayfish opener, and this effect was blocked by the specific GABA<sub>A</sub> receptor antagonist phaclofen. In addition, we have confirmed that the more potent antagonist 2-OH-saclofen is effective against baclofen at the crayfish GABA<sub>B</sub> receptors, as was originally shown by Fischer and Parnas (1996a). In particular, we have established the concentration-dependent depression of excitatory transmitter release by baclofen at the crayfish opener neuromuscular system, with an EC<sub>50</sub> value of 30 μM and a maximum depression of 87% at 150 μM. We made two different estimates of potency for phaclofen as an antagonist at the crustacean GABA<sub>B</sub> receptors, both of which gave a mean value of pA<sub>2</sub> = 4.3, close to the pA<sub>2</sub> value of 4.0 previously obtained for phaclofen in the mammal (Kerr et al. 1990). In addition, we found that the phosphonic analogue of GABA, 3-APPA, itself partly reduced transmitter release, yet attenuated the action of baclofen when coapplied. Such actions are consistent with partial agonist/antagonist properties of 3-APPA at these crustacean GABA<sub>B</sub> receptors, as also found in the rat brain (Drew et al. 1990; Kerr et al. 1989). Thus, in relation to the agents examined so far (GABA, baclofen, 3-APPA, phaclofen and 2-OH-saclofen), the pharmacology of the crustacean GABA<sub>B</sub> receptor closely resembles that of its mammalian counterpart. We did not attempt to use the more recent, potent agonists or antagonists for GABA<sub>B</sub> receptors, based on P-substituted phosphinic analogues of GABA (Froestl and Mickel 1997), because many of these have been found to have significant affinity for GABA<sub>A</sub> receptors, which crustacean GABA<sub>A</sub> receptors closely resemble (Johnston 1997).

In all experiments, we found that there was a delay of some minutes before the depressant action of baclofen was exerted on transmitter release, and recovery was slow after wash out, suggesting that this baclofen action is mediated through a G-protein rather than through a rapidly acting channel-linked mechanism, although we have no formal proof for this. In general, G-protein–coupled inhibitory receptors, including GABA<sub>B</sub> receptors, are blocked by pertussis toxin (Thalmann 1987), as was originally shown by Miwa et al. (1990), who found that the conductance change induced by baclofen in crustacean motor fibers was abolished by treatment with pertussis toxin. It could be argued that the delayed onset of the depressant action of baclofen in our preparation is somehow related to its slow diffusion from the fluid in the perfusion chamber to the receptors beneath the macropatch electrode; but we discount this because the seal resistance at the macropatch electrode is low (≈200 Ω), so that materials in solution can easily diffuse below the rim of the electrode to reach the receptors. Instead, the evidence is more consistent with the notion that the GABA<sub>B</sub> receptors involved are G-protein coupled to some mechanism controlling excitatory transmitter release. Indeed, such pertussis toxin–sensitive G-proteins, likely involved, are highly conserved across vertebrates and invertebrates as common transducing elements linked to a variety of ligands to intracellular effectors (Simon et al. 1991). For instance, in the crustacean neuromuscular junction, different presynaptic receptors are coupled to G-proteins, including glutamate (Miwa et al. 1987), serotonin (Dixon and Atwood 1989), and GABA (Miwa et al. 1990). With the presynaptic GABA<sub>B</sub> receptors in the crayfish, the ultimate action is a longer lasting and possibly a tonic reduction in excitatory transmitter release.

At higher concentrations, phaclofen removed a fraction of tonic inhibition, resulting in a substantial reduction in the basal level of inhibition (“over antagonism”). As seen in Figs. 6 and 7, 200 μM phaclofen antagonized the depressant action of 100 μM baclofen, raising the quantal content to 78% over the initial basal control. A similar increase in the basal level of release was seen when 200 μM phaclofen was added alone. Likewise, 200 μM of 2-OH-saclofen, on its own, significantly increased basal release (Fig. 7A). It seems that a presynaptic tonic inhibitory action of GABA on release of glutamate from excitatory terminals must be present at the crayfish opener muscle system, acting on a high-affinity state of the GABA<sub>B</sub> receptor and displaceable only by high concentrations of phaclofen or 2-OH-saclofen. In the crayfish opener system, these underlying levels of GABA were readily removed by destruction of the inhibitory axon along with its terminals, using intra-axonal pronase, as confirmed by electron microscopy studies (N. Feinstein and I. Parnas, unpublished observations). Here, such destruction enabled us to uncover a new tonic presynaptic effect of the inhibitory transmitter present at the synaptic cleft. The actual concentration of GABA in the synaptic cleft was never determined, but it is estimated to be in the submicromolar range, since Fischer and Parnas (1996a,b) found that a concentration of 2 μM GABA already produced some detectable GABA<sub>B</sub> receptor–mediated presynaptic inhibition. It should be noted that for GABA to act at such low concentrations to produce tonic inhibition, two conditions must be met. The presynaptic receptors must be of a high affinity to GABA, and they should not show desensitization at these or even higher concentrations (Fischer and Parnas 1996a,b). The tonic inhibitory effect is substantial because its removal increased the basal level of release by ~50%, which suggests that, in systems where presynaptic inhibition exists, there is an additional securing mechanism to prevent release operating as long as the excitatory axon is not activated. It is also interesting to note that, when a smaller fraction of tonic inhibition was removed by lower concentrations of phaclofen, we could detect a depressant response to lower concentrations of baclofen in the presence of phaclofen. The latter not only shifted the baclofen concentration-response curve to the right (Fig. 4), but also lowered the minimal detectable response to baclofen, which evidently was normally occluded by endogenous GABA in the absence of phaclofen.

Modulation of glutamate release through GABA<sub>B</sub> receptor–activated G-proteins as seen here could involve a decreased Ca<sup>2+</sup> influx at the terminal or might be due to some action at the release mechanism itself, independent of alterations in K<sup>+</sup> conductance as discussed recently by Zhang et al. (1996). In this regard, the present method for studying modulation of release offers several advantages, because the detection of release does not depend on propagation of an action potential or on its amplitude. The focal depolarization technique detects only effects on the region below the macropatch electrode and thus provides direct access to the presynaptic receptors on excitatory boutons. The latter raises the feasibility of directly examining the influence of modulators such as GABA on intracellular Ca<sup>2+</sup> concentration, and associated changes in
transmitter release at single release boutons, independently of alterations in K⁺ conductance, as was done recently by Parnas et al. (1996) and Ravin et al. (1997). Thus our present technique may profitably be used for future characterization of the receptors involved.

We are grateful to the Goldie Anna fund for continuous support. We thank Prof. R. H. Prager for the synthesis of phaclofen and 2-hydroxy-saclofen, and R. Ravin for continuous discussions and help. Professor I. Parnas is the Greenfield Professor of Neurobiology.

This work was supported by an SFB 391 grant from the Deutsche Forschungsgemeinschaft, Germany, to Drs. Dudel and Parnas. J. Ong was the recipient of an Australian Research Council Senior Research Fellowship.

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Received 24 September 1998; accepted in final form 3 November 1998.

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