Contribution of Ca\(^{2+}\)-Permeable AMPA/KA Receptors to Glutamate-Induced Ca\(^{2+}\) Rise in Embryonic Lumbar Motoneurons In Situ

FRIEDRICH METZGER,\(^2\) ANNA KULIK,\(^1\) MICHAEL SENDTNER,\(^2\) AND KLAUS BALLANYI\(^1\)

\(^1\)II. Physiologisches Institut, Universität Göttingen, D-37073 Göttingen; and \(^2\)Klinische Forschergruppe Neuroregeneration, Neurologische Klinik, Universität Würzburg, D-97080 Würzburg, Germany

Metzger, Friedrich, Anna Kulik, Michael Sendtner, and Klaus Ballanyi. Contribution of Ca\(^{2+}\)-permeable AMPA/KA receptors to glutamate-induced Ca\(^{2+}\) rise in embryonic lumbar motoneurons in situ. J. Neurophysiol. 83: 50–59, 2000. Intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) was fluormetrically measured with fura-2 in lumbar motoneurons of acutely isolated spinal cord slices from embryonic rats. In ester-loaded cells, bath-applied glutamate (3 \(\mu\)M to 1 mM) evoked a [Ca\(^{2+}\)]\(_i\) increase by up to 250 nM that was abolished by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) plus 2-amino-5-phosphonovalerate (APV). CNQX or APV alone reduced the response by 82 and 25%, respectively. The glutamatergic agonists kainate (KA), quisqualate (QUI), and S-\(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxalone (S-AMPA) evoked a similar [Ca\(^{2+}\)]\(_i\) transient as glutamate. N-methyl-n-aspartate (NMDA) was only effective to increase [Ca\(^{2+}\)]\(_i\) in Mg\(^{2+}\)-free saline, whereas [1S,3R]-1-amino-2-cyclopentane-1,3-dicarboxylic acid ([1S,3R]-CPA) had no effect. The glutamate-induced [Ca\(^{2+}\)]\(_i\) rise was suppressed in Ca\(^{2+}\)-free superfusate. Depletion of Ca\(^{2+}\) stores with cyclopiazonic acid (CPA) did not affect the response. Thirty-six percent of the [Ca\(^{2+}\)]\(_i\) increase in response to membrane depolarization induced by a 50 mM K\(^+\) solution consisted on combined application of the voltage-gated Ca\(^{2+}\) channel blockers nifedipine, \(\omega\)-conotoxin-GVIA and \(\omega\)-agatoxin-IVA. In fura-2 dialyzed motoneurons, the glutamate-induced [Ca\(^{2+}\)]\(_i\) increase was attenuated by ~70% after changing from current to voltage clamp. Forty percent of the remaining [Ca\(^{2+}\)]\(_i\), transient and 20% of the concomitant inward current of 0.3 nA were blocked by Joro spider toxin-3 (JSTX). The results show that voltage-gated Ca\(^{2+}\) channels, including a major portion of R-type channels, constitute the predominant component of glutamate-induced [Ca\(^{2+}\)]\(_i\) rises. NMDA and Ca\(^{2+}\)-permeable KA/AMPA receptors contribute about equally to the remaining component of the Ca\(^{2+}\) rise. The results substantiate previous assumptions that Ca\(^{2+}\) influx through JSTX-sensitive KA/AMPA receptors is involved in (trophic) signaling in developing motoneurons.

INTRODUCTION

It is established that glutamate receptor–associated rises of the free intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) are of ultimate importance for neuronal excitability (Collingridge and Singer 1990; Finkbeiner and Greenberg 1998; Hollmann and Heinemann 1994; Mayer and Westbrook 1987) and pathophysiological processes (Choi and Rothman 1990; Krieger et al. 1996). But glutamate-mediated [Ca\(^{2+}\)]\(_i\) increases also seem to play a crucial role in the outgrowth of dendrites and axons and thus influence neuronal differentiation during development of the brain (Collingridge and Singer 1990). Local glutamate-induced [Ca\(^{2+}\)]\(_i\) increases, influence postsynaptic phosphorylation of microtubule-associated proteins (e.g., MAP-2) (Quinlan and Halpain 1996). Such phosphorylation might initiate structural changes by modulation of microtubule stability and bundling during neuritogenesis (Avila et al. 1994). In particular glutamate receptors that are activated by kainate (KA) or \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxalone (AMPA) (Hollmann and Heinemann 1994) seem to be involved in the trophic effects of glutamate. Activation of KA/AMPA receptors has been demonstrated to reduce outgrowth and mobility of hippocampal neurons (Mattson et al. 1988). These ionotropic glutamate receptors were also found to influence developing motoneurons that are among the earliest neurons to differentiate within the CNS. As an example, KA/AMPA receptors are responsible for suppression of neurite outgrowth in spinal motoneurons from embryonic rodents (Metzger et al. 1998; Owen and Bird 1997). The highly selective blocker of the Ca\(^{2+}\)-permeable subtype of KA/AMPA receptors Joro spider toxin-3 (JSTX) (Blaschke et al. 1993; Meucci and Miller 1998) was found to inhibit this response (Metzger et al. 1998). This led to the hypothesis that the latter trophic effect of glutamate on spinal motoneurons is causally related to localized elevation of [Ca\(^{2+}\)]\(_i\), that follows Ca\(^{2+}\) influx through the JSTX-inhibitable KA/AMPA receptors (Metzger et al. 1998).

Despite the central role of Ca\(^{2+}\)-permeable KA/AMPA receptors for synaptic, trophic, and pathological glutamatergic processes, there is only sparse information exclusively from studies on cell cultures or expression systems on the effects of the toxin on glutamate receptor-associated neuronal Ca\(^{2+}\) transients (Meucci and Miller 1998; Savidge and Bristow 1998; Ying et al. 1997). According to our knowledge, the contribution of JSTX-sensitive KA/AMPA receptors to glutamate-evoked [Ca\(^{2+}\)]\(_i\) increases of neurons in functionally intact brain slices has not been studied so far. To test the hypothesis that Ca\(^{2+}\)-permeable KA/AMPA receptors are involved in glutamatergic trophic modulation of developing motoneurons (Metzger et al. 1998), we have analyzed the effects of JSTX on glutamate receptor–induced [Ca\(^{2+}\)]\(_i\) rises in lumbar motoneurons of acutely isolated spinal cord slices from rats at embryonic days 17–18. Within this time period, glutamatergic synapses are formed (Furuyama et al. 1993; Gao and Ziskind-Conhaim 1995; Kudo et al. 1993; Ziskind-Conhaim 1990), and glutamate exerts an inhibitory action on dendrite outgrowth of these cells (Metzger et al. 1998; Owen and Bird 1997). The motoneurons that were functionally identified by antidromic stimulation were either filled with the Ca\(^{2+}\)-sensitive dye fura-2 by exposure of the slices to fura-2 acetoxyethyl (AM)-
ester or by dialysis via the patch electrode (Ballanyi and Kulik 1998). The results show that even saturating concentrations of glutamate or ionotropic receptor agonists evoke only a moderate (<250 nM) rise of [Ca^{2+}]. That is mediated at least by 70% via voltage-gated Ca^{2+} channels. About 50% of the remaining Ca^{2+} influx is caused by both Ca^{2+} influx through NMDA and JSTX-sensitive KA/AMPA receptors. It is hypothesized that these KA/AMPA receptors play an important role in Ca^{2+}-dependent (trophic) neuronal signaling.

**METHODS**

**Preparation of spinal cord slices**

The experiments were performed on lumbar motoneurons of transverse spinal cord slices that were acutely isolated from 17- to 18-day-old Wistar rat fetuses. Pregnant rats were anesthetized with ether and killed by cervical dislocation. The fetuses were removed by Caesarean section and decapitated. The lumbar part of the spinal cord was dissected together with surrounding tissue and meninges and transferred to ice-cold solution (for composition, see Solutions and superfusion system). Afterward, the spinal cord was embedded into 2% agarose solution at ~35°C (Gao and Ziskind-Conhaim 1999) and subsequently cooled down on ice. The agarose block including the spinal cord was glued to the stage of a vibratome (Vibracut, FTB, Weinheim, Germany). Transverse 300 μm thick slices were cut from the spinal cord together with the surrounding agarose. Before transfer to the recording chamber, the slices were stored at 30°C (Ballanyi 1999).

**Solutions and superfusion system**

After transfer and immobilization of individual slices with a net, the recording chamber (volume 3 ml) was superfused at 30°C with oxygenated standard solution (flow rate 5 ml/min) of the following composition (in mM): 118 NaCl, 3 KCl, 1 MgCl2, 1.5 CaCl2, 25 NaHCO3, 1.2 NaH2PO4, and 10 d-glucose. The pH was adjusted to 7.4 by gassing with 95% O2 and 5% CO2. In a high K+ solution, 50 mM NaCl was substituted by KC1. Ca^{2+}-free standard solution contained 1 mM ethylene-glycol-bis-β-aminoethyl ester)-N,N,N',N'-tetraacetic acid (EGTA) and 5 mM MgCl2. In the Mg^{2+}-free solution, Mg^{2+} was substituted by 1 mM Ca^{2+}. Drugs purchased from Sigma (Deisenhofen, Germany), RBI/Biotrend (Köln, Germany), Calbiochem (Bad Soden, Germany), or Tocris/Biotrend (Köln, Germany) were added to the superfuse from stock solutions. The following stock solutions (in standard saline) were used (in mM): 100 glutamate, 10 KA, 10 quisquulate (QU), 10 N-methyl-D-aspartate (NMDA), 10 2-amino-5-phosphonovalerate (APV), 10 (+)-a-methyl-4-carboxyphenylglycine ( MCPG), 1 tetrodotoxin (TTX), 1 JSTX, 100 Co^{2+}, 100 Ni^{2+}, 1 ω-agatoxin-IVA, and 1 ω-conotoxin-GVIA. Cyclopiazonic acid (CPA, 50 mM), 6-cyano-7-nitroquinolaxine-2,3-dione (CNQX, 10 mM), nifedipine (50 mM), methoxyverapamil (D-600, 100 mM), and the active enantiomer of AMPA, S-AMPA (50 mM), were dissolved in DMSO. One millimolar 15,3R-1-aminocyclo-pentane-1,3-dicarboxylic acid ([1S,3R]-ACPD) was dissolved in 0.1 M NaOH. Agarose was dissolved in standard solution by heating to 100°C. Fura-2 acetoxymethylster (fura-2-AM), fura-2 pentapotassium salt (fura-2), and pluronic acid were obtained from Molecular Probes (Eugene, Oregon).

**Flurometric measurements of [Ca^{2+}]i**

[Ca^{2+}]i was fluorometrically measured using an upright microscope [Axioskop (digital imaging system) or Standard-16 (photomultiplier system), Zeiss, Oberkochen, Germany] that was equipped with epifluorescence optics, a monochromator (Polychrome II, T.I.L.L. photomics, Planegg, Germany) and either a charge-coupled device (CCD) camera (T.I.L.L. photomics) or a photomultiplier (Luigs und Neumann, Ratingen, Germany). For fura-2 excitation, cells were exposed to alternating wavelengths (360 and 380 nm), whereas emission was measured at 515 nm. Images were acquired at a frequency of 1 Hz, and exposure time was 20 ms. Fluorescence ratios of the fura-2 signals were converted into [Ca^{2+}]i by using Eq. 1 [Eq. 1, $\frac{F_{\text{max}}}{F_{\text{min}}}-R_{\text{min}} = (R - F_{\text{min}})/R_{\text{max}}$] (Gryniewicz et al. 1985), in which R is the fluorescence ratio (360 nm/380 nm) and K is the effective dissociation constant of fura-2. In vivo calibrations to determine R_{\text{min}}, R_{\text{max}} and K were performed according to the method described by Neher (1989). Briefly, measurements were performed with three different pipette solutions (in mM): 1) 130 KCl, 1 MgCl2, 10 K-1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), and 1 Na2ATP (low calcium; R_{\text{min}}); 2) 130 KCl, 1 MgCl2, 3 CaCl2, 4 K-BAPTA, 10 HEPES, and 1 Na2ATP [intermediate calcium; 300 mM, according to a K+ of 107 mM for BAPTA (Tsien 1980)]; and 3) 130 KCl, 1 MgCl2, 10 CaCl2, 10 HEPES, and 1 Na2ATP (high calcium; R_{\text{max}}), to each solution 100 μM fura-2 was added. The resulting intracellular fluorescence ratios were calculated according to Eq. 1. K was calculated as $K = 300 \times (R_{\text{max}} - R) / (R - R_{\text{min}})$. Cells were loaded with fura-2 either by incubation of slices in standard solution containing 10 μM fura-2-AM, 5 μM pluronic F-127, and 0.1% albumin (bovine) for 30 min at 37°C or by dialyzing an individual cell via a patch pipette with a solution containing 100 μM fura-2.

**Electrophysiological recording and stimulation**

For intracellular recording from lumbar motoneurons, patch pipettes were obtained from borosilicate glass capillaries (GC 150TF, Clark Electromedical Instruments, Pangbourne, UK) using a horizontal electrode puller (Zeitz, München, Germany). The standard patch pipette solution contained (in mM) 140 potassium gluconate, 1 MgCl2, 10 HEPES, and 1 Na2ATP, pH 7.3–7.4. The resistance of the patch electrodes ranged from 4 to 6 MΩ. Whole cell recordings were performed on superficial neurons under visual control, using an EPC-9 patch-clamp amplifier (HEKA, Lambrecht, Germany). Membrane conductance (g_m) was measured by application of hyperpolarizing currents (5–10 pA) or voltage pulses (–20 mV) with a duration of 500 ms.

For functional identification of lumbar motoneurons, suction electrodes were positioned close to the ventrolateral surface of the spinal cord slices in the vicinity of ventral nerve rootlets (Fig. 1A). In eight of nine whole cell recorded cells from slices of five different animals, single pulse stimulation (10–80 V, 100 μs) via the suction electrode evoked an action potential of 60 to 100 mV amplitude (Fig. 1B). The short latency (200–500 μs) of these spikes and also the observation that no postsynaptic potentials were observed on decreasing the stimulation voltage to 90% of activation threshold (90%) suggests antidromic activation of motoneurons as the cause of these responses (Fig. 1B) (for details, see Lev-Tov and O’Donovan 1995; O’Donovan et al. 1994; Ziskind-Conhaim 1988). In the neuron that did not respond to antidromic stimulation, spiking could also not be evoked by injection of depolarizing current pulses (not shown). In the other cells, repetitive spike discharge was elicited by injection of depolarizing current pulses (30–40 pA). These action potentials were blocked by addition of 1 μM TTX to the superfusate (Fig. 1C). In the latter motoneurons, tetanic antidromic stimulation (50 Hz, 2 s, 10 – 40 V, single pulse duration 100 μs) evoked a [Ca^{2+}]i increase of between 50 and 130 nM that was abolished by TTX (not shown).

**Data analysis**

Electrophysiological and microfluorometrical signals were sampled into a Macintosh PowerPC (Apple Computers, Cupertino, CA) using Pulse/Pulsefit and X-Chart/Fura extension from HEKA. Current and voltage signals were also digitized (Instrutech VR-100A, Elmont,
RESULTS

Glutamate-evoked \([\text{Ca}^{2+}]_i\) rises in functionally identified motoneurons

In a first series of experiments, slices were loaded with fura-2-AM to assess the population response to glutamate of motoneurons within the ventrolateral aspect of gray matter. Similar to the high probability of antidromic activation of individual whole cell recorded motoneurons (Fig. 1, A–C, see METHODS), \(>70\%\) of the fura-2 ester-loaded cells (Fig. 1D) responded to tetanic stimulation with a rise of \([\text{Ca}^{2+}]_i\) by 50–100 nM from a baseline of between 50 and 150 nM (83 \(\pm\) 6 nM, mean \(\pm\) SE, \(n=23\); Fig. 1E). The stimulus-evoked \([\text{Ca}^{2+}]_i\), transients in these cells from slices of five animals were not due to direct effect of the electrical stimulation because they were abolished by 1 \(\mu\)M TTX (Fig. 1E). Furthermore, as indicated in the scheme of Fig. 1A, no \([\text{Ca}^{2+}]_i\) rises were evoked after positioning the stimulation electrode by \(\sim200\) \(\mu\)m more medially or laterally without changing the overall distance to the recorded neurons. The assumption that by far most of the neurons in this region of the slices are motoneurons within the ventrolateral aspect of gray matter. Similar to the high probability of antidromic activation of individual whole cell recorded motoneurons (Fig. 1, A–C, see METHODS), \(>70\%\) of the fura-2 ester-loaded cells (Fig. 1D) responded to tetanic stimulation with a rise of \([\text{Ca}^{2+}]_i\) by 50–100 nM from a baseline of between 50 and 150 nM (83 \(\pm\) 6 nM, mean \(\pm\) SE, \(n=23\); Fig. 1E). The stimulus-evoked \([\text{Ca}^{2+}]_i\), transients in these cells from slices of five animals were not due to direct effect of the electrical stimulation because they were abolished by 1 \(\mu\)M TTX (Fig. 1E). Furthermore, as indicated in the scheme of Fig. 1A, no \([\text{Ca}^{2+}]_i\) rises were evoked after positioning the stimulation electrode by \(\sim200\) \(\mu\)m more medially or laterally without changing the overall distance to the recorded neurons. The assumption that by far most of the neurons in this region of the slices are motoneurons gained further support from the size of the cell somata (\(>15\) \(\mu\)m diam) and the shape of dendrites (Figs. 1D and 2A). All cells in which antidromic activation produced a \([\text{Ca}^{2+}]_i\) increase also responded to bath application of 300 \(\mu\)M glutamate for 15 s. In contrast to the stimulus-evoked intracellular \([\text{Ca}^{2+}]_i\) rises, the magnitude of the glutamate-evoked \([\text{Ca}^{2+}]_i\) increase (210 \(\pm\) 19 nM, \(n=20\)) was not substantially reduced by TTX (88.4 \(\pm\) 5.6\% of control; Fig. 1E). All cells responded with a very similar \([\text{Ca}^{2+}]_i\), increase to different concentrations of glutamate (Fig. 2, A and B). The dose-dependent mean rise of \([\text{Ca}^{2+}]_i\), saturated at values of \(\sim200\) nM above baseline at glutamate concentrations of 0.3–1 mM (Fig. 2, C and D). The concentration threshold of the glutamate-induced \([\text{Ca}^{2+}]_i\), rise was \(\sim3\) \(\mu\)M, and a half-maximal rise occurred at a glutamate concentration of 64 \(\pm\) 13 \(\mu\)M (Fig. 2D). On wash out, intracellular \([\text{Ca}^{2+}]_i\) returned to baseline with monoeponential kinetics [time constant (\(t_{1/2}\)) 55.5 \(\pm\) 5 s, \(n=20\)].

Glutamate receptor pharmacology

In a further series of experiments, the pharmacology of the effects of glutamate on \([\text{Ca}^{2+}]_i\), was investigated. The \([\text{Ca}^{2+}]_i\), response to 300 \(\mu\)M glutamate (15 s) was suppressed to 18.4 \(\pm\) 2.6\% (\(n=16\)) of control after 5 min of preincubation of the slices with 50 \(\mu\)M CNQX, a blocker of the KA/AMPA subtype of glutamate receptors (Fig. 3, A and C). In contrast, preincubation with the NMDA receptor antagonist APV (100 \(\mu\)M) reduced the peak of the glutamate-induced intracellular \([\text{Ca}^{2+}]_i\) rise to 74.7 \(\pm\) 2.7\% (\(n=16\)) of control (Fig. 3, B and C). The \([\text{Ca}^{2+}]_i\), response to glutamate was almost abolished on combined application of both antagonists (Fig. 3, A and C). The KA/AMPA receptors agonists S-AMPA (20 and 5 \(\mu\)M), KA (50 and 100 \(\mu\)M; Fig. 4A), and QUI (100 \(\mu\)M; Fig. 4B) increased intracellular \([\text{Ca}^{2+}]_i\) to a similar moderate level as seen during glutamate on bath application for 15 s (for statistics, see Fig. 4D). As exemplified in Fig. 4B for QUI, the \([\text{Ca}^{2+}]_i\), rises evoked by the KA/AMPA receptor agonists were attenuated to 14.5 \(\pm\) 3.1\% (\(n=12\)) of control after preincubation with CNQX. In contrast to the KA/AMPA receptor agonists, bath application of NMDA (50 and 200 \(\mu\)M) had only a minor effect on \([\text{Ca}^{2+}]_i\), in standard solution. In Mg\(^{2+}\)-free saline, however, the drug evoked intracellular \([\text{Ca}^{2+}]_i\) rises of 116 \(\pm\) 7 nM (\(n=15\)), and these responses were effectively blocked by 100 \(\mu\)M APV (Fig. 4, C and D).
In contrast to the inhibitory action of CNQX and APV, the broad spectrum metabotropic glutamate receptor antagonist MCPG (200 μM) had no effect on the glutamate-induced intracellular Ca^{2+} changes (n = 12; not shown). Furthermore, bath application of the metabotropic glutamate receptor agonist [1S,3R]-ACPD (100 μM, 5 min), did not change [Ca^{2+}], (n = 9; not shown). As further indication that metabotropic receptors, which act on IP3-sensitive Ca^{2+} stores, have a negligible contribution to glutamate-induced [Ca^{2+}], rises, the effects of the endoplasmatic reticulum Ca^{2+}-ATPase blocker CPA were tested. Bath application of 50 μM of the drug led to a rise of [Ca^{2+}] by between 10 and 40 nM (n = 15). However, CPA did not change the [Ca^{2+}] transient in response to 300 μM glutamate (Fig. 5A). The conclusion from these results, that the glutamate-induced Ca^{2+} signal results from Ca^{2+} influx from the extracellular space, is supported by the finding that the [Ca^{2+}] responses were reversibly abolished by Ca^{2+}-free superfusate (n = 15; Fig. 5B).

**Blocks of voltage-gated Ca^{2+} channels**

For determination of a possible contribution of voltage-gated Ca^{2+} channels to the glutamate-induced [Ca^{2+}], rises in the lumbar motoneurons, the effects of blockers of these channels were tested. The L-type Ca^{2+} channels blockers D-600 (200 μM) and nifedipine (50 μM) reduced the glutamate-evoked [Ca^{2+}], increase to 75 and 73% of control, respectively (Table 1). The P- (and Q-) type Ca^{2+} channel antagonist ω-agatoxin-IVA (200 nM) and the blocker of N-type Ca^{2+} channels ω-conotoxin-GVIA (1 μM) had a similar modest attenuating effect on the glutamate-evoked Ca^{2+} transients (Fig. 6A; Table 1). Combined administration of ω-agatoxin-IVA, ω-conotoxin-GVIA, and nifedipine decreased the glutamate-induced [Ca^{2+}], transient by ~50% (Table 1).

To investigate whether the remaining Ca^{2+} transient represents Ca^{2+} influx exclusively through glutamate receptors after block of voltage-gated Ca^{2+} channels, the effects of the drugs were tested on depolarization-induced [Ca^{2+}], rises. Superfusion for 1 min of a solution that contained 50 mM K^+ led to a mean rise of [Ca^{2+}], by 101 ± 6 nM (n = 21). Similar with the above finding that TTX produced only a modest attenuation of the glutamate-evoked [Ca^{2+}], rise, TTX only slightly reduced the K^+-induced Ca^{2+} rise.
(87.8 ± 8.1% of control, n = 12). This excludes that the K^+-mediated neuronal depolarization initiates a major release of glutamate or other neuroactive substances that act on intracellular Ca^2+.

Because combined application of selective blockers of L-, N-, and P-type voltage-gated Ca^2+ channels was not effective to abolish Ca^2+ influx due to K^+-induced depolarization (see also Bacsakai et al. 1995; Magnelli et al. 1998; Plant et al. 1998; Regan et al. 1991; Wu et al. 1999), the effects of nonspecific blockers were analyzed. Co^2+ (0.5 mM) reduced the glutamate- and the K^+-evoked Ca^2+ rise to 49 and 36% of control, respectively (Table 1). Finally, the K^+-induced intracellular Ca^2+ transients were reduced to 4% of the control response by 2 mM Ni^2+ (Fig. 6B). However, also the glutamate-associated [Ca^{2+}]_i transient was blocked to 6% of control (n = 12) with Ni^2+ (Fig. 6B, Table 1). As revealed by [Ca^{2+}]_i measurements in four whole cell recorded cells (see [Ca^{2+}]_i rises due to JSTX-sensitive KA/AMPA receptors in voltage clamp), Ni^2+ not only abolished the glutamate-induced Ca^2+ transient, but also strongly attenuated the concomitant inward current by 78% (Fig. 6. C and D; Table 1). In summary, these results showed that voltage-gated Ca^2+ channel blockers are not useful for analysis of the relative contribution of NMDA and Ca^2+-permeable KA/AMPA receptors to the glutamate-induced Ca^2+ transients in ester-loaded spinal cord neurons from embryonic rats.

[Ca^{2+}]_i rises due to JSTX-sensitive KA/AMPA receptors in voltage clamp

To exclude the contribution of Ca^2+ channels to the glutamate response, motoneurons that were dialyzed with fura-2 via the patch electrode were studied under voltage-clamp conditions. In current clamp, a mean resting potential of −55.2 ± 1.5 mV was revealed. Injection of depolarizing current pulses evoked action potentials with an amplitude of up to 100 mV (compare Fig. 1; n = 8). Application of 300 μM glutamate (15 s) induced a mean depolarization to −21.8 ± 1.9 mV and elevated [Ca^{2+}] by 271 ± 4 nM (n = 4; Fig. 6D). In these motoneurons, switching to voltage clamp revealed a glutamate-induced inward current of −180 ± 4 pA (at a holding potential of −60 mV). The concomitant [Ca^{2+}]_i rises were significantly (P < 0.01) smaller (69 ± 1 nM, i.e., <30% of control) than under current clamp (Fig. 6D). These results indicate that a major part of the glutamate-evoked [Ca^{2+}]_i elevation is secondary due to activation of voltage-gated Ca^2+ channels.

To test whether the remaining component of glutamate-
and JSTX-sensitive Ca\textsuperscript{2+}-permeable glutamate receptors have a noticeable contribution. These results are consistent with recent observations that neurite outgrowth in developing motoneurons is regulated by glutamate via Ca\textsuperscript{2+} influx through JSTX-sensitive KA/AMPA receptors.

\[ \text{[Ca}\textsuperscript{2+}] \text{, rises in antidromically activated lumbar motoneurons} \]

Previous studies on intracellular Ca\textsuperscript{2+} in identified motoneurons in situ were primarily devoted to analysis of Ca\textsuperscript{2+} buffering mechanisms (Lips and Keller 1998; Palaecek et al. 1999) or of dynamic [Ca\textsuperscript{2+}], changes during (spontaneous) activity (Bacskai et al. 1995; Lev-Tov and O’Donovan 1995; O’Donovan et al. 1994). In agreement with the latter reports, we found that repetitive stimulation of ventral roots evokes [Ca\textsuperscript{2+}], increases in the vast majority of neurons within the ventrolateral aspect of the gray matter in the spinal cord slices. The observation that these Ca\textsuperscript{2+} transients were blocked with TTX and could only be evoked in the close vicinity of the ventral roots suggests that they were secondary due to antidromic action potentials leading to activation of voltage-gated Ca\textsuperscript{2+} channels in the cell soma. The recorded embryonic motoneurons of the present study were electrically excitable as spike discharge was observed on injection of depolarizing current via the patch electrode (see also Ziskind-Conhaim 1988, 1990). Bath-applied glutamate evoked rather stereotypic [Ca\textsuperscript{2+}], increases in all cells that responded to antidromic stimulation with a Ca\textsuperscript{2+} transient. Therefore it is very likely that the glutamate-induced [Ca\textsuperscript{2+}], transients observed in this study occurred in motoneurons and not interneurons (Bacskai et al. 1995; Gao and Ziskind-Conhaim 1995; Lev-Tov and O’Donovan 1995; O’Donovan et al. 1994; Ziskind-Conhaim 1990).

Mediation of glutamate-induced [Ca\textsuperscript{2+}], rises by ionotropic receptors

APV and, to a considerably larger extent, CNQX reduced the glutamate-induced [Ca\textsuperscript{2+}], transients in the lumbar motoneurons. This agrees well with previous studies that suggested that KA/AMPA as well as NMDA receptors are expressed and contribute to synaptic processes in spinal motoneurons of 17- to 18-day-old rat embryos (Gao and Ziskind-Conhaim 1995; Mandler et al. 1990; Ziskind-Conhaim 1990). This view is supported by the finding that NMDA (in the absence of extracellular Mg\textsuperscript{2+}) and also KA, QUI, or

<p>| TABLE 1. Reduction of [Ca\textsuperscript{2+}], rises in ester-loaded lumbar motoneurons by blockers of voltage-gated Ca\textsuperscript{2+} channels (% of control remaining) |
|---------------------------------|-----------------|-----------------|</p>
<table>
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<tr>
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<th>GLU (300 μM)</th>
<th>K\textsuperscript{+} (50 mM)</th>
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<tr>
<td>D-600 (200 μM)</td>
<td>75 ± 3</td>
<td>47 ± 4</td>
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<tr>
<td>Nifedipine (50 μM)</td>
<td>73 ± 4</td>
<td>40 ± 4</td>
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<tr>
<td>ω-Agatoxin-IVA (0.2 μM)</td>
<td>77 ± 5</td>
<td>71 ± 2</td>
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<tr>
<td>ω-Conotoxin-GVIA (1 μM)</td>
<td>73 ± 4</td>
<td>67 ± 3</td>
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<td>ω-Agatoxin-IVA + ω-conotoxin-</td>
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<tr>
<td>VIA + Nifedipine</td>
<td>46 ± 3</td>
<td>36 ± 3</td>
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<tr>
<td>Ca\textsuperscript{2+} (0.5 mM)</td>
<td>49 ± 2</td>
<td>36 ± 3</td>
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<tr>
<td>Ni\textsuperscript{2+} (2 mM)</td>
<td>6 ± 1</td>
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</table>

Values are means ± SE; n > 10.
S-AMPA elevated [Ca\(^{2+}\)]\(_{i}\) to values similar to those induced by glutamate. Combined administration of CNQX and APV as well as Ca\(^{2+}\)-free superfusate abolished the glutamate-induced [Ca\(^{2+}\)]\(_{i}\) transient (see also Fryer et al. 1999; Hubert et al. 1998; Tymianski et al. 1993). The latter results indicate that Ca\(^{2+}\) release from IP\(_3\)-sensitive stores due to activation of metabotropic glutamate receptors (Murphy and Miller 1988; Nicoletti et al. 1996) had no major contribution to the Ca\(^{2+}\) response to glutamate. This assumption gains support from the observations that the metabotropic glutamate receptor antagonist MCPG did not change the glutamate-evoked [Ca\(^{2+}\)]\(_{i}\) rise, whereas the broad spectrum metabotropic agonist [1S,3R]-ACPD had no effect on the Ca\(^{2+}\) baseline (see also Hubert et al. 1998). Nevertheless, Ca\(^{2+}\) stores appear to be operating in the embryonic motoneurons as administration of the Ca\(^{2+}\) store uptake blocker CPA evoked a noticable Ca\(^{2+}\) rise. However, CPA did not attenuate the glutamate-induced [Ca\(^{2+}\)]\(_{i}\) transient.

**Magnitude of glutamate-induced peak [Ca\(^{2+}\)]\(_{i}\), rises**

In the spinal motoneurons of the present study, ionotropic glutamate receptor agonists or saturating concentrations of glutamate (300 μM) elevated [Ca\(^{2+}\)]\(_{i}\) on average by ~200 nM. The relatively large size of the motoneuron somata (15–20 μm) is not responsible for these moderate peak amplitudes. In the embryonic motoneurons, whereas glutamate-induced Ca\(^{2+}\) rises were considerably larger in cultured cerebellar granule cells (Hubert et al. 1998). Also a further report revealed a modest glutamate-evoked [Ca\(^{2+}\)]\(_{i}\) rise in cultured embryonic spinal motoneurons (Fryer et al. 1999). In contrast, peak responses of intracellular Ca\(^{2+}\) exceeding 1 μM were revealed in two other studies (Carriedo et al. 1996; Tymianski et al. 1993). Species-specific differences between mice (Carriedo et al. 1996; Tymianski et al. 1993) and rats (Fryer et al. 1999; Hubert et al. 1998; present study) might be responsible for such differences in the magnitude of glutamate-induced Ca\(^{2+}\) transients (see also Ballanyi 1999).

Differences in the maturational state of the motoneurons could also underlie the discrepancies between the peak values of the glutamate receptor–evoked [Ca\(^{2+}\)]\(_{i}\) transients in the above studies. Large [Ca\(^{2+}\)]\(_{i}\), rises due to glutamate (receptor agonists) were revealed in motoneurons that were kept in culture for >2 wk before the measurements (Carriedo et al. 1996; Tymianski et al. 1993). In contrast, a modest elevation of intracellular Ca\(^{2+}\) was seen in motoneurons that were either investigated within 1 to 3 days after isolation from rats at embryonic day 15 (Fryer et al. 1999; Hubert et al. 1998) or within hours after preparation of acute spinal cord slices from rats of embryonic days 17–18 (our study). A different maturational state of the motoneurons in the latter reports would be in agreement with the observation that embryonic motoneurons are more susceptible to glutamate-mediated excitotoxicity after 2 wk in culture (Regan and Choi 1991; Stewart et al. 1991; Tymianski et al. 1993). The presumed age dependence of both the peak value of [Ca\(^{2+}\)]\(_{i}\) rises, and the concomitant vulnerability due to glutamate thus appears causally related to a developmental change in the magnitude of glutamate-induced depolarization (Seno et al. 1984; Ziskind-Conhaim 1990). Age-dependent differences in the peak amplitude of the response to glutamate might lead to activation of different types (and/or numbers) of high-voltage–activated Ca\(^{2+}\) channels (see Role of voltage-gated Ca\(^{2+}\) channels). Indeed, the recent observation that the high-voltage–activated component of Ca\(^{2+}\) channels is progressively increased in embryonic motoneurons within several days of culture fits into this proposed scenario (Magnelli et al. 1998).
GLUTAMATE-INDUCED $\text{Ca}^{2+}$ RISE IN FETAL LUMBAR MOTONEURONS

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Combined block of $\text{L}_{\text{v}}$, $\text{N}_{\text{v}}$, and $\text{P/Q}$-type $\text{Ca}^{2+}$ channels was found to reduce the $[\text{Ca}^{2+}]$, transient due to application of 300 $\mu \text{M}$ glutamate by $>50\%$. This shows that $\text{Ca}^{2+}$ entry from the interstitial space via high-voltage-activated $\text{Ca}^{2+}$ channels has a major contribution to $\text{Ca}^{2+}$ rises in spinopelvic motoneurons in situ on stimulation of ionotropic glutamate receptors. The relevance of $\text{Ca}^{2+}$ channels might be even underestimated as administration of a mixture of $\omega$-agatoxin-IVA, $\omega$-conotoxin-GVIA, and nifedipine at supramaximal concentrations reduced $\text{K}^{+}$-evoked $[\text{Ca}^{2+}]$, rises by $<65\%$. This indicates that in addition to $\text{N}_{\text{v}}$, $\text{L}_{\text{v}}$, and $\text{P/Q}$-type $\text{Ca}^{2+}$ channels the so-called “$\text{R}_{\text{v}}$-type” isofrom constitutes a major population of voltage-gated $\text{Ca}^{2+}$ channels in the embryonic lumbar motoneurons in situ as was suggested also for cultured motoneurons (Hivert et al. 1995; Magnelli et al. 1998; Scamps et al. 1998), facial motoneurons of slices (Plant et al. 1998), and other central neurons (Bacskai et al. 1995; Regan et al. 1991; Wu et al. 1999). In addition to involvement of $\text{R}_{\text{v}}$-type voltage-gated $\text{Ca}^{2+}$ channels in the glutamate-induced $[\text{Ca}^{2+}]$, rises, the amount of depolarization that is evoked by glutamate or high $\text{K}^{+}$ solutions needs to be considered. This is due to the fact that the magnitude of membrane depolarization is critical for the relative contribution of different types of $\text{Ca}^{2+}$ channels to depolarization-induced intracellular $\text{Ca}^{2+}$ rises. For example, it was found that $\omega$-conotoxin-GVIA blocks $\sim 50\%$ of current through voltage-gated $\text{Ca}^{2+}$ channels on depolarization of cultured motoneurons to 0 mV, whereas the drug only has an attenuating effect of $7\%$ during depolarization to $-30$ mV (Viana et al. 1997). This might explain the relatively small effect of the toxin on the $\text{K}^{+}$- and glutamate-induced $[\text{Ca}^{2+}]$, rises in the present study because the cells were solely depolarized to about $-20$ mV by both these procedures.

A pivotal role of voltage-gated $\text{Ca}^{2+}$ channels in the $[\text{Ca}^{2+}]$, rises in response to high concentrations of glutamate or ionotropic agonists is also suggested by the finding that these $\text{Ca}^{2+}$ transients were reduced by $\sim 70\%$ after switching from current to voltage clamp during whole cell recording. Also under these conditions, the quantitative contribution of $\text{Ca}^{2+}$ channels might be underestimated due to possibly imperfect voltage control of the glutamate-induced inward current secondary to space-clamp problems (Magnelli et al. 1998). That voltage-gated $\text{Ca}^{2+}$ channels constitute the predominant route of glutamate-induced $[\text{Ca}^{2+}]$, entry into embryonic motoneurons was recently also suggested by Hubert et al. (1998). The latter study presented evidence that $>80\%$ of the glutamate-induced $[\text{Ca}^{2+}]$, rise in cultured motoneurons is caused by $\text{L}_{\text{v}}$-type $\text{Ca}^{2+}$ channels, whereas the results of Tymianski et al. (1993) suggested a negligible contribution of nonligand gated $\text{Ca}^{2+}$ channels to the glutamate response.

Ca$^{2+}$ influx through KA/AMPA and NMDA receptors

In the present study, voltage-clamp techniques revealed that JSTX reversibly inhibits $\sim 50\%$ of the remaining S-AMPA-induced $[\text{Ca}^{2+}]$, response. The toxin was also found to reduce KA- or AMPA-induced $\text{Ca}^{2+}$ accumulation in hippocampal cell cultures from rats (Ying et al. 1997). JSTX also attenuated fluorometrically monitored $[\text{Ca}^{2+}]$, transients in cultured granule cells and transfected HEK cells that were induced by the latter glutamate receptor agonists (Meucci and Miller 1998; Savidge and Bristow 1998). In the present study, JSTX had a relatively smaller (25%) blocking effect on the S-AMPA-induced inward current than on the concomitant $[\text{Ca}^{2+}]$, rise (50%). This indicates that $\text{Ca}^{2+}$-permeable KA/AMPA receptors that lack the GluR2 subunit (Hollmann et al. 1991; Hollmann and Heinemann 1994) are co-expressed with $\text{Ca}^{2+}$-impermeable KA/AMPA receptors in individual motoneurons (see also Jonas and Burnashev 1995; Meucci and Miller 1998; O’Brien et al. 1997; Pellegrini-Giampietro et al. 1997). In this context, it was found that JSTX reduces AMPA receptor–mediated $[\text{Ca}^{2+}]$, rises in cultured cerebellar granule cells by 65%, whereas KA receptor–mediated responses are largely insensitive (Savidge and Bristow 1998). With respect to the potential physiological role of $\text{Ca}^{2+}$-permeable KA/AMPA receptors in developing spinal motoneurons, it should be noted that JSTX reduced the $[\text{Ca}^{2+}]$, rise in response to both S-AMPA or glutamate.

Under voltage clamp, APV attenuated the glutamate-evoked $[\text{Ca}^{2+}]$, increase by 32%. This shows that $\text{Ca}^{2+}$ influx via the...
Role of Ca\(^{2+}\)-permeable KA/AMPA receptors in embryonic motoneurons

It was recently shown for embryonic rat motoneurons that glutamate has a specific regulatory effect on outgrowth of dendrites, but not axons (Metzger et al. 1998). This effect was antagonized by diverse blockers of KA/AMPA receptors including JSTX. Ca\(^{2+}\)-permeable glutamate receptor subtypes presumably represent only a small part of the total KA/AMPA receptor population in neurons (Pellegrini-Giampietro et al. 1997). But the blocking effect of JSTX in the present study shows that these receptors provoke a noticable rise of intracellular Ca\(^{2+}\) of neurons in situ. Such Ca\(^{2+}\) influx might provide a robust intracellular signal (Mainen et al. 1999) that initiates the signal cascade involved in the JSTX-sensitive trophic effects of glutamate (Metzger et al. 1998). As recently reviewed (Kennedy 1997; Kirsch 1999), NMDA as well as AMPA receptors are clustered at postsynaptic densities to amplify signals that pass the synaptic cleft. Among other proteins, calmodulin and the Ca\(^{2+}\)-calmodulin–dependent protein kinase II (CAMKII) are protein components of the postsynaptic densities (Kirsch 1999; Strack et al. 1997). Furthermore, GluR1 and GluR2/3 receptor subtypes are phosphorylated by CAMKII, thus enhancing their responsiveness (Hayashi et al. 1997). This might enable Ca\(^{2+}\)-permeable KA/AMPA receptors to locally transform albeit small Ca\(^{2+}\) increases into Ca\(^{2+}\)-independent signals of the subsequent signal cascade involved in trophic actions of glutamate receptors. Thus these Ca\(^{2+}\)-dependent regulatory proteins may trigger very specific functions independent on those evoked by large and widespread Ca\(^{2+}\) increases via voltage-dependent Ca\(^{2+}\) channels.

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