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TEMPOROSPATIAL COUPLING OF NETWORKED SYNAPTIC ACTIVATION OF AMPA-TYPE GLUTAMATE RECEPTOR CHANNELS AND CALCIUM TRANSIENTS IN CULTURED MOTONEURONS

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Abstract—AMPA-type glutamate receptor (GluR) channels provide fast excitatory synaptic transmission in the CNS, but mediate also cytotoxic insults. It could be shown that AMPA-type GluR channel–mediated chronic excitotoxicity leads to an increased intracellular calcium concentration and plays an important role in neurodegenerative diseases like for example amyotrophic lateral sclerosis (ALS). As calcium is an important mediator of various processes in the cell and calcium signals have to be very precise in the temporospatial resolution, excessive intracellular calcium increases can seriously impair cell function. It is still unclear if AMPA-type receptors can directly interact with the intracellular calcium homeostasis or if other mechanisms are involved in this process. The objective of this study was therefore to investigate the calcium homeostasis in rat motoneurons under physiological stimulation of AMPA-type GluR channels using calcium imaging techniques and patch-clamp recordings simultaneously. It was found that spontaneous excitatory postsynaptic currents of cultured motoneurons did not elicit significant intracellular calcium transients. Large intracellular calcium transients occurred only when preceding fast sodium currents were observed. Pharmacological experiments showed that activation of AMPA-type GluR channels during synaptic transmission has a great functional impact on the calcium homeostasis in motoneurons as all kinds of activity was completely blocked by application of the selective kainate- and AMPA-type GluR channel blocker 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). Furthermore we suggest from our experiments that calcium transients of several hundred milliseconds' duration result from release of calcium from the endoplasmic reticulum via activation of ryanodine receptors (calcium-induced calcium release, CICR). Our results help to understand the regulatory function of AMPA-type GluR channels in the intracellular calcium homeostasis which is known to be disturbed in neurodegenerative diseases.

Key words: action potentials, excitatory postsynaptic currents, calcium induced calcium release, motoneuron culture, calcium imaging, excitotoxicity.

Specific processes in the cell like gene expression, enzyme activation, transmitter release or apoptosis are mediated by pathways which depend on calcium as a mediator (Tully and Treistman, 2004). It could be shown that excitotoxicity leads to an increased intracellular calcium concentration and as a consequence to a dysfunction of various metabolic pathways (Arundine and Tymianski, 2003). Chronic excitotoxicity is known to play an important role in neurodegenerative diseases like for example amyotrophic lateral sclerosis (ALS) (Cleveland and Rothstein, 2001; Ikonomidou et al., 1996) and is mainly mediated by activation of calcium permeable AMPA-type glutamate receptor (GluR) channels in motoneurons (Carriedo et al., 1995, 1996). AMPA-type GluR channels are calcium-permeable unless they contain GluR2 subunits which are edited at the Q/R editing site (Burnashev et al., 1995; Dingledine et al., 1999). Vandenberghe et al. (2000a,b) found almost the same amount of mRNA for GluR2 and a similar permeability of AMPA-type receptors for calcium in motoneurons and dorsal horn neurons of rats but they could show a higher density of AMPA receptor currents in motoneurons. In humans it was found that the proportion of GluR2 mRNA in spinal motoneurons was the lowest among several tested neuronal subsets. However, motoneurons of ALS patients showed no differences in the expression level of GluR2 mRNA in comparison to a control group (Kawahara et al., 2003). Tully and Treistman (2004) found that calcium entry through N-type voltage-gated calcium channels (VGCC) leads to calcium induced calcium release (CICR) via an activation of co-localized ryanodine receptors on the endoplasmic reticulum (ER) in pheochromocytoma cells (PC12). Palecek et al. (1999) showed that activation of VGCC leads to a long lasting increase of the somatic calcium concentration of hypoglossal motoneurons of brainstem slices. They found a clear dependence between the membrane depolarization and the intracellular calcium accumulation. However, it is still unclear how activation of AMPA-type receptors can lead to alterations of the intracellular calcium concentration.

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Abbreviations: ALS, amyotrophic lateral sclerosis; AUC, area under the curve; CICR, calcium induced calcium release; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; CPA, cyclopiazonic acid; DRG, dorsal root ganglion; EPSCs, excitatory postsynaptic currents; ER, endoplasmic reticulum; GluR, glutamate receptor; NMDA, *N*-methyl-D-aspartate; ROI, region of interest; SERCA, sarcoplasmatic reticulum/endoplasmic reticulum calcium ATPase; VGCC, voltage-gated calcium channel.

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To get an activation of the correct intracellular pathways, calcium signals have to be very precise in the temporospatial resolution. Therefore intracellular calcium release from and uptake into different subcellular compartments like the ER and mitochondria has an important impact on energy production, protein synthesis and can even trigger cell death (Paschen, 2003; Jacobson and Duchen, 2004).

The aim of the present study was to investigate the effect of AMPA-type GluR activation on intracellular calcium concentration during physiological synaptic activity in motoneurons.

EXPERIMENTAL PROCEDURES

Long term cultures of embryonic rat spinal cord motoneurons (Haastert et al., 2005) were investigated. Motoneurons formed functional networks *in vitro* as indicated by spontaneous synaptic activity of these neurons. Due to the synaptic connections via AMPA-type GluR channels we were able to measure the intracellular calcium transients of motoneurons and excitatory postsynaptic currents (EPSCs) simultaneously.

Cell cultures

Spinal motoneurons and dorsal horn neurons were cultured as previously described (Haastert et al., 2005). For harvesting of cells, a minimal number of rats were housed and sacrificed according to German law on animal care. Harvesting of tissue for scientific use was approved by the Bezirksregierung Hannover. In brief, on gestational days 14/15 lumbar regions of ventral and dorsal spinal cords of Sprague–Dawley rat embryos were harvested. After tissue dissociation the motoneurons were enriched by gradient density centrifugation using OptiPrep (Sigma-Aldrich, Steinheim, Germany). Cells were differentiated in four-well-plates on glass cover slips in a co-culture system with neonatal rat Schwann cells.

Identification of motoneurons

According to previous studies (Haastert et al., 2005; Vandenberghe et al., 2000b) in ventral horn preparations cells have to exceed at minimum a diameter of 20 μm to be considered as motoneurons.

In dorsal horn preparations neurons exceeding a diameter of 37.5 μm had a probability of about 80% to be motoneurons. This was found in additional experiments in which dorsal horn preparations were double stained with antibodies to βIII-tubulin (Upstate, Inc., Charlottesville, VA, USA) (Fig. 1A) to estimate the total neuronal population and with SMI 32 antibodies (Steinberger Monoclonals Inc., Baltimore, MD, USA) to the non-phosphorylated neurofilament H/M at DIV 10. In the given cultures SMI 32 specifically labels motoneurons (Fig. 1B). The use of Cy2- and Cy3-labeled secondary antibodies, respectively, allowed counting of both cell populations using fluorescent microscopy. The diameter of neurons which were SMI 32-positive and of those which were βIII-tubulin-positive but SMI 32-negative were determined. Finally the percentage of SMI 32-positive neurons (Fig. 1C, solid columns) and the percentage of βIII-tubulin-positive but SMI 32-negative cells (Fig. 1C, hatched columns) in relation to total neuronal number (all βIII-tubulin positive cells) were calculated for certain cell diameter intervals. Counts were done in nine wells of three different preparations (three wells per each preparation).

Electrophysiology

Whole-cell voltage-clamp experiments were performed at room temperature. Pipettes were placed on the soma and had a resistance of 3–5 MΩ when filled with intracellular solution. Cells were included in the study if they exceeded a minimum whole cell resistance of 120 MΩ and showed a series resistance $<$20 MΩ. Responses were recorded by an EPC 9 amplifier (HEKA Electronics, Lambrecht, Germany). Membrane currents were sampled at 10 kHz and filtered with 5 kHz. The intracellular solution consisted of Hepes 2.38 g/l, potassium gluconate 32.79 g/l, $MgCl_2$ 0.19 g/l, Na_2-ATP 2.2 g/l and Na-GTP 0.21 g/l. The extracellular solution contained Hepes 2.76 g/l, KCl 0.44 g/l, $MgCl_2$ 0.11 g/l, NaCl 7.54 g/l, glucose 2.07 g/l and $CaCl_2$ 0.35 g/l. pH was adjusted with NaOH to 7.3. Cells were perfused with extracellular solution with 2 ml/min.

For estimation of the physiological membrane potential the current clamp mode was used. For voltage clamp measurements cells were held at a membrane potential of $-$80 mV.

Calcium imaging

High spatiotemporal resolution FURA-2 AM calcium imaging was used to monitor cytosolic calcium transients in individual neurons (20 Hz, Till Vision Imaging System by TillPhotonics, Graefelfing, Germany). Cells cultured on glass coverslips were incubated for 20 min with the membrane permeable ester form of FURA 2 AM, which mainly accumulates in the cytosol. For the analysis of calcium transients, background subtraction was used and subcellular regions of interest (ROI) were defined over the nucleus, perinuclear region, cytosol, neurite base and neurite.

Patch clamp recordings at $-$80 mV membrane potential and calcium imaging were used simultaneously.

Substances

All chemicals were purchased from Sigma-Aldrich unless otherwise indicated. 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX), a selective blocker of kainate-and AMPA-type GluR channels, was diluted in extracellular buffer to achieve a concentration of 10 μM. The *N*-methyl-D-aspartate (NMDA) blocker MK-801 (Tocris, Ellisville, MO, USA) was applied at concentrations of 10 and 100 μM. Cyclothiazide (Tocris), an AMPA desensitization blocker, was used at a concentration of 100 μM. The sarcoplasmatic reticulum/endoplasmic reticulum calcium ATPase (SERCA) pump inhibitor cyclopiazonic acid (CPA) was added at a concentration of 10 μM and dantrolene, a blocker of ryanodine receptors, at a concentration of 30 μM.

The experimental values are given as mean$\pm$s.e.m. in the Results section.

RESULTS

In long-term cultured embryonic rat motoneurons spontaneous inward currents and simultaneous whole cell cytosolic fast calcium transients were observed from DIV 10 on, indicating functional synaptic connections *in vitro*.

In ventral horn preparations it had previously been shown that neurons have to exceed a diameter of at least 20 μm to be a motoneuron (Haastert et al., 2005; Vandenberghe et al., 2000b). In dorsal horn preparations we found that only neurons with a diameter $>$37.5 μm can be considered as motoneurons (Fig. 1). Ventral horn neurons included in the study had diameters between 24 and 50 μm and dorsal horn neurons had diameters between 38 and 62 μm. Therefore all included neurons were most probably motoneurons.

Cells in our cell culture system showed a resting potential of $-$72.4 mV$\pm$2.1 mV (n=20). The patch pipette was positioned on the cell soma (see Fig. 2A). Under