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 μ M muscimol. After destruction of the microtubular system with nocodazol, 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; Wyszynski 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., 1995; Nobes and Hall, 1995). Thus, RhoA controls the formation of cytosolic actin fibers, whereas Rac1 organizes the formation of the cortical actin. Indeed, NMDA receptors in cultured hippocampal neurons showed a rapid rundown after the specific inactivation of RhoA by the C3 toxin of *C. botulinum* (Aktories et al., 1989; Nörenberg et al., 1999).

Glycine-gated anion channels are anchored in the membrane via microtubules and the linker protein gephyrin (Prior et al., 1992; Kirsch and Betz, 1995), whereas actin fibers organize the density of the receptor clusters (Kirsch and Betz, 1995). Apparently, type A receptors for GABA also bind to gephyrin. Thus, the protein has been found at GABAergic postsynaptic membranes (Sassoe-Pognetto et al., 1995; Todd et al., 1995; Craig et al., 1996). Moreover, GABA_A receptor subtypes that contain the γ 2 subunit form clusters with the help of gephyrin (Essrich et al., 1998). In addition,

the protein GABARAP, which is similar or even identical to microtubule-associated protein 1B, seems to connect GABA_A and GABA_C receptors to microtubules (Hanley et al., 1999; Wang 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 α 1 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 a humidified atmosphere of 95% air and 5% CO₂ 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): NaCl 162, KCl 2.4, CaCl₂ 1.2, MgCl₂ 1, HEPES 10, and glucose 11 (~320 mOsm, pH 7.3, with NaOH). Tetrodotoxin (0.5 μ M) was added to inhibit action potentials. The pipette (internal) solution contained (in mM): CsCl 140, CaCl₂ 1, MgCl₂ 2, HEPES 10, and EGTA 11 (pH 7.2, ~300 mOsm). The calculated free Ca²⁺ concentration was 11 nM (Program WinMAXC;

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C. Patton, Stanford University, Pacific Grove, CA). All solutions were used at room temperature. All membrane potential values were corrected for the liquid junction potential (5 mV) (Barry, 1994). For amphotericin-perforated patches, patch pipettes were front-filled by dipping for ~1 sec in filtered pipette solution. Thereafter, they were back-filled with the pipette solution that contained 240 $\mu\text{g/ml}$ amphotericin B. The borosilicate patch pipettes (GB 150–8P; Science Products, Hofheim, Germany) had a resistance of 1.5–3.5 M Ω , when filled with pipette solution.

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, i.e., 20 min after gaining whole-cell access, control settings were 29.0 ± 1.0 pF for C_m and 12.4 ± 0.4 M Ω 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 pF and 12.2 ± 0.8 M Ω ; $n = 24$). Again, recording conditions remained stable over time. Current records were filtered at 3 kHz, digitized at 1 kHz (CED 1401; 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 diffusion 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 μM) was applied by means of a fast-flow pressurized superfusion system (DAD-12; Adams and List, New York, NY), which completely exchanges the bath medium in the vicinity of the investigated cells within <200 msec (Kügelgen et al., 1997).

Preparation of clostridial cytotoxins and recombinant proteins. Toxin B and lethal toxin from *C. difficile* and *C. sordellii*, respectively, were prepared as described previously (Hofmann et al., 1997). C2I protein, the enzymatic component of C2 toxin from *C. botulinum*, and C3 toxin from *C. limosum* were purified as described previously (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 (Rac1N17), and wild-type Cdc42 (Cdc42) were subcloned into the pGEX-2T vector (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 *Escherichia coli* BL21 cells. After induction of the expression with 100 μM isopropyl- β -D-thiogalactopyranoside (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 $\times$ g, and GST fusion proteins were purified from the supernatants on glutathione Sepharose 4B (Amersham Pharmacia Biotech) according to the instructions of the manufacturer. Cleavage of the desired proteins from the immobilized GST was achieved by incubating the sample with the protease thrombin (Sigma). Thereafter, thrombin was removed from the eluted protein by absorption onto *p*-aminobenzamidine-agarose. Finally the purified proteins were checked by SDS-PAGE.

Staining of microtubules. Immunocytochemistry for β -tubulin III was used to analyze microtubules. After fixation with paraformaldehyde (4%), cells were washed and incubated with a monoclonal mouse anti- β -tubulin III antibody (Sigma). The resulting immune complex was visualized with CyTM3-conjugated F(ab')₂ fragment goat anti-mouse IgG (Dianova, Hamburg, Germany).

GABA_A receptor immunofluorescence. Immunocytochemistry for the α 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°C, cells were washed with PBS, blocked with 4% normal serum, and incubated with a polyclonal pig anti- α 2 subunit antiserum (Dr. M. Fritschy, Department of Pharmacology, University of Zurich, Zurich, Switzerland). The resulting immune complex was visualized with CyTM3-conjugated F(ab')₂ fragment goat anti-guinea pig IgG (Dianova, Hamburg, Germany).

Confocal microscopy. Neurons were imaged using a Bio-Rad (Hercules, CA) MRC 1024 (version 3.2) confocal system with a krypton–argon laser and a Zeiss (Oberkochen, Germany) Axiovert 135TV microscope. CyTM3 fluorescence was measured using an excitation wavelength of 554 nm and an emission filter set at 576 nm. A 63 $\times$ water objective lens was used, and the laser intensity, photomultiplier gain, and pinhole aperture were kept constant for all experiments. Images were obtained using LaserSharp 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 (mean $\pm$ SEM of n trials). Differences between means were tested for significance by the Kruskal–Wallis test, followed by Mann–Whitney *U* test. In some cases, a modified *t* test (Bonferroni–Dunn) for multiple comparisons was used, $p < 0.05$ was the accepted level of significance.

Materials. Muscimol was from Tocris Cookson (Bristol, UK). If not mentioned otherwise, all drugs and agents used were from Sigma.

Figure 1. Intracellular ATP prevents rundown of GABA_A receptor currents in rat hippocampal neurons during repetitive stimulation. Inward currents were evoked by 3 sec pressure application of 1 μM muscimol at 30 sec intervals over 25 min. *A, B*, Superimposed inward currents after the first (T_0) and last (T_{25}) application of muscimol. Currents were recorded at a holding potential of –70 mV with pipette solutions without (*A*) or with (*B*) 4 mM Mg-ATP. The solid, horizontal lines above the current traces indicate the periods of muscimol pressure application; the dotted lines indicate the zero current level. *C*, Grouped mean data showing the time course of GABA_A receptor current rundown. Currents were expressed as percent of the first muscimol application at T_0 ($n = 7$ –8; means $\pm$ SEM). The filled squares show the effects of muscimol (1 μM) when applied only twice, i.e., at T_0 and T_{25} .

RESULTS

Neurons with pyramidal morphology were used for the experiments (see Fig. 2*B*). In all hippocampal neurons tested ($n = 185$), a concentration of muscimol >0.03 μM elicited inward currents at a holding potential of –70 mV. This holding potential was used in all experiments. The peak response to the first challenge with 1 μM muscimol ranged from –587 to –3379 pA in cells from different preparations but rarely differed in neurons from the same batch.

As was to be expected for GABA-gated Cl[–] channels, the current reversal potential (V_{rev}) was -4.2 ± 2.1 mV ($n = 5$) and thus close to chloride equilibrium potential (–3.7 mV). Furthermore, V_{rev} responded to changes in the external Cl[–] concentration like a Nernstian Cl[–] electrode (data not shown).

The inward currents induced by muscimol were concentration-dependent and reached a plateau at ~ 10 μM . The Hill slope was 1.2, and the EC_{50} was 0.8 μM ($n = 9$). The GABA_A receptor-selective antagonist bicuculline (10 μM) (Quian and Dowling, 1994; Bormann and Feigenspan, 1995) reduced the currents induced by 1 μM muscimol by $97.0 \pm 1.4\%$ ($n = 5$). Therefore, muscimol apparently activated GABA_A and not GABA_C receptors in the hippocampal neurons.

To test the stability of the GABA_A receptor currents, the cultured hippocampal neurons were repetitively stimulated with 1 μM muscimol for 25 min, i.e., twice per minute for 3 sec. In the absence of ATP in the patch pipette, there was a time-dependent decrease in GABA_A receptor currents (Fig. 1*A, C*). The current decreased to $47.4 \pm 5.5\%$ ($n = 8$) of the first response after 25 min of stimulation. A similar decline was found when cells were only stimulated twice, i.e., at the beginning and at the end of the 25 min period ($34.5 \pm 7.7\%$; $n = 7$) (Fig. 1*C*). Mg-ATP (4 mM) in the

Table 1. Effects of ATP and lethal toxin on desensitization of GABA_A receptor channels in rat hippocampal neurons

Pipette	Desensitization (%)			
	T_0	T_{10}	T_{20}	T_{25}
No ATP	10.0 ± 2.0	10.2 ± 1.7	10.0 ± 1.1	10.2 ± 1.6
No ATP	11.0 ± 2.0			11.3 ± 2.2
ATP	9.5 ± 2.2	10.2 ± 2.3	10.6 ± 2.4	10.6 ± 2.0
ATP + lethal toxin	9.4 ± 3.6	11.6 ± 2.7	11.5 ± 2.2	12.5 ± 3.1

Muscimol (1 μ M) 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_0), muscimol was applied either regularly at every 30 sec over 25 min or twice, once at the beginning (T_0) and once at the end (T_{25}) of the experiment. The extent of current desensitization is shown at the start of the experiment (T_0) and 10 (T_{10}), 20 (T_{20}), and 25 (T_{25}) 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 $\pm$ SEM are shown. The desensitization values did not differ.

pipette solution largely prevented the time-dependent decrease (Fig. 1*B,C*). The current amplitudes were now $81.0 \pm 4.0\%$ 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_A 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_A and GABA_C receptors, because the latter show an ATP-induced decrement in current amplitude (Filippova et al., 1999). GABA_A 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_A receptor currents observed during repetitive stimulation was attributable to cumulative desensitization, which trapped a progressively increasing fraction of GABA_A receptor channels in the nonconductive state. In our experiments, currents desensitized by $\sim 10\%$ 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. 1*C*). Hence, current desensitization and current rundown seemed to be independent of each other.

Microtubules are involved in the maintenance of GABA_A receptor activity

First, we tested the possible role of tubulin structures in GABA_A receptor activity. Because colchicine can act as a competitive antagonist at GABA_A receptors, we used nocodazol to destroy the microtubules (Samson et al., 1979; Weiner et al., 1998). Nocodazol (2 μ M) 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 $73.5 \pm 2.9\%$ of the control value measured at 0 min ($n = 6$) (Fig. 2*A*). To evaluate the destructive effect of nocodazol on the microtubules, we used immunocytochemistry for β -tubulin III. When neurons were treated with 10 μ M nocodazol for 30 min, no change of the tubular cytoskeleton was observed (Fig. 2*C*). When cells were preincubated with 10 μ M nocodazol for 4 hr, however, the microtubular network was completely destroyed (Fig. 2*D*). Under these conditions, the muscimol-induced currents showed a pronounced rundown ($21.9 \pm 3.4\%$ of control; $n = 6$) (Fig. 2*A*). These findings indicated that intact microtubules were essential for the activity of GABA_A receptors.

Actin filaments are also necessary for GABA_A receptor activity

Next, we tested whether actin fibers affected the activity of GABA_A receptors. The C2 toxin of *C. botulinum* selectively ADP-

Figure 2. Microtubules, as well as actin filaments, are involved in the maintenance of GABA_A receptor activity in rat hippocampal neurons. *A*, Time course of GABA_A 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 μ M), and Mg-ATP plus nocodazol (2 μ M, open triangles; 10 μ M, filled triangles). Parameters for repetitive stimulation with 1 μ M muscimol correspond to those of Figure 1. In the experiment with 10 μ M nocodazol, the culture was also preincubated with 10 μ M nocodazol for at least 4 hr. Currents were expressed as percent of the first muscimol application at T_0 ($n = 5$ –6 each; means $\pm$ SEM). Immunohistochemistry for β -tubulin III in hippocampal neurons in controls (*B*), after incubation with nocodazol (10 μ M) for 0.5 hr (*C*) and for 4 hr (*D*).

ribosylates actin monomers and thereby prevents their polymerization (Aktories et al., 1986; Vandekerckhove et al., 1988; Aktories and Wegner, 1992). Because actin filaments are continuously rebuilt, C2 toxin ultimately causes their breakdown. When applied

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 μM) 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 GABA_A receptors.

The GTPase Rac1 maintains the GABA_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 GABA_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 $85.7 \pm 2.1\%$ ($n = 6$) (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 GABA_A receptors.

To test whether the toxin B-induced current reduction depended on the repetitive stimulation of the GABA_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 GABA_A receptors were repetitively stimulated.

Next, we applied toxin B (5 ng/ml) together with latrunculin B (2 μM), 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 $24.7 \pm 5.6\%$ 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 GABA_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 GABA_A receptor desensitization (Table 1).

Finally, we tested whether the lethal toxin (50 ng/ml) from *C. sordellii* affected the activity of the GABA_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 μM muscimol. The current responses under these conditions were -1426 ± 320 and -1309 ± 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 GABA_A receptor activity during the period of repetitive stimulation.

Figure 3. Inactivation of Rho GTPases by clostridial toxins induces a use-dependent GABA_A receptor rundown in rat hippocampal neurons. *A–C*, Superimposed 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 $\mu\text{g/ml}$); *C*, lethal toxin (50 ng/ml). Parameters for repetitive stimulation with 1 μM 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 GABA_A receptor current rundown. Filled squares show effects of muscimol (1 μM) 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 $\pm$ 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 regulated the GABA_A receptors. We applied recombinant GTPase proteins via the patch pipette together with 0.3 mM GTP γ 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 γ 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 GABA_A receptor activity.

Figure 4. Effects of recombinant Rac1 on GABA_A receptor currents in rat hippocampal neurons. Pipette solutions contained Mg-ATP (4 mM) or Mg-ATP plus the recombinant GTPases Rac, its constitutively inactive form Rac_i together with GTPγS, and Cdc42 together with GTPγS (0.3 mM). Parameters for repetitive stimulation with 1 μM muscimol corresponded to those in Figure 1. Currents were normalized by comparison to the first muscimol application at T_0 ($n = 5$ –7; means $\pm$ SEM).

Inactivation of Rac does not affect GABA_A receptor affinity for muscimol

The inactivation of Rac1 by clostridial toxins may diminish GABA_A 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 $32.4 \pm 8.4\%$ after 25 min) (Fig. 5*A*, inset). Muscimol concentrations of 1, 10, and 100 μM were chosen because they represented the EC_{50} (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_{50} 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. 5*A*). Thus, a reduction in GABA_A 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_A channels, which is one determinant of the GABA_A 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_{rev} . When fast voltage ramps were used to measure the V_{rev} of muscimol-induced currents during the repetitive stimulation protocol in the presence of intracellular lethal toxin (50 ng/ml) (Fig. 5*B*), V_{rev} 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 $35.9 \pm 10.0\%$; $n = 5$) (Fig. 5*B*, inset). Thus, inhibition of Rac1 did not alter the driving force through open GABA_A receptor channels, suggesting that the electrochem-

Figure 5. *A*, Lethal toxin of *C. sordellii* does not affect the affinity of GABA_A receptors to muscimol. Half-maximal (1 μM), maximal (10 μM), and supramaximal (100 μM) muscimol concentrations were tested in neurons immediately before and after the rundown of GABA_A receptor currents (see arrows in inset), which was induced by intracellular lethal toxin (50 ng/ml) plus repetitive stimulation with 1 μM muscimol. Currents were reduced to $32.4 \pm 8.5\%$ after 25 min of stimulation. The resulting current amplitudes were normalized by comparison to the maximal response obtained with 100 μM muscimol in the respective individual experiments before induction of rundown. Shown are means $\pm$ SEM ($n = 5$). *B*, Lethal toxin of *C. sordellii* does not affect the reversal potential (V_{rev}) of GABA_A receptor-mediated currents. In this experiment, rundown of GABA_A receptor currents was also induced by intracellular lethal toxin (50 ng/ml) plus repetitive stimulation with 1 μM muscimol. A representative recording of five experiments shows how V_{rev} of GABA_A receptor-mediated currents was assessed (left panel). Fast voltage ramps, 200 msec from the holding potential of -70 to $+15$ mV, were applied immediately before every application of muscimol (T_0 to T_{25}), as well as at approximately each peak response to the agonist (T_0 to T_{25} ; bottom trace). Also shown are superimposed current traces obtained in response to the voltage-ramps imposed before (*I*) and during (*II*) the application of muscimol at the first (T_0) and last (T_{25}) challenge with muscimol (left panel, top trace). Under these conditions, the current was decreased to $35.9 \pm 10.0\%$ (inset; $n = 5$). The respective current–voltage (I – V) relationships are shown in the right panel. I – V curves obtained in response to the first (T_0) and last (T_{25}) challenge were superimposed. Curves were obtained by subtracting current responses to the voltage ramp in the absence of muscimol from those in its presence (II – I).

ical Cl[−] gradient over the cell membrane, as well as the selective Cl[−] permeability of the channel pore, remained unchanged.

GABA_A receptor clusters in the neuronal membrane depend on the cytoskeleton

Continuous stimulation with GABA_A 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_A receptor immunocytochemistry whether the nocodazol and lethal toxin affected the

Figure 6. Nocodazol and lethal toxin of *C. sordellii* enhance the reduction in GABA_A receptor clusters induced by repetitive stimulation with muscimol. Immunohistochemistry for $\alpha 2$ subunit of GABA_A receptors after destruction of microtubular or actin cytoskeleton with or without subsequent muscimol application. *A*, Controls; *B*, muscimol (1 μ M for 25 min); *C*, nocodazol (10 μ M for 4 hr); *D*, lethal toxin (50 ng/ml for 1 hr); *E*, nocodazol (10 μ M for 4 hr) plus subsequent muscimol (1 μ M for 25 min); *F*, lethal toxin (50 ng/ml for 1 hr) plus subsequent muscimol (1 μ M for 25 min). Scale bar, 25 μ m.

organization of GABA_A receptors in the plasma membrane. Because hippocampal neurons are known to express GABA_A receptors, which contain the $\alpha 2$ subunit (Essrich et al., 1998; Kannenberg et al., 1999), we used a respective antiserum. Untreated hippocampal neurons showed granular immunoreactivity in dendritic and somatic membranes (Fig. 6*A*). When the neurons were treated for 25 min with 1 μ M muscimol, the number of these granules appeared to be reduced (Fig. 6*B*). In contrast, treatment of the neurons with lethal toxin for 1 hr did not reduce the number of GABA_A receptor clusters (Fig. 6*D*). After pretreatment with lethal toxin and subsequent stimulation with muscimol, however, hardly any clusters were observed in the somatic membrane (Fig. 6*F*). To test the involvement of microtubules in GABA_A receptor organization, a respective experiment was performed with nocodazol. Treatment of the neurons with nocodazol for 4 hr did not affect the clusters (Fig. 6*C*), whereas the subsequent treatment with muscimol strongly reduced the number of receptor clusters in the somatic membrane (Fig. 6*E*). 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_A 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_A receptors in an unspecific

Figure 7. Insulin prevents the ATP-independent rundown of GABA_A receptor currents in rat hippocampal neurons. Inward currents were evoked by 3 sec pressure application of 1 μ M muscimol at 30 sec intervals over 25 min. Representative inward currents evoked at the holding potential of -70 mV in amphotericin B perforated patch recording configuration after first (T_0) and last (T_{25}) application of muscimol were superimposed and are shown in *A–C*. *A*, Control; *B*, insulin (10 μ g/ml) was present in the incubation medium during a 12 hr preincubation period, as well as during the actual experiment; *C*, protocol (*B*) was used, and in addition, lethal toxin (50 ng/ml) was added 1 hr before the start of the experiments. The solid, horizontal lines above the current traces indicate the period of muscimol pressure application; the dotted lines indicate the zero current level. The respective time courses of GABA_A receptor currents are shown in *D*. In addition, the effects of muscimol (1 μ M) are shown when it was applied only twice, i.e., at T_0 and T_{25} , in the absence of extracellular insulin (filled circles). Currents were normalized by comparison to the first muscimol application at T_0 ($n = 7$ each; means $\pm$ SEM).

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 ($96.3 \pm 9.6\%$; $n = 7$) (Fig. 7*D*). Repetitive stimulation with 1 μ M muscimol, however, reduced the current within 25 min to $43.4 \pm 3.2\%$ of the initial value ($n = 5$) (Fig. 7*A,D*).

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 μ g/ml) for 12 hr before and during the actual experiment prevented the use-dependent reduction in muscimol-induced current ($n = 5$) (Fig. 7*B,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 muscimol-induced current was $30.0 \pm 7.6\%$ 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 nocodazol, which breaks down microtubules. Latrunculin B and the C2 toxin of *C. botulinum* were used to depolymerize F-actin and thus break down cytoskeletal actin fibers (Spector et al., 1983, 1989; Aktories et al., 1986; Allison et al., 1998). After these treatments, muscimol-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 microfilaments. These actin fibers seem to be under the control of the small GTPase Rac1, inactivation of which also facilitated the muscimol-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 depolymerization has been shown previously to inhibit the muscimol-induced uptake of Cl[−] 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 receptor activity may be attributable to the kinetics of tubular reorganization in neurons. Thus, destruction of the microtubules could not be induced by a 30 min but only by a 4 hr pretreatment with nocodazol, which indeed facilitated the muscimol-induced rundown.

Data on the connections of actin filaments with GABA_A receptors are also sparse. Here, biochemical findings show that actin coprecipitates with $\alpha 1$ subunits of GABA_A receptors extracted from bovine brain (Kannenberg et al., 1997). It is unknown, however, whether other α subunit isoforms, such as the $\alpha 2$ 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 regulate the organization of actin fibers in cells. Toxin B from *C. difficile* and lethal toxin from *C. sordellii* inactivate the GTPases via glucosylation 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 muscimol. 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 dependent 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 muscimol nor the Cl[−] driving force through open GABA_A receptor channels. It also seems unlikely that inactivation of Rac1 with lethal toxin induced a state of cumulative receptor desensitization, which then caused the rundown, because 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 muscimol but enhanced the rundown caused by repetitive stimulation with the agonist. Similarly, treatment of hippocampal neurons with lethal toxin or nocodazol did not reduce the density of GABA_A receptor clusters in the somatic membrane as shown by immunocytochemistry (Allison et al., 1998) but facilitated the disappearance of receptor clusters caused by continuous receptor stimulation with muscimol. 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 $\gamma 2$ 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 internalization caused the disappearance of the clusters and the reduction of receptor activity.

There is growing evidence that Rac1 and actin fibers are involved in membrane organization and recycling (Bretscher, 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 clathrin-coated vesicles is induced by repetitive stimulation with an agonist and can result in a significant reduction in membrane-located GABA_A receptors, i.e., downregulation (Tehrani and Barnes, 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 muscimol-induced receptor endocytosis, resulting in the reduction of muscimol-induced currents. 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 inhibiting receptor exocytosis (Chapell et al., 1998; Connolly et al., 1999; Filippova et al., 2000). It is doubtful that destruction of the cytoskeleton diminished receptor exocytosis in our experiments, because inhibition of constitutive exocytosis should have resulted in reduced receptor clusters already in the absence of muscimol.

Endocytosis and exocytosis of membrane proteins, such as the Na⁺/H⁺ exchanger and/or the transferrin receptor, are under the control of phosphatidylinositol 3-kinase (PI3-K) (Martys et al., 1996; Kurashima et al., 1998). This has been also shown for

GABA_A receptors (Connolly et al., 1999). Because PI3-K is an effector of Rac1 (Hartwig et al., 1995; Tolia et al., 1995), these findings present indirect evidence for a control of the GABA_A receptor recycling by the GTPase. There is further evidence for such a role of Rac1. Insulin, which activates Rac1 (Nishiyama et al., 1994), has been shown to recruit functional GABA_A 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_A receptors, NMDA receptors do not seem to be cycled into and out of the synaptic membrane (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_A receptor density and function. If the GTPase indeed regulates GABA_A 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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