AMPARs and NMDARs are expressed by differentiating Xenopus spinal neurons.

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Gleason, Evanna L. and Nicholas C. Spitzer. AMPA and NMDA receptors expressed by differentiating Xenopus spinal neurons. J. Neurophysiol. 79: 2986–2998, 1998. N-methyl-D-aspartate (NMDA) receptors are often the first ionotropic glutamate receptors expressed at early stages of development and appear to influence neuronal differentiation by mediating Ca$^{2+}$ influx. Although less well studied, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors also can generate Ca$^{2+}$ elevations and may have developmental roles. We document the presence of AMPA and NMDA class receptors and the absence of kainate class receptors with whole cell voltage-clamp recordings from Xenopus embryonic spinal neurons differentiated in vitro. Reversal potential measurements indicate that AMPA receptors are permeable to Ca$^{2+}$ both in differentiated neurons and at the time they first are expressed. The $P_{Ca}/P_{Na}$, of 1.9 is close to that of cloned Ca$^{2+}$-permeable AMPA receptors expressed in heterologous systems. Ca$^{2+}$ imaging reveals that Ca$^{2+}$ elevations are elicited by AMPA or NMDA in the absence of Mg$^{2+}$. The amplitudes and durations of these agonist-induced Ca$^{2+}$ elevations are similar to those of spontaneous Ca$^{2+}$ transients known to act as differentiation signals in these cells. Two sources of Ca$^{2+}$ amplify AMPA- and NMDA-induced Ca$^{2+}$ elevations. Activation of voltage-gated Ca$^{2+}$ channels by AMPA- or NMDA-mediated depolarization contributes ~15 or 30% of cytosolic Ca$^{2+}$ elevations, respectively. Activation of either class of receptor produces elevations of Ca$^{2+}$ that elicit further release of Ca$^{2+}$ from thapsigargin-sensitive but ryanodine-insensitive stores, contributing an additional ~30% of Ca$^{2+}$ elevations. Voltage-clamp recordings and Ca$^{2+}$ imaging both show that these spinal neurons express functional AMPA receptors soon after neurite initiation and before expression of NMDA receptors. The Ca$^{2+}$ permeability of AMPA receptors, their ability to generate significant elevations of [Ca$^{2+}$]i, and their appearance before synapse formation position them to play roles in neural development. Spontaneous release of agonists from growth cones is detected with glutamate receptors in outside-out patches, suggesting that spinal neurons are early, nonsynaptic sources of glutamate that can influence neuronal differentiation in vivo.

INTRODUCTION

Ionotropic glutamate receptors play critical roles in neuronal development in addition to mediating synaptic transmission and plasticity in mature neurons. Early synaptic activity involving N-methyl-D-aspartate (NMDA) receptors is important for refinement of sensory maps (Constantine-Paton 1990; Fox et al. 1996; Li et al. 1994; Rabacchi et al. 1992; Schnupp et al. 1995) and Ca$^{2+}$ influx through NMDA receptors has been suggested to underlie this refinement. Ionotropic glutamate receptors also are expressed before synaptogenesis, raising the possibility that these receptors influence even earlier aspects of neuronal differentiation (Komuro and Rakic 1993; Zheng et al. 1996). Although most work has focused on NMDA receptors, non-NMDA receptors also can participate in Ca$^{2+}$ signaling, either by their ability to depolarize cells and activate voltage-gated Ca$^{2+}$ channels or by virtue of their own Ca$^{2+}$ permeability. DNA synthesis and neurite outgrowth have both been shown to be regulated by activation of non-NMDA receptors (LoTurco et al. 1995; Mattson et al. 1988).

Embryonic Xenopus spinal neurons use elevations of intracellular Ca$^{2+}$ as differentiation signals (Bixby and Spitzer 1984a; Desarmenien and Spitzer 1991; Gu and Spitzer 1995; Gu et al. 1994; Holliday and Spitzer 1990; Spitzer et al. 1993). One class of Ca$^{2+}$ elevation is triggered by spontaneous activation of voltage-gated Ca$^{2+}$ channels. These Ca$^{2+}$ elevations influence neurotransmitter expression and maturation of voltage-gated potassium currents. A second class of Ca$^{2+}$ elevation regulates neurite extension. Because activation of ionotropic glutamate receptors can increase cytosolic Ca$^{2+}$, glutamate-mediated Ca$^{2+}$ elevations also may serve as signals for other aspects of differentiation in spinal neurons. Although Xenopus oocytes are widely used for expression studies of cloned receptors, relatively little is known about the endogenous ionotropic glutamate receptors in this species. Both NMDA and non-NMDA receptors are used by these cells later in development (Sands and Barish 1989; Sillar and Roberts 1988) but the specific types of ionotropic glutamate receptors expressed at early stages and their functional properties have not been examined.

We have investigated the role played by ionotropic glutamate receptors in the development of Xenopus spinal neurons by studying properties of these receptors on cells differentiating in culture. Because we are interested primarily in the functional properties of these ligand-gated channels during neuronal differentiation, we undertook a physiological analysis using electrophysiology and calcium imaging techniques rather than a pharmacological or molecular approach. Voltage and ligand-gated channels studied thus far in this system differentiate on a timetable indistinguishable from that observed in vivo (Baccaglini and Spitzer 1977; Bixby and Spitzer 1982, 1984b; Spitzer and Lamborghini 1976). Here we identify the subtypes of ionotropic glutamate receptors expressed by developing spinal neurons and determine their Ca$^{2+}$ permeability. To understand how expression of these receptors may influence development of spinal neurons, we determine when specific classes of receptors are first expressed. We examine the effectiveness of receptor activation in elevating cytosolic Ca$^{2+}$ and elucidate amplifi-
cation mechanisms involved in these responses. We hypothesize that glutamatergic spinal neurons are a source of agonist for these receptors. In support of this idea, outside-out membrane patches containing ionotropic glutamate receptors detect spontaneous release of agonist from growth cones, indicating that these receptors may be activated at early stages of development in vivo.

**METHODS**

**Cell culture**

Primary cultures were prepared from neural plate embryos (stage 15) (Nieuwkoop and Faber 1967) as previously described (Desarmenien and Spitzer 1991; Holiday and Spitzer 1990; Spitzer and Lamborghini 1976). Tissue of the presumptive spinal cord, including underlying mesoderm, was excised and dissociated in divalent cation-free medium, containing (in mM) 116.7 NaCl, 0.7 KCl, 0.4 EDTA, and 4.6 tris (hydroxymethyl) aminomethane (Tris), pH adjusted to 7.8 with HCl, for 20–25 min and then plated on tissue culture plastic (Falcon or Costar). Culture medium contained (in mM) 117.0 NaCl, 0.7 KCl, 1.3 MgSO$_4$, 2.0 CaCl$_2$, and 4.6 Tris, pH adjusted to 7.8 with HCl. Characterization of receptors and mechanisms of amplification of Ca$^{2+}$ signals was carried out on neurons between 18 and 24 h in culture, when cells are largely electrically mature, unless otherwise indicated; these cells are designated 1-day neurons. For developmental studies, cells were examined at four times after plating: from 6 to 9 h (when outgrowth standard external solution to solution containing agonist for 3 ± 5 s was estimated by examining the kinetics of current change due to agonist internal solution contained 77 (in mM) current-clamp configurations using an Axopatch 1-D amplifier.

**Electrophysiology**

Culture dishes were mounted on the stage of a Zeiss inverted microscope, and cells were observed with phase contrast optics. Neurons were chosen for recording on the criterion that they were electrically compact, having neurite lengths less than two to three soma diameters (Desarmenien and Spitzer 1991; O’Dowd et al. 1988). Pipettes were pulled from glass capillary (Drummond Scientific; Broomall, PA) on a Flaming Brown micropipette puller (Sutter Instruments; Novato, CA) to resistances of 2.5–4.0 MΩ for whole cell and outside-out patch recordings. Electrodes were coated with silicone elastomer (Sylgard; Dow Corning, Midland, MI) for outside-out patch recordings. Pipettes were filled with one of two recording solutions. Standard internal solution contained (in mM) 100 KCl, 3.0 MgCl$_2$, 0.1 CaCl$_2$, 1.1 ethylene glycol-bis(β-aminoethyl ether)-N,N′,N′,N′-tetraacetic acid (EGTA), 10.0 N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES), and 1.0 NaATP, pH adjusted to 7.6 with KOH. Cesium methanesulfonate internal solution contained (in mM) 90.0 CsMeSO$_4$, 10.0 CsCl, 0.1 CaCl$_2$, 1.1 EGTA, 10.0 HEPES, and 1.0 NaATP, pH adjusted to 7.6 with CsOH. A silver chloride reference electrode was connected to the bath with an agar bridge (2% in M KC1) positioned adjacent to the perfusion outflow. All recordings were made at room temperature (21 ± 2°C).

Cells were bathed in one of three external solutions. Standard external solution had no added Mg$^{2+}$ and contained (in mM) 116.7 NaCl, 0.67 KCl, 2.0 CaCl$_2$, and 4.6 Tris, pH adjusted to 7.6 with HCl. One millimolar Ca$^{2+}$ external solution contained 77 (in mM) NaCl, 3.0 KCl, 1.0 CaCl$_2$, 30.0 N-methyl-d-glucamine Cl (NMG Cl), 10.0 tetraethylammonium (TEA) Cl, and 5.0 HEPES, pH adjusted to 7.6 with NaOH. For 20 mM Ca$^{2+}$ external solution, NMG Cl was replaced with 20.0 mM CaCl$_2$. For reversal potential measurements, 1 and 20 mM Ca$^{2+}$ external solutions contained 300 mM tetrodotoxin (TTX) and 200 mM Cd$^{2+}$ to block voltage-gated Na$^+$ and Ca$^{2+}$ channels, respectively. For all experiments, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) was applied in the presence of 1.3–2.0 mM Mg$^{2+}$; NMDA was applied in the absence of Mg$^{2+}$ and with its coagonist glycine (10 μM) and 10 μMstrychnine to block activation of glycine receptors. AMPA, NMDA, 2-amino-5-phosphonovaleric acid (APV) and 15,3R-1-amino cyclopentane-1,3-dicarboxylic acid (ACPD) were obtained from Research Biochemicals International (Natick, MA). GYKI 53655, a 2,3 benzodiazepine compound and a highly selective noncompetitive antagonist of AMPA-preferring receptors (Donovan and Rogawski 1993; Paternain et al. 1995; Zurumski et al. 1993), has been used to reveal kainate-preferring receptors on cells that co-express AMPA-preferring receptors (Paternain et al. 1995). GYKI was a gift from Dr. J. David Leander at Lilly Research Laboratories (Indianapolis, IN). Thapsigargin and ryanodine were acquired from Calbiochem (San Diego, CA). All other reagents were obtained from Sigma (St. Louis, MO).

 Cultures were superfused continuously throughout all experiments at 3–4 ml/min, unless otherwise indicated, with a 1-mm ID gravity-driven inflow. Solution changes were executed by three methods. **Bath application:** for detection of receptor expression, the perfusion solution (3–4 ml/min) was switched manually from standard external solution to solution containing agonist for 3–5 s using a Hamilton valve. The agonist concentration was typically 100 μM; this concentration of AMPA and NMDA activates 80% (not shown) and 65% (Sands and Barish 1989) of the maximum activatable currents, respectively. Agonist was delivered at intervals of ≥1 min. This method was least disruptive to the integrity of cells during whole cell recording. **Rapid application:** for detection of receptor desensitization, application of agonists ± antagonist was achieved with an array of 250 μm ID tubes positioned ~100 μm from the neuron. Flow of test solution was initiated by opening a solenoid switch, which produced a swath of test solution that covered the entire cell. The rate of application with this method was estimated by examining the kinetics of current change due to the shift in junction potential produced by application of 0.1 times external solution onto an open pipette containing standard internal solution, with standard internal solution in the bath. The mean time to peak of responses ranged from 2 to 10 ms and averaged ~5 ms. Repeated applications of agonist were separated by >1 min to allow recovery from desensitization. **Puffer application:** for determination of reversal potentials, agonists were puffed onto cells through a 2- to 3-MΩ puffer positioned ~30 μm from the cell body during constant perfusion of standard external solution. Because a single puffer pipette was used to deliver the agonist, only one Ca$^{2+}$ concentration was examined for each cell. For most experiments, AMPA and NMDA were both used at 100 μM. Agonist was delivered at 4-s intervals. For stimulus-evoked agonist release-detection experiments, the 100 mM KCl puffer solution contained phenol red so that the size of the puff directed at the neuron could be adjusted to cover only the cell body. Because puffing this solution directly onto outside-out patches elicited a small, slow, inward current, the position of the KCl puff directed toward cell bodies was adjusted until no KCl-induced current was detected in the patch. For spontaneous agonist release-detection experiments, patch current was recorded continuously for two to five periods of 1–2 min each. For both evoked and spontaneous release experiments slow (~1 ml/min) perfusion of standard external solution was maintained throughout.

Whole cell recordings were made in both voltage-clamp and current-clamp configurations using an Axopatch 1-D amplifier.
Calcium imaging

Fluo-3 AM (Molecular Probes, Eugene, OR) stock solution was prepared in dimethyl sulfoxide (DMSO; 50 \mu g/16 \mu l) containing 4% Pluronic. Stock solution was added to the culture medium to achieve a final concentration of 1–2 \mu M fluo-3 AM. Cultures were incubated for 30 min at room temperature in the dark, washed with fluo-3-free medium (3 times, 10 min), and mounted on the microscope stage. Cells were imaged using a Bio-Rad MRC600 argon laser confocal system on a Zeiss upright microscope with a x20 water immersion objective. Images were acquired at 5-s intervals, for on-line analysis with a QuickCapture frame grabber board and the IMAGE program (W. Rashband, National Institutes of Health). Pixel intensity was sampled and averaged over a rectangular region of the cell body. Changes in fluorescence intensity of each region of interest were normalized to baseline fluorescence intensity (F/F0). Amplitudes were measured at the peak of the responses and durations at the base of the responses. Comparisons of response amplitudes are always for data collected from the same cell.

In imaging experiments, all solutions were applied by gravity-driven bath perfusion at 5–7 ml/min, and solutions were changed by manually switching a valve. To standardize the timing of drug delivery from experiment to experiment, valve switches were synchronized with opening of the shutter regulating illumination. Unless otherwise indicated, drugs were applied for ~10 s. The slow onset of bath-applied agonist and variations in duration of application precluded a detailed analysis of kinetic properties of responses. Standard (Mg2+-free) external solution was the same as described for voltage-clamp experiments. Voltage-gated Ca2+ channels were activated by depolarization with application of 50 mM K+, which contained (in mM) 66.7 NaCl, 50.0 KCl, 2.0 CaCl2, and 4.6 Tris, pH adjusted to 7.6 with HCl. Zero external Na+ (in mM) 116.7 choline Cl, 0.67 KCl, 2.0 CaCl2, and 4.6 Tris, pH adjusted to 7.6 with HCl. For imaging experiments in which voltage-gated Ca2+ channels were blocked with Cd2+, cells were not pre-exposed to Cd2+ because prolonged exposure resulted in irreversible increases in fluorescence. Brief (10–15 s) applications of 200 \mu M Cd2+ did not produce this change in fluorescence. Twenty millimolar caffeine external solution was prepared by replacement of 10 mM NaCl to maintain constant osmolarity. This concentration of caffeine produces maximal responses in these neurons (Holliday et al. 1991). For examination of contributions of Ca2+ stores to ionotropic glutamate receptor-mediated Ca2+ responses, only cells having an initial caffeine response >200% of baseline were analyzed. In initial imaging experiments, coapplication of caffeine and NMDA selectively and reversibly reduced NMDA response amplitudes. A reduction in NMDA-induced current also was seen in voltage-clamp experiments but was completely reversible within 1 min. Accordingly, experiments involving caffeine were designed so that caffeine was not coapplied with agonist.

In pilot experiments, we tested the possibility that the 5 min exposure to thapsigargin used in some imaging protocols might directly alter AMPA and/or NMDA receptors such that their current amplitude, and therefore Ca2+ influx, would be affected. Because it was not feasible to record from these cells in whole cell voltage clamp for the extended duration of thapsigargin imaging experiments, we mimicked the conditions of imaging experiments by exposing a population of cells to thapsigargin for 5 min followed by a 5-min wash and comparing the mean whole cell current density in response to AMPA or NMDA with that in an untreated population of cells. No significant difference was observed between mean current densities in control and thapsigargin-treated neurons (AMPA: control, −192 ± 20 fA/\mu m2, mean ± SE; n = 35; thapsigargin, −243 ± 33 fA/\mu m2, n = 11, P = 0.2; NMDA: control, −66 ± 5 fA/\mu m2, n = 29; thapsigargin, −55 ± 12 fA/\mu m2, n = 11, P = 0.3). In other preliminary experiments we determined the time for the intracellular Ca2+ concentration to return to baseline after thapsigargin, because elevated cytosolic Ca2+ can inhibit NMDA currents (Legendre et al. 1993; Rosenmund et al. 1995). Recovery of baseline Ca2+ concentration usually was achieved after ~5 min.

Data are reported as means ± SE; n is the total number of neurons examined unless otherwise indicated. The two-tailed t-test was used for statistical analysis.

RESULTS

AMPA and NMDA receptors are expressed on spinal neurons

Given the diversity in functional properties of different ionotropic glutamate receptors, we first determined which of these receptors are expressed at 18–24 h in vitro when most voltage-gated currents are expressed in their mature form. Xenopus spinal neurons have both AMPA and NMDA classes of glutamate receptors. Agonists were applied by bath application because it is more likely that the release of agonist at early stages of differentiation, before synaptogenesis, is mimicked by the relatively slow onset of bath perfusion than by rapid application. Bath application of AMPA generated currents ranging in density from −11 to −505 fA/\mu m2 with a mean of −192 ± 20 fA/\mu m2 (n = 35), when cells were voltage clamped at −70 mV (Fig. 1A). In the absence of Mg2+, NMDA elicited smaller currents when neurons were held at the same potential (Fig. 1B), with...
AMPA responses are generated by gating of AMPA-prefer-

Fig. 1. Neurons express both α-amino-3-hydroxy-5-methyl-4-isoxazo-

leproionic acid (AMPA) and N-methyl-D-aspartate (NMDA) classes of

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densities from −10 to −120 fA/μm² and a mean of −33 ± 5 fA/μm² (n = 29). At 1 day in culture, 59% (n = 61) of
eurons tested with both agonists had AMPA- and NMDA-gated currents. For most cells, AMPA-induced current was
substantially larger in peak amplitude than NMDA-induced current recorded from the same neuron (I_{AMPA}/I_{NMDA} =
9.4 ± 1.6, n = 26). AMPA- and NMDA-induced currents also were examined in the perforated-patch configuration to
minimize internal dialysis that might alter current ampli-
tudes. Peak amplitudes for currents induced by both agonists
fell within the same range as those recorded in the whole
cell configuration, and the relative amplitude of responses
was not significantly different from that found in whole cell
recordings (I_{AMPA}/I_{NMDA} = 13.2 ± 3.9, n = 4; P = 0.7).

Rapid application of AMPA was used to test whether
AMPA responses are generated by gating of AMPA-prefer-
ing receptors, by kainate-prefering receptors, or by both.
Although rapid application of agonist reveals a fast, desensi-
tizing component when AMPA binds its preferred receptor
or when kainate binds its preferred receptor, no desensitiza-
tion is seen in the presence of the nonpreferred agonist
(Lerma et al. 1993; Partin et al. 1993; Paternain et al. 1995;
Seeburg 1993). In all cases, rapid application of AMPA
produced a current response with a rapidly desensitizing
component, whereas application of kainate produced a non-
desensitizing response (n = 15). To determine whether a
small population of rapidly desensitizing kainate receptors
was masked by the large current produced by kainate activa-
tion of AMPA receptors, we recorded responses to rapidly
applied agonist in the presence of GYKI 53655, a selective
AMPA receptor blocker. GYKI (300 μM) reversibly
blocked 97 ± 1% of the AMPA-induced current (n = 5,
Fig. 1C). GYKI (300 or 50 μM) also blocked responses to
either 200 μM or 1 mM kainate by 99 ± 1% (n = 7, Fig.
1D), and in no case was a rapidly desensitizing response to
kainate revealed. These results also suggest that AMPA is
not activating NMDA receptors, given the selectivity of
GYKI for AMPA receptors.

AMPA receptors are calcium permeable

These AMPA-gated currents demonstrate significant Ca²⁺
permeability. Relative Ca²⁺ permeabilities of AMPA and
NMDA receptors were examined by puffing agonist onto
neurons during a series of voltage steps and measuring the
reversal potential of sustained agonist-induced current. We
analyzed the sustained rather than the fast inactivating por-
tion of the AMPA current because this is the current compo-
nent most likely to be activated by the slower, nonsynaptic
delivery of agonist. Voltage-gated inward currents were
blocked pharmacologically. In saline containing 1 mM Ca²⁺,
AMPA-induced current reversed at −10.6 ± 0.6 mV (n = 10),
whereas in saline containing 20 mM Ca²⁺, it reversed at
0.0 ± 0.5 mV (n = 18; Fig. 2, A–C) at 1 day in culture.
This 10-mV positive shift demonstrates that AMPA receptor
channels are permeable to Ca²⁺. The small variance suggests
that these neurons express chiefly Ca²⁺-permeable AMPA
receptors, but these data do not exclude the existence of a
small population of Ca²⁺-impermeable receptors. Assuming
K⁺, Cs⁺, and Na⁺ are equally permeant (Mayer and West-
brook 1987), the permeability ratio for Ca²⁺ to monovalent
cations (P_Ca/P_monocat) was estimated from the constant field
equation (Mayer and Westbrook 1987). Using ionic activi-
ties (see METHODS), the mean reversal potentials in both 1
and 20 mM Ca²⁺ yield an estimated P_Ca/P_monocat of 1.9 for
AMPA receptors.

Because AMPA receptor subunit composition might
change during development, producing a change in Ca²⁺
permeability (Burnashev et al. 1992b), we also assessed the
Ca²⁺ permeability of AMPA receptors at 6–9 h in culture
when they are first expressed (see below) at the time of
initial neurite extension. These experiments were performed
using 20 mM Cu²⁺ because reversal potential measurements
in 1 mM Ca²⁺ do not clearly distinguish different P_Ca/P_monocat
values (compare 1 mM Ca²⁺ reversal potential measure-
ments for AMPA and NMDA receptors, in the following
text). Reversal potentials measured for young neurons were
not significantly different from those measured at 1 day in
culture (20 mM Ca²⁺; −1.6 ± 0.7 mV, n = 4, P = 0.1),
showing that AMPA receptors can participate in Ca²⁺ signal-
ing by directly admitting Ca²⁺ as soon as they are present.

As expected, and in agreement with Sands and Barish
(1989), NMDA-gated currents in neurons demonstrate a
high relative Ca²⁺ permeability. These currents reversed at
−9.4 ± 0.7 mV (n = 5), and at +11.1 ± 1.1 mV (n = 11;
Fig. 2, D–F) in 1 and 20 mM Ca²⁺, respectively, at 1 day
in culture. We calculate a P_Ca/P_monocat of 5.8 for NMDA
Both AMPA and NMDA receptors are Ca\(^{2+}\) permeable at 1 day in culture. AMPA (100 \(\mu\)M; A and B) and NMDA (500 \(\mu\)M; D and E) were puff sprayed onto neurons during a series of voltage steps in 10-mV increments in the presence of either 1 or 20 mM Ca\(^{2+}\). Bars indicate duration of puffs. Slow decay of current results from the slow rate of bath perfusion. Data from 4 different neurons. C and F: current-voltage relationships for agonist-induced currents shown in A, B, D, and E. Data were collected in Mg\(^{2+}\)-free external solutions, using CsMeSO\(_4\) internal solution. Scale bars are 40 pA and 100 ms.

Because transient elevations of Ca\(^{2+}\) are developmental signals in these neurons (Bixby and Spitzer 1984a; Desarmenien and Spitzer 1991; Gu and Spitzer 1995; Gu et al. 1994; Holliday and Spitzer 1990; Spitzer et al. 1993) and activation of ionotropic glutamate receptors elevates intracellular Ca\(^{2+}\) (Connor et al. 1987; Hockberger et al. 1989; Leinders-Zufall et al. 1994), we loaded neurons with the Ca\(^{2+}\) indicator fluo-3AM and determined responses to 10-s bath application of NMDA (without Mg\(^{2+}\)) or AMPA at 1 day in culture. Paralleling results from voltage-clamp recordings, 60% of cells responded to both agonists at 100 \(\mu\)M (Fig. 3A, \(n = 130\) ). Activation of AMPA receptors produces Ca\(^{2+}\) signals comparable in amplitude to those produced by 50 mM KCl. This is a functionally significant index for these neurons because KCl-induced elevations of intracellular Ca\(^{2+}\) mimic spontaneous elevations of intracellular Ca\(^{2+}\) that regulate neuronal differentiation (Gu and Spitzer 1995). Because glutamate is the presumed agonist for these receptors in vivo, we also examined Ca\(^{2+}\) elevations induced by glutamate in the presence of 2 mM Mg\(^{2+}\). Glutamate elicits elevations of Ca\(^{2+}\) that are similar in waveform to those elicited by KCl or by activation of either AMPA or NMDA receptors alone (Fig. 3B, \(n = 8\) ). Application of 10-s pulses of APCD, which activates types I and II metabotropic glutamate receptors (Pin and Duvoisin 1995), had no effect on the level of intracellular Ca\(^{2+}\) (\(n = 40\), not shown).

For neurons with well-loaded processes, typically \(\sim 80 \mu\)m in length, Ca\(^{2+}\) elevations were detectable in the somata.
neurites and growth cones within one sampling interval. Because the propagation rate of spontaneous Ca\(^{2+}\) waves in these neurons is about 1 \(\mu\)m/s (Gu et al. 1994), consistent with diffusion of Ca\(^{2+}\) (Allbritton et al. 1992), simultaneous observation of elevation of [Ca\(^{2+}\)] in the cell body, neurite, and growth cone with a 0.2-Hz sampling rate implies that these Ca\(^{2+}\) elevations are due to the presence of receptors on growth cones and processes as well as the cell body and not due to diffusion from cell body to growth cone or vice versa. This conclusion is in agreement with the demonstration that glutamate applied to growth cones directs process outgrowth in *Xenopus* spinal neurons (Zheng et al. 1996).

Peak amplitudes of AMPA-induced Ca\(^{2+}\) responses were only 1.3 ± 0.9-fold larger than NMDA-induced Ca\(^{2+}\) responses recorded in the same cell (n = 90). The similarity in amplitudes of AMPA and NMDA responses was unexpected, even considering that NMDA-gated channels are several times more permeable to Ca\(^{2+}\) than AMPA-gated channels, because AMPA-evoked whole cell currents are ninefold larger than NMDA-evoked currents. This observation raised the possibility that AMPA and NMDA signals are differentially amplified.

### Amplification mechanisms for calcium signals: voltage-gated Ca\(^{2+}\) channels

Amplification of the Ca\(^{2+}\) signal produced by Ca\(^{2+}\) influx through AMPA and NMDA receptors could occur in several ways. Activation of AMPA and NMDA receptors produces inward current at the resting potential that may depolarize cells sufficiently to activate voltage-gated Ca\(^{2+}\) channels (VGCCs). To address this possibility, we reduced the depolarization produced by receptor activation by replacing the predominant inward current carrier (Na\(^{+}\)) with an impermeant cation (choline\(^{+}\)). In current-clamp recordings, replacement of external Na\(^{+}\) with choline\(^{+}\) reduced voltage responses to agonists by about 90% and prevented the membrane voltage from entering the activation range for Na\(^{+}\) and Ca\(^{2+}\) channels (Fig. 4A). In imaging experiments, replacement of extracellular Na\(^{+}\) with choline\(^{+}\) caused a transient (<3 min) increase in cytosolic Ca\(^{2+}\) presumably because of inhibition of the Na\(^{+}\)/Ca\(^{2+}\) exchanger. After recovery of baseline Ca\(^{2+}\) concentration, responses of neurons to AMPA and NMDA were compared with those measured in the same neurons in the presence of external Na\(^{+}\) (Fig. 5, A and B) at 1 day in culture. In Na\(^{+}\)-free external solution, AMPA responses were reduced by 15 ± 5% (n = 9) and NMDA responses by 27 ± 8% (n = 9). The greater effect on NMDA response amplitudes was unanticipated because small currents produced by NMDA (Fig. 1B) would not be expected to depolarize these cells sufficiently, although it is consistent with the observation that choline\(^{+}\) can block NMDA receptors (Ascher and Nowak 1988; however, see below).

Contributions of VGCCs also were examined by testing the effects of Cd\(^{2+}\), an effective blocker of VGCCs in these cells (Barish 1991; Gu and Spitzer 1993), because dihydropyridines do not completely block these VGCCs and the block by \(\omega\)-conotoxin is partly reversible (see also McCleskey et al. 1987). Cadmium ions were shown to inhibit current through NMDA receptors in some systems (Mayer et al. 1989, but see Ascher and Nowak 1988; Mayer et al. 1984); however, in voltage-clamp recordings, 200 \(\mu\)M Cd\(^{2+}\) had little effect on NMDA-evoked current (0.0 ± 5.0%; n = 8) although AMPA-evoked currents were reduced by 13 ± 5% (n = 11, Fig. 4B). In imaging experiments, responses of neurons to AMPA at 1 day in culture were reduced in amplitude by 16 ± 3% (n = 13) when AMPA was coapplied with Cd\(^{2+}\); NMDA responses were suppressed by 25 ± 4% (n = 13; Fig. 5, C and D). The similar reduction of AMPA responses when contributions of Ca\(^{2+}\) channels are suppressed by 0 Na\(^{+}\) or by 10 Na\(^{+}\) to eliminate action potentials or by 15 Na\(^{+}\) to directly block Ca\(^{2+}\) channels (Fig. 5E) suggests that the reduction in AMPA-evoked current produced by Cd\(^{2+}\) may not greatly influence peak amplitudes of AMPA-induced Ca\(^{2+}\) responses. The comparable reduction of NMDA responses when contributions of Ca\(^{2+}\) channels are suppressed by 0 Na\(^{+}\) or by 10 Na\(^{+}\) to eliminate action potentials or by 15 Na\(^{+}\) to directly block Ca\(^{2+}\) channels (Fig. 5F) suggests that little or none of the reduction in Ca\(^{2+}\) elevation is due to direct blockade of NMDA receptors by choline\(^{+}\) (Ascher et al. 1988). In addition, the similar reduction of AMPA or...
NMDA responses by either treatment suggests that factors such as nonindependence of Na\(^+\) and Ca\(^{2+}\) permeation or permeation of Cd\(^{2+}\) through these channels are unlikely to contribute significantly to these results (Fig. 5, E and F).

The effect of either 0 Na\(^+\) or +Cd\(^{2+}\) on NMDA-induced Ca\(^{2+}\) responses was variable, ranging from little effect on response amplitude (<10% reduction, 36% of neurons) to a substantial effect (>50% reduction, 14% of neurons).

Although the reductions of AMPA- and NMDA-induced Ca\(^{2+}\) elevations in 0 Na\(^+\) and +Cd\(^{2+}\) experiments did not differ significantly, the consistently greater suppression of NMDA-induced responses led us to compare the depolarization produced by activation of NMDA and AMPA receptors. In current-clamp recordings NMDA elicited multiple action potentials in 18% of neurons, paralleling the incidence with which suppression of the contribution of VGCCs substantially suppressed NMDA-induced Ca\(^{2+}\) responses. In contrast, the effect of either 0 Na\(^+\) or +Cd\(^{2+}\) on AMPA-induced Ca\(^{2+}\) responses was more consistent; only 10% of neurons generated an action potential, and no repetitive firing was observed in these cells (compare Fig. 5, G and H). Although the mechanism by which activation of VGCCs by NMDA is nearly twice that achieved by AMPA remains to be addressed, these experiments reveal that VGCCs make a consistently modest contribution to AMPA Ca\(^{2+}\) responses and a greater but variable contribution to NMDA Ca\(^{2+}\) responses.

Amplification mechanisms for Ca\(^{2+}\) signals: intracellular Ca\(^{2+}\) stores

*Xenopus* spinal neurons have stores that amplify Ca\(^{2+}\) elevations originating from Ca\(^{2+}\) influx across the plasma membrane (Barish 1991; Holliday et al. 1991). NMDA receptor activation efficiently elevates cytosolic Ca\(^{2+}\) despite the relatively small NMDA receptor currents. This observation raised the possibility that, in addition to a greater relative Ca\(^{2+}\) permeability and the ability of a subset of neurons to produce action potentials, Ca\(^{2+}\) entering through NMDA receptors also might have preferential access to Ca\(^{2+}\) stores. We used thapsigargin to determine the contributions of Ca\(^{2+}\) stores to Ca\(^{2+}\) elevations induced by activation of each type of ionotropic glutamate receptor. Thapsigargin is an irreversible inhibitor of ATP-dependent Ca\(^{2+}\) pump activity in the endoplasmic reticulum (ER) that spares Ca\(^{2+}\) transporters in mitochondria and on the plasma membrane (Thastrup et al. 1990). Thapsigargin itself elicited a transient elevation in Ca\(^{2+}\), presumably due to the slow leak of Ca\(^{2+}\) that is normally offset by the action of the ER Ca\(^{2+}\) pump. AMPA and NMDA responses were elicited from neurons at 1 day in culture both before and subsequent to a 5-min exposure to 100 nM thapsigargin after Ca\(^{2+}\) had returned to baseline (Fig. 6, A and B). Responses to 20 mM caffeine also were elicited before and after thapsigargin treatment to confirm its effectiveness in emptying caffeine-sensitive stores. Thapsigargin treatment abolished responses to caffeine in all cells examined (n = 18).

Thapsigargin treatment reduced peak amplitudes of responses to AMPA and NMDA in all cases by \(-35 \pm 7\%\) (n = 17) and \(-30 \pm 3\%\) (n = 18), respectively. As a control for rundown of agonist responses with time, the results of these experiments were compared with others in which AMPA and NMDA responses were recorded over the same time period but without addition of thapsigargin (Fig. 6B). Because thapsigargin has no long-term effect on AMPA-
and NMDA-induced current amplitudes (see METHODS), we conclude that the effects on AMPA and NMDA responses in imaging experiments are due specifically to the action of thapsigargin on Ca\textsuperscript{2+} store pumps. Furthermore, this result indicates that release of Ca\textsuperscript{2+} from thapsigargin-sensitive stores contributes approximately equally to peak amplitudes of Ca\textsuperscript{2+} responses elicited by activation of either AMPA or NMDA receptors.

Ca\textsuperscript{2+} responses in neurons at 1 day in culture also were tested for their dependence upon release of Ca\textsuperscript{2+} from the caffeine- and ryanodine-sensitive store. Low concentrations of ryanodine (1–10 µM) lock the ryanodine receptor-channel open in a use-dependent manner (Smith et al. 1988). Responses to repeated applications of caffeine at 1.5-min intervals were reduced by 44 ± 2% at steady state in the presence of 10 µM ryanodine (Fig. 6C) but were not eliminated (Fig. 6D, n = 6). This reduction was not merely due to the inability of caffeine stores to refill between caffeine applications because there was no diminution in amplitude of responses to repeated caffeine applications at 1.5-min intervals in control experiments in the absence of ryanodine (n = 8, not shown). Unexpectedly, AMPA and NMDA responses after ryanodine and caffeine treatment were not consistently different from pre-ryanodine responses and were not significantly different from control responses recorded during the same interval (Fig. 6, C and D).

Similar experiments were performed with repetitive applications of caffeine in the presence of 100 µM ryanodine, a concentration that traps the ryanodine receptor in the closed state (Smith et al. 1988). This concentration of ryanodine abolished the caffeine response in all neurons tested (n = 12) but was still without significant effect on amplitudes of AMPA and NMDA responses (Fig. 6E). These findings indicate that although an intracellular, thapsigargin-sensitive Ca\textsuperscript{2+} store contributes to AMPA and NMDA responses, under these conditions the caffeine- and ryanodine-sensitive store is not involved.

In Xenopus spinal neurons it is not known whether IP\textsubscript{3}- and caffeine-sensitive Ca\textsuperscript{2+} stores overlap or are physically distinct (Golovina and Blaustein 1997). Thapsigargin is effective in emptying IP\textsubscript{3}-sensitive stores in other cells (Irving et al. 1992; Law et al. 1990; Robinson and Burgoyne 1991; Thastrup et al. 1990). Because thapsigargin and ryanodine affect functionally separate stores in these neurons, IP\textsubscript{3} and caffeine-sensitive Ca\textsuperscript{2+} stores appear to be operationally distinct.

**AMPA receptors are expressed before NMDA receptors**

AMPA receptors are the first ionotropic glutamate receptors to be functionally expressed by these neurons, appearing in ~40% of cells by 9 h in culture when assessed both by fluo-3 Ca\textsuperscript{2+} imaging and whole cell voltage clamp (Fig. 7, A and B). This period, from 6–9 h in culture, corresponds...
to the time of initial neurite outgrowth, and examination of data from this period in 1 h increments indicates that AMPA receptors are not detectable at 6 h in culture. No responses to 100 µM NMDA (Mg²⁺-free, n = 25) were elicited between 6 and 9 h in either imaging or voltage-clamp experiments despite sensitivity allowing detection of fluorescence increases ≥10% and currents ≥10 pA. Ten cells examined with 400 µM NMDA also failed to show a current response (not shown). Both AMPA and glutamate (100 µM) produced Ca²⁺ elevations with similar amplitudes to those produced by 50 mM KCl. Because stimulation with KCl mimics elevations of intracellular Ca²⁺ that regulate neuronal differentiation (Gu and Spitzer 1995), these observations indicate that Ca²⁺ elevations elicited by early activation of AMPA receptors could act as developmental regulatory signals.

NMDA receptors were first detectable between 12 and 15 h in ~45% of neurons tested. The small NMDA current at early stages of differentiation could result from block of NMDA receptors by endogenous Zn²⁺ or a low Pₒₐ₆₆ due to subunit composition (Paollett et al. 1997). By 1 day in culture, when neurons express a largely mature complement of voltage-gated channels, ~60% of cells are responsive to NMDA, whereas ~90% are responsive to AMPA. After 2 days in culture, about 95% respond to both NMDA and AMPA. γ-Aminobutyric acid receptors are first expressed at approximately the same stage both in vitro and in vivo (Bixby and Spitzer 1982, 1984a); thus it seems likely that the appearance of glutamate receptors in culture reflects primary development rather than regeneration. Voltage-clamp data generally show a slightly larger percentage of responsive cells, suggesting that small currents may not produce detectable Ca²⁺ signals. The density of AMPA- and NMDA-evoked currents increased with time in culture, reaching a plateau at 1 day (Fig. 7, C and D).

**Growth cones spontaneously release glutamate receptor agonist**

Because AMPA receptors are expressed early in development and their activation produces elevations of intracellular Ca²⁺, we were interested in potential sources of agonist, presumably glutamate, in the early developing embryo. We tested the possibility that growth cones of spinal neurons are a source of agonist using outside-out patches of membrane from neuron cell bodies at 1 day in vitro as detectors of agonist release (Copenhagen and Jahr 1989; Hume et al. 1983; Young and Poo 1983). Patches first were stimulated with bath-applied glutamate to ensure that they contained increasing amounts of glutamate receptors and then positioned close to growth cones of other neurons in the dish. In some experiments, we evoked release by depolarization with a ~10 µM solution of 100 mM KCl puffed onto cell bodies of test cells (Fig. 8A). In other experiments, growth cones were tested without depolarization of the cell body, and spontaneous bursts of channel activity were detected in patches when they were positioned near a growth cone (Fig. 8B). Channel activity in these patches was completely eliminated either by coapplication of APV (100 µM) and GYKI (50 µM) or by moving the patch away from the growth cone. We observed evoked release of agonist from two of three neurons and spontaneous agonist release from two of five neurons examined for 1- to 2-min intervals. Because spontaneous release occurs in bursts, longer periods of recording would be expected to increase the incidence of observation. These results indicate that an agonist, presumably glutamate, is released from the growth cones of growing neurites. Although spontaneous Ca²⁺ elevations observed in dissociated neurons differentiating at low density in vitro are not affected by glutamate receptor blockers (Gu et al. 1994), growth cones provide a source of glutamate receptor agonist that

**FIG. 7.** AMPA receptors precede NMDA receptors developmentally. A and B: percentages of cells responding to applications of 100 µM AMPA, or NMDA in the absence of extracellular Mg²⁺. are plotted as a function of developmental age. □, data from Ca²⁺ imaging experiments; ▪, data from voltage-clamp recordings. Numbers above the bars indicate the number of separate experiments; ±3 neurons were examined for each condition. * Absence of response to NMDA at 6–9 h. Nine of the 15 cells that gave no current response to 100 µM NMDA at 12–15 h also were tested with 500 µM NMDA and 3 of these cells produced just detectable responses (7–11 fA/µm²). C and D: increases in density of currents evoked by 100 µM AMPA or NMDA in the absence of extracellular Mg²⁺. Data from ≥3 experiments were pooled for each time point, and numbers above the bars indicate the total number of neurons examined. Data from cells that did not respond to the agonists were not included in this analysis. All data were collected in standard external solution, and for voltage-clamp experiments standard internal solution was used.
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Comparison of Xenopus and mammalian ionotropic glutamate receptors

We identified Xenopus ionotropic glutamate receptors based on the assumption that Xenopus neurons express sub-units homologous to those cloned from mammalian preparations (for review see Hollmann and Heinemann 1994). Receptors expressed on Xenopus spinal neurons have properties similar to those of cloned as well as native mammalian glutamate receptors. Their ED50 values for kainate and quisqualate on non-NMDA receptors and for NMDA on NMDA receptors match closely those described for rat brain receptors expressed in oocytes (Sands and Barish 1989).

Our results also demonstrate functional similarities between Xenopus and mammalian receptors. The estimated P_{Ca}/P_{monocat} of 5.8 for NMDA receptors is close to that obtained for rat hippocampal neurons, for which a comparable analysis gave an estimated P_{Ca}/P_{monocat} of 5 (Jahr and Stevens 1993). For mammalian AMPA receptors, low Ca2+-permeability is conferred by expression of the GluR2 subunit in an edited form. This subunit, in combination with any of the other subunits (GluR1, 3 or 4), forms Ca2+-impermeable channels (Hollmann et al. 1991; Hume et al. 1991). For AMPA receptors on Xenopus spinal neurons we estimate a P_{Ca}/P_{monocat} of 1.9. This value falls within the range estimated for channels formed by expression of GluR2 in its unedited, and therefore Ca2+-permeable, form (Hume et al. 1991; Jonas and Burnashev 1995). If Xenopus subunits are homologous to mammalian subunits, then the high P_{Ca}/P_{monocat} estimated for AMPA receptors implies that edited GluR2 subunits are expressed at low levels, if at all, in these neurons.

There is no clear rectification of the I-V relationship for AMPA-induced current in these Xenopus neurons, although cloned and expressed mammalian AMPA receptors demonstrate strong rectification in the absence of edited GluR2 expression (Boulter et al. 1990; Burnashev et al. 1992b; Hume et al. 1991). Native Ca2+-permeable AMPA receptors studied in other preparations are heterogeneous with respect to rectification (Burnashev et al. 1992a; Gilbertson et al. 1991; Iino et al. 1990; Jonas et al. 1994; Leinders-Zufall et al. 1994), suggesting that rectification may not be a property intrinsic to the channel. Polyamines such as spermine can modulate the degree of rectification of AMPA-prefering receptors (Bowie and Mayer 1995). Thus differences in cytosolic constituents among cell type and variability in dialysis of these factors in the whole cell recording configuration may underlie differences in rectification properties reported for native receptors.

Identity of internal stores

Activation of both AMPA and NMDA receptors leads to mobilization of Ca2+ from internal stores that constitutes ~30% of the elevation of intracellular Ca2+. We conclude that ER-associated stores are involved in amplification of responses to AMPA and to NMDA because thapsigargin was effective in reducing their amplitude. The degree to which ER store depletion alters response amplitudes varies among preparations. NMDA-mediated responses in cerebellar granule cells are reduced by ~43% (Kocsis et al. 1994), a value similar to that observed here for both AMPA and NMDA receptor activation. In contrast, NMDA responses in hippocampal neurons are more substantially affected (63%) (Kocsis et al. 1993).

The best characterized mechanism of Ca2+-induced Ca2+...
release involves opening of ryanodine receptor channels by elevated cytosolic Ca\(^{2+}\). However, repeated activation of ryanodine receptors by caffeine in the presence of high concentrations of ryanodine did not change the responses to AMPA and NMDA. A similar result has been reported for pituitary cells (Law et al. 1990). One possibility is that IP\(_3\) receptor-activated stores are the source of elevated cytosolic Ca\(^{2+}\). Because Ca\(^{2+}\) acts as a coagonist for this receptor (Bezprozvanny et al. 1991; Finch et al. 1991), Ca\(^{2+}\) alone could act as a trigger for activation of these receptors if resting IP\(_3\) is sufficiently high in these cells (Berridge 1993; Finch et al. 1991; Simpson et al. 1993). Specific, cell-permeant inhibitors of IP\(_3\) receptors would facilitate investigation of this possibility. Both anatomic localization and physiological studies have indicated that the degree of physical overlap of IP\(_3\) and ryanodine receptors varies among cell types (Malgaroli et al. 1990; Robinson and Burgoyne 1991; Verma 1990) and can vary regionally within a single cell (Golovina and Blaustein 1997; Sharp et al. 1993; Walton et al. 1991). Our results indicate that stores distinct from caffeine-sensitive stores normally contribute to elevations of intracellular Ca\(^{2+}\) by AMPA and NMDA in Xenopus spinal neurons.

Functional significance

These spinal neurons begin to express ionotropic glutamate receptors soon after initiation of neurite outgrowth. AMPA receptors are detected first, then NMDA receptors become detectable after a delay of several hours. The same temporal sequence of expression of these receptors has been observed in hypothalamic neurons (van den Pol et al. 1995). This delay in NMDA receptor expression is economical because NMDA receptor activation alone in the normal presence of extracellular Mg\(^{2+}\) produces only small, brief elevations of cytosolic Ca\(^{2+}\) that may not be sufficient to activate 

Ca\(^{2+}\)-dependent mechanisms in these cells (Gu and Spitzer 1995). The early appearance of Ca\(^{2+}\)-permeable AMPA receptors may allow substantial glutamate-induced Ca\(^{2+}\) elevations to occur before synaptogenesis. Whole cell recordings from interneurons in the intact spinal cord indicate that AMPA receptors have substantial Ca\(^{2+}\) permeability in vivo (J. Rohrbough and N. C. Spitzer, unpublished data). Although slow, nonsynaptic activation of these receptors probably would desensitize the fast component of the current, our imaging results using slow agonist application indicate that the substantial nonactivating component of AMPA-induced current is sufficient to permit Ca\(^{2+}\) signaling.

The percentage of cells responding to AMPA and NMDA increases substantially between 6 and 18 h in culture. Spontaneous, transient elevations of intracellular Ca\(^{2+}\) also occur during this period and influence several aspects of differentiation, including the rate of neurite extension, neurotransmitter expression and activation kinetics of voltage-gated potassium channels (Gu and Spitzer 1995; Gu et al. 1994). Because these neurons are sensitive to Ca\(^{2+}\) elevations over this period, activation of Ca\(^{2+}\)-permeable glutamate receptors has the potential to influence their development. If these receptors are expressed on the same time table in vivo as in culture, as preliminary evidence suggests (E. L. Gleason, T. M. Gomez, and N. C. Spitzer, unpublished observations), AMPA receptors are expressed before the majority of synapse formation (Hayes and Roberts 1973, 1974) and during a period of significant neurite outgrowth (Jacobson and Huang 1985; Taylor and Roberts 1983) in the intact spinal cord. A gradient of glutamate has been shown to influence the growth trajectory of cultured Xenopus spinal neurons (Zheng et al. 1996), suggesting that glutamate may function as a guidance signal in vivo. The origin of endogenous glutamate in the developing spinal cord that could act as a signal is presently unknown, but potential sources include spinal neurons themselves. In support of this idea, our results indicate that growth cones of Xenopus spinal neurons release a glutamate receptor agonist both spontaneously and in response to stimulation. Different information may be encoded by different patterns of release in vivo, occurring spontaneously or via the Ca\(^{2+}\) elevations achieved by spontaneous Ca\(^{2+}\) spikes or Ca\(^{2+}\) waves (Gu and Spitzer 1995; Gu et al. 1994).

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