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$GABA_A$ AND STRYCHNINE-SENSITIVE GLYCINE RECEPTORS MODULATE *N*-METHYL-D-ASPARTATE–EVOKED ACETYLCHOLINE RELEASE FROM RAT SPINAL MOTONEURONS: A POSSIBLE ROLE IN NEUROPROTECTION

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Abstract—Increasing experimental and clinical evidence suggests that abnormal glutamate transmission might play a major role in a vast number of neurological disorders.

As a measure of glutamatergic excitation, we have studied the acetylcholine (ACh) release induced by *N*-methyl-D-aspartate (NMDA) receptor stimulation in primary cultured rat ventral horn spinal neurons and we have evaluated the possibility to limit the consequences of the hyperactivation of glutamatergic receptors, by recruiting the inhibitory transmission mediated by GABA and glycine. For this purpose, we have exposed cell cultures, previously loaded with [^{3}H]choline, to NMDA, which increased the spontaneous tritium efflux in a concentration-dependent manner. Tritium release is dependent upon external Ca^{2+}, tetrodotoxin, Cd^{2+} ions and ω-conotoxin GVIA, but not on ω-conotoxin MVIIC nor nifedipine, suggesting the involvement of N-type voltage-sensitive calcium channels. NMDA-mediated [^{3}H]ACh release was completely prevented by MK-801, 5,7-diclorokynurenic acid and ifenprodil, while it was strongly inhibited by a lower external pH, suggesting that the involved NMDA receptors contain NR1 and NR2B subunits. Muscimol inhibited NMDA-evoked [^{3}H]ACh release and its effect was antagonized by SR95531 and potentiated by diazepam, indicating the involvement of benzodiazepine-sensitive $GABA_A$ receptors. Also glycine, via strychnine-sensitive receptors, inhibited the effect of NMDA.

It is concluded that glutamate acts on the NMDA receptors situated on spinal motoneurons to evoke ACh release, which can be inhibited through the activation of $GABA_A$ and glycine receptors present on the same neurons. These data suggest that glutamatergic overload of receptors located onto spinal cord motoneurons might be decreased by activating $GABA_A$ and glycine receptors.

Key words: primary cultures, ALS, radiolabeled neurotransmitter, calcium channels, NMDA receptor subunit.

Glutamate receptor channels mediate most of the fast excitatory synaptic transmission in the mammalian CNS and play important roles in various physiological functions, such as synaptic plasticity and synapse formation underlying memory, learning and formation of neural networks during development. However, excessive synaptic release of glutamate or its inadequate uptake can result in an increase of extracellular concentrations of glutamate, leading to excitotoxicity, a well known phenomenon implicated in a variety of acute and chronic neurological disorders (Yamakura and Shimoji, 1999; Rocha et al., 2005; Baptiste and Fehlings, 2006; Fan and Raymond, 2007; Sheldon and Robinson, 2007).

Glutamate can induce neuronal cell death through the activation of different classes of ionotropic glutamate receptors (iGluRs): AMPA/kainate (KA) and *N*-methyl-D-aspartate (NMDA) receptors (Hara and Snyder, 2007). An increasing amount of experimental results has suggested that NMDA receptors directly mediate motoneuron degeneration (Van Den Bosch and Robberecht, 2000; Urushitani et al., 2001; Van Westerlaak et al., 2001), while other studies have proposed that motor neurons are highly vulnerable to injury triggered by the AMPA/KA receptor activation (Carriedo et al., 1996, 2000; Van Damme et al., 2003). However, it is important to note that spinal neurons express high levels of both NMDA (MacLean and Schmidt, 2001) and AMPA receptors (Williams et al., 1997).

In the spinal cord, the inhibition of synaptic activity is assured by both GABA and glycine, mainly through the activation of $GABA_A$ and strychnine-sensitive glycine receptors (Rekling et al., 2000). Several immunocytochemical and electrophysiological studies have underpinned the presence of the abovementioned receptors on the motoneurons (Marchetti et al., 2002; Lorenzo et al., 2006) and in the terminal boutons of subpopulations of spinal interneurons, where the receptors are expressed at the same synapse (Örnung et al., 1994; Todd et al., 1996). Hence, GABA and glycine may be released as cotransmitters on the postsynaptic targets (Todd et al., 1996; Jonas et al., 1998), while they are known to interact presynaptically in the spinal cord, regulating each other's release through the

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Abbreviations: ACh, acetylcholine; ACM, astrocyte-conditioned medium; ALS, amyotrophic lateral sclerosis; ANOVA, analysis of variance; DMEM, Dulbecco's modified Eagle's medium; EC_{50} value, concentration of drug producing 50% of the maximum effect; FCS, fetal calf serum; HMs, hypoglossal motoneurons; IC_{50} value, concentration of drug producing 50% of the maximum inhibition; iGluRs, ionotropic glutamate receptors; KA, kainate; NMDA, *N*-methyl-D-aspartate; SR95531, 2-(3-carboxypropyl)-3-amino-6-methoxyphenyl-pyridazinium bromide; TTX, tetrodotoxin; VSCCs, voltage-sensitive calcium channels; 5,7-DCKY, 5,7-dichlorokynurenic acid.

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GABA and glycine transporters that coexist on the same axon terminals (Raiteri et al., 1992). The pictured frame agrees with the presence of a sophisticated inhibitory system, that relies on pure GABAergic, pure glycinergic and mixed GABA/glycine synapses connecting interneurons and motoneurons (Jonas et al., 1998; Nicoll and Malenka, 1998).

Although the presence of glycine and $GABA_A$ receptors in ventral horns is well known, playing a crucial role in modulating motoneuron excitability and firing rates (Rekling et al., 2000), only few studies are yet completed on the inhibitory synaptic transmission in the spinal cord. In addition, the release of acetylcholine (ACh), the main motoneuronal neurotransmitter, has received little attention in functional neurochemistry studies and even the involvement of NMDA receptors in such effect has not been explored. In the present work, we have investigated the direct effects of NMDA receptor activity on the ACh release in cultured rat ventral horn spinal cord neurons, as a feasible read-out measure of the glutamate receptor over-activation that leads to excitotoxicity.

It was recently reported that the NMDA receptor-dependent rhythmic activity originating from a discrete increase of glutamate concentration is the hallmark of runaway excitation that triggers motoneuronal death (Sharifullina and Nistri, 2006). These authors conclude that the intense motoneuron activity, resulting from fast synaptic transmission, is correlated to excitotoxicity. Such correspondence enables us to indirectly predict, from the level of neurotransmitters released, the possible toxic effects of NMDA receptor activation.

The NMDA concentration that was here used (100 μM) is the one reported to kill about 40% of cells, when applied for 1 h to either purified motor neurons (Fryer et al., 1999) or mixed spinal cord cultures (Sen et al., 2008). The same concentration produced a 50% cell death in primary spinal cord cultures after 8 h of continuous NMDA exposure, as monitored by the assay of the lactate dehydrogenase activity (Wells et al., 1994).

Furthermore, we have examined the activation of a subpopulation of GABA and glycine receptors, in an attempt to limit the NMDA-evoked ACh release.

EXPERIMENTAL PROCEDURES

Cell culture and release experiments

In accordance with the National Institutes of Health guidelines and the Italian act Decreto Legislativo 27/1/92 n. 116 (implementing the European Community directives n. 86/609 and 93/88), experiments were performed on cultures obtained from embryos of Sprague–Dawley rats (Charles River, Calco, Italy). All efforts were made to reduce the number of animals used and to minimize animal suffering. The experimental setup was the same as described by Fontana et al. (2001).

Briefly, pregnant rats were narcotized with CO_2, decapitated and the spinal ventral horns from 14 to 15 day-old rat embryos were rapidly excised, then treated with trypsin (0.125% for 30 min at 37 °C) and finally mechanically dissociated. Cell suspension was plated at $0.40-0.50\times10^6$ cells/cm^2 on glass coverslips, previously coated with poly-L-ornithine (20 μg/mL), in a Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 2 mM glutamine, 6 g/L glucose. The cultures were maintained at 37 °C in a humidified 5% CO_2/95% air atmosphere. Culture medium was replaced with (1:1) Neurobasal medium/astrocyte-conditioned medium (ACM) supplemented with B27 (2%) and 0.5 mM L-glutamine, within 24 h. The culture medium was renewed after 1 week and every 3 days thereafter. In these cultures, approximately 25% of cells are motoneurons, as previously demonstrated by comparing immunocytochemical staining for ChAT and the DAPI staining (Fontana et al., 2001). Our results are in accordance with those reported by others (Wong et al., 1993).

Astroglial secondary cultures were prepared from mixed primary glial cultures obtained from cerebral cortex of 1–2 day-old Sprague–Dawley rats and grown in DMEM supplemented with 10% FCS, as described by Levi et al. (1993). Astroglial subcultures were plated (2.5×10^4 cells/cm^2) and grown in Neurobasal medium containing 2.5% FCS and 0.5 mM L-glutamine. After 3 days, time at which they reached confluence, cultures were used to obtain ACM. This latter was prepared with the addition of Neurobasal medium containing 2.5% FCS and 0.5 mM L-glutamine to plates of astrocytes and then collecting it after 24 h. This procedure was repeated on the same cultures another three to four times.

Release experiments

After 14–16 days, culture medium was removed from each well and cultured neurons were rinsed three times with a standard medium, kept at 37 °C, having the following composition (mM): NaCl 125, KCl 3, $MgSO_4$ 1.2, $CaCl_2$ 1.2, $NaHCO_3$ 22, NaH_2PO_4 1, glucose 10, bubbled with O_2/CO_2 (95:5), pH 7.4. The cultures were then incubated with 0.03 μM [^{3}H]choline for 15 min at 37 °C. Each glass coverslip was then transferred into a small chamber and superfused at 37 °C with a standard medium containing 3-hemicholinium (0.01 mM) at a flow rate of 0.5 mL/min. The spontaneous tritium efflux, calculated as a fractional rate, was 0.51 ± 0.06 ($n=30$), corresponding to 505 ± 21 c.p.m./sample. At the end of the experiment, the radioactivity remaining in the cells was extracted with Triton X-100 (0.1%). Tritium in the superfusate samples and in the cell extracts was measured by liquid scintillation counting.

Calculations

The efflux of radioactivity collected in each fraction has been calculated as a percentage of the total radioactivity present in the tissue. Data are expressed as a ratio between the efflux in the fraction with the maximum effect and the one at basal level. The dose-response curves plotted in Fig. 1 have been obtained using a fitting function routine (software Sigma Plot, version 5.0; Systat Software Inc., San Jose, CA, USA) and adopting the following four-parameter logistic equation: $Y=a+((b-a)/(1+(10^c/10^x)^d))$ where a is the minimum effect of the agonist, b the maximum effect, c the value at inflection point and d the slope of the curve. All data were expressed as mean$\pm$S.D., where n represents the number of experiments performed.

Data were first classified in parametric and non-parametric values, using a normality test. Then, parametric data were analyzed using Student's t-test (paired or unpaired) to compare two groups of data, or ANOVA (analysis of variance) for more than two groups. Alternatively, for non-parametric values we used the Mann-Whitney test for two groups or the ANOVA on ranks test, followed by a post hoc test (Dunnett test) for groups >2. Results were considered significant when $P<0.05$.

Chemicals

[^{3}H]choline (specific activity 83 Ci/mmol) was purchased from the Amersham Radiochemicals Centre (Buckinghamshire, UK).