Regulation of Somatodendritic GABA$_A$ Receptor Channels in Rat Hippocampal Neurons: Evidence for a Role of the Small GTPase Rac1

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The role of the cytoskeleton in the activity of GABA$_A$ receptors was investigated in cultured hippocampal neurons. Receptor currents were measured with the whole-cell patch-clamp technique during repetitive stimulation with 1 μM muscimol. After destruction of the microtubular system with nocodazole, muscimol-induced currents showed a rundown by 78%. A similar rundown was observed when actin fibers were destroyed with latrunculin B or C2 toxin of Clostridium botulinum. Because the small GTPases of the Rho family RhoA, Rac1, and Cdc42 are known to control the organization of actin fibers, we investigated their possible involvement. Inactivation of the GTPases with clostridial toxins, as well as intracellular application of recombinant Rho GTPases, indicated that active Rac1 was necessary for full GABA$_A$ receptor activity. Immunocytochemical labeling of the receptors showed that the disappearance of receptor clusters in the somatic membrane as induced by muscimol stimulation was enhanced by Rac1 inactivation. It is suggested that Rac1 participates in the regulation of GABA$_A$ receptor clustering and/or recycling.

Key words: GABA$_A$ receptor rundown; actin cytoskeleton; microtubules; hippocampal neurons; Rac1 GTPase; receptor clusters

The open probability and open times of ligand-gated ion channels are regulated by agonists and modulators. In addition, the cytoskeleton can regulate ligand-gated ion channels by mediating their anchorage and organization within the cell membrane. Both tubulin and actin structures seem to be involved. Thus, glutamate receptors of the NMDA type are anchored in the membrane via actin fibers and the linker protein α-actinin-2 (Rosenmund and Westbrook, 1993; Ehlers et al., 1996; Wyszyński et al., 1997; Allison et al., 1998). Depolymerization of the actin fibers by cytochalasin D or the C2 toxin of Clostridium botulinum causes the rapid rundown of the NMDA receptor-mediated currents (Rosenmund and Westbrook, 1993).

The involvement of actin fibers indicates a role of the small GTPases of the Rho family RhoA, Rac1, and Cdc42 in the control of NMDA receptor activity. They act as molecular switches and organize the actin cytoskeleton (Hall, 1994, 1998; Mackay et al., 1999). It is unclear, however, whether microtubules affect the activity of GABA$_A$ receptors.

The association of GABA$_A$ receptors with actin structures is still controversial. Although actin has been found to coprecipitate with α1 subunits of the GABA$_A$ receptors extracted from bovine brain (Kannenberg et al., 1997), the destruction of actin fibers does not affect the clustering of the receptors (Allison et al., 1998). Although there is yet no evidence that actin fibers influence the activity of GABA$_A$ receptors, their destruction induced the rundown of GABA$_C$ receptor-mediated currents (Filippova et al., 1999).

Here, we have investigated whether microtubules and actin fibers regulate the activity of somatodendritic GABA$_A$ receptors in whole-cell voltage-clamped hippocampal neurons in primary culture. The destruction of microtubules, as well as of actin fibers, caused a rundown in GABA$_A$ receptor currents. Further studies with use of clostridial cytotoxins to inactivate the GTPases of the Rho family, as well as with recombinant GTPases, showed that Rac1 organized the actin fibers that are functionally connected with GABA$_A$ receptor.

MATERIALS AND METHODS

Cultures of hippocampal neurons. Primary cultures of neurons were prepared from hippocampi of newborn Wistar rats (postnatal day 0) as described previously (Benz et al., 1998). The dissociated neuronal and astroglial cells were seeded on laminin-coated coverslips and cultured with DMEM-F12 medium that contained insulin, selenite, and transferrin, as well as B27 supplement. Cultures were incubated at 37°C in an humidified atmosphere of 95% air and 5% CO$_2$ for 8–10 d.

Electrophysiology. Experiments were performed on hippocampal neurons with pyramidal cell-like morphology. Membrane currents through GABA$_A$ receptors were recorded in the whole-cell configuration. In some experiments, amphotericin-perforated patch-clamp recordings were made (Rae et al., 1991). The external (bath) solution contained (in mM): KCl 162, KC1 2.4, CaCl$_2$ 1.2, MgCl$_2$ 1, HEPES 10, and glucose 11 (–320 mOsm, pH 7.3, with NaOH). Tetrodotoxin (0.5 μM) was added to inhibit action potentials. The pipette (internal) solution contained (in mM): CsCl 140, CaCl$_2$ 1, MgCl$_2$ 2, HEPES 10, and EGTA 11 (pH 7.2, –360 mOsm). The calculated free Ca$^{2+}$ concentration was 11 nM (Program WinMAXC;
At that time, test. In some cases, a modified

whole-cell currents were recorded, and cell capacitance \( C_m \) and series resistance \( R_s \) were partially compensated (60–80\%) with an EPC-7 amplifier (List, Darmstadt, Germany). At the beginning of the recording period, access, 20 min after gaining whole-cell access, cell capacitance was 29.0 ± 1.0 pF for \( C_m \) and 12.4 ± 0.4 MΩ for \( R_s \) in conventional whole-cell measurements \((n = 161)\). At the end of the experiment, i.e., 25 min later, these values had not changed significantly, indicating that the recording conditions had remained stable. In amphotericin-perforated patches, stable \( R_s \) values were consistently achieved within 20 min after seal formation. At that time, \( C_m \) and \( R_s \) values found in perforated patches were similar to those in whole-cell recordings \( (27.2 ± 1.9 \text{ pF and } 12.2 ± 0.8 \text{ MΩ}; n = 24)\). Again, recording conditions remained stable over time. Current records were filtered at 3 kHz, digitized at 1 kHz \((\text{CED } 1401); \text{Cambridge Electronic Devices, Cambridge, UK)}\), and analyzed with a laboratory computer using software from Cambridge Electronic Devices.

Unless stated otherwise, experimental agents were introduced into the cells by pressure from the patch pipette. Therefore, the system was allowed to equilibrate for at least 20 min after whole-cell access had been achieved. To investigate GABA\(_A\) receptor currents, the agonist muscimol \((0.03–100 \mu M)\) was applied by means of a fast-flow pressurized superfusion system \((\text{DAD-12}; \text{Adams and List, New York, NY)}\), which completely exchanges the bath medium in the vicinity of the investigated cells within <200 msec \((\text{Kügelgen et al., 1997)}\).

Preparation of cisternal cytotoxins and recombinant proteins. Toxin B and lethal毒素 from \( C. \text{ difficile} \) and \( C. \text{ sordellii} \), respectively, were prepared as described previously \((\text{Hofmann et al., 1997); C21 protein, the enzymatic component of C2 toxin from C. botulinum, and C3 toxin from C. limosum}\) were purified as described previously \((\text{Böhmer et al., 1996; Barth et al., 1998a,b)}\). If not mentioned otherwise, the toxins were applied intracellularly via the patch pipette.

Full-length cDNAs coding for wild-type Rac1, a dominant negative form of Rac1 with Thr at position 17 mutated to Asn \((\text{Rac1N17}); \text{and wild-type Cdc42}) \) were subcloned into the pGEX-2T vector \((\text{Amersham Pharmacia Biotech, Uppsala, Sweden}). The resulting pGEX-Rac1 and Cdc42 plasmids were used to obtain the glutathione S-transferase (GST) fusion proteins by expression into \( \text{Escherichia coli BL21} \) cells. After induction of the expression with 100 \( \mu M \) isopropyl-\( \beta \)-thiogalactopyranoside \((\text{Sigma, Deisenhofen, Germany})\) for 20 hr at 29°C, cells were resuspended in PBS containing 0.1% Triton X-100 and then lysed by sonication. Lysates were centrifuged for 10 min at 12,000 \( \times \text{g} \), and GST fusion proteins were purified from the supernatants on glutathione Sepharose 4B \((\text{Amersham Pharmacia Biotech, Buckinghamshire, UK})\) according to the instructions of the manufacturer.

Clavage of the cleared proteins from the immobilized GST was achieved by incubating the sample with the protease thrombin \((\text{Sigma}). \) Thereafter, thrombin was removed from the eluted protein by absorption onto \( \alpha \)-aminobenzamidine-agarose. Finally the purified proteins were checked by SDS-PAGE.

Staining of microtubules. Immunochemistry for \( \beta \)-tubulin III was used to analyze microtubules. After fixation with paraformaldehyde (4%), cells were washed with the fusion medium and incubated with a polyclonal guinea pig anti-\( \beta \)-tubulin antibody \((\text{Dr. M. Fritschy, Department of Pharmacology, University of Zurich, Zurich, Switzerland}). The resulting immune complex was visualized with \( \text{CyTM3-conjugated F(ab')}^2 \) fragment goat anti-mouse IgG \((\text{Dianova, Hamburg, Germany)}\).

GABA\(_A\) receptor immunofluorescence. Immunochemistry for the \( \alpha_2 \) subunit of GABA\(_A\) receptors was used to localize GABA\(_A\) receptors in the neuronal membrane. After fixation with precooled methanol for 10 min at \(-20^\circ \text{C}, cells were washed with PBS, blocked with 4% normal serum, and incubated with a polyclonal guinea pig anti-\( \alpha_2 \) subunit antiserum \((\text{Dr. M. Fritschy, Department of Pharmacology, University of Zurich, Zurich, Switzerland}). The resulting immune complex was visualized with \( \text{CyTM3-conjugated F(ab')}^2 \) fragment goat anti-guinea pig IgG \((\text{Dianova, Hamburg, Germany)}\).

Confocal microscopy. Neurons were imaged using a Bio-Rad \((\text{Hercules, CA})\) MRC 1024 (version 3.2) confocal system with a krypton–argon laser and a Zeiss \((\text{Oberkochen, Germany})\) Axiovert 135TV microscope. \( \text{CyTM3 fluorescence was measured using an excitation wavelength of 554 nm and an emission objective lens set at 570 nm}. \) A 6% water objective lens was used, and the laser intensity, photomultiplier gain, and pinhole aperture were kept constant for all experiments. Images were obtained using Laser Sharp 2.1T software and processed using Corel Photopaint.

Data evaluation. Current amplitudes were measured at the peak response (average of 10 data points). The current amplitudes at 10 and 25 min were expressed as percent of controls, i.e., the response at 0 min \( (\text{mean ± SEM of n trials}). \) Differences between means were tested for significance with a \( \text{Kruskal–Wallis} \) test followed by the Mann–Whitney \( U \) test. In some cases, a modified \( t \) test \((\text{Bonferroni–Dunn}) \) was used for multiple comparisons was used. \( p < 0.05 \) was the accepted level of significance.

Materials. Muscimol was from Tocris Cookson \((\text{Bristol, UK}). \) If not mentioned otherwise, all drugs and agents used were from Sigma.
pipette solution largely prevented the time-dependent decrease (Fig. 1B,C). The current amplitudes were now 81.0 ± 4.0% of controls after 25 min of repetitive stimulation (n = 7; p > 0.05). All further experiments were performed with 4 mM Mg-ATP in the patch pipette.

Although these findings confirmed that intracellular ATP is necessary for maintaining GABA<sub>A</sub> receptor function (Gyenes et al., 1988, 1994; Stelzer et al., 1988; Sweetnam et al., 1988; Chen et al., 1990), they also emphasized the difference between GABA<sub>A</sub> and GABA<sub>B</sub> receptors, because the latter show an ATP-induced decrement in current amplitude (Filippova et al., 1999). GABA<sub>A</sub> receptor channels can undergo desensitization, which has been defined on the macroscopic level as current decay in the presence of an agonist (Jones and Westbrook, 1995; Berger et al., 1998). We also considered the possibility that the decrease in GABA<sub>A</sub> receptor currents observed during repetitive stimulation was attributable to cumulative desensitization, which trapped a progressively increasing fraction of GABA<sub>A</sub> receptor channels in the nonconductive state. In our experiments, currents desensitized by ~10% at the beginning of repetitive stimulation and after 25 min (Table 1). Moreover, the extent of desensitization was independent of the absence or presence of intracellular Mg-ATP, which strongly affected rundown. Also, neurons stimulated either 51 times or only twice showed the same extent of desensitization (Table 1, Fig. 1C). Hence, current desensitization and current rundown seemed to be independent of each other.

**Microtubules are involved in the maintenance of GABA<sub>A</sub> receptor activity**

First, we tested the possible role of tubulin structures in GABA<sub>A</sub> receptor activity. Because colchicine can act as a competitive antagonist at GABA<sub>A</sub> receptors, we used nocodazol to destroy the microtubules (Samson et al., 1979; Weiner et al., 1998). Nocodazol (2 μM) had no effect when applied into neurons via the patch pipette for 20 min before and during the experiment. After 25 min of repetitive stimulation with muscimol, the current peak amplitudes were 73.5 ± 2.9% of the control value measured at 0 min (n = 6) (Fig. 2A). To evaluate the destructive effect of nocodazol on the microtubules, we used immunocytochemistry for β-tubulin III. When neurons were treated with 10 μM nocodazol for 30 min, no change of the tubular cytoskeleton was observed (Fig. 2C). When cells were preincubated with 10 μM nocodazol for 4 hr, however, the microtubular network was completely destroyed (Fig. 2D). Under these conditions, the muscimol-induced currents showed a pronounced rundown (21.9 ± 3.4% of control; n = 6) (Fig. 2A). These findings indicated that intact microtubules were essential for the activity of GABA<sub>A</sub> receptors.

**Actin filaments are also necessary for GABA<sub>A</sub> receptor activity**

Next, we tested whether actin fibers affected the activity of GABA<sub>A</sub> receptors. The C2 toxin of *C. botulinum* selectively ADP-ribosylates actin monomers and thereby prevents their polymerization (Aktories et al., 1986; Vandenkerckhove et al., 1988; Aktories and Wegner, 1992). Because actin filaments are continuously rebuilt, C2 toxin ultimately causes their breakdown. When applied

**Table 1. Effects of ATP and lethal toxin on desensitization of GABA<sub>A</sub> receptor channels in rat hippocampal neurons**

<table>
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<tr>
<th>Pipette</th>
<th>Desensitization (%)</th>
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<tr>
<td></td>
<td>T&lt;sub&gt;0&lt;/sub&gt;</td>
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<tr>
<td>No ATP</td>
<td>10.0 ± 2.0</td>
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<tr>
<td>No ATP</td>
<td>11.0 ± 2.0</td>
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<tr>
<td>ATP</td>
<td>9.5 ± 2.2</td>
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<tr>
<td>ATP + lethal toxin</td>
<td>9.4 ± 3.6</td>
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Muscimol (1 μM) was pressure applied for 3 sec to cultured rat hippocampal neurons, and current amplitudes were measured at the peak response, as well as at the end of the 3 sec application period by means of the whole-cell patch clamp technique. Starting 20 min after gaining whole-cell access (T<sub>0</sub>), muscimol was applied either regularly at every 30 sec over 25 min or twice, once at the beginning (T<sub>10</sub>) and once at the end (T<sub>25</sub>) of the experiment. The extent of current desensitization is shown at the start of the experiment (T<sub>0</sub>) and 10 (T<sub>10</sub>), 20 (T<sub>20</sub>), and 25 (T<sub>25</sub>) min after the first agonist application. It is expressed as the percentage by which current amplitudes decayed within the 3 sec of muscimol application. Data were obtained from the experiments shown in Figures 1 and 3. Mean ± SEM are shown. The desensitization values did not differ.

**Figure 2.** Microtubules, as well as actin filaments, are involved in the maintenance of GABA<sub>A</sub> receptor activity in rat hippocampal neurons. A, Time course of GABA<sub>A</sub> receptor current rundown after repetitive stimulation with muscimol. Pipette solutions contained Mg-ATP (4 mM) alone, Mg-ATP plus C2 toxin (10 ng/ml), Mg-ATP plus latrunculin B (2 μM), and Mg-ATP plus nocodazol (2 μM, open triangles; 10 μM, filled triangles). Parameters for repetitive stimulation with 1 μM muscimol correspond to those of Figure 1. In the experiment with 10 μM nocodazol, the culture was also preincubated with 10 μM nocodazol for at least 4 hr. Currents were expressed as percent of the first muscimol application at T<sub>0</sub> (n = 5–6 each; means ± SEM). Immunohistochemistry for β-tubulin III in hippocampal neurons in controls (B), after incubation with nocodazol (10 μM) for 0.5 hr (C), and for 4 hr (D).
via the patch pipette, C2 toxin (10 ng/ml) reduced the current amplitudes induced by repetitive stimulation with muscimol. After 10 min of stimulation, the current was reduced to $45.2 \pm 5.9\%$ of the initial value. After 25 min, it declined further to $33.4 \pm 3.9\%$ ($n = 6$) (Fig. 2A). In contrast, in controls, the current was only reduced to $83.7 \pm 5.1\%$ of the initial value after 25 min of stimulation ($n = 5$) (Fig. 2A). Next, we used latrunculin B, which also prevents actin polymerization. Within 25 min of repetitive stimulation with muscimol, latrunculin B (2 \(\mu\)M) reduced the current to $42.3 \pm 5.4\%$ of the initial value ($n = 6$) (Fig. 2A). Together, these findings indicated that actin filaments were essential for the activity of the GAB\(A\)\(_A\) receptors.

The GTPase Rac1 maintains the GAB\(A\)\(_A\) receptor activity

GTPases of the Rho family control the polymerization of actin but do not affect tubulin fibers (Hall, 1994, 1998; Mackay et al., 1995; Nobes and Hall, 1995; Best et al., 1996). To test whether Rho GTPases regulate the function of GAB\(A\)\(_A\) receptors, we used toxin B from C. difficile, which inactivates Rho, Rac1, and Cdc42 (Just et al., 1995). When applied via the patch pipette, toxin B reduced within 25 min the muscimol-induced current amplitudes to $25.9 \pm 4.2\%$ ($n = 7$) (Fig. 3A,D), whereas in controls, the currents were reduced to only $88.7 \pm 2.1\%$ ($n = 6$) (Fig. 3D). Not only was the extent of the toxin B-induced reduction similar to the effects of C2 toxin and latrunculin B, but so was the time course (compare with Fig. 2). Apparently, Rho GTPases were indeed involved in maintaining the activity of GAB\(A\)\(_A\) receptors.

To test whether the toxin B-induced current reduction depended on the repetitive stimulation of the GAB\(A\)\(_A\) receptors, we applied muscimol only twice, i.e., at the beginning and at the end of the 25 min period. Under these conditions, the second current amplitude was $78.5 \pm 4.1\%$ ($n = 6$) of the initial value, despite the presence of toxin B in the cytosol (Fig. 3D). Thus, toxin B only decreased the currents if the GAB\(A\)\(_A\) receptors were repetitively stimulated.

Next, we applied toxin B (5 ng/ml) together with latrunculin B (2 \(\mu\)M), to test whether toxin B had effects that were independent of the actin fibers. The combined application reduced the muscimol currents within 25 min to $24.7 \pm 5.1\%$ of the initial value ($n = 6$) (Fig. 3D). This decrease was not significantly different from the reduction observed with toxin B alone (Fig. 3).

Because toxin B inactivates Rho, Rac1, and Cdc42, we next tried to identify the GTPase involved. The possible role of Rho A was tested with C3 toxin from C. botulinum, which selectively inactivates this GTPase (Aktories et al., 1989). However, C3 toxin did not reduce the muscimol-induced currents ($76.5 \pm 4.6\%$ after 25 min of repetitive stimulation; $p > 0.05$; $n = 7$) (Fig. 3B,D). Because Rac1 or Cdc42 seemed to be involved, we next applied the lethal toxin of C. sordellii, which is known to inactivate these two GTPases (Just et al., 1996). Indeed, in the presence of lethal toxin, the GAB\(A\)\(_A\) receptor-mediated current declined during repetitive stimulation with muscimol (Fig. 3C,D). It was $20.8 \pm 5.8\%$ of the initial value at the end of the 25 min period (Fig. 3D), whereas the maximal reduction was observed after 15 min ($n = 7$) (Fig. 3D).

In contrast to its effects on current peak amplitudes, lethal toxin had no influence on the current decay during repetitive muscimol applications. Hence, inactivation of Rac1 and/or Cdc42 did not seem to interfere with GAB\(A\)\(_A\) receptor desensitization (Table 1).

Finally, we tested whether the lethal toxin (50 ng/ml) from C. sordellii affected the activity of the GAB\(A\)\(_A\) receptors during the usual waiting period before the stimulation. For this purpose, neurons from the same preparation were dialyzed with normal or lethal toxin containing pipette solution for 20 min in the whole-cell configuration. Thereafter, the cells were stimulated once with 1 \(\mu\)M muscimol. The current responses under these conditions were $-1426 \pm 320$ and $-1309 \pm 252$ pA, respectively ($n = 6$ each; $p > 0.05$). Together, these experiments showed that inactivation of the GTPases Rac1 and/or Cdc42 by clostridial toxins reduced GAB\(A\)\(_A\) receptor activity during the period of repetitive stimulation.

Figure 3. Inactivation of Rho GTPases by clostridial toxins induces a use-dependent GAB\(A\)\(_A\) receptor rundown in rat hippocampal neurons. The dotted lines indicate the periods of intracellular toxin pressure application; the dotted lines indicate the zero current level. The solid, horizontal lines above the current traces indicate the periods of muscimol pressure application. A–C. Repetitive inward currents evoked at $T_0$ and $T_{25}$. Toxins were added to pipette solutions containing 4 mM Mg-ATP. A. Toxin B (5 ng/ml); B, C3 toxin (6 \(\mu\)g/ml); C, lethal toxin (50 ng/ml). Parameters for repetitive stimulation with 1 \(\mu\)M muscimol correspond to those of Figure 1. The solid, horizontal lines above the current traces indicate the periods of muscimol pressure application; the dotted lines indicate the zero current level. D. Grouped mean data showing the time course of GAB\(A\)\(_A\) receptor current rundown. Filled squares show effects of muscimol (1 \(\mu\)M) applied only twice, i.e., at $T_0$ and $T_{25}$, in the presence of intracellular toxin B. Currents were expressed as percent of the first muscimol application at $T_0$ ($n = 6$–7; means ± SEM).

Because there are no toxins available that selectively inactivate Rac1 or Cdc42, a different approach was used to determine which of the two GTPases regulate the GAB\(A\)\(_A\) receptors. We applied recombinant GTPase proteins via the patch pipette together with 0.3 mM GTP-\(\gamma\)S, which is necessary for their activation. After 25 min of stimulation with muscimol, currents were $81.5 \pm 3.5\%$ ($n = 5$) of the initial value in the presence of intracellular Mg-ATP alone. When GTP-\(\gamma\)S (0.3 mM) was added, they declined to $78.4 \pm 5.4\%$ ($n = 6$; $p > 0.05$). Recombinant Cdc42 (100 ng/ml) also had no effect on the muscimol-induced currents ($72.5 \pm 6.5\%$ after 25 min of stimulation; $n = 6$) (Fig. 4). However, 100 ng/ml recombinant Rac1 significantly elevated the currents as a result of repetitive stimulation. An increase over the initial value was observed within 5 min of stimulation (Fig. 4). After the 25 min stimulation period, the current was still $114.8 \pm 4.2\%$ of the initial value [compared with $81.5 \pm 3.5\%$ in controls ($p < 0.05$; $n = 7$)] (Fig. 4). In contrast, application of a dominant inactive form of recombinant Rac1 reduced the current to $43.8 \pm 12.9\%$ ($p < 0.05$) of the initial value ($n = 6$) (Fig. 4). Together, these findings indicated that Rac1 was involved in the control of GAB\(A\)\(_A\) receptor activity.
Inactivation of Rac does not affect GABA<sub>A</sub> receptor affinity for muscimol

The inactivation of Rac1 by clostridial toxins may diminish GABA<sub>A</sub> receptor activity by disturbing ligand–receptor interactions or the resulting downstream events. Next, we tested whether inactivation of Rac1 by lethal toxin affected the ligand–receptor interaction. For this purpose, two concentration–response curves with muscimol were made in the presence of intracellular lethal toxin (50 ng/ml). The first curve was done at the beginning of the stimulation period when the effect of the lethal toxin was not yet observed. The second curve was made at the end of the usual 25 min period of repetitive stimulation, when the effect of the lethal toxin was pronounced (rundown to 32.4 ± 8.4% after 25 min) (Fig. 5A, inset). Muscimol concentrations of 1, 10, and 100 μM were chosen because they represented the EC<sub>50</sub> (1 μM), as well as maximal and supramaximal concentrations. Before and after repetitive stimulation, muscimol concentrations of 10 and 100 μM produced maximal responses, the absolute values of which differed. In both curves, 1 μM muscimol caused a half-maximal response, i.e., the EC<sub>50</sub> was independent of treatment with lethal toxin. However, lethal toxin plus repetitive stimulation lowered the current amplitudes induced by 10 or 100 μM muscimol to ~40% of the respective pretreatment values (n = 5) (Fig. 5A). Thus, a reduction in GABA<sub>A</sub> receptor affinity was not the reason for the observed rundown.

Next, we tested whether the inactivation of Rac1 by lethal toxin affected the driving force through open GABA<sub>A</sub> channels, which is one determinant of the GABA<sub>A</sub> receptor-mediated peak current. This parameter is determined by the difference between membrane potential V (voltage-clamped at −70 mV) minus the reversal potential for the current, V<sub>rev</sub>. When fast voltage ramps were used to measure the V<sub>rev</sub> of muscimol-induced currents during the repetitive stimulation protocol in the presence of intracellular lethal toxin (50 ng/ml) (Fig. 5B), V<sub>rev</sub> did not change; at the start it was −6.2 ± 3.4, and at the end of the repetitive stimulation it was −5.6 ± 2.5 mV. Under these conditions, the muscimol-induced currents showed the usual rundown (to 35.9 ± 10.0%; n = 5) (Fig. 5B, inset). Thus, inhibition of Rac1 did not alter the driving force through open GABA<sub>A</sub> receptor channels, suggesting that the electrochemical Cl<sup>−</sup> gradient over the cell membrane, as well as the selective Cl<sup>−</sup> permeability of the channel pore, remained unchanged.

GABA<sub>A</sub> receptor clusters in the neuronal membrane depend on the cytoskeleton

Continuous stimulation with GABA<sub>A</sub> receptor agonists has been shown to reduce the current amplitude and, in addition, receptor density in the plasma membrane (Tehrani and Barnes, 1991). Therefore, we investigated with use of GABA<sub>A</sub> receptor immunocytochemistry whether the nocodazol and lethal toxin affected the...
tors, which contain the a2 subunit of GABA<sub>A</sub> receptors after destruction of microtubular or actin cytoskeleton with or without subsequent muscimol application. A, Controls; B, muscimol (1 μM for 25 min); C, nocodazol (10 μM for 4 hr); D, lethal toxin (50 ng/ml for 1 hr); E, nocodazol (10 μM for 4 hr) plus subsequent muscimol (1 μM for 25 min); F, lethal toxin (50 ng/ml for 1 hr) plus subsequent muscimol (1 μM for 25 min). Scale bar, 25 μm.

Figure 6. Nocodazol and lethal toxin of C. sordellii enhance the reduction in GABA<sub>A</sub> receptor clusters induced by repetitive stimulation with muscimol. Immunohistochemistry for a2 subunit of GABA<sub>A</sub> receptors, which contain the a2 subunit (Essrich et al., 1998; Kanno et al., 1999), was used as a respective antiserum. Untreated hippocampal neurons showed granular immunoreactivity in dendritic and somatic membranes (Fig. 6A). When the neurons were treated for 25 min with 1 μM muscimol, the number of these granules appeared to be reduced (Fig. 6B). In contrast, treatment of the neurons with lethal toxin for 1 hr did not reduce the number of GABA<sub>A</sub> receptor clusters (Fig. 6D). After pretreatment with lethal toxin and subsequent stimulation with muscimol, however, hardly any clusters were observed in the somatic membrane (Fig. 6F). To test the involvement of microtubules in GABA<sub>A</sub> receptor organization, a respective experiment was performed with nocodazol. Treatment of the neurons with nocodazol for 4 hr did not affect the clusters (Fig. 6C), whereas the subsequent treatment with muscimol strongly reduced the number of receptor clusters in the somatic membrane (Fig. 6E). Together, these data showed that destruction of the actin, as well as of the microtubular cytoskeleton alone, did not affect the number of receptor clusters in the somatic membrane, although it facilitated the muscimol-induced reduction.

Lethal toxin of C. sordellii also affects GABA<sub>A</sub> receptors in perforated patches

A relatively high negative pressure is necessary to disrupt the cell membrane to gain whole-cell access. Consequently, cytoskeletal structures may be disturbed (Rosenmund and Westbrook, 1993), which may affect the activity of GABA<sub>A</sub> receptors in an unspecified manner. In addition, cytosolic components may be lost by diffusion, when the whole-cell patch mode is applied. Such a loss of ATP may have contributed to the decreased response to muscimol observed in Figure 1. Because the cytoskeleton is much less disturbed in the perforated patch configuration, we used this procedure for further analysis. Amphotericin was applied to form membrane pores that allow the diffusion of small ions but not of larger molecules (Cass et al., 1970; Holz and Finkelstein, 1970). In such hippocampal neurons, there was no decrease in muscimol-induced current when cells were stimulated only twice (96.3 ± 9.6%; n = 7) (Fig. 7D). Repetitive stimulation with 1 μM muscimol, however, reduced the current within 25 min to 43.4 ± 3.2% of the initial value (n = 5) (Fig. 7D).

To also test the possible involvement of Rac1 in this model, we used insulin, which has been reported to activate Rac1 (Nishiyama et al., 1994). Incubation of the cells with insulin (10 μg/ml) for 12 hr before and during the actual experiment prevented the use-dependent reduction in muscimol-induced current (n = 5) (Fig. 7B,D). This effect of insulin was blocked when the cells were
additionally incubated with lethal toxin (50 ng/ml) for 60 min before the experiment (Fig. 7C,D). Under these conditions, the
musculin-induced current was 30.0 ± 7.6% of the initial value (n = 7). However, when insulin was added to the bath medium after 12.5
min of repetitive stimulation, i.e., when the rundown was already
established, it did not reverse the rundown (data not shown).

These results indicated that Rac1 also regulated GABA_{A} receptor
activity in cells in which the intracellular milieu, as well as the
cytoskeleton, remained relatively undisturbed.

**DISCUSSION**

The present study tested the hypothesis that microtubules and
microfilaments are relevant for the function of GABA_{A} receptors.
Hippocampal neurons were treated with nocodazole, which breaks
down microtubules. Latrunculin B and the C2 toxin of C. botulinum
were used to depolymerize F-actin and thus break down cytoskel-
etal actin fibers (Spector et al., 1983, 1989; Aktories et al., 1986;
Alliston et al., 1998). After these treatments, musculin-induced
currents showed a rundown of 60–82% during 25 min of repetitive
stimulation. Together, these data show that the function of
GABA_{A} receptors is connected to microtubules, as well as micro-
filaments. These actin fibers seem to be under the control of the
small GTPase Rac1, inactivation of which also facilitated the
musculin-induced rundown of GABA_{A} receptor activity. In view of
our previous finding that glutamate receptors of the NMDA
type are functionally coupled to RhoA-dependent actin fibers
(Nörenberg et al., 1999), we present the first evidence that different
GTPases of the Rho family may regulate different types of ligand-
gated ion channels.

Until now, there was only indirect evidence that the activity of
GABA_{A} receptors is affected by the cytoskeleton. The lack of
direct evidence is surprising because microtubular depolymeriza-
tion has been shown previously to inhibit the musculin-induced
uptake of Ca^{2+} into cerebral cortical microsacs (Whatley et al.,
1994). Moreover, biochemical data suggest a connection between
GABA_{A} receptors and tubulin via the linker proteins gephyrin and
GABARAP (Item and Sieghart, 1994; Essrich et al., 1998; Wang et
al., 1999). The previous lack of electrophysiological data on recep-
tor activity may be attributable to the kinetics of tubular reorgani-
zation in neurons. Thus, destruction of the microtubules could not be
induced by a 30 min but only by a 4 hr pretreatment with nocodazole,
which indeed facilitated the musculin-induced rundown.

Data on the connections of actin filaments with GABA_{A} recep-
tors are also sparse. Here, biochemical findings show that actin
coprecipitates with α1 subunits of GABA_{A} receptors extracted from
bovine brain (Kannenberg et al., 1997). It is unknown, how-
ever, whether other α subunit isoforms, such as the α2 subunit
found in rat hippocampal neurons (Essrich et al., 1998), are also
coupled to actin filaments. Thus, to our knowledge, the present
data are the first evidence that GABA_{A} receptor activity is coupled
to actin fibers.

The GTPases of the Rho family RhoA, Rac1, and Cdc42 regu-
late the organization of actin fibers in cells. Toxin B from C. difficile
and lethal toxin from C. sordellii inactivate the GTPases via glu-
cosylation and galactosylation, respectively (Just et al., 1995, 1996).
Toxin B inhibits all GTPases of the Rho family, whereas lethal
toxin blocks Rac1 and Cdc42. Both toxins facilitated the rundown
of GABA_{A} receptor activity. When toxin B was applied together
with latrunculin B, there was no synergistic effect. Therefore,
we conclude that inactivation of the GTPase, which was subsequently
identified as Rac1, induced the rundown of GABA_{A} receptor
activity via degradation of microfilaments. This identification was
performed with recombinant Rho proteins. Upon intracellular
application via the patch pipette, the wild-type form of Cdc42
proved to be inactive, whereas that of Rac1 enhanced the current
induced by musculin. Some Rho GTPase effector molecules, such as
proteins of the PAK family or p70^{S6K}, seem to be shared by Rac1
and Cdc42 (for review, see Ridley, 1996). The selective effect of
Rac1 indicated, however, that these effectors were not involved in
the control of GABA_{A} receptor activity. The additional finding
that the constitutively inactive form of Rac1 also decreased
GABA_{A} receptor activity corroborated the involvement of this
GTPase, because inactive forms of the GTPases can block the
respective endogenous proteins by binding and thereby trapping
GDP/GTP exchange factors, which are necessary for activation
(Seasholtz et al., 1999).

Several mechanisms were excluded by which Rac1 and the de-
pendent actin fibers may have reduced GABA_{A} receptor activity.
Thus, inactivation of Rac1 by lethal toxin decreased neither the
affinity of the GABA_{A} receptors to musculin nor the Cl^{-}
ion driving force through open GABA_{A} receptor channels. It also seems un-
likely that inactivation of Rac1 with lethal toxin induced a state of
cumulative receptor desensitization, which then caused the run-
down (Collins et al., 1998). Desensitization was not affected by the toxin.
It remains possible, however, that inactivation of Rac1 may have
reduced the unitary conductance of the channel and/or the channel
open probability. The latter mechanism is of particular interest in
view of reports that cytoskeletal elements and Rho GTPases can
regulate the open probability of some K^{+} channels (Benz et al.,
1998; Cachero et al., 1998), as well as of NMDA receptor channels
(Ehlers et al., 1996).

Inhibition of Rac1 with lethal toxin or destruction of actin or
tubulin fibers did not affect the GABA_{A} currents induced by the
initial pulses of musculin but enhanced the rundown caused by
repetitive stimulation with the agonist. Similarly, treatment of
hippocampal neurons with lethal toxin or nocodazole did not reduce
the density of GABA_{A} receptor clusters in the somatic membrane
as shown by immunocytochemistry (Allison et al., 1998) but facili-
tated the disappearance of receptor clusters caused by continuous
receptor stimulation with musculin. Thus, the disappearance of
GABA_{A} receptor clusters was related to the measured loss of
activity.

It is presently unknown whether disassembly of the clusters into
single receptors already caused their loss of activity. There is some
indirect evidence in favor of this speculation. Thus, GABA_{A}
receptors that lack the γ2 subunit do not cluster and show a reduced
conductance (Günther et al., 1995; Essrich et al., 1998). Moreover,
cluster formation depends on the presence of gephyrin in the
membrane, indicating that the cytoskeleton is involved (Essrich et
al., 1998). However, it cannot be excluded that receptor internal-
ization caused the disappearance of the clusters and the reduction
of receptor activity.

There is growing evidence that Rac1 and actin fibers are in-
volved in membrane organization and recycling (Bretschler, 1996;
Radhakrishna et al., 1999). The process of receptor recycling
seems to consist of two steps that are regulated separately, i.e.,
endocytosis of receptors and their subsequent reinsertion into the
membrane (exocytosis). Endocytosis of GABA_{A} receptors via
cellmembrane-coated vesicles is induced by repetitive stimulation
with an agonist and can result in a significant reduction in membrane-
lcoated GABA_{A} receptors, i.e., downregulation (Tehrani and Bar-
nes, 1991; Tehrani et al., 1997; Connolly et al., 1999). The present
data are in accord with the hypothesis that destruction of the actin
or tubulin cytoskeleton facilitated the musculin-induced receptor
endocytosis, resulting in the reduction of musculin-induced cur-
rents. This hypothesis would imply that an intact cytoskeleton
reduces receptor endocytosis.

Recently, it has been shown that GABA_{A} receptors are not only
internalized but also recycled (Filippova et al., 2000). Activation of
protein kinase C facilitates rundown of GABA_{A} receptor currents
and receptor downregulation after repetitive stimulation by inhib-
iting receptor exocytosis (Chapell et al., 1998; Connolly et al., 1999;
Filippova et al., 2000). It is doubtful that destruction of the cy-
toskeleton diminished receptor exocytosis in our experiments,
because inhibition of constitutive exocytosis should have resulted
in reduced receptor clusters already in the absence of musculin.

Endocytosis and exocytosis of membrane proteins, such as the
Na^{+}/K^{+} exchanger and/or the transferrin receptor, are under the
control of phosphatidylinositol 3-kinase (PI3-K) (Marty et al.,
1996; Kurashima et al., 1998). This has been also shown for
GABA<sub>A</sub> receptors (Connolly et al., 1999). Because PI3-K is an effector of Rac1 (Hartwig et al., 1995; Tolias et al., 1995), these findings present indirect evidence for a control of the GABA<sub>A</sub> receptor recycling by the GTPase. There is further evidence for such a role of Rac1. Insulin, which activates Rac1 (Nishiyama et al., 1997), has been shown to recruit functional GABA<sub>A</sub> receptors into the membrane within 10 min of application (Wan et al., 1997). In our perforated patch experiments, pretreatment with insulin prevented the use-dependent reduction in current. This effect was abolished by lethal toxin. However, insulin did not reverse the use-dependent reduction once it was established, indicating that receptor exocytosis did not occur within the observation period.

In contrast to GABA<sub>A</sub> receptors, NMDA receptors do not seem to be controlled into thin lipid membranes (Carroll et al., 1999). This observation may explain our previous finding that NMDA receptor activity is independent of Rac1 activity. Instead, it is functionally coupled to RhoA-dependent actin fibers (Nörenberg et al., 1999), which may provide anchorage within the membrane (Rosenmund and Westbrook, 1993).

Our previous finding that Rac1 is strongly expressed in pyramidal neurons of the hippocampus of the adult rat (Olenik et al., 1997) indicates that the GTPase may have important functions in differentiated neurons. The present findings suggest that Rac1 is involved in the control of GABA<sub>A</sub> receptor density and function. If the GTPase indeed regulates GABA<sub>A</sub> receptor clustering and/or endocytosis, other receptors that undergo recycling could also be under the control of Rac1. Such a role requires that the GTPase itself is regulated in its activity. Among several candidates, insulin deserves some attention because it is found in relevant concentrations in the brain (Havrankova et al., 1978, 1979).
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