Glycine Receptors Involved in Synaptic Transmission Are Selectively Regulated by the Cytoskeleton in Mouse Spinal Neurons

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van Zundert, Brigitte, Francisco J. Alvarez, Gonzalo E. Yevenes, Juan G. Carcamo, Juan Carlos Vera, and Luis G. Aguayo. Glycine receptors involved in synaptic transmission are selectively regulated by the cytoskeleton in mouse spinal neurons. J Neurophysiol 87: 640–644, 2002; 10.1152/jn.00455.2001. Using whole cell patch-clamp recordings, we examined the effect of colchicine, a microtubule disruptor, on the properties of glycine receptors (GlyRs) in cultured spinal cord neurons. Confocal microscopy revealed that colchicine treatment effectively altered microtubule bundles and neuronal morphology. Application of colchicine via the culture media or the patch-pipette, however, did not affect the whole cell current rundown (73 ± 6% of control after 1 h), the sensitivity of the GlyR to glycine (EC50 = 29 ± 1 μM), or strychnine inhibition (47 ± 5% of control after 100 nM strychnine). On the other hand, colchicine dialyzed for 25 min via the patch pipette selectively reduced the quantal amplitude of spontaneous glycineric miniature inhibitory postsynaptic currents (mIPSCs) to 68 ± 5% of control. This effect was specific for GlyRs since synaptic events mediated by α-amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid (AMPA) and GABA(A) receptors were unchanged. In conclusion, this study indicates that microtubules can regulate the function of GlyRs involved in inhibitory synaptic transmission.

INTRODUCTION

Several studies have suggested the existence of a structural relationship between cytoskeletal elements and ligand-activated ion channels, such as the N-methyl-D-aspartate receptor (NMDA-R), α-amino-3-hydroxy-5-methyl-4-isoxazolopropionic acid receptor (AMPA-R), nACH receptor (nACH-R), and GABA(A) receptor (GABA(A)-R) (Allison et al. 1998; Shoop et al. 2000; Wang et al. 1999). Moreover, the state of cytoskeleton polymerization appears to play an important role in regulating NMDA-R and GABA(A)-R channel activity (Rosenmund and Westbrook 1993; Whatley et al. 1994). Thus, these studies showed that receptor-evoked current rundown and ligand sensitivity were acutely affected by alkaldoids known to disrupt selective elements of the cytoskeleton (Rosenmund and Westbrook 1993; Whatley et al. 1994). These results were interpreted as a cytoskeleton-dependent increase in receptor internalization and modifications on ligand affinity. Previous studies have analyzed the effect of cytoskeletal disrupters on the cellular localization of gephyrin, a GlyR-associated anchoring protein that binds directly to microtubules and is also indirectly associated to actin filaments (reviewed in Kneussel and Betz 2000). Disruption of microtubules was able to alter the organization of gephyrin and GlyR clusters in spinal neurons (Kirsch and Betz 1995), although gephyrin distribution in cultured hippocampal neurons was not affected by microtubule depolymerization (Allison et al. 2000). These results suggest that microtubules could regulate the function of the GlyR in certain neurons, but no data on the physiological properties of GlyRs in spinal neurons after microtubule disruption are available. Therefore to assess the role of microtubules on GlyR function, we induced microtubule depolymerization with colchicine and analyzed the current evoked by exogenous glycine (Iglycine). In addition, we analyzed the current obtained from the spontaneous release of this neurotransmitter by recording spontaneous miniature inhibitory postsynaptic currents (mIPSCs).

METHODS

Cell culture

Embryonic (13–14 days old) C57/BL mouse spinal cord neurons were cultured as previously described (Tapia and Aguayo 1998). Briefly, spinal neurons obtained from five or six embryos were plated at 300–350,000 cells/ml into 35-mm tissue culture dishes coated with poly-L-lysine (MW >350 kDa; Sigma). Serum containing media was changed every 3 days. The microtubule disrupter colchicine and its inactive analogue γ-lumicolchicine (Liebman 1986) were applied as previously reported (Johnson and Byerly 1993; Kirsch and Betz 1995; Rosenmund and Westbrook 1993). Colchicine (20 μM) was applied to the bath for 3 h before either fixing or recording the cells, or was internally dialized into single cells for 25 min via the internal solution of the patch pipette.

Immunostaining

Neurons were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 30 min and permeabilized with 0.25% Triton X-100 for 15 min. Microtubules were labeled with a monoclonal antibody against tubulin (1:1,000; clone DM1α, Sigma) and visualized with FITC (1:50; Jackson Labs). Samples from two experiments were analyzed using an Olympus Fluoview confocal microscope.

Electrophysiology

GLYCINE-EVOKED CURRENTS. Whole cell patch-clamp recordings in 5–12 days in vitro (DIV) neurons were performed and analyzed as
previously described (Tapia and Aguayo 1998). Briefly, the cell was clamped at −60 mV and rapid onset applications of glycine were used to activate GlyRs. Data were excluded if the uncompensated series resistance (<8 MΩ) or the leak current (<75 pA) increased by >15% during the long-term recordings. Patch electrodes were filled with (in mM): 140 KCl, 10 BAPTA, 10 HEPES (pH 7.4), 4 MgCl₂, and 2 ATP-Na₂. The external solution contained (in mM): 150 NaCl, 10 KCl, 2.0 CaCl₂, 1.0 MgCl₂, 10 HEPES (pH 7.4), and 10 glucose.

MINIATURE IPSCs. Spontaneous glycinergic mIPSCs were isolated by the addition of 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; 2 μM), bicuculline (2 μM), TTX (0.1 μM), and MgCl₂ (2 mM) to the external solution. Strychnine (750 nM) blocked all the events that remained after application of these two antagonists. Synaptic currents were recorded using Axotape 7.0 software (Axon Instruments, Union City, CA) for off-line analysis. Every identified synaptic event encountered during a 3-min period, and having an amplitude above the background noise (12–15 pA), was analyzed with MiniAnalysis 5.0 software (Synaptosoft, Leonia, NJ). Cursors were automatically set to measure the peak amplitude and rise-time and decay-time constants. To build the amplitude histogram, the amplitudes of six cells were arranged into 1-pA bins and combined to obtain a single histogram. To analyze whether this alkaloid causes alterations on its subunit composition and GlyR was previously suggested (Kirsch and Betz 1995). The amplitude of the glycine-activated Cl⁻ current ran down by 10.2 ± 0.3% on October 25, 2016 http://jn.physiology.org/ Downloaded from

Properties of evoked glycine current after microtubule disruption

The amplitude of the glycine-activated Cl⁻ current ran down to 73 ± 6% (n = 8) after 60 min of recording in control neurons (Fig. 2A). The current rundown was not affected by the addition of 20 μM colchicine to the internal solution (80 ± 3%, n = 5), nor after 3 h with 20 μM colchicine in the media (71 ± 9%, n = 3). Concentration-response curves in control neurons resulted in graded glycine responses with an EC₅₀ of 29 ± 1 μM (Fig. 2C, n = 10) and a Hill coefficient of 1.8 ± 0.1. Neither EC₅₀ values nor Hill coefficients were altered when colchicine was added to the patch-pipette (24 ± 2 μM, 1.6 ± 0.2, n = 4) or to the media (31 ± 1 μM, 2.0 ± 0.1, n = 6). Next, we examined whether the sensitivity of the Iglycine to the antagonist strychnine changes after colchicine treatment to analyze whether this alkaloid causes alterations on its subunit stoichiometry (Schmieden et al. 1992). However, colchicine at a concentration that clearly altered microtubule organization and cellular morphology had no effect on Iglycine properties. Strychnine (100 nM) inhibited Iglycine by 53 ± 5% (n = 5) in control neurons (Fig. 2D). The Iglycine amplitude was inhibited by 47 ± 8% (n = 5) and 57 ± 7% (n = 4) when colchicine was added to the internal solution or to the culture media, respectively.

Disruption of microtubules reduced the quantal amplitude of spontaneous glycinergic mIPSCs

The lack of colchicine effect on the whole cell Iglycine was surprising since a structural interaction between microtubules and GlyRs was previously suggested (Kirsch and Betz 1995). Therefore we analyzed the effect of colchicine on spontaneous glycinergic postsynaptic currents to determine whether microtubules are able to modify the function of synaptic GlyRs. The synaptic events recorded under our experimental conditions are assumed to arise from the spontaneous release of single vesicles and are referred to as quantal or mIPSCs. The amplitude of mIPSCs in cultured spinal neurons was 34.7 ± 7 pA (n = 6). In addition, these mIPSCs were confirmed as glycinergic because they were blocked by a low concentration (750 nM) of strychnine. It is interesting to note that very similar mIPSC properties were found in brain stem motoneurons (O’Brien and Berger 1999).

Synaptic current properties, such as peak amplitude, frequency, rise time, and decay time remained highly stable for 25 min under control whole cell recordings (Fig. 3A and Table 1). On the other hand, a time-dependent alteration of synaptic properties occurred during colchicine (20 μM) dialysis inside the cells (Fig. 3B and Table 1). After 25 min, the mean peak amplitude, frequency, and decay time were significantly decreased. Dialysis with the inactive analogue γ-lumicolchicine (20 μM; n = 5) was unable to significantly alter the average amplitude (93 ± 9%) or frequency (85 ± 17%). Similarly, external application of colchicine (20 μM, n = 3) for 20 min did not significantly change the frequency (94 ± 7%) or amplitude (93 ± 7%) of mIPSCs.

Colchicine specifically decreases spontaneous glycinergic synaptic currents

To address whether the inhibitory effect of intracellular colchicine on GlyRs was selective, we examined the effect of
this alkaloid on the overall synaptic activity of 8–10 DIV neurons. Figure 3C, for example, shows overall spontaneous synaptic activity (left trace) and glycinergic mIPSCs pharmacologically isolated in the presence of bicuculline (2 μM) and CNQX (2 μM; right trace). Recordings after 25 min of intracellular colchicine dialysis revealed that the glycinergic activity was strongly inhibited (Fig. 3C2, right trace), but the overall synaptic activity remained basically unchanged (left trace). Pharmacologically isolated GABAergic mIPSCs (with 1 μM strychnine and 2 μM CNQX) during colchicine dialysis showed no change in mean peak amplitudes; 61 ± 19 and 60 ± 21 pA at minutes 1 and 25, respectively (n = 3 neurons). Similarly, the corresponding mean frequencies of GABA mIPSCs were 1.3 ± 0.3 and 1.1 ± 0.3 Hz at minutes 1 and 25. These results are interesting because GlyRs and GABA<sub>A</sub>-Rs co-localize in a large proportion of inhibitory synapses in cultured spinal neurons (Dumoulin et al. 2000) and suggest that GlyRs were preferentially affected by colchicine treatment at synaptic sites containing both receptors. Furthermore, the average peak amplitude of AMPAergic miniature excitatory postsynaptic currents (mEPSCs; isolated with 1 μM bicuculline and 2 μM CNQX) following a similar colchicine treatment was 43 ± 6 and 43 ± 8 pA at 1 and 25 min, respectively. Mean frequency of AMPA mEPSCs remained highly stable for 25 min (91 ± 10% of minute 1) in the presence of colchicine (n = 3).

DISCUSSION

Previous studies have shown that the selective disruption of cytoskeletal elements can modify several functional properties of NMDA-Rs and GABA<sub>A</sub>-Rs (Rosenmund and Westbrook 1993; Whatley et al. 1994). Similarly, GlyRs are believed to be tightly associated to the peripheral cytoskeleton through gephyrin, a microtubule-binding protein (Kirsch and Betz 1995), but the consequences of microtubule disruption on GlyR function were unknown. Our results showed a selective effect of colchicine over synaptic transmission mediated by GlyRs, but not on AMPA- or GABA<sub>A</sub>-mediated synaptic activity. Therefore microtubules appear more tightly associated to synaptic GlyRs than to AMPA-Rs or GABA<sub>A</sub>-Rs. Morphological studies also suggests that the maintenance of core components of AMPA and GABA<sub>A</sub> postsynatic receptor clusters are independent of the microtubule state (Allison et al. 2000).

We also found that microtubule disruption did not affect the glycine whole cell current. The results agree with the specific localization of gephyrin at synaptic sites in spinal neurons (Alvarez et al. 1997; Triller et al. 1985) and suggest that the GlyRs responsible for the I<sub>glycine</sub> current are most likely extrasynaptic and do not interact with microtubules.

Recent findings have suggested a positive correlation between gephyrin cluster size and glycinergic mIPSC amplitude (Lim et al. 1999; Oleskevich et al. 1999). Therefore the reduc-
tion on glycinergic mIPSC amplitude could be due to a disorganization of the postsynaptic gephyrin cluster and parallel reduction in the number of postsynaptic receptors. Alterations in gephyrin-mediated postsynaptic clustering and an increase in the lateral mobility of GlyRs and their disappearance from postsynaptic sites has been reported in spinal neurons treated with the microtubule disrupter demecolcine (Kirsch and Betz 1995). However, others have failed to observe any alteration in gephyrin cluster structure or number after microtubule depolymerization in hippocampal neurons (Allison et al. 2000). The reduction on glycinergic inhibitory efficacy that we found agrees with a decrease in the number of postsynaptic receptors, with the microtubule disrupter demecolcine (Kirsch and Betz 1995). However, others have failed to observe any alteration in gephyrin cluster structure or number after microtubule depolymerization in hippocampal neurons (Allison et al. 2000). The reduction on glycinergic inhibitory efficacy that we found agrees with a decrease in the number of postsynaptic receptors,

| TABLE 1. Comparison of glycinergic mIPSC amplitudes and kinetics in control and in the presence of colchicine |
|---------------------------------------------------------------|-------|-------|----------------|----------------|-------|-------|----------------|----------------|
|                   | Control |                   | Colchicine |                   |       |       |                   |       |
|                   | Amplitude, pA | Frequency, Hz | Rise time T10-90 %, ms | Decay time to 37%, ms | Amplitude, pA | Frequency, Hz | Rise time T10-90 %, ms | Decay time to 37%, ms |
| Minute 1          | −34.7 ± 7 | 1.13 ± 0.2 | 1.79 ± 0.03 | 4.06 ± 1.5 | −34.9 ± 9 | 1.49 ± 0.5 | 1.73 ± 0.08 | 4.68 ± 1.0 |
| Minute 25         | −37.4 ± 9 | 1.08 ± 0.3 | 1.77 ± 0.06 | 4.63 ± 1.9 | −24.0 ± 6 | 0.25 ± 0.2 | 1.74 ± 0.01 | 3.09 ± 0.6 |
| Minute 25/minute 1, % | 107 ± 7 | 84 ± 15 | 99 ± 2 | 114 ± 6.6 | 68 ± 5* | 18 ± 11* | 101 ± 5 | 66 ± 3* |

In Minute 1 and Minute 25, values are means ± SE from 6 neurons. In Minute 25/minute 1 values represent means ± SE obtained from averaging individual effects in the 6 neurons studied. Significance of treatment at minute 25 was tested against minute 25 of control by paired t-test. mIPSC, miniature postsynaptic current. * P < 0.001 by Student t-test.
but future experiments are needed to correlate the results described here with possible alterations in the number and/or sensitivity of postsynaptic GlyRs in these cultures.

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