The Effects of Excitatory Amino Acids on Intracellular Calcium in Single Mouse Striatal Neurons *in vitro*

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Using microspectrofluorimetry and the calcium-sensitive dye fura-2, we examined the effect of excitatory amino acids on $[Ca^{2+}]_i$ in single striatal neurons *in vitro*. *N*-methyl-D-aspartic acid (NMDA) produced rapid increases in $[Ca^{2+}]_i$. These were blocked by DL-2-amino-5-phosphonovaleric acid (AP5), by Mg^{2+} , by phencyclidine, and by MK801. The block produced by Mg^{2+} and MK801 could be relieved by depolarizing cells with veratridine. When external Ca^{2+} was removed, NMDA no longer increased $[Ca^{2+}]_i$. Furthermore, the effects of NMDA were not blocked by concentrations of La^{3+} that blocked depolarization induced rises in $[Ca^{2+}]_i$. Substitution of Na^+ by Li^+ did not block the effects of NMDA. Concentrations of L-glutamate $\geq 10^{-6}$ M also increased $[Ca^{2+}]_i$. The effects of moderate concentrations of glutamate were blocked by AP5 but not by La^{3+} or by substitution of Na^+ by Li^+ . The effects of glutamate were blocked by removal of external Ca^{2+} but were not blocked by concentrations of Mg^{2+} or MK801 that completely blocked the effects of NMDA. The glutamate analogs kainic acid (KA) and quisqualic acid also increased $[Ca^{2+}]_i$. The effects of KA were blocked by removal of external Ca^{2+} but not by La^{3+} , Mg^{2+} , MK801, or replacement of Na^+ by Li^+ . Although AP5 was able to block the effects of KA partially, very high concentrations were required. These results may be explained by considering the properties of glutamate-receptor-linked ionophores. Excitatory amino acid induced increases in $[Ca^{2+}]_i$ are consistent with the possibility that Ca^{2+} mediates excitatory amino acid induced neuronal degeneration.

L-Glutamate or a similar molecule is probably the most widely used excitatory neurotransmitter in the brain. The electrophysiological effects of glutamate are complex, as it is hypothesized to act as an agonist at more than one type of receptor site (Foster and Fagg, 1984; Mayer and Westbrook, 1984, 1987a; O'Brien and Fischbach, 1986). It is thought that these diverse receptor types allow glutamate to transmit both rapid excitatory signals and also other forms of transsynaptic information that may result in the long-term alteration of synaptic contacts (Lynch and Baudry, 1984). In addition to its physiological effects, it has

also often been observed that administration of glutamate or its structural analogs directly into the brain is highly toxic and results in the destruction of neurons whose cell bodies are close to the site of administration (Olney, 1969; Schwarcz et al., 1984; Rothman, 1985). Apart from being a useful lesioning technique, such effects may be extremely important in certain pathological conditions. For example, it is thought that the neuronal degeneration associated with conditions such as cerebral ischemia, epilepsy, Alzheimer's disease, and Huntington's chorea may result from the release of abnormal quantities of glutamate-like agonists *in situ* under certain conditions (Schwarcz et al., 1984). Neuronal death can also be observed following the addition of glutamate-like agonists to nerve cells *in vitro* (Rothman, 1985; Hajos et al., 1986; Olney, 1986; Choi et al., 1987a, b). It is clearly important to elucidate the mechanisms underlying the toxic effects of excitatory amino acids ("excitotoxicity") and their relationship to the normal physiological actions of these substances.

One major hypothesis suggests that excitotoxicity may be due to agonist-induced Ca^{2+} overloading in neurons (Garthwaite and Garthwaite, 1986a; Garthwaite et al., 1986; Hajos et al., 1986; Choi et al., 1987). The toxic effects of abnormally high levels of free Ca^{2+} , $[Ca^{2+}]_i$, would be analogous to similar toxic effects of high $[Ca^{2+}]_i$ associated with the atrophy of cardiac muscle cells (Shen and Jennings, 1972). In support of this idea, several studies have demonstrated that neurons can be protected from excitotoxic effects by reducing $[Ca^{2+}]_i$ (Choi, 1985, 1987; Garthwaite and Garthwaite, 1986a; Garthwaite et al., 1986; Hajos et al., 1986), although this is not true in every case (Price et al., 1985; Rothman, 1985; Kleinschmidt et al., 1986). Furthermore, administration of excitatory amino acids directly into the brain or to brain slices appears to lead to an influx of Ca^{2+} as assessed by an increase in Ca^{2+} uptake (Ichida et al., 1982; Retz et al., 1982; Berdichevsky et al., 1983; Retz and Coyle, 1984; Harris, 1985; Pastuszko and Wilson, 1985; Wroblewski et al., 1985, 1987; Crowder et al., 1986; Garthwaite and Garthwaite, 1986b; Lazarewicz et al., 1986; Riveros and Orrego, 1986) or a decrease in $[Ca^{2+}]_i$ measured with Ca^{2+} -sensitive microelectrodes or by other means (Heinemann and Pumain, 1980, 1981; Marciani et al., 1982; Buhle and Sonnhof, 1983; Zanotto and Heinemann, 1983; Korf and Postema, 1984; Pumain and Heinemann, 1985; Ashton et al., 1986; Hamon and Heinemann, 1986; Sakamoto et al., 1986). Unfortunately, however, the cellular elements responsible for such effects are not usually precisely identified.

The mechanisms by which glutamate increases $[Ca^{2+}]_i$ are also unclear. The best-defined receptor for glutamate is characterized by the agonist effects of the amino acid, *N*-methyl-D-

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aspartate (NMDA). The ionophore activated by such agonists appears to be permeable to monovalent cations such as Na^+ (Mayer et al., 1984; Nowak et al., 1984; Mayer and Westbrook, 1985b, 1987a; Jahr and Stevens, 1987) and is also appreciably permeable to Ca^{2+} (Mayer and Westbrook, 1985a, 1986; Ascher and Nowak, 1986; MacDermott et al., 1986; Jahr and Stevens, 1987; Mayer et al., 1987b). Interestingly, in normal physiological medium, $I-V$ curves in the presence of NMDA exhibit a region of negative slope conductance at membrane potentials between about -30 to -80 mV (Mayer and Westbrook, 1984, 1987a; Westbrook and Mayer, 1984; O'Brien and Fischbach, 1986). The reason for this is unusual. Rather than possessing voltage-dependent gating properties per se, the NMDA-linked ionophore is blocked in a voltage-dependent manner by low concentrations of Mg^{2+} (Crunelli and Mayer, 1984; Mayer et al., 1984; Nowak et al., 1984; Mayer and Westbrook, 1987a). This block is relieved if the cell depolarizes. In the absence of Mg^{2+} , NMDA-induced currents increase as the membrane is hyperpolarized. Glutamate also acts at other non-NMDA receptors (Foster and Fagg, 1984). Such receptors are typified by the actions of agonists such as kainic acid (KA) or quisqualic acid (QA). Cellular depolarization resulting from the action of these agonists is associated with an increase in membrane conductance (Mayer and Westbrook, 1984, 1987b; Nowak and Ascher, 1984; Westbrook and Mayer, 1984; O'Brien and Fischbach, 1986). Under voltage-clamp conditions the currents induced by KA increase with increasing hyperpolarization. Thus, the ionophore linked to the KA receptor does not exhibit a voltage-dependent block by Mg^{2+} . Furthermore, in contradistinction to the NMDA-linked channel, this ionophore is reported to be virtually impermeable to Ca^{2+} (MacDermott et al., 1986; Mayer et al., 1987). The exact number of non-NMDA-linked glutamate receptor types is unclear at this point.

One area of the CNS in which the normal and toxic effects of excitatory amino acids have been well studied is the striatum (Nowak and Ascher, 1984, 1985; Nowak et al., 1984; Ascher and Nowak, 1986; Beal et al., 1986; Silverstein et al., 1986). Recently we have developed a technique for the measurement of changes in $[\text{Ca}^{2+}]_i$ in single neurons in primary culture. In the present series of experiments, we have examined the effects of NMDA and non-NMDA receptor agonists on $[\text{Ca}^{2+}]_i$ in single striatal neurons. We find that both types of glutamate agonists produce large increases in $[\text{Ca}^{2+}]_i$. We have also attempted to elucidate the mechanisms by which these effects are produced.

Materials and Methods

Cell culture. Neurons were cultured as described in Thayer et al. (1986). Briefly, striatal cells were dissected from mice embryos (C57BL/6J) on day E14. The cells were dissociated and grown as monolayer cultures on glass coverslips coated with polylysine and laminin. Standard cultures were grown at a density of 1200 cells/mm². Cells were used after 11 d in culture, at which time the effects of all agonists were maximal (unpublished observations).

All measurements were made on single cells. It was important to distinguish neuronal cells from glial cells. Most glial cells were distinguishable as large flat cells that covered the coverslip, and neurons could be distinguished on morphological grounds (Panula et al., 1979). In the first 120 cells used, each experiment was terminated by raising $[\text{K}^+]_o$ to 50 mM in order to investigate the presence of voltage-sensitive Ca^{2+} channels. We have found that glial cells respond weakly ($\Delta[\text{Ca}^{2+}]_i < 100$ nM) or not at all to this depolarizing stimulation; in contrast, neurons nearly always raise cytosolic calcium >200 nM (Thayer et al., 1986). After testing 120 cells and obtaining 119 positive results, the test was no longer considered necessary.

Measurement of $[\text{Ca}^{2+}]_i$. Cytosolic free Ca^{2+} was determined by using a microspectrofluorimeter, which has been described previously (Perney et al., 1984; Thayer et al., 1986) to monitor the Ca^{2+} -sensitive, fluorescent chelator, fura-2. Neurons were loaded with the dye by incubation in 5 μM fura-2 acetoxymethyl ester, which is membrane permeant, for 1 hr at 37°C in HEPES-buffered Hanks' balanced salt solution, pH 7.45. During the loading incubation, the dye ester is hydrolyzed by cytosolic esterases to the membrane-impermeant polycarboxylate anion that is fura-2. Following the incubation, the cells were washed twice in HEPES-Hanks' solution and incubated for an additional 15 min. Complete hydrolysis of the fura-2 ester to fura-2 was confirmed by comparing excitation spectra run on individual dye-loaded cells (Fig. 1A). Cells were treated with 10 μM ionomycin to raise $[\text{Ca}^{2+}]_i > 30$ μM (where the calcium chelator is saturated). EGTA, 20 mM, was then added to the standard HEPES-Hanks solution, lowering the $[\text{Ca}^{2+}]_o$ to very low levels. Notice that the spectrum of the fura-2 ester peaked at wavelengths slightly greater than 380 nm and was Ca^{2+} insensitive. Spectra of ionomycin-treated, dye-loaded cells peaked at less than 350 nm, as expected for the fura-2-free acid in the presence of Ca^{2+} , and fluoresced at an intensity near background ($<10\%$ of intensity at 340 nm) at wavelengths greater than 380 nm, indicating that no significant concentration of the dye ester was present in these loaded and washed striatal neurons.

The coverslips containing the loaded and washed cells were mounted in a flow-through chamber for viewing. The chamber consisted of a 3 ml stainless-steel-lined well, surmounted by a rubber O-ring, against which the inverted coverslip was sealed by pressure. The bottom of the well was covered by a Plexiglas plate for substage illumination to localize cells. The temperature in the well was monitored by a thermocouple probe and maintained at 37°C by circulating warm water in a separate surrounding cavity. The cells were usually maintained in a bath of HEPES-buffered Hanks' balanced salt solution (pH 7.45), which contains (in mM): NaCl, 137; KCl, 5.4; MgSO_4 , 0.41; MgCl_2 , 0.49; CaCl_2 , 1.26; KH_2PO_4 , 0.44; $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 0.64; NaHCO_3 , 3; glucose, 5.5; HEPES, 20. Exceptions were Mg^{2+} -free experiments, in which Mg^{2+} was eliminated from the medium; La^{3+} experiments, where all anions were replaced with an equal normal of Cl^- ; Na^+ -free experiments in which equal molar amounts of Li^+ or *N*-methyl-D-glucamine (NMDG) was substituted; and Ca^{2+} -free experiments, where Ca^{2+} was removed and 20 μM EGTA added to the solution. All washing was done using a flow rate of 3 ml/min.

Fura-2 fluorescence from individual cells was monitored with a microspectrofluorimeter operating in the epi-illumination mode. Light from a xenon arc lamp was passed through a double-grating monochromator to the Ploem illuminator of a Leitz Orthoplan microscope. A $\times 54$ fluorite objective was used, and a rectangular diaphragm was set to limit fluorescence measurements to individual cells. The fluorescence emission was analyzed by a second double-grating monochromator and a Leitz MPV I photomultiplier tube coupled to a Princeton Applied Research photon counter. Each cell was alternately excited with 340 and 380 nm light every 30 sec. Emissions were collected every second with the photomultiplier tube, digitized with the photon counter, and stored in the memory of an LSI 11/03 minicomputer. The 1 sec readings were usually averaged over each 30 sec time period, and a ratio was calculated for every 30 sec. This allowed free cytosolic calcium concentrations to be obtained every 30 sec using the calibration curve shown in Figure 1B. The fura-2 fluorescence intensity measured at 340 nm increases upon binding Ca^{2+} , whereas the 380 nm intensity reflects dye that has not bound Ca^{2+} ; therefore, the ratio of these 2 measurements is related to the free Ca^{2+} concentration in a manner that is not dependent on optical pathlength, dye concentration, or dye bleaching. A standard curve was determined for the fura-2 pentapotassium salt in calibration buffer (pH 7.05, 37°C—which contains (in mM): KCl, 120; NaCl, 5; KH_2CO_3 , 1; NaHCO_3 , 5; HEPES, 20; EGTA, 20)—containing CaEGTA and K_2EGTA in ratios that were calculated using the stability constant $3.06 \times 10^6 \text{ M}^{-1}$ (Bers, 1982) to give Ca^{2+} concentrations ranging from 0 to 1000 nM. In these buffers we assume that the dye behaves like the intracellular dye. The standard curve was fit with the equation $K[(R - R_{\min})/(R_{\max} - R)]$, in which K is a calibration constant, R is the 340/380 nm fluorescence ratio, and $R_{\min}$ and $R_{\max}$ are the ratios in the absence of calcium and in saturating concentrations of calcium, respectively.

Experimental procedure. Cells were mounted in the microspectrofluorimeter as shown in Figure 1C. Note the image of the back-lit photomultiplier diaphragm, which defines the area from which photon counts were recorded. $[\text{Ca}^{2+}]_i$ was measured for 2 min prior to agonist application; neurons with resting $[\text{Ca}^{2+}]_i$ above 120 nM or whose resting

Figure 1. Fura-2 spectral and calibration curves. Excitation spectra were run on a single striatal neuron (*A*) treated with 1 μ M ionomycin in the presence of 1.2 mM Ca^{2+} (solid squares) followed by 20 mM EGTA (open squares). Also shown is the spectrum of fura-2 acetoxymethyl ester determined in the same chamber (open triangles). The spectral shift of fura-2 upon binding calcium enabled the determination of the calibration curve shown in *B*, where the Y-axis is μ moles/liter of calcium in cytosolic buffer. *C*, Photomicrograph of a striatal neuron mounted in the microspectrofluorimeter.

$[\text{Ca}^{2+}]_i$ fluctuated more than ± 20 nM were not used. These conditions occurred rarely and seemed to be related to poor cell viability. Agonists and antagonists were applied with rapid injections into the chamber using Hamilton syringes. Similar volumes of solvents were often injected prior to agonist application to test for injection artifacts. All antagonists were applied 3 min before agonist application, except for MK801, which was applied 1 hr before agonist application. Desensitization experiments were always performed with 4 min of wash time, followed by a minute of wait time (allowing a stable basal $[\text{Ca}^{2+}]_i$ reading to be taken) before the second agonist application. Those cells whose $[\text{Ca}^{2+}]_i$ did not return to within 40 nM of its preagonist value were not used.

Calculations. The dose-response curves of all 3 agonists tested showed a strong concentration dependence and were fit with the equation $[\text{Ca}^{2+}]_i = K_1(K_2A + 2K_2K_3A^2)/(1 + K_2A + K_2K_3A^2)$, where A is the concentration of agonist and K_1 , K_2 , K_3 are constants that are varied to provide the best fit. This classical cooperative binding equation allowed for the steep dose dependence to be fit satisfactorily for a more accurate determination of EC_{50} 's. Statistical tests on experiments where the effects of antagonists and ion substitution were tested were paired with control experiments of that same day. The SE shown on the control dose in the figures represents the largest SE of any controls paired with experiments of that figure. Illustrated error bars represent SEs.

Materials. MK801 was a kind gift of Drs. L. Iversen and G. Woodruff, Merck Sharp & Dohme, UK. PCP was obtained from the National Institutes of Health. Dioxadrol isomers were gifts of S. R. Zukin. GAMS was a gift of J. Ferkany, NOVA, USA. Fura-2 was purchased from Molecular Probes Inc., Eugene, OR. All other drugs were purchased from Sigma.

Results

Cell culture

Striatal neurons in primary culture were routinely used between day 11 and 21 unless specified. Although the magnitude of the excitatory amino acid-induced changes in $[\text{Ca}^{2+}]_i$ we observed did differ from cell to cell, we did not find any obvious subcategories of neurons that could be defined according to response size. Resting $[\text{Ca}^{2+}]_i$'s were 56 ± 27 nM (mean $\pm$ SD). It is our impression that virtually all neurons in these striatal cultures will respond to excitatory amino acids, although using other criteria, the cultures are quite heterogeneous, as might be expected. Thus, populations of neurons stained positively with antisera against GABA, choline acetyltransferase, substance P, methionine enkephalin, and somatostatin. Of these the GABA-containing cells were by far in greatest abundance, as in the mature striatum (Panula et al., 1980). Experiments were carried out in 550 single striatal neurons, selected as discussed in Materials and Methods.

Action of NMDA

When NMDA (10^{-6} – 10^{-3} M) was added to cells in normal physiological medium, only very small increases in $[\text{Ca}^{2+}]_i$ were observed (Fig. 2). However, the situation altered dramatically when Mg^{2+} was removed from the medium. Now, addition of NMDA at concentrations above 3×10^{-6} M produced a rapid increase in $[\text{Ca}^{2+}]_i$ (Fig. 2). Maximal effects of NMDA were observed at or above 3×10^{-5} M. If NMDA remained in the incubation medium, $[\text{Ca}^{2+}]_i$ tended to remain elevated over several minutes, sometimes declining slowly (Fig. 2). If NMDA was washed out, $[\text{Ca}^{2+}]_i$ tended to decline more rapidly, although the rate of decline varied considerably from cell to cell. The response to 10 μ M NMDA had a tendency to desensitize. Thus, following an initial response and washout, a second challenge with the same concentration of agonist produced a considerably attenuated response (Fig. 2). The effects of NMDA were blocked by prior addition of the putative competitive NMDA receptor blocker DL-2-amino-5-phosphonovaleric acid (AP5) (Fig. 3). Other classes of drugs have been reported to block the effects of NMDA by interacting with the receptor-linked ionophore rather than by competing with NMDA for its recognition site. These include PCP-like compounds (Anis et al., 1983; Honey et al., 1985). As can be seen from Figure 3, PCP caused a dose-dependent, noncompetitive decrease in the effect of NMDA ($\text{IC}_{50} = 2 \times 10^{-7}$ M). The block produced by these drugs was stereospecific. Thus, the D-isomer of the enantiomeric pair dextro-diol and levo-diol was a potent blocker, whereas the L-isomer was ineffective at 2×10^{-7} M (Fig. 3). Furthermore, the newly described compound MK-801, which also appears to act at the same site on the ionophore as PCP (Wong et al., 1986), also powerfully blocked the effect of NMDA (Fig. 3).

The effects of both Mg^{2+} and MK-801 appeared to be voltage dependent (Fig. 4). Normally, in the presence of Mg^{2+} (9×10^{-4} M) NMDA produced no effect (*vide supra*). We added low concentrations of veratridine (5×10^{-7} M) to cells in order to depolarize them. As shown in Figure 5, this concentration of veratridine was insufficient to depolarize cells enough to evoke Ca^{2+} entry through voltage-sensitive Ca^{2+} channels. However, after adding veratridine, NMDA produced large increases in $[\text{Ca}^{2+}]_i$ even in the presence of Mg^{2+} . In a similar paradigm it can be seen that veratridine also relieved the block of the NMDA

Figure 2. NMDA-induced increases in $[Ca^{2+}]_i$. In Mg^{2+} -free buffer, NMDA stimulated a rise in $[Ca^{2+}]_i$ in a dose-dependent manner as shown in *A* (solid squares). In the presence of 0.9 mM Mg^{2+} , the response was essentially blocked (open triangles). A curve was fit through the Mg^{2+} -free points using the equation described in Materials and Methods and the parameters $K_1 = 161.6$, $K_2 = 0.0022$, $K_3 = 2.52$ ($4 < n < 10$ for all points). *B*, Using the paradigm described in Materials and Methods, the second response (Mg^{2+} -free media) was attenuated by 91% ($n = 5$). *C*, Typical response as well as an attenuated second response. *D*, The reversible nature of the Mg^{2+} block is shown in the trace. The asterisk designates significantly different from control with $p < 0.01$.

effect produced by MK801, although the NMDA responses were somewhat attenuated over time. In contrast, the blocking effects of AP5 were not voltage dependent and were still manifest in the presence of veratridine.

We attempted to elucidate the mechanism of the NMDA-induced increase in $[Ca^{2+}]_i$. Figure 5 presents the various possibilities that we considered. The simplest explanation is that Ca^{2+} enters through the NMDA-receptor-linked ionophore. A second possibility is that Na^+ and Ca^{2+} enter via this ionophore, depolarizing the cell and opening voltage-sensitive Ca^{2+} channels. The major route of Ca^{2+} entry might then be through such channels. A further possibility is that Na^+ entering through the ionophore reduces the gradient for Na^+/Ca^{2+} exchange. Calcium may enter by the exchanger operating in reverse or calcium may accumulate with the exchanger unavailable for extrusion of calcium that may leak into the cell. It is also possible that the increase in $[Ca^{2+}]_i$ represents mobilization of intracellular bound Ca^{2+} stores using an inositol trisphosphate (IP_3)-linked mechanism and does not depend on Ca^{2+} entry at all. Furthermore, entry of a small amount of Ca^{2+} might be necessary, and this could trigger the further release of Ca^{2+} from intracellular stores. Such Ca^{2+} -triggered Ca^{2+} release mechanisms are known to exist

in skeletal and cardiac muscle for example, and they may also exist in peripheral neurons (MacDermott and Weight, 1982; Smith et al., 1983; Neering and McBurney, 1984).

Figure 6 shows that when $[Ca^{2+}]_o$ was completely removed, NMDA was unable to increase $[Ca^{2+}]_i$. Thus, the effect of NMDA was dependent on the influx of Ca^{2+} . We attempted to assess the role of Ca^{2+} -induced Ca^{2+} release by examining the effects of methylxanthines. Intracellular Ca^{2+} stores that participate in this type of process are normally sensitive to the actions of high concentrations of caffeine or theophylline (MacDermott and Weight, 1982; Smith et al., 1983; Neering and McBurney, 1984; Saida and Van Breemen, 1984). Theophylline, 10 mM, evoked a slow sustained increase in $[Ca^{2+}]_i$. However, the methylxanthine was found to be ineffective when $[Ca^{2+}]_o$ was lowered. Thus, in these cells the rise in $[Ca^{2+}]_i$ provoked by theophylline seems to be the result of increased Ca^{2+} permeability rather than mobilization of intracellular bound Ca^{2+} stores. It therefore seems unlikely that Ca^{2+} -induced Ca^{2+} release operates in these central neurons. However, we have not systematically investigated this point, and so it remains possible that the rise in $[Ca^{2+}]_i$ is amplified in this fashion to some extent.

It is important to try and distinguish Ca^{2+} entry through ag-

Figure 3. Noncompetitive and competitive antagonisms are shown for the NMDA receptor-linked ionophore in *A* and *B*, respectively. MK801 and the isomers of dioxadrol are presumptive noncompetitive antagonists. NMDA was applied at either 10 μM (filled bars) or 100 μM (open bars) in Mg²⁺-free media. The dagger (†) and asterisk (*) designate significantly different from control with $p < 0.05$ and $p < 0.01$, respectively; $n > 3$ for all values. PCP, phencyclidine; DEX, dexodrol; LEV, levodrol.

onist-regulated and voltage-sensitive Ca²⁺ channels. As we have previously demonstrated, when cells are depolarized by raising [K⁺]_o, Ca²⁺ enters through voltage-sensitive Ca²⁺ channels (Thayer et al., 1986). This can be blocked by 3×10^{-5} M La³⁺ (Fig. 6). (It should be noted that divalent channel blockers such as Cd²⁺ produce spurious effects on the fura-2 fluorescence and so cannot be used.) La³⁺ had no effect on the increase in [Ca²⁺]_i produced by NMDA. Thus, voltage-sensitive Ca²⁺ channels do not seem to participate in this response. Li⁺ has been reported not to participate in Na⁺/Ca²⁺ exchange (Baker and DiPolo, 1984). When all Na⁺ was replaced by Li⁺, there was no rise in [Ca²⁺]_i, suggesting that normal resting [Ca²⁺]_i does not rely on the activity of this exchange system. Removing Na⁺ had no effect on NMDA- or depolarization-induced rises in [Ca²⁺]_i. Replacement of external Na⁺ with the organic cation *N*-methyl-D-glucamine (NMDG) also did not have an inhibitory effect on the action of NMDA (Fig. 6).

The most parsimonious explanation of the data is that NMDA activates an ionophore that allows the entry of Ca²⁺ into the

cell. This ionophore is blocked in a voltage-dependent fashion by Mg²⁺ and MK801. Voltage-sensitive Ca²⁺ channels and Na⁺/Ca²⁺ exchange do not seem to participate in this case.

The action of glutamate

Glutamate is also an agonist at the NMDA receptor. When glutamate was added to striatal neurons in the absence of Mg²⁺, it produced effects that closely resembled those produced by NMDA (Fig. 7). Glutamate was more potent, however, producing significant effects at concentrations of 10⁻⁶ M.

A clear distinction between the actions of NMDA and glutamate was seen however with respect to the effects of Mg²⁺. In normal physiological medium, which contains sufficient Mg²⁺ to completely block the effects of NMDA, the effects of glutamate were still manifest. Glutamate was slightly less potent in the presence of Mg²⁺ than in its absence, and its dose-response curve rose more steeply. However, it was still equally efficacious. The time course of the effects of glutamate observed in the presence of Mg²⁺ was usually similar to that observed in Mg²⁺-

Figure 4. Effects of veratridine depolarization on antagonism of the NMDA response. *A*, In the presence of 0.9 mM Mg²⁺, NMDA failed to elicit an increase in [Ca²⁺]_i (open squares). However, treatment with a dose of veratridine that did not activate voltage-sensitive calcium channels (VSCCs) relieved the voltage-dependent block of Mg²⁺ (solid diamonds). A similar paradigm was used to test the drugs MK801 and AP5 for voltage-sensitive effects. As shown in *B*, 1 μM MK801 is a potent inhibitor of the NMDA response in Mg²⁺-free media (open squares), and this can be reversed by veratridine (solid diamonds). *C*, In contrast, the inhibition produced by 1 mM AP5 in Mg²⁺-free media is not reversed by veratridine (solid diamonds). A control response (with veratridine) is represented by the open squares.

Figure 5. Possible routes of calcium entry into the cell induced by excitatory amino acids.

free medium, although occasionally onset of a response was somewhat delayed. The effects of glutamate in Mg^{2+} were only slightly blocked by MK801 at concentrations that completely blocked the effects of NMDA (Fig. 8). However, although Mg^{2+} and MK801 were not very effective in blocking the actions of moderate concentrations glutamate, AP5 was a very effective blocker (Figs. 7 and 8). Indeed, the actions of glutamate could be almost completely inhibited by AP5 in a reversible manner. Interestingly, when very high concentrations of glutamate were used (10^{-4} M), its effects became resistant to the actions of AP5 (Fig. 8). We also attempted to inhibit the effects of glutamate with other putative blockers of receptors for excitatory amino acids (Fig. 8). Unfortunately, at the concentrations at which these substances are usually employed, we found that most of them produced substantial increases in $[Ca^{2+}]_i$ by themselves (see also Kudo and Ogura, 1986). At the highest concentrations at which these increases were not seen, only γ -D-glutamyl-aminomethylsulphonate (GAMS) produced any inhibition of the glutamate effect. In contrast to NMDA, the effects of 10 μ M glutamate exhibited little desensitization. This was the case whether glutamate was used in the presence or absence of Mg^{2+} .

We attempted to elucidate the mechanism by which glutamate (3×10^{-5} M) increased $[Ca^{2+}]_i$ when it was added in the presence of Mg^{2+} . We considered the possibilities previously explored in the case of NMDA. In addition, however, we must also consider the possibility that glutamate can act upon other non-NMDA

glutamate receptors and that these may play a role in the production of the response. As with NMDA, the effects of glutamate were virtually completely blocked when all $[Ca^{2+}]_o$ was removed, indicating that the response was dependent on the influx of Ca^{2+} (Figs. 7 and 9). The effects of glutamate were not blocked by 3×10^{-5} La^{3+} or altered by replacement of all $[Na^+]_o$ by Li^+ (Fig. 9). Furthermore, the effects of glutamate were not blocked by TTX (10^{-6} M). However, a clear difference between the effects of glutamate and those of NMDA were noted with respect to the effects of replacement of $[Na^+]_o$ with NMDG. Such replacement had relatively minor effects on the action of NMDA (*vide supra*); however, the effects of glutamate were substantially inhibited.

These data suggest that the mechanism by which glutamate produces increases in $[Ca^{2+}]_i$ differs somewhat from that discussed above for NMDA. The response seems to require Ca^{2+} influx and does not seem to involve voltage-sensitive Ca^{2+} channels or Na^+/Ca^{2+} exchange. Moreover, the effects of glutamate (10^{-5} M) are blocked by AP5 and therefore presumably involve the NMDA receptor/ionophore system. However, other observations such as the lack of effect of Mg^{2+} and MK801 and the increased inhibition on substitution of $[Na^+]_o$ by NMDG indicate that other factors are also involved. We shall discuss a possible mechanism below.

Action of KA and QA

KA was also able to provoke large increases in $[Ca^{2+}]_i$ (Fig. 10). As in the case of glutamate, the actions of KA were not altered by the presence of Mg^{2+} . Relatively high concentrations of KA ($\geq 3 \times 10^{-5}$ M) were required to produce responses both in the presence and absence of Mg^{2+} . The actions of KA were not inhibited by MK801 but were partially blocked by AP5 (Figs. 10 and 11). However the effects of KA appeared more resistant to the blocking actions of AP5 than those of glutamate or NMDA. Nevertheless, at high concentrations of AP5 the effects of KA were reduced. The responses to KA also showed a tendency to desensitize, as observed for NMDA (Fig. 10). The effects of KA were abolished on removal of Ca^{2+} and were not blocked by 3×10^{-5} M La^{3+} or affected when Na^+ was replaced by Li^+ (Fig. 12). As with responses to glutamate, however, the effects of KA were substantially reduced when $[Na^+]_o$ was replaced by NMDG.

We also performed a limited number of studies using the glutamate analog QA (Fig. 13). The effects of QA resembled

Figure 6. Effects of ion substitution on calcium influx stimulated by 50 mM K^+ or 100 μ M NMDA (in Mg^{2+} -free media). Control responses (solid bars) were compared to responses obtained in media in which 20 μ M EGTA was substituted for calcium (labeled in figure), 30 μ M La^{3+} was added (stippled bars), Na^+ was replaced with 137 mM choline (wide hatched bars), Na^+ was replaced with 137 mM Li^+ (hatched bars), and Na^+ was replaced by 137 mM NMDG (open bars). Dagger (†) and asterisk (*) designate significantly different from control with $p < 0.05$ and $p < 0.01$, respectively; $n > 4$ for all values.

Figure 7. Glutamate-induced increases in $[Ca^{2+}]_i$. Glutamate stimulated a rise in $[Ca^{2+}]_i$ in a dose-dependent manner in both the presence (*open triangles*) and absence (*solid squares*) of 0.9 mM Mg^{2+} . The curve was fit as described in Materials and Methods with the parameters $K_1 = 136.6$, $K_2 = 0.00053$, $K_3 = 219.0$ through the Mg^{2+} -free points ($4 < n < 19$ for all points). **B**, Attempts to generate a second response using double applications of 10 μM glutamate as described in Materials and Methods resulted in only a slight attenuation of the response ($n = 8$). **C**, Typical response. **D**, The reversible nature of the block by AP5. **E**, The trace illustrates the requirement of the glutamate response for external calcium.

those of glutamate and KA. Relatively high concentrations of QA were required to elicit these responses ($\geq 10^{-4}$ M). QA was effective in the presence of Mg^{2+} and MK801. As in the case of glutamate and KA, the responses to QA could be blocked by AP5. However, as with KA, high concentrations of AP5 were required, and only a partial block was achieved even at these concentrations. The QA analog α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) produced effects that resembled those of QA.

Discussion

The major finding in the present study is that glutamate and various types of glutamate agonists substantially increase $[Ca^{2+}]_i$ in striatal neurons. This is true for agonists that activate NMDA and non-NMDA receptors alike. These findings are consistent

with the proposal that excitotoxicity is associated with an increase in $[Ca^{2+}]_i$ (Choi, 1985, 1987; Garthwaite and Garthwaite, 1986; Garthwaite et al., 1986; Hajos et al., 1986). Clearly, however, the notion that an increase in $[Ca^{2+}]_i$ is actually responsible for excitotoxicity is neither supported or negated by our observations. We shall now discuss a model to explain these various observations. For convenience we adopt the nomenclature suggested by O'Brien and Fischbach (1986), who designated the NMDA-type glutamate receptor as G_1 and the non-NMDA glutamate receptor as G_2 . Clearly, however, we realize that this may ultimately prove to be an oversimplification and that there may either be more than one type of G_2 receptor or that all types of excitatory amino acid receptors may actually be separate functional manifestations of a single type of ionophore complex gated in diverse ways (Cull-Candy and Usowicz, 1987; Jahr and Stevens, 1987).

Figure 8. Antagonism of glutamate-stimulated increases in $[Ca^{2+}]_i$. *A*, Antagonists for the glutamate receptor. Glutamate was used at 10 μ M. *B*, The maximum inhibition of various glutamate concentrations—3 μ M (hatched bar), 10 μ M (filled bar), or 100 μ M (open bar)—obtained with increasing doses of AP5. Dagger (†) and asterisk (*) designate significantly different from control with $p < 0.05$ and $p < 0.01$, respectively; $n > 3$ for all values.

Effects of NMDA

NMDA clearly produced rapid increases in $[Ca^{2+}]_i$ in striatal neurons. Such results are consistent with some very recently reported measurements using fura-2 microspectrofluorimetry and single hippocampal neurons (Kudo and Ogura, 1986), in which NMDA was also effective in raising $[Ca^{2+}]_i$. Our results are also quite consistent with several previous studies on the properties of the G_i receptor/ionophore complex. The ionophore activated by G_i agonists has a conductance of about 50 pS (Nowak et al., 1984; Cull-Candy and Ogden, 1985; O'Brien and Fischbach, 1986; Cull-Candy and Usowicz, 1987; Jahr and Stevens, 1987) and appears to be permeable to a variety of cations, including Ca^{2+} (Ascher and Nowak, 1986; MacDermott et al., 1986; Mayer and Westbrook, 1987a; Jahr and Stevens, 1987; Mayer et al., 1987). Of particular interest is the well-established observation that this ionophore can be blocked in a voltage-dependent fashion by Mg^{2+} (Mayer et al., 1984; Nowak et al., 1984; Mayer and Westbrook, 1987b) and other divalent cations (e.g., Ni^{2+} , Co^{2+} , and Mn^{2+}) at submillimolar concentrations (Nowak and Ascher, 1985; Mayer and West-

brook, 1986, 1987b). Interestingly, low concentrations of Cd^{2+} are rather ineffective blockers (Mayer et al., 1984; Mayer and Westbrook, 1987a), an observation that clearly distinguishes this ionophore from the various types of voltage-sensitive Ca^{2+} channels that are potently blocked by Cd^{2+} (Hagiwara and Byerly, 1981). High concentrations of Ca^{2+} and Ba^{2+} ($> 5 \times 10^{-3}$ M) can also act as NMDA antagonists, decreasing single-channel conductance (Nowak and Ascher, 1985). The fact that the G_i -linked ionophore exhibits an appreciable permeability to Ca^{2+} suggests that it may be the primary route for Ca^{2+} entry seen in our studies. Indeed, all our observations support this proposal. The effect of NMDA is clearly blocked by Mg^{2+} in a voltage-dependent fashion. It should be noted that the Mg^{2+} -dependent block can be relieved by very low concentrations of veratridine, which depolarizes cells by allowing sustained Na^+ entry via voltage-sensitive Na^+ channels. The concentration of veratridine employed clearly does not depolarize the neurons sufficiently to allow Ca^{2+} entry via voltage-sensitive Ca^{2+} channels. This is consistent with previous electrophysiological studies. Thus if our striatal neurons have a resting potential of about -70 mV, a depolarization of 20–30 mV would be sufficient to

Figure 9. Effects of ion substitution on calcium influx stimulated by 30 μ M glutamate in Mg^{2+} -containing media. The control response (solid bar) is compared to responses in which 20 μ M EGTA was substituted for calcium (filled hatched bars), 30 μ M La^{3+} was added (stippled bars), Na^+ was replaced with 137 mM Li^+ (hatched bars), Na^+ was replaced by 137 mM NMDG (open bars), and 1 μ M TTX was added (wide hatched bars). Dagger (†) and asterisk (*) designate significantly different from control with $p < 0.05$ and $p < 0.01$, respectively; $n > 4$ for all values.

Figure 10. *A*, Kainic acid-induced increases in $[Ca^{2+}]_i$. Increasing concentrations of kainate stimulated a rise in $[Ca^{2+}]_i$ in both the presence (*open triangles*) and absence (*solid squares*) of 0.9 mM Mg^{2+} . The curve was fit as described in Materials and Methods with the parameters $K_1 = 121.5$, $K_2 = 8.9 \times 10^{-5}$, $K_3 = 29.2$ using the values obtained in Mg^{2+} -containing media ($4 < n < 19$ for all points). *B*, Attempts to generate a second response, as described in Materials and Methods, resulted in a 61% attenuated response ($p < 0.05$, $n = 5$). *C*, Typical response. *D*, Reversible nature of the block by 5 mM AP5.

relieve the block of the G_i ionophore by Mg^{2+} but not to activate voltage-sensitive Ca^{2+} channels that require stronger depolarizations (Nowycky et al., 1985). Block of the NMDA response by PCP/MK801-like agents also seems to be voltage dependent, supporting previous suggestions that the locus of action of these compounds is at the level of the ionophore rather than the NMDA recognition site (Honey et al., 1985). The effect of NMDA seems to depend on the influx of Ca^{2+} , as completely removing all $[Ca^{2+}]_o$ eliminates the response. Such data is consistent with other studies that show that NMDA increases the uptake of $^{45}Ca^{2+}$ by brain cells in primary culture or in slice preparations (Ichida et al., 1982; Retz et al., 1982; Retz and Coyle, 1984; Harris, 1985; Wroblewski et al., 1985, 1987; Riveros and Orrego, 1986). It is conceivable that this initial entry of Ca^{2+} is now amplified if it triggers a secondary release of Ca^{2+} from intracellular bound stores. However, although such mechanisms may occur in some peripheral neurons (MacDermott and Weight, 1982; Smith et al., 1983; Neering and McBurney, 1984), we found little evidence for such effects in our studies. Furthermore, abolition of the effects of NMDA on removal of $[Ca^{2+}]_o$ also suggests that NMDA does not function through its reported degradation of phosphatidyl inositol biphosphate and generation of inositol triphosphate (Nicoletti et al., 1986a, b, 1987; Sugiyama et al., 1987). As the G_i ionophore is also permeable to Na^+ (Nowak et al., 1984; Mayer and Westbrook 1987a; Jahr and Stevens, 1987), we also examined the potential role of Na^+ in the NMDA response. Cell depolarization due to the entry of Na^+ (and also Ca^{2+}) could lead to the opening of voltage-sen-

Figure 11. Inhibition of the kainate response by AP5 and MK801. Antagonists were tested against 30 μM (*solid bars*) or 100 μM kainic acid (*open bars*); $n > 3$ for all values.

Figure 12. Effects of ion substitution on calcium influx stimulated by 100 μM kainate. The control response (solid bar) was compared to responses obtained in media in which 20 μM EGTA was substituted for calcium (filled hatched bars), 30 μM La^{3+} was added (stippled bars), Na^+ was replaced with 137 mM Li^+ (hatched bars), and Na^+ was replaced by 137 mM NMDG (open bars). Dagger ($\dagger$) and asterisk (*) designate significantly different from control with $p < 0.05$ and $p < 0.01$, respectively; $n > 4$ for all values.

sitive Ca^{2+} channels and the subsequent entry of Ca^{2+} . However, in the presence of a concentration of La^{3+} that blocked voltage-dependent Ca^{2+} entry, NMDA-stimulated Ca^{2+} uptake was fully maintained. Furthermore, NMDA-linked Na^+ entry could reverse $\text{Na}^+/\text{Ca}^{2+}$ exchange or at least decrease Ca^{2+} extrusion, leading to an increase in $[\text{Ca}^{2+}]_i$. Complete substitution of all Na^+ by Li^+ , however, failed to reduce the response to NMDA or increase basal $[\text{Ca}^{2+}]_i$. In squid axon, at any rate, Li^+ cannot utilize the $\text{Na}^+/\text{Ca}^{2+}$ system (Baker and DiPolo, 1984). Substitution of Na^+ by NMDG also failed to reduce the response to NMDA. This supports the interpretation that depolarization of the membrane by sodium is not involved in the actions of NMDA in Mg^{2+} -free media, since large organic cations are not believed to pass through the G_i ionophore (Nowak and Ascher, 1984). Overall, the most parsimonious interpretation of our data is that NMDA activates an ionophore that is Ca^{2+} permeable and blocked in a voltage-dependent manner by Mg^{2+} and that this is the route of NMDA-activated Ca^{2+} uptake.

Effects of glutamate

Glutamate acts as an agonist at G_i receptors (Mayer and Westbrook, 1984, 1987a; Westbrook and Mayer, 1984; O'Brien and Fischbach, 1986). Thus, in the absence of Mg^{2+} the glutamate-induced increase in $[\text{Ca}^{2+}]_i$, presumably reflects activation of the

same mechanisms discussed above for NMDA. However, there are several important differences in the responses to NMDA and glutamate. The most striking of these is that Mg^{2+} does not block the effects of glutamate. An obvious interpretation of such an observation is that glutamate can activate another type of ionophore (G_2 ?) in addition to the G_i receptor and that this second ionophore is also Ca^{2+} permeable. However, some properties of the ionophore(s) activated by G_2 agonists have been reported. It has a smaller conductance than the G_i -linked ionophore (O'Brien and Fischbach, 1986; Cull-Candy and Usowicz, 1987; Jahr and Stevens, 1987). Although it appears to be permeable to monovalent cations, it has been reported not to be appreciably permeable to Ca^{2+} (MacDermott et al., 1986; Mayer et al., 1987). Thus, increasing $[\text{Ca}^{2+}]_o$ causes a depolarizing shift in the reversal potential for NMDA-activated currents in CNS neurons but only a slight shift for KA-induced currents (MacDermott et al., 1986; Mayer and Westbrook, 1987b). Let us start by considering a separate mechanism. It may be supposed that G_i and G_2 receptors coexist on the vast majority of glutamate-sensitive striatal neurons. We have not systematically investigated this question, as we wished to avoid the complicating effects of desensitization. However, other studies have clearly supported this contention (Nowak et al., 1984; Mayer and Westbrook, 1987a; Jahr and Stevens, 1987). Glutamate acts

Figure 13. A, Dose-response curve for the effects of quisqualate in Mg^{2+} -containing media ($3 < n < 7$ for all values). The curve was fit as described in Materials and Methods with the parameters $K_1 = 86.6$, $K_2 = 3.5 \times 10^{-7}$, $K_3 = 1089$. B, Typical response to the agonist AMPA.

as an agonist at both these receptor types. The effects of adding glutamate will therefore be functionally very similar to those of adding NMDA and veratridine simultaneously in the presence of Mg^{2+} . Glutamate acting at the G_2 site will activate a Na^+ ionophore (the G_2 ionophore) and produce depolarization, i.e., the same ultimate result as activation of the voltage-sensitive Na^+ channel by veratridine. The degree of depolarization produced by moderate concentrations of glutamate might, in fact, be expected to be in the range of 20–30 mV (e.g., Rothman, 1985; Rothman and Samaie, 1985). This would be enough to reduce the voltage-dependent block of G_1 by Mg^{2+} but not enough to activate voltage-dependent Ca^{2+} channels. Indeed, the effects of glutamate are not blocked by La^{3+} . As glutamate can also activate G_1 receptors, it will simultaneously be able to evoke Ca^{2+} uptake via the G_1 -linked ionophore. In effect the action of glutamate at G_2 exerts a permissive effect for the simultaneous action of glutamate at G_1 . Several of our observations are consistent with this model. The most striking is that even in the presence of Mg^{2+} the effects of moderate concentrations of glutamate are completely blocked by AP5, suggesting the involvement of the G_1 complex. Second, it can be seen that substitution of Na^+ by Li^+ does not reduce the effects of glutamate, whereas substitution by NMDG has quite a strong suppressive effect. It may well be that Li^+ can permeate the G_2 -linked ionophore and thus participate in G_2 -linked depolarization as can Na^+ . However, if NMDG is not permeable, depolarization would not be produced, and the permissive effect of G_2 activation would not occur. (It is also possible, however, that NMDG may have a blocking effect at the G_2 ionophore, an effect that would also be consistent with our observations.) The effects of glutamate were also not blocked by MK801. This could also be explained by the voltage dependence of the action of this drug at G_1 (*vide supra*). One problematic series of experiments concerns studies on the effects of agents that are supposed to act as G_2 or G_1/G_2 antagonists. We found that when we used these agents at concentrations at which others have used them, most of them caused increases in $[Ca^{2+}]_i$ by themselves. At concentrations where this did not occur, most of these agents were ineffective. The exception was GAMS, which did block some of the glutamate effect. Our experience has apparently been shared by Kudo and Ogura (1986), who observed similar “nonspecific” effects of these so-called blockers in hippocampal pyramidal cells. In addition these authors also reported that AP5 produced similar effects. In general, however, we have not found such spurious actions of AP5 up to concentrations of 5 mM. Thus, our observations are all consistent with the participation of the G_1 complex in the response to glutamate in the presence of Mg^{2+} . Other observations such as the lack of effect of La^{3+} and the effect of the removal of $[Ca^{2+}]_o$ are also consistent with this model.

The alternative explanation would be that the G_2 ionophore does have a significant permeability to Ca^{2+} . It could be supposed that although the G_2 permeability to Ca^{2+} was much less than that for G_1 , G_2 receptors were found in much greater abundance than G_1 receptors, and thus the net cellular rise in $[Ca^{2+}]_i$ might be the same. In this case, the blocking effects of AP5 would have to be mediated at the G_2 site. Again, however, there is no evidence for such a proposal. Indeed, O'Brien and Fischbach (1986) demonstrated that even at 1 mM AP5 had no effect whatsoever on the conductance activated by G_2 agonists in chick motoneurons *in vitro*. Thus, for moderate concentrations of glutamate, the mechanism we have discussed seems the most likely. It should be noted, though, that when very high concentrations

of glutamate are used, the response becomes much less sensitive to blockade by AP5, suggesting that alternative mechanisms may also come into play. The lack of effect of AP5 cannot be explained solely on the basis that higher AP5 concentrations would be needed to compete with higher concentrations of glutamate. Recently, Jahr and Stevens (1987) and Cull-Candy and Usowicz (1987) have suggested that G_1/G_2 receptors may, in fact, form a functionally linked complex. They suggest that the different actions of G_1 and G_2 agonists merely reflect the stabilization of distinct conductance states in this complex. Large, medium, and small conductance states are found. Of these, the large conductance state clearly corresponds to that previously identified with the G_1 ionophore. Interestingly, although G_2 agonists tend to stabilize the smaller Ca^{2+} -impermeable conductance states, they do also produce some openings of the large, Ca^{2+} -permeable conductance state, although much less frequently than G_1 agonists. Clearly, therefore, according to this model, G_2 agonists might be expected to cause some Ca^{2+} entry directly. According to Jahr and Stevens, lower concentrations of glutamate tend to behave like NMDA and produce mostly large conductance state openings; however, high concentrations of glutamate favor the smaller, relatively Ca^{2+} -impermeable states.

Effects of KA and QA

In most respects the effects of KA and, in so far as we have pursued them, of QA resemble those of glutamate. This is true with respect to their resistance to the blocking effects of Mg^{2+} and MK801, the effect of various ionic manipulations, substitutions, etc. We propose that the same 2 mechanisms operate here as with the glutamate receptor. The NMDG/AP5-blockable parts of a response represent activation of the G_1 receptor. The most significant problem with this mechanism is that it necessitates agonist effects of KA and QA at G_1 , as well as G_2 . Is such a proposal reasonable? It should be noted that the effects of KA and QA seen in our studies require relatively high concentrations of these agonists. Is there any evidence for G_1 effects of KA and QA even at elevated concentrations? Electrophysiological studies are difficult to judge in this respect, although many studies might be interpreted as suggesting a small G_1 contamination of the basically G_2 effects of KA and QA (Mayer and Westbrook, 1987a). In MacDermott et al. (1986; Fig. 3), this could also explain the small rise in $[Ca^{2+}]_i$ evoked by 10^{-4} M KA. The most accurate data should come from ligand binding studies. Unfortunately, although their pharmacology is relatively sophisticated, G_1 receptors have been difficult to identify unequivocally in biochemical paradigms. Until recently no consensus had been reached with respect to such studies (Foster and Fagg, 1984). Recently, however, a novel ligand 3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid (CPP) has been introduced that appears to have been used successfully by at least 2 groups for labeling G_1 sites (Olverman et al., 1986; Murphy et al., 1987). Interestingly, Olverman et al. (1986) observed that the K_i values for the inhibition of 3H -CPP binding to G_1 sites in rat cortical membranes were 10^{-4} M for QA and 2.8×10^{-4} M for KA. These are clearly in the same range as the concentrations found to be effective in the present studies. Unfortunately, the K_i for AMPA is about 10^{-4} M, and we have shown that lower concentrations of this QA analog are also effective in raising $[Ca^{2+}]_i$. Responses resistant to NMDG substitution and high doses of AP5 could represent Ca^{2+} permeability through the G_2 ionophore. In the case of KA and QA,

the observations of Jahr and Stevens (1987) and Cull-Candy and Usowicz (1987) are particularly useful, as they help to explain the situation. Thus, presumably the AP5-resistant portion of the effects observed could be due to the activation of the large conductance Ca^{2+} -permeable state of the channel by the G_2 rather than the G_1 receptor. However, one wishes to view the situation, the conclusion must be that under the conditions of our experiments all the amino acids we have used induce Ca^{2+} uptake predominately via receptor-linked ionophores rather than by other routes.

Relationship to other studies and implications for excitotoxicity

We have suggested a model that provides a mechanism for the excitatory amino acid-induced increase in $[\text{Ca}^{2+}]_i$ observed in our studies. However, is this indeed the manner in which excitatory amino acids causes increases in $[\text{Ca}^{2+}]_i$ *in vivo* or even in other systems *in vitro*? The most comparable study is that by Kudo and Ogura (1986), who used fura-2 microspectrofluorimetry to analyze the effects of glutamate on $[\text{Ca}^{2+}]_i$ in hippocampal pyramidal cells *in vitro*. There is excellent agreement between the results of that study and those reported here. This applies to values for the resting $[\text{Ca}^{2+}]_i$ in neurons and to the size of the increment in $[\text{Ca}^{2+}]_i$ produced by glutamate. These authors also found no effect on blocking voltage-sensitive Ca^{2+} channels or substitution of Na^+ . Several other studies have utilized radiotracers to analyze the effects of excitatory amino acids on neuronal Ca^{2+} uptake (Ichida et al., 1982; Retz et al., 1982; Retz and Coyle, 1984; Harris, 1985; Wroblewski et al., 1985, 1987; Riveros and Orrego, 1986). In most cases, these results are difficult to interpret. An exception is a recent study by Wroblewski et al. (1987) on primary cultures of cerebellar granule cells. These authors observed that NMDA and KA could increase $^{45}\text{Ca}^{2+}$ uptake by these cultures. The effects of KA were somewhat reduced by AP5, as in the present study. Rather than suggesting the mechanism we have proposed, Wroblewski et al. suggest a reasonable alternative. They propose that the KA effect is mediated presynaptically and involves the secondary release of a glutamate-like neurotransmitter, which could then activate G_1 -linked channels (Ferkany et al., 1982).

Although such an explanation is clearly reasonable, we do not believe that it explains the effects we observed. In several instances we have observed the effects of NMDA, glutamate, KA, and QA on isolated cells that clearly did not form contacts with other neurons. $^{45}\text{Ca}^{2+}$ uptake studies only monitor events in a population of cells and cannot distinguish between the mechanisms proposed by us and by Wroblewski et al. However, the ability to analyze $[\text{Ca}^{2+}]_i$ in single cells allows us to make this distinction. It is, of course, conceivable that different mechanisms underlie the events observed in cerebellar granule cells and those observed in striatal neurons. In general, it is encouraging to note that results from 3 culture systems using striatal, hippocampal, and cerebellar neurons are extremely compatible. Several studies have also analyzed the effects of excitatory amino acids on Ca^{2+} movements *in situ* or in brain slices by monitoring the removal of Ca^{2+} from the extracellular space using ion-sensitive electrodes. Excitatory amino acids of all classes are able to provoke a large apparent uptake of Ca^{2+} in the cortex, hippocampus, and spinal cord (Heinemann and Pumain, 1980, 1981; Marciani et al., 1982; Buhle and Sonnhof, 1983; Zanotto and Heinemann, 1983; Heinemann et al., 1984; Korf and Postema, 1984; Pumain and Heinemann, 1985; Ashton et al., 1986;

Hamon and Heinemann, 1986; Sakamoto et al., 1986). The authors of these studies have usually concluded that the uptake is due to the influx of Ca^{2+} through both ligand-gated and voltage-sensitive ionophores. However, such conclusions are generally based on the blocking effects of divalent cations. As we have discussed, such cations generally block the G_1 -linked ionophore in addition to voltage-sensitive Ca^{2+} channels, and so such conclusions may be unwarranted. Nevertheless, it is quite conceivable that, under other *in vitro*, *in situ*, or certain pathological conditions, excitatory amino acids may depolarize cells sufficiently to allow Ca^{2+} influx by routes other than the G_1/G_2 -linked ionophores (e.g., Mayer et al., 1987).

If indeed our hypothesis correctly explains some aspects of excitatory amino acid-induced Ca^{2+} uptake occurring *in situ* and if such an increase is actually responsible for excitotoxicity, then certain predictions should be testable. The first is that drugs such as MK801 should protect against NMDA-induced toxicity much more effectively than KA-induced toxicity. This is certainly found to be the case in the striatum and hippocampus (Foster et al., 1986). Furthermore, we would expect AP5 to protect somewhat against KA-induced toxicity. In a recent study of the toxic effects of excitatory amino acids on the retina *in vitro*, Olney et al. (1986) observed that AP5 did partially block KA-induced toxicity.

It is interesting to note that the absolute increases in $[\text{Ca}^{2+}]_i$ observed in this study and elsewhere are not that enormous and seldom approach 10^{-6} M. One may wonder whether these increases in $[\text{Ca}^{2+}]_i$ are sufficient to produce toxicity, but it should be noted that the increases in $[\text{Ca}^{2+}]_i$ are sustained, which may be an important consideration. Moreover, it has been observed (Marciani et al., 1982; Hamon and Heinemann, 1986) that excitatory amino acids stimulate Ca^{2+} uptake into both the cell bodies and dendrites of hippocampal neurons. These authors calculated that, although the net uptake of Ca^{2+} observed in the stratum pyramidal was greater than in the dendritic layers, the absolute concentration of Ca^{2+} achieved within the dendrites would probably be higher.

Finally, it should be noted that, although we have considered our results in light of the proposed role of Ca^{2+} in the excitotoxic effects of amino acids, the observed increases in $[\text{Ca}^{2+}]_i$ may normally play a much more important role in phenomena such as long-term potentiation, long-term depression, or other forms of neuronal activity (Lynch and Baudry, 1984; Kano and Kato, 1987).

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