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Glutamate Receptor-Mediated Calcium Entry in Neurons Derived From P19 Embryonal Carcinoma Cells

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We have examined the control of calcium elevation by glutamate in neurons derived from the mouse P19 embryonal carcinoma cell line. Following transient exposure to retinoic acid, P19 cells differentiate into neurons that express both NMDA and non-NMDA glutamate receptor subtypes. Fluorescence videomicroscopy using the indicator fura-2 revealed concentration-dependent elevation in cytosolic calcium levels with exposure to NMDA or kainate. Replacement of extracellular sodium with *N*-methylglucamine significantly reduced the action of kainate. Exposure to high K^+ medium also elicited an elevation of cytosolic calcium in P19 cells, which was partially inhibited by the calcium channel antagonist nimodipine. These experiments suggest that the elevation in calcium produced by kainate involves the activation of voltage-gated calcium channels as a consequence of membrane depolarization, in contrast to direct calcium entry through NMDA receptor channels.

Whole-cell recordings revealed that P19 NMDA receptors were highly permeable to calcium (P_{Ca} / P_{Na} = 5.6 ± 0.2). In most cells, channels gated by kainate displayed low permeability to calcium; the median permeability ratio, P_{Ca}/P_{Na}, was 0.053 (range 0.045 to 0.132). Activation of peak currents by NMDA, glycine, and kainate was half-maximal at 24 μM, 240 nM, and 81 μM, respectively. In addition, cadmium-sensitive currents through voltage-gated calcium channels were recorded in P19 cells bathed in barium/TEA chloride. Staining with antibodies directed against AMPA receptor subunits revealed widespread immunoreactivity for anti-GluR-B/C and anti-GluR-B/D. About half of the P19 cells were stained with antibodies selective for GluR-D but there was little or no immunoreactivity for the GluR-A subunit.

Key words: permeation, excitotoxicity, N-methyl-D-aspartate, mouse, fura-2

INTRODUCTION

Changes in intracellular calcium are known to underlie a variety of developmental processes, including the control of growth cone motility and the plasticity of mature synapses (Lipton and Kater, 1989; Malenka, 1994). In addition, excessive elevation of intracellular calcium has been implicated in neuronal pathology leading to cell death (Choi, 1992). In the central nervous system, glutamate receptors provide a number of different pathways that can lead to calcium elevation (Mayer and Miller, 1990). NMDA receptor channels mediate a direct route for calcium entry into neurons. These channels are blocked by magnesium ions but are permeable to monovalent cations and to some divalents, including calcium (Nowak et al., 1984; Mayer and Westbrook, 1987). Their relative permeability to calcium versus monovalents is quite high; permeability ratios (P_{Ca}/P_{Na}) typically fall in the range from 4 to 6 (Mayer and Westbrook, 1987; Ascher and Nowak, 1988; Iino et al., 1990; Jahr and Stevens, 1993), or 10 to 12 when corrections for ionic activities have been employed (Mayer and Westbrook, 1987; Zarei and Dani, 1994). It is estimated that as much as 15 to 20% of the current through NMDA receptor channels may be carried by calcium under physiological conditions (Jahr and Stevens, 1993; but see Schneggenburger et al., 1993).

Agonists selective for non-NMDA receptors can also lead to elevation of intracellular calcium (Mayer and Miller, 1990). In many cell types, this entry of calcium is due to depolarization of the cell membrane and subsequent activation of voltage-gated calcium channels (Murphy and Miller, 1989). Direct measurement of cal-

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cium permeability has shown that most central neurons express non-NMDA receptors with relatively low conductance to calcium (Mayer and Westbrook, 1987; Iino et al., 1990); however a number of recent studies indicate that specific cell populations posses channels with higher calcium permeability (Iino et al., 1990; Gilbertson et al., 1991; Turetsky et al., 1994; Brorson et al., 1994; Jonas et al., 1994). A third mechanism for glutamate to increase intracellular calcium is through the activation of metabotropic receptors and subsequent release of calcium from intracellular stores (Murphy and Miller, 1988). All of these pathways for calcium elevation are thought to play a role in the normal operation of the nervous system or in the excitotoxic destruction of neurons that is associated with a variety of pathological conditions (Mayer and Miller, 1990).

P19 cells have emerged as a compelling model for CNS neurons (Bain et al., 1994). In their undifferentiated state, the cells are open to genetic manipulation; transient exposure to retinoic acid leads them to adopt a neuronal phenotype (Jones-Villeneuve et al., 1982). Previous work (Turetsky et al., 1993a; Morley et al., 1995) has shown that P19 cells become sensitive to excitatory amino acids within 6 to 10 days after retinoic acid treatment. Virtually all of the cells express both NMDA and non-NMDA receptors and exposure to glutamate receptor agonists produces widespread toxicity. In order to further characterize the receptor properties and possible mechanisms of glutamate toxicity we have examined the entry of calcium elicited by NMDA and kainate into P19 derived neurons. An abstract describing portions of this work has appeared (Turetsky et al., 1993b).

METHODS

Cell Culture

P 19 cells were induced to differentiate into neurons as previously described (Turetsky et al., 1993a). Undifferentiated cells obtained from the American Type Culture Collection were propagated in minimum essential medium (MEM), α-formulation (Life Technologies, Gaithersburg, MD), supplemented with 10% fetal bovine serum (FBS). For differentiation, 1×10^6 cells were suspended in 10 ml of αMEM containing 5% FBS and 500 nM retinoic acid, and seeded on a 10 cm bacteriological culture dish. After 4 days of retinoic acid treatment, aggregates were dissociated, resuspended in αMEM plus 10% FBS, and plated onto confluent primary glial cultures at a density of 1×10^6 cells per 35 mm dish. On the second day after plating, cells were switched to Eagle's MEM (Earl's salts) supplemented with 20 mM glucose, 5% FBS and 5% horse serum. Cytosine arabinoside (Ara-C) was added at a final concentration of 20 μM to inhibit division of non-neuronal cells. Cultures were fed every 2 to 3 days with Eagle's MEM plus 20 mM glucose, 5% FBS, and 5% HS, without Ara-C. Dissociated cultures of rat cortical neurons used for calcium permeation measurements were prepared as previously described (Huettner and Baughman, 1986).

Cytosolic Ca^{2+} Measurements

Intracellular free calcium was monitored using fura-2 fluorescence videomicroscopy. P19 cells were plated onto a pre-existing bed of mouse cortical glia in 35 mm glass bottom dishes (Mat-Tek, Ashland, MA); calcium measurements were taken 7 to 9 days after plating. Cells were loaded by a 30 min incubation at room temperature in 5 μM fura-2 AM (Molecular Probes, Inc., Eugene, OR) and 0.2% Pluronic F-127 dissolved in HEPES buffered Salt Solution (HBSS): 120 mM NaCl, 5.4 mM KCl, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 15 mM glucose, and 20 mM HEPES, pH adjusted to 7.4 with NaOH. Following a brief rinse with HBSS, the cells were maintained at room temperature for 30 min to allow for hydrolysis of the fura ester. Experiments were performed at room temperature under constant perfusion with HBSS at 2 ml/min on the stage of a Nikon Diaphot equipped with a 75 W Xenon lamp and a Nikon 40× 1.3 N.A. oil immersion objective. All drugs were delivered through the perfusion system. Fura-2 (ex.: $\lambda = 340$ nm and 380 nm, em.: $\lambda = 510$) ratio images were acquired with an intensified CCD camera (Quantex, Santa Clara, CA) and were digitized (256 × 512 pixels) by an Image 1 system (Universal Imaging Corp, Westchester, PA). Background fluorescence was determined for a cell-free region of the coverslip and subtracted from each experimental image. Calibrated values were obtained according to Grynkiewicz et al. (1985) by determining f_{min} and f_{max} in situ using zero calcium plus 2 mM EGTA for f_{min} and 10 mM calcium plus 10 μM ionomycin for f_{max}. All calculations assumed a K_d of 225 nM for the interaction of calcium with fura-2 (Grynkiewicz et al., 1985).

Electrophysiology

Pipettes for whole-cell recording were pulled from boralex capillaries, coated with sylgard, and filled with 140 mM $CsCH_3SO_3$ or CsF, 5 mM CsCl, 10 mM EGTA, and 10 mM HEPES, pH adjusted to 7.4 with CsOH. The open tip resistance ranged from 1 to 5 MOhm. The culture dish was perfused at a rate of 1 to 2 ml per min with Tyrode's solution: 150 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose, and 10 mM HEPES, pH adjusted to 7.4 with NaOH. An agar bridge equilibrated with 3 M KCl connected the bath to a chamber filled with internal solution for the ground electrode. Drug solutions were applied to the cells using an array of microcapillary tubes. The time constant for exchange of the external solution was approximately 30 to 50 msec.

For most experiments, excitatory amino acids were dissolved in 160 mM NaCl, 2 mM $CaCl_2$, 0.5 μM tetrodotoxin (TTX, Sigma, St. Louis, MO), and 10 mM HEPES, pH adjusted to 7.4 with NaOH. Concentration-response experiments were performed and analyzed as described (Swartz et al., 1992).

For calcium permeation experiments, agonists were dissolved in 160 mM NaCl, 0.5 mM $CaCl_2$, 0.5 μM TTX, and 10 mM HEPES, pH adjusted to 7.4 with NaOH, or in 110 mM $CaCl_2$, 0.5 μM TTX, and 10 mM HEPES, pH adjusted to 7.4 with CaOH. The internal solution for calcium permeability experiments was 100 mM CsCl, 10 mM EGTA, 1 mM ATP (Mg salt), 0.3 mM GTP (trisma salt), and 40 mM HEPES, pH adjusted to 7.4 with CsOH. Currents in agonist-free and agonist-containing external solution were recorded with an Axopatch 200 amplifier, filtered at 1 to 5 kHz (−3 db, 4 pole Bessel), and digitized at 5 to 10 kHz. Whole-cell currents recorded during agonist applications were compressed for storage and analysis by averaging 3 msec of data at 0.1 to 1 sec intervals.

Steady-state current-voltage relations were obtained by holding the cell at a given potential and applying the agonist. In addition, a pulse protocol was employed to determine current-voltage relations quickly over a wide range of potentials. Cells were clamped at −20 mV to inactivate residual voltage-dependent currents and stepped from +60 to −90 mV in 10 mV increments. Pulses to each potential lasted 20 msec. The entire staircase was repeated six times in control solution and six times in agonist. The mean current during the final 3 msec (10 kHz sampling rate) of the six pulses in agonist was determined and the mean for the six pulses in control was subtracted to yield the agonist-dependent current at each test potential. In general, there was very close agreement between current-voltage relations obtained using this pulse protocol and I-V relations determined from steady-state applications. Permeability ratios were calculated using the extended Goldman-Hodgkin-Katz (GHK) equation (Fatt and Ginsborg, 1958; Lewis and Stevens, 1979):

$$\frac{P_{Ca}}{P_{Na}} = \frac{[Na]_o(\exp(FV_{Ca}/RT))(1 + \exp(FV_{Ca}/RT))}{4[Ca]_o(\exp(FV_{Na}/RT))}, \quad (1)$$

where V_{Ca} and V_{Na} are the zero current potentials in 110 mM calcium and 160 mM sodium, respectively. All calculations employed the free concentrations of each ion, which have not been corrected for ion activity (see Tsien, 1983). Values are reported as the mean ± SEM, except as indicated in the text.

Voltage-gated calcium channel current was recorded as described by Bean (1992). The external solution was 5 mM $BaCl_2$, 150 mM tetraethylammonium (TEA) chloride, 2 mM $MgCl_2$, 0.1 mM EGTA, 1 μM TTX, and 10 mM HEPES, pH adjusted to 7.4 with TEA hydroxide. For most experiments, pipettes were filled with the CsCl internal solution used to study calcium permeability. In the text, membrane potentials have been corrected for the junction potential between the internal solution and the Tyrode's solution in which seals were formed. This correction, determined as described by Neher (1992), was −10 mV for pipettes containing cesium methanesulfonate while for CsF and the CsCl solution used in calcium permeability studies the junction potential was −7 mV. All recordings were obtained at room temperature.

Immunocytochemistry

P19 cells were fixed for 30 min with 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4. Cultures were rinsed three times in PBS and incubated for 2 hr at room temperature in 10% normal goat serum (NGS) to block non-specific binding. Glutamate receptor antibodies were diluted 1:20 in 2% NGS in PBS and incubated for 48 hr at 4°C. After washing with PBS, biotinylated goat anti-rabbit IgG (or horse anti-mouse for GluR2/4) was added for 45 min at room temperature, followed by avidin-biotin-peroxidase reagent (Vector Laboratories, Burlingame, CA) for 45 min. Staining was visualized with the chromagen 3-amino-9-ethylcarbazole. Immunostaining was performed 8 to 10 days after P19 cells were plated. Affinity purified antibodies to GluR-A, GluR-B/C, and GluR-D were obtained from Chemicon International, Inc. (Temecula, CA). Monoclonal antibodies to GluR-B/D were purchased from PharMingen (San Diego, CA).

RESULTS

Elevation of Intracellular Calcium by NMDA and Kainate

Changes in cytoplasmic calcium concentration $[Ca^{2+}]_i$ evoked by excitatory amino acids were monitored using the fluorescent indicator fura-2. Brief exposure to NMDA or to kainate produced a concentration-dependent rise of $[Ca^{2+}]_i$ in all of the P19 cells examined. Figures 1A and 2A show the concentration-dependent increase in $[Ca^{2+}]_i$ elicited by 1 min applications of 10 μM to 100 μM NMDA (Fig. 1A) or kainate (Fig. 2A). The figures plot the average $[Ca^{2+}]_i$ determined in 60 cells. Data from three such experiments is summarized in Figures 1B and 2B, which plot the peak $[Ca^{2+}]_i$ as well as an estimate of the net $[Ca^{2+}]_i$ (area under the curves in part A during 5 min following agonist onset) as a function of the agonist concentration. Application of agonist concentrations higher than 100 μM

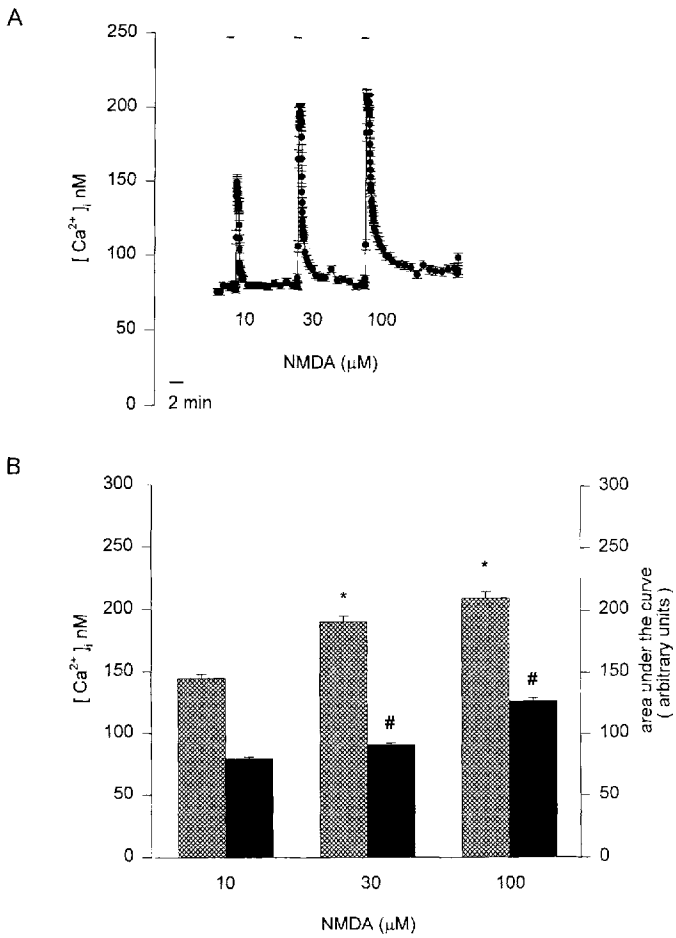


Fig. 1. NMDA-induced increase of $[Ca^{2+}]_i$. **A:** Concentration-dependent increase of $[Ca^{2+}]_i$ evoked by increasing concentrations of NMDA in the presence of glycine (10 μM) applied for 1 min. Each point is the mean ± SE of the calibrated calcium level from 60 cells in a representative experiment. **B:** Concentration-response relations for peak calcium concentration (gray bars) and cumulative calcium levels (black bars) from three separate experiments each involving 60 to 100 cells. Cumulative levels were calculated as the area beneath each stimulus using a trapezoidal rule (Sigma Plot). *, # Indicates values different from the respective lower concentration determined by one-way ANOVA and Student-Newman-Keuls test, $P<0.05$.

elicited larger changes in $[Ca^{2+}]_i$, which did not recover to the initial baseline (data not shown).

In order to determine whether the increase in cytoplasmic calcium produced by kainate was dependent on direct calcium entry through AMPA/kainate receptors, several experiments were performed in a sodium-free solution. As shown in Figure 2C, replacement of sodium by equimolar N-methylglucamine 2 min before and after agonist exposure significantly blunted the elevation of cytoplasmic calcium by kainate; thus much of the calcium elevation elicited by kainate appears to be secondary to sodium-dependent membrane depolarization. Figure 3 demonstrates that direct depolarization by high K^+ medium is effective at producing an elevation of cytoplasmic calcium that is on a par with that evoked by kainate. Co-incubation with the L-type calcium channel blocker, nimodipine (10 μM), resulted in a 25 to 30% decrease in the calcium elevation produced by high K^+, suggesting that L-type channels may contribute to the influx of calcium during periods of depolarization.

Calcium permeability of NMDA receptor channels

The relative permeability of P19 cell NMDA receptors was determined from the steady-state current-voltage relations obtained from whole-cell recordings in isotonic sodium and calcium solutions. NMDA (100 μM) and glycine (1 μM) were applied to cells while holding at various membrane potentials to determine the potential at which currents changed from inward to outward (Fig. 4A). In order to allow comparison to earlier work on neuronal NMDA receptors, we determined the permeability ratio, P_{Ca}/P_{Na}, for P19 neurons and for cultured rat cortical neurons using identical solutions. In both cell types, the reversal potential for NMDA receptor current shifted from near 0 mV in 160 mM NaCl to roughly +30 mV in the presence of 110 mM $CaCl_2$ (Fig. 4B). This positive shift in reversal potential upon substitution of calcium ions for sodium ions indicates that in both cell types the channels gated by NMDA are more permeable to Ca than to Na. Quantitatively, the results shown in Figure 4 indicate a permeability ratio, P_{Ca}/P_{Na}, of 5.9. The average permeability ratio for three P 19 cells was 5.6 ± 0.2 and for three cortical neurons 6.0 ± 0.2 (difference not significant at $P = 0.05$ by Student's t-test). This high permeability to calcium was confirmed in 12 additional P19 cells and 4 additional cortical neurons for which we were not able to complete a full set of steady-state applications, but instead, obtained rapid I-V relations for NMDA plus glycine using a series of voltage steps. In addition to shifting the reversal potential, exposure to 110 mM $CaCl_2$ also caused a dramatic reduction in the amplitude of whole-cell currents evoked by NMDA; however, unlike the change in reversal potential, this decrease in current amplitude did not recover when P 19 cells or cortical neurons were returned to 160 mM NaCl (Fig. 4C).

Calcium Permeability of Non-NMDA Receptors

In contrast to the high calcium permeability observed for NMDA receptor channels, whole-cell currents activated by kainate displayed much lower apparent permeability to calcium (Fig. 5). In all cases, the current elicited by kainate reversed from inward to outward near 0 mV in 160 mM NaCl, as has been previously reported (Mayer and Westbrook, 1987; Iino et al., 1990). In most

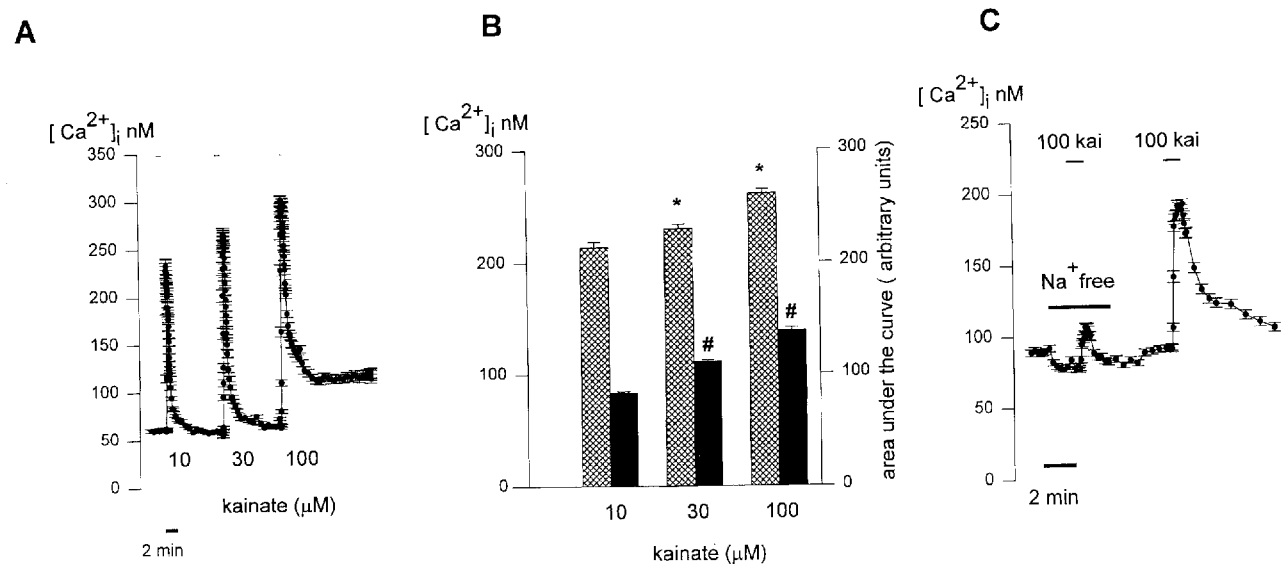


Fig. 2. Kainate-induced increase of $[Ca^{2+}]_i$. **A:** Concentration-dependent increase of $[Ca^{2+}]_i$ elicited by increasing concentrations of kainate applied for 1 min in the presence of MK-801 (10 μM) to block NMDA receptor channels. Each point represents the mean ± SE of the calibrated $[Ca^{2+}]_i$ levels obtained from 60 cells in a representative experiment. **B:** Concentration-response relations for peak (gray bars) and cumulative (black bars) calcium levels from three experiments as shown in A. Cumulative levels were calculated as the area beneath each stimulus using a trapezoidal rule (Sigma Plot). *, # Indicates values different from the respective lower concentration determined by one-way ANOVA and Student-Newman-Keuls test, $P<0.05$. **C:** Calcium elevation evoked by kainate in Na-free and Na-containing medium. NaCl was replaced by an equimolar concentration of N-methyl-D-glucamine (130 mM) for 2 min before and after the exposure to kainate (100 μM for 1 min), as indicated by the Na^+ free bar.

of the P19 cells and cortical neurons that we studied, the current evoked by kainate in 110 mM $CaCl_2$ reversed at a holding potential between −40 mV and −60 mV (Fig. 5A,C). This negative shift in reversal potential compared to isotonic NaCl indicates that in these cells Na and Cs are much more permeable than calcium through the non-NMDA channels. However, experiments on a total of eight P19 cells and eight cortical neurons revealed a skewed distribution of reversal potentials in isotonic $CaCl_2$ for both cell types. In P19 cells, the reversal potential for current activated by kainate in 110 mM $CaCl_2$ ranged from -56 to -34 mV, with a median value of −48 mV. The median permeability ratio, P_{Ca}/P_{Na}, in P19 cells was 0.053. Our sample of cortical neurons displayed a wider range of reversal potentials in 110 Ca, from −62 to 0 mV. The median value (−57 mV) was more negative than that obtained in P19-derived neurons. As a result, the median permeability ratio for cortical neurons (P_{Ca}/P_{Na} = 0.046) was slightly lower than for P19 cells.

Concentration-Response Relations for NMDA, Glycine, and Kainate

Concentration-response relations were determined for activation of whole-cell currents by NMDA, glycine, and kainate (Nakanishi et al., 1992; Swartz et al., 1992). Figure 6A shows currents evoked by increasing concentrations of NMDA plus a fixed level of glycine (1 μM). Steady-state current was half-maximal at a concentration of 24 μM NMDA (Fig. 6C). The currents in Figure 6B were elicited by increasing doses of glycine with a fixed concentration of NMDA (100 μM). The EC_{50} for glycine under these conditions was 240 nM. Current activated by kainate was half-maximal at a concentration of 81 μM.

Voltage-Gated Calcium Channel Current

Whole-cell current through voltage-gated calcium channels was recorded using barium as the charge carrier. Figure 7A shows an example of current elicited by a voltage step from -70 mV to -10 mV in a P19 derived neuron that was bathed in a solution composed of 150 mM TEA-Cl, 5 mM $BaCl_2$, 2 mM $MgCl_2$, 0.1 mM EGTA, 10 mM HEPES, and 1 μM TTX. Similar results were obtained in eight additional cells from two separate platings. In most cells the currents displayed relatively little inactivation during a 100 msec step from −70 mV, although holding for prolonged periods at depolarized potentials did produce a steady reduction in current amplitude. Addition of 3 mM $CdCl_2$ resulted in complete block of inward current in all cells tested (n = 4). The I-V relationship for Cd-sensitive current for test potentials from −60 mV to +70 mV is shown in part B of Figure 7. Inward current was elicited for test potentials

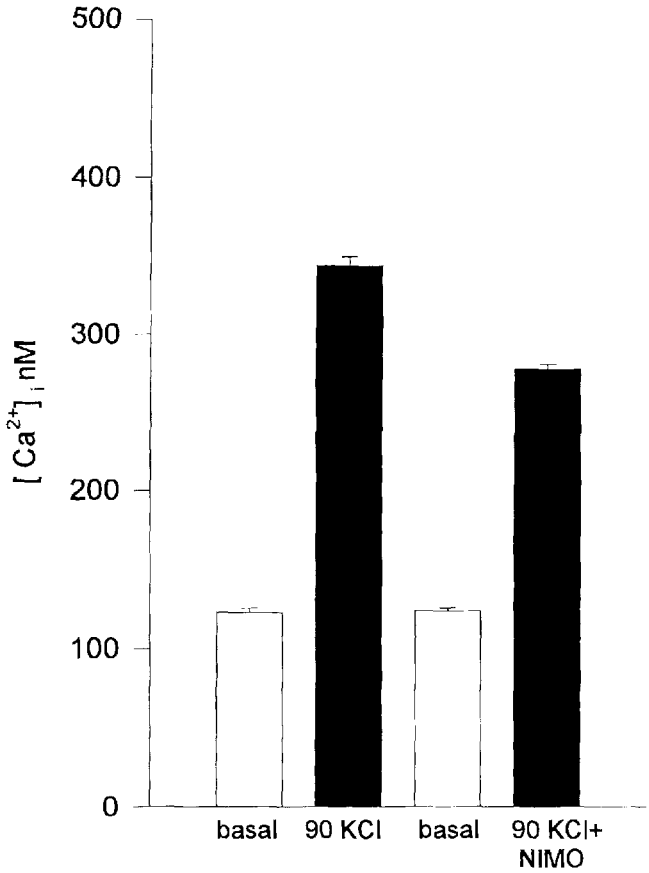


Fig. 3. Depolarization-induced increase of $[Ca^{2+}]_i$ in P19 cells. Cells were exposed for 30 sec to 90 mM KCl with or without nimodipine (10 μM). The non-competitive NMDA antagonist MK-801 (10 μM) was added to the perfusion medium to avoid the possible activation of NMDA receptors following the depolarization. Each column represents the mean ± SE of the peak $[Ca^{2+}]_i$ level in three different experiments.

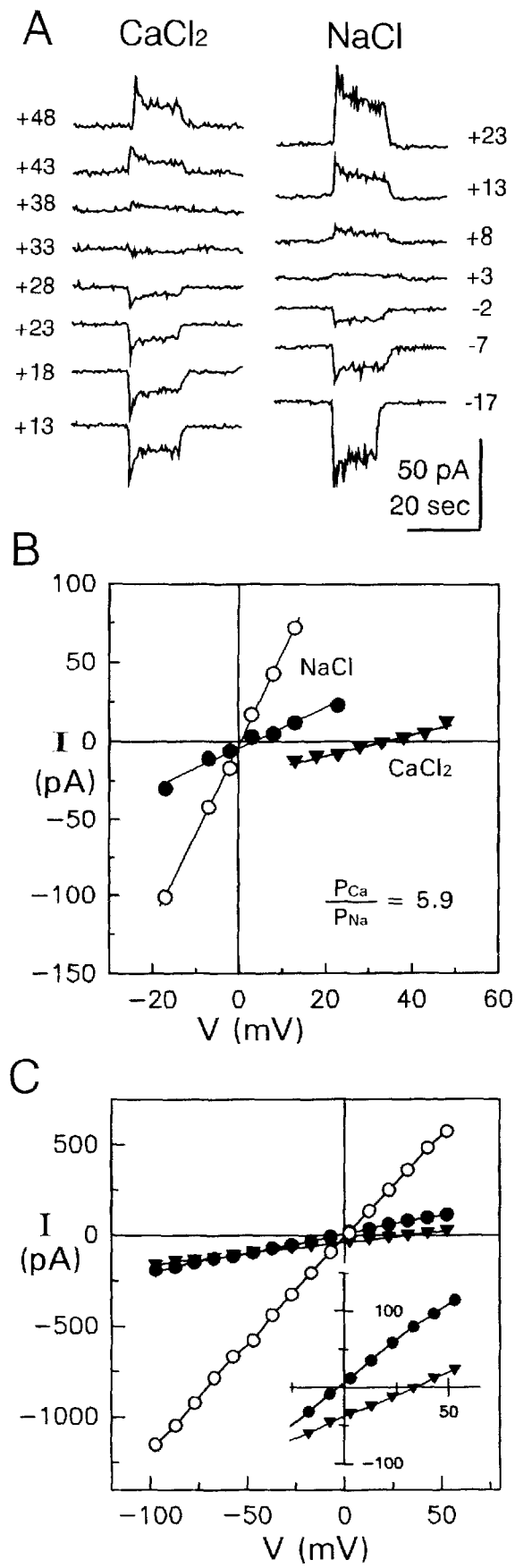
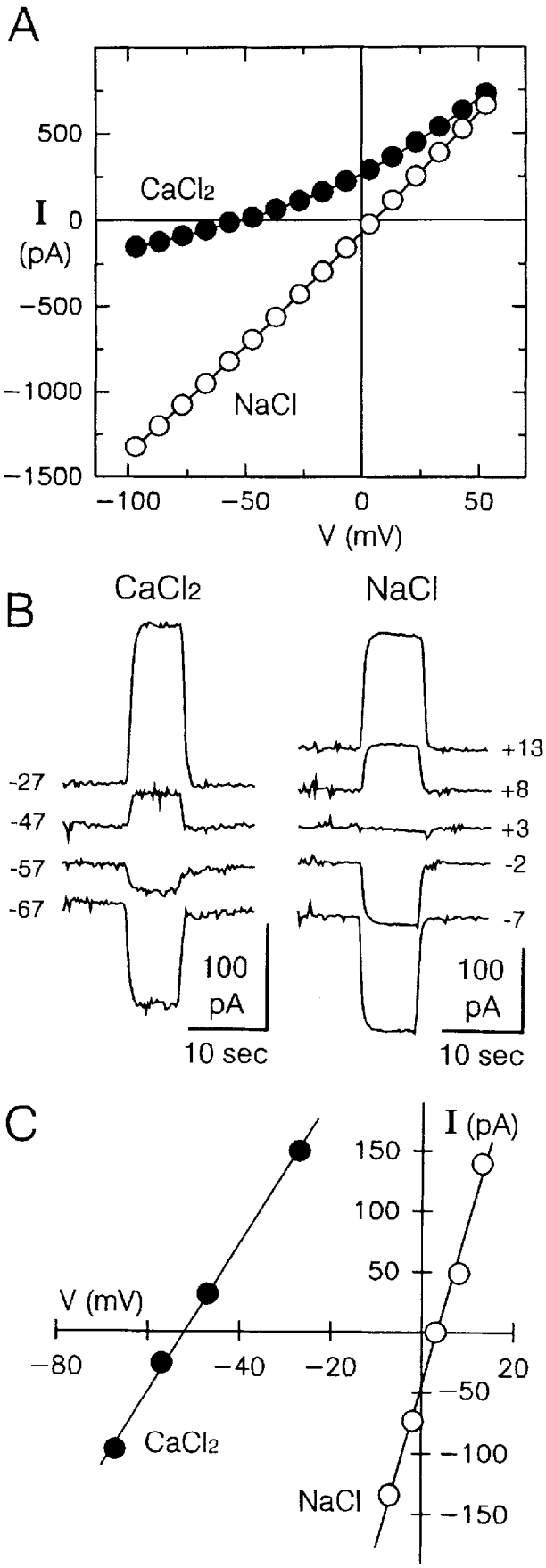


Fig. 4. Calcium permeation through NMDA receptor channels in P19 derived neurons. **A:** Currents activated by 100 μM NMDA and 1 μM glycine in 110 mM $CaCl_2$ (left) or in 160 mM NaCl (right). Numbers next to each trace indicate the holding potential. The traces have been displaced relative to the zero current level for clarity. **B:** Steady-state current activated by NMDA plus glycine is plotted vs. holding potential. Currents recorded in 110 mM Ca are plotted as inverted triangles, while circles show the current recorded in 160 mM Na, either before (○) or after (●) exposure to $CaCl_2$. **C:** Current voltage-relations for NMDA receptor current obtained by holding at −27 mV and stepping from +53 to −97 mV in 10 mV increments (see Methods). Current in NaCl before (O) and after (●) exposure to 110 mM Ca (▼). Inset: An expanded view of the voltage axis to show the reversal potentials for current in NaCl (●) and $CaCl_2$ (▼).



positive to −50 mV and reached a maximum for steps to 0 mV. With Cs as the main internal cation the current reversed from inward to outward near +60 to +70 mV (Fenwick et al., 1982; Lee and Tsien, 1982).

Expression of AMPA Receptor Subunits

Affinity purified polyclonal antibodies were used to visualize immunoreactivity for AMPA receptor subunits. Figure 8 presents immunostaining of P19 cultures with three different antibodies. The most widespread staining was observed with antibodies that recognize both GluR-B and GluR-C subunits. As shown in Figure 8B, virtually all of the P19-derived neurons were strongly positive for GluR-B/C. Similarly, most of the neurons were stained with a monoclonal antibody that reacts with a common epitope on GluR-B and GluR-D (data not shown). A much smaller fraction of the cells, roughly 50%, were visualized with antibodies that are selective for the GluR-D subunit (Fig. 8C). Finally, staining of cultures with antibodies selective for the GluR-A subunit resulted in very weak labeling of a small percentage of cells (Fig. 8A).

Parallel experiments on cultured mouse cortical neurons revealed a similar staining pattern with anti-GluR-B/C, anti-GluR-B/D, and anti-GluR-D (unpublished observations); however, expression of GluR-A was much more prevalent in the cortical neurons. In particular, strongly staining GluR-A neurons, which have been shown to possess calcium permeable AMPA receptors in mouse cortical cultures (Yin et al., 1994) were completely absent in the P19 cultures. Consistent with this, kainate activated cobalt uptake (Pruss et al., 1991), a histochemical marker for calcium permeable AMPA receptors, was not detected in P19-derived neurons (data not shown).

DISCUSSION

Control of intracellular calcium levels is critical to the survival and normal functioning of mammalian neurons. Previous work (reviewed by Mayer and Miller, 1990) has emphasized the important role that glutamate

Fig. 5. Calcium permeability of non-NMDA receptors in P 19 derived neurons. **A:** Currents activated by 100 μM kainate in 160 mM NaCl (○) or 110 mM $CaCl_2$ (●) is plotted as a function of membrane potential. Currents were obtained by holding at −27 mV and stepping from +53 to −97 mV in 10 mV increments (see Methods). **B:** Currents activated by kainate in 110 mM Ca or 160 mM Na while holding at a fixed potential. Numbers next to each trace indicate the holding potential. **C:** Steady-state current activated by kainate is plotted vs. holding potential.

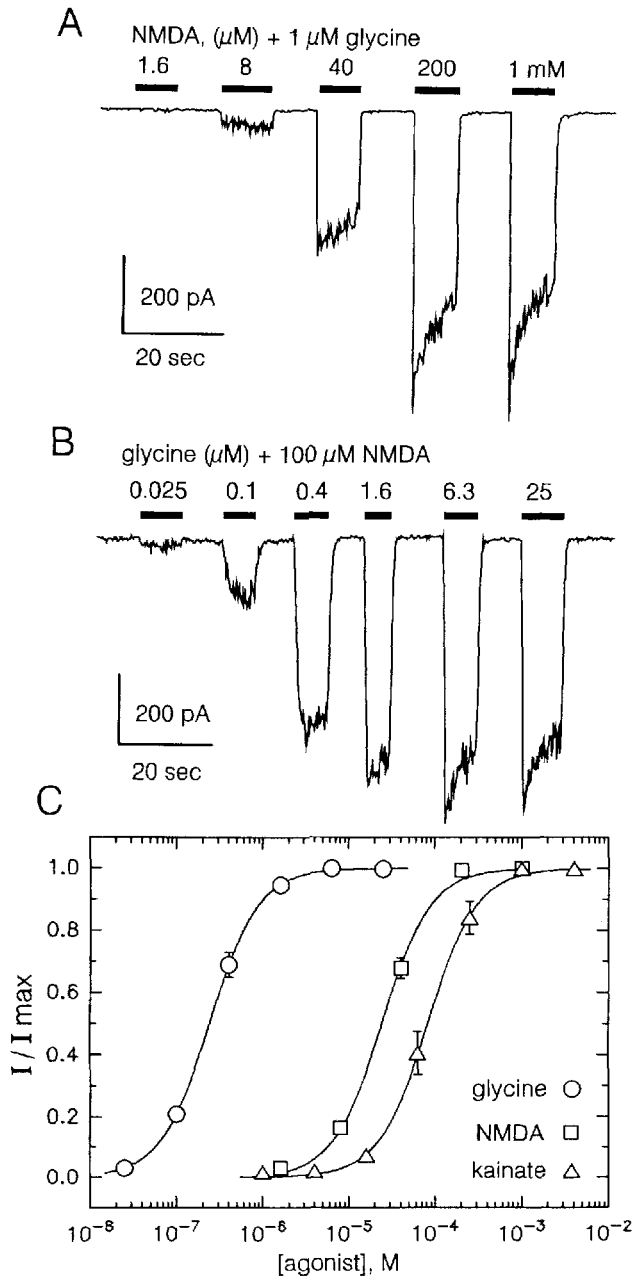


Fig. 6. Concentration-response relations for NMDA, glycine, and kainate in P19 cells. **A:** Current activated by 1.6 μM to 1 mM NMDA in the presence of 1 μM glycine. Holding potential, −70 mV. **B:** Current evoked by 100 μM NMDA with 25 nM to 25 μM glycine added. Holding potential, −70 mV. **C:** Concentration-response relations for kainate (three cells), glycine plus 100 μM NMDA (four cells), and NMDA plus 1 μM glycine (seven cells). Points, mean ± SE of the normalized current (I/I_{max}). Smooth curves, best fits of $I/I_{max} = 1/(1 + (EC_{50}/[agonist])^n)$ to all of the data points. For kainate EC_{50} = 81 μM and n = 1.5; NMDA EC_{50} = 24 μM and n = 1.5; and glycine EC_{50} = 240 nM and n = 1.5.

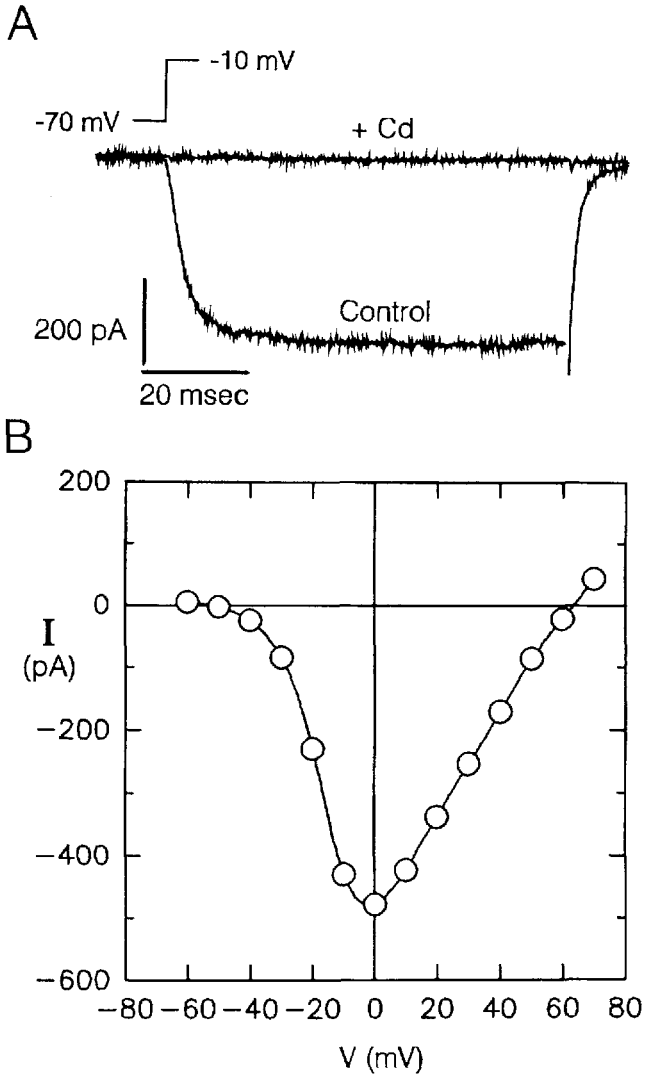


Fig. 7. Whole-cell barium current through voltage-gated calcium channels. **A:** Currents elicited by a 100 msec step from −70 mV to −10 mV in barium/TEA chloride with and without 3 mM $CdCl_2$. Individual traces corrected for leak and capacity current are shown (0.5 msec has been blanked at the beginning and end of the voltage step). **B:** Cd-sensitive current elicited from a holding potential of −70 mV. Peak current is plotted vs. test potential.

receptors play in changing the cytosolic concentration of calcium in CNS neurons. In this study, we have examined the pathways for glutamate-mediated calcium entry in neurons derived from the P19 embryonal carcinoma cell line. P19 cells differentiate in response to retinoic acid to adopt a phenotype very similar to neurons from the CNS (Jones-Villeneuve et al, 1982; Bain et al., 1994). The differentiated cells express functional NMDA and AMPA-type receptors as well as RNA for the GluR-5, −6, −7 and KA-1, −2 subunits (Ray and Gottlieb, 1993), which contribute to kainate-type receptors (Seeburg, 1993). Because the undifferentiated P19

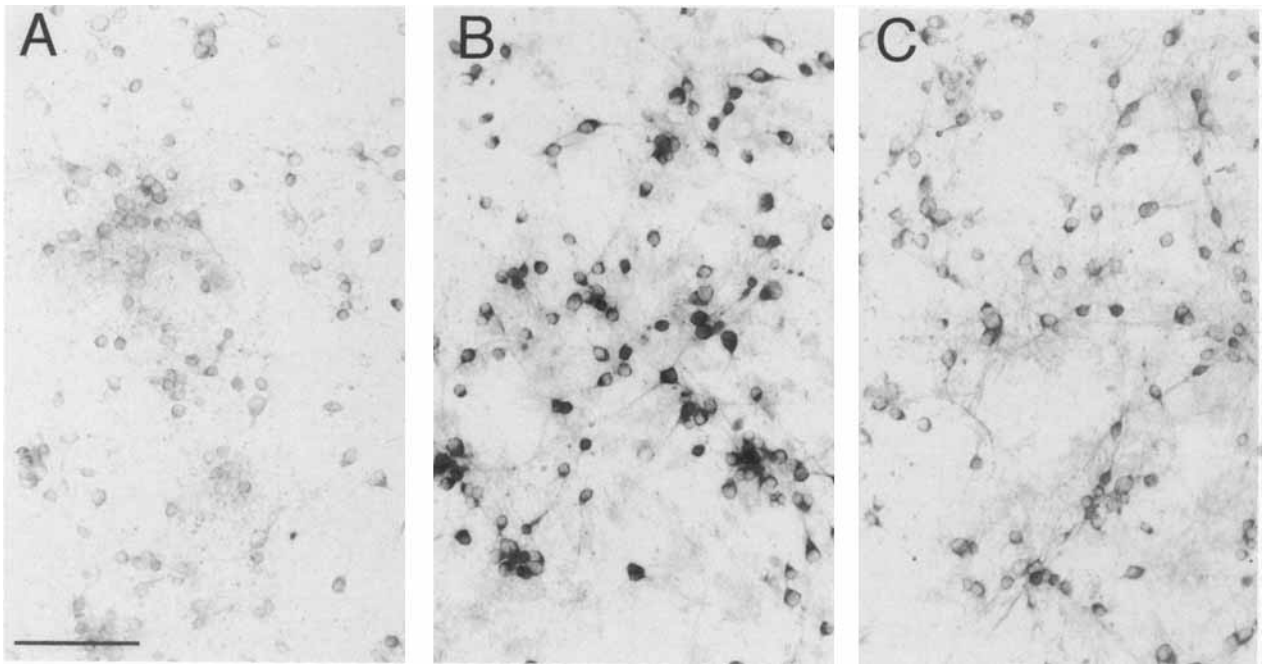


Fig. 8. Immunostaining for AMPA receptor subunits in P19 neurons. Brightfield photomicrographs of immunoperoxidase staining for GluR-A (**A**), GluR-B/C (**B**), and GluR-D (**C**). Scale bar, 100 μm.

cells are open to genetic manipulation, this system may be ideal for investigating the downstream response pathways which are activated by calcium elevation under both normal and pathological or neurotoxic conditions.

NMDA Receptors

Calcium entry through NMDA receptors is recognized as a necessary step in several forms of synaptic plasticity, including long-term potentiation and long-term depression (Malenka, 1994). Previous work on a variety of different neurons has demonstrated the high permeability of neuronal NMDA receptors to calcium (Mayer and Westbrook, 1987). In addition, molecular studies have identified specific amino acids in the primary structure of NMDA receptor subunits that are thought to underlie the permeation properties of these channels (Seeburg, 1993). Our results with P19 cells and cortical neurons are in close agreement with previous estimates (Mayer and Westbrook, 1987; Jahr and Stevens, 1993; Zarei and Dani, 1994) of the relative permeability of neuronal NMDA receptors to calcium and monovalent cations. Calculations based on molar concentrations indicate that the channels are approximately 6-fold more permeable to calcium than sodium. The strong calcium signals detected with fura-2 during exposure to NMDA are consistent with the widespread expression of functional, calcium-permeable NMDA receptors by virtually all of the P19 derived neurons.

In addition to shifting the reversal potential for NMDA-gated current, exposure to isotonic calcium also reduced the current amplitude. This effect is likely to result from calcium-dependent receptor inactivation described by Westbrook and colleagues (Legendre et al., 1993; Rosenmund and Westbrook, 1993). In our experiments, switching from NaCl to $CaCl_2$ caused a 3- to 5-fold reduction in the steady-state current elicited by NMDA. There was relatively little evidence for recovery from inactivation once cells were returned to the low calcium solution, although prolonged exposure to high calcium made it difficult to maintain recordings during the recovery period.

Non-NMDA Receptors

In contrast to NMDA receptors, most non-NMDA receptors exhibit relatively low permeability to calcium. For AMPA-type receptors, this low calcium permeability is dependent on a posttranscriptional modification of the messenger RNA for the GluR-B subunit (Sommer et al., 1991). In heterologous cells that express high levels of the recombinant edited GluR-B(R) subunit, the I-V relationship for AMPA current is nearly linear and the permeability to calcium is low ($P_{Ca}/P_{monovalent} \cong 0.05$; Hollmann et al., 1991; Burnashev et al., 1992). AMPA-type channels in cells that lack the GluR-B subunit exhibit much higher calcium permeability and display strongly rectifying I-V relations ($P_{Ca}/P_{monovalent} \cong 1$–$2$; Hollmann et al., 1991; Burnashev at al., 1992). Intermediate levels of GluR-B(R) expression result in rela-

tively linear I-V relations and intermediate permeability values ($P_{Ca}/P_{monovalent} \cong 0.5$; Burnashev et al., 1992; Jonas et al., 1994).

In the present study, both P19 cells and cultured cortical neurons were found to express AMPA-type receptors with low permeability to calcium; however, in both cell types the reversal potential values in isotonic calcium were skewed toward depolarized potentials. In all cells tested, the I-V relations for AMPA receptor-mediated current in both NaCl and $CaCl_2$ solutions were relatively linear. The median permeability to calcium versus sodium was 0.053 in P19 cells and 0.046 in cortical neurons. In both populations a few of the cells had distinctly higher apparent permeability. Values were more tightly clustered about the median for P19 cells; cortical cells displayed a much wider range of relative permeabilities. These electrophysiological results are in close agreement with fura-2 measurements of cytosolic calcium. P19 cells showed a uniform increase in cytosolic calcium when exposed to kainate, but in all cells this increase was significantly depressed under conditions that reduced the activation of voltage-sensitive calcium channels. In contrast, previous work (Turetsky et al., 1994) on cortical neurons in culture has shown that approximately 10 to 15% of the cells express AMPA receptor channels that are directly permeable to calcium. The fact that P19 cell AMPA receptors exhibit low calcium permeability provides strong evidence that they express and edit GluR-B appropriately. Although the exact molecular composition of P19 AMPA-type receptors remains to be established, our immunocytochemical results suggest that they are likely to include GluR-B, together with the GluR-C and/or GluR-D subunits. The GluR-A subunit, by contrast, appears to be only weakly expressed.

Voltage-Gated Calcium Channels

Previous work (Kubo, 1989; Cheun and Yeh, 1991; Morley et al., 1995) on P19 cells has suggested that they exhibit little or no voltage-gated calcium current, although both low and high threshold calcium current has been demonstrated in other embryonic carcinoma cells upon neuronal differentiation (e.g., PCC4-Aza1-ECA2 cells; Kubo, 1989). Our recordings of whole-cell Ba^{2+} current, together with the elevation of intracellular calcium evoked by KCl depolarization, provide strong evidence for the widespread expression of voltage-gated calcium channels in P19 cells maintained under our culture conditions. The ability of nimodipine to reduce the change in $[Ca^{2+}]_i$ during stimulation with KCl suggests that L-type channels contribute to the influx of calcium, but further work will be needed to determine whether the cells express additional channel subtypes. At this point it is not clear what factors allow for calcium channel expression in our cultures. One difference in culture conditions is our use of primary astrocytes as a substrate for the P19 cells, but whether this is explains the resulting phenotype remains to be explored.

Implications for Excitotoxicity

Both NMDA and non-NMDA receptors are known to contribute to excitotoxic cell death in native neurons (Choi, 1992) and neurons derived from the P19 embryonic carcinoma line (Turetsky et al., 1993a). Calcium entry, either directly through glutamate-gated channels or indirectly through voltage-gated calcium channels, is thought to play a key role in triggering neuronal death (Choi, 1992). Our results show that P19-derived neurons resemble the vast majority of native CNS neurons in that they express NMDA receptors with high intrinsic calcium permeability and non-NMDA receptors with relatively low permeability to calcium. Elevation of cytosolic calcium by non-NMDA receptor agonists in P19 cells and most neurons appears to require indirect activation of voltage-dependent calcium currents.

Although much recent attention has focused on neuronal populations that express AMPA receptors with relatively high intrinsic calcium permeability (e.g., Iino et al, 1990; Gilbertson et al., 1991; Turetsky et al., 1994; Brorson et al., 1994; Bochet et al., 1994; Yin et al., 1994), these cell types are clearly the exception, rather than the rule among CNS neurons; most neurons exhibit low or intermediate levels of calcium permeability through AMPA receptors (Mayer and Miller, 1990). Much remains to be learned about the downstream pathways that underlie glutamate receptor-mediated toxicity; however, the fact that NMDA and non-NMDA receptor antagonists provide neuroprotection in animal models of excitotoxicity has greatly stimulated research in this area. Our results suggest that further mechanistic studies of excitotoxic cell death in P19-derived neurons may yield information that is applicable to the majority of native neurons.

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