Excitatory Amino Acid Regulation of Intracellular Ca^{2+} in Isolated Catfish Cone Horizontal Cells Measured under Voltage- and Concentration-Clamp Conditions

C. P. Linn and B. N. Christensen

Department of Physiology and Biophysics, University of Texas Medical Branch, Galveston, Texas 77550

$[\text{Ca}^{2+}]_i$ was measured using fura-2-loaded isolated catfish horizontal cells in the presence of L-glutamate and the glutamate analogs kainate (KA), quisqualate (QA), and NMDA. Caffeine was used to release Ca^{2+} from intracellular stores. Cell membrane potential was controlled with a voltage clamp to prevent activation of voltage-dependent Ca^{2+} channels in the presence of agonist. All excitatory amino acid agonists produced a rapid and sustained rise in $[\text{Ca}^{2+}]_i$ with the order of potency being $\text{QA} > \text{Glu} > \text{KA} > \text{NMDA}$. The agonist-induced $[\text{Ca}^{2+}]_i$ increase was blocked in reduced $[\text{Ca}^{2+}]_o$ and by 6-cyano-7-nitroquinoxaline-2,3-dione and 2-amino-5-phosphonopentanoate, which are specific blockers for QA/KA and NMDA receptors, respectively. The metabotropic receptor agonist *trans*-1-amino-1,3-cyclopentanedicarboxylic acid (ACPD; 10–200 μM) had no effect on $[\text{Ca}^{2+}]_i$. Hill coefficients from curves fitted to concentration–response data suggested an amplification of the Ca^{2+} signal that was interpreted as calcium-induced calcium release (CICR) from intracellular Ca^{2+} stores. Caffeine (10 mM) produced a rapid transient rise in $[\text{Ca}^{2+}]_i$, confirming the existence of a Ca^{2+} -sensitive store. Following caffeine-induced depletion of Ca^{2+} from intracellular stores, agonists were still able to produce increases in $[\text{Ca}^{2+}]_i$, confirming Ca^{2+} influx through the agonist-gated channel. The agonist-induced increase in $[\text{Ca}^{2+}]_i$ was decreased following caffeine-induced depletion, confirming a process of CICR.

These results are consistent with the hypothesis that excitatory amino acids can produce direct modulation of $[\text{Ca}^{2+}]_i$ by influx through the agonist-gated channel and by CICR from intracellular stores.

It is currently believed that the excitatory amino acid neurotransmitter glutamate produces its effect by acting on several separate glutamate receptor subtypes. These are designated according to the agonist selectivity as the non-NMDA kainate (KA) or quisqualate (QA) receptors and the NMDA receptor (for review, see Collingridge and Lester, 1989). Activation of these receptors leads to the gating of a variety of ion channels that exhibit different permeabilities to monovalent and divalent

cations (MacDermott et al., 1986; Mayer et al., 1987; Mayer and Westbrook, 1987; Murphy et al., 1987; Ascher and Nowak, 1988) or to mobilization of Ca^{2+} from intracellular stores as a subsequent action of Ca^{2+} permeation through the agonist-gated channel (Benavides et al., 1988; Holopainen et al., 1989; Norden et al., 1991). In addition, direct receptor-mediated increases in inositol 1,4,5-trisphosphate (IP_3) production via a QA-prefering metabotropic receptor that may be linked to a G-protein have been demonstrated (Nicoletti et al., 1986; Sugiyama et al., 1987; Murphy and Miller, 1988, 1989; Schoepp and Johnson, 1988; Furuya et al., 1989; Manzoni et al., 1990).

Previous studies have suggested that the NMDA receptor is primarily responsible for Ca^{2+} influx through glutamate-activated channels. Whole-cell voltage-clamp experiments on cultured CNS neurons have demonstrated that the reversal potential of the current response to NMDA is shifted in the depolarized direction by raising extracellular Ca^{2+} concentration. However, the reversal potentials of the KA and QA responses are much less affected (MacDermott et al., 1986; Mayer and Westbrook, 1987; Mayer et al., 1987; Gilbertson et al., 1991). The cellular events that occur subsequent to an increase in $[\text{Ca}^{2+}]_i$ include activation of enzyme systems important for protein phosphorylation (Chad and Eckert, 1986) that may result in ion channel regulation (Murphy et al., 1987; Murphy and Miller, 1988; Holopainen et al., 1989, 1990), changes in cell pathology (for review, see Choi and Rothman, 1990), and activation of long-term potentiation (for review, see Nicoll et al., 1988).

Two major non-mitochondrial intracellular Ca^{2+} stores have been identified in neurons that sequester Ca^{2+} and provide for a site from which rapid Ca^{2+} mobilization and exchange can occur. One has been shown to be sensitive to IP_3 (Berridge and Irvine, 1989), which is increased by hydrolysis of phosphatidylinositol 4,5-bisphosphate following receptor activation in the plasma membrane. Recent evidence suggests that Ca^{2+} may act in concert with IP_3 to release Ca^{2+} (Finch et al., 1991). IP_3 acts on specific receptors that have now been purified (Supattapone et al., 1988; Chadwick et al., 1990), cloned (Furuichi et al., 1989), expressed in a stable cell line (Miyawaki et al., 1990), and found to be a functional ligand-gated Ca^{2+} channel in cerebellum and aortic smooth muscle (Ehrlich and Watras, 1988; Miyawaki et al., 1990).

A second neuronal intracellular Ca^{2+} store is sensitive to Ca^{2+} and drugs such as caffeine and ryanodine. These stores have been previously described in muscle (Fill and Coronado, 1988), sensory and sympathetic neurons (Lipscombe et al., 1988; Thayer et al., 1988a,b) and sea urchin eggs (Norden et al., 1991) and appear to be functionally (Thayer et al., 1988b; Cheek et al.,

Received June 20, 1991; revised Nov. 12, 1991; accepted Jan. 9, 1992.

This research was supported by Department of Health and Human Services Grant NEI-EY-01897. C.P.L. was supported by NRSA EY-06346. We thank Drs. Aileen Ritchie and Mae Huang for much useful discussion and commenting on the manuscript.

Correspondence should be addressed to Burgess N. Christensen at the above address.

Copyright © 1992 Society for Neuroscience 0270-6474/92/122156-09\$05.00/0

1990; Malgaroli et al., 1990), pharmacologically (Palade et al., 1989), and anatomically (Burgoyne et al., 1989) distinct from IP_3 -sensitive stores.

Intracellular Ca^{2+} can also increase following influx through voltage-dependent Ca^{2+} channels in those cells that express these channels. In many studies, the dihydropyridine family of Ca^{2+} channel blockers has been used to separate effects of open Ca^{2+} channels from those of ligand-gated channels on Ca^{2+} homeostasis. However, the use of pharmacological agents suffers from incomplete antagonist action, changing conditions that could relieve the antagonistic effect, and the possibility that an agent believed to be specific for one channel might affect another. In order to gain a better understanding of the role that excitatory amino acids play in regulating $[Ca^{2+}]_i$ in neurons, we have investigated the effects of glutamate receptor activation on intracellular free Ca^{2+} levels in isolated catfish horizontal cells using the Ca^{2+} -sensitive dye fura-2. Catfish cone horizontal cells express NMDA as well as non-NMDA glutamate subtype receptors (Hals et al., 1986; O'Dell and Christensen, 1986, 1989). These receptors can be functionally isolated from the voltage-dependent L-type Ca^{2+} channel by voltage clamping near the cell resting potential and preventing the voltage change that would occur in the presence of agonist-induced membrane current. We have found that the permeation of Ca^{2+} ion occurs through all the glutamate-activated channels, leading to an increase in intracellular free Ca^{2+} . This Ca^{2+} rise subsequently leads to release of Ca^{2+} from caffeine-sensitive intracellular stores, resulting in an amplification of the Ca^{2+} signal.

Some of these results have appeared previously in abstract form (Linn and Christensen, 1990).

Materials and Methods

Cell isolation procedure and loading with fura-2. Catfish cell isolation procedures have been described in previous publications (O'Dell and Christensen, 1989). Briefly, dark-adapted Texas channel catfish (*Ictalurus punctatus*) were anesthetized with tricaine methanesulfonate (100 mg/liter) until the animal no longer reacted to tactile stimulation. Both eyes were then excised and the overlying tissues removed, and the remaining vitreous fluid covering the retina was digested for 3 min with hyaluronidase (0.1 mg/ml, 5270 U/mg) in low- Ca^{2+} catfish saline containing (in mM) 126 NaCl, 4 KCl, 0.3 $CaCl_2$, 1 $MgCl_2$, 15 dextrose, and 10 HEPES; pH adjusted to 7.4 with NaOH. The eyecups were then rinsed in low- Ca^{2+} catfish saline and subsequently placed in a cysteine-activated papain solution for 4 min (0.7 mg/ml papain, 27 U/mg, prepared 30 min prior to use). Following this procedure, the retina was stripped from the eyecup, cut into quarters, and dissociated for another 4 min in fresh papain/cysteine solution. The retinal pieces were then kept in normal catfish saline (NCFS; same as above but with 3 mM Ca^{2+}) and 1 mg/ml bovine serum albumin at 5°C until used (within 48 hr).

Prior to recording, a piece of retina was further dissociated to yield isolated cone horizontal cells. In this process, the retina was gently triturated in a small volume of NCFS (0.5 ml) through a series of fire-polished Pasteur pipettes of progressively smaller tip diameters. The membrane-permeable ester form of fura-2 (5 μ M fura-2 in NCFS containing dimethyl sulfoxide and 0.02% pluronic F-127 detergent) was added to a cell suspension, making the final fura-2 loading concentration 2 μ M. Cells were allowed to incubate at room temperature for 30 min and then were transferred to a recording chamber mounted on the stage of a Nikon inverted phase-contrast microscope. The recording chamber was subsequently filled with NCFS.

Cone horizontal cells were identified by their characteristic morphology (Shingai and Christensen, 1986). Following identification, cells were voltage clamped using the single-patch electrode in the whole-cell mode (Hamill et al., 1981) and held near the resting membrane potential (−65 mV) using an Iwazumi Mark II patch-clamp amplifier. Electrodes were pulled from a Sutter model P-87 horizontal puller using 1.65 mm o.d. round-bore hard glass (Corning 7052); resistances ranged from 2

to 7 M Ω . Patch electrode solutions contained 140 mM K-gluconate, 2 mM $MgCl_2$, and 11 mM HEPES (pH adjusted to 7.4 using KOH). Neither Ca^{2+} nor EGTA was added to the patch electrode.

Drug application. Solutions were applied to voltage-clamped horizontal cells using a modification of the concentration-clamp technique (Akaike et al., 1986). Clamped cells were transferred from the bottom of the recording chamber to a superfusion chamber by passing the electrode tip through a rubber aperture covering a hole drilled into the side of a saline-filled Plexiglas tube. The aperture was sufficiently small to form a fluid tight seal around the electrode shaft, thus preventing fluid from exchanging between the superfusion chamber and the larger chamber holding the loaded cells. The bottom of the superfusion chamber was a coverslip that provided an excellent optical pathway to the inside of the superfusion chamber. The cell was positioned in the superfusion chamber by manipulating the electrode until the cell lay near the coverslip. A 40 $\times$ oil objective lens (1.3 NA) was then focused on the cell, and fluorescence intensity was measured by a photomultiplier tube connected to one of the photographic ports of the microscope. Measurements were made from the entire cell soma and proximal dendrites. One end of the superfusion tube connected to a small well into which solutions were introduced. The opposite end of the tube connected to a flexible tubing attached to a solenoid valve. Opening of the solenoid served to exchange solutions by siphoning through the tube. In these experiments, slow continuous superfusion rates were used so that movement of the cell positioned in the tube was minimized. Complete exchange of solution was achieved within 5 sec.

Measurement of intracellular Ca^{2+} . Fluorescence measurements using fura-2 take advantage of the shift in fluorescence intensity toward shorter excitation wavelengths with increasing $[Ca^{2+}]_i$. In this study, cells were illuminated at wavelengths of 340 and 380 nm by switching light to two monochromators via a revolving chopper positioned in front of a single 75 W xenon light source (Deltascan, Photon Technology International). Intracellular Ca^{2+} concentrations were calculated according to the equation (Grynkiewicz et al., 1985)

$$[Ca^{2+}]_i = K_d (R - R_{min}/R_{max} - R)(F_o/F_s),$$

where K_d is the equilibrium dissociation constant for fura-2 at 20°C (135 nM) (Grynkiewicz et al., 1985), F_o/F_s is the fluorescence ratio obtained at low and saturating levels with 380 nm excitation, and R is the fluorescence ratio using 340 nm and 380 nm excitation (i.e., $R = F_{340}/F_{380}$). Every attempt was made to measure R_{min} and R_{max} at the end of recording from each cell by superfusion of a buffered 10 nM Ca^{2+} catfish saline with 10 μ M ionomycin followed by 3 mM Ca^{2+} with 10 μ M ionomycin. However, this was not always possible since some recordings were lost before all measurements could be made. In this instance, only the ratio was measured. Absolute $[Ca^{2+}]_i$ values are given in the text when available. Basal $[Ca^{2+}]_i$ was near 100 nM in these cells.

Autofluorescence can be a significant factor if not corrected. In the catfish horizontal cells, autofluorescence measured from unloaded cells was quite low but was subtracted at the two wavelengths before forming the intensity ratio.

Results

Agonist-induced increases in intracellular Ca^{2+}

The excitatory amino acid agonists used in these experiments all depolarize the cell membrane potential, and this depolarization would activate voltage-sensitive Ca^{2+} channels in these cells. To provide evidence that none of the changes in $[Ca^{2+}]_i$ in the presence of agonist could be associated with the activation of a voltage-sensitive Ca^{2+} channel, a few experiments were done before and after blocking both the sodium channel with TTX and the Ca^{2+} channel with the dihydropyridine nimodipine. Only the L-type Ca^{2+} channel has been identified in catfish cone horizontal cells. This channel is activated near −40 mV and is blocked by dihydropyridines (Shingai and Christensen, 1986).

The two top traces shown in Figure 1A are the membrane current during a voltage step to 0 mV from a holding potential of −65 mV before (top trace) and after (bottom trace) exposing the cell to 1 μ M TTX and 5 μ M nimodipine. Figure 1B shows the change in the fluorescence intensity at 380 nm and 340 nm during application of 20 μ M KA in this same cell before (first

Figure 1. *A*, Effect of 5 μ M nimodipine and 1 μ M TTX on voltage-sensitive currents recorded from isolated catfish horizontal cells. Cells were voltage clamped in the whole-cell mode and stepped to 0 mV from a holding potential of -65 mV. The *top trace* illustrates a typical control response. The resulting inward current is due to an initial voltage-sensitive sodium current and a subsequent voltage-sensitive Ca^{2+} current. The *bottom trace* illustrates the effect of nimodipine and TTX on these voltage-sensitive currents. Calibration, 10 msec, 0.2 nA. *B*, Nimodipine and TTX have no effect on KA-induced $[Ca^{2+}]_i$ increase. The *solid trace* shows fluorescence intensity at 380 nm, and the *broken trace*, at 340 nm. The response that begins at the *first up arrow* represents the change in fluorescence to 10 μ M KA before blocking sodium and Ca^{2+} channels. (The *down arrows* indicate the time when KA was washed out and nimodipine and TTX added to the solution.) The ratio increased from 0.87 to 2.0. The *next up arrow* is a repeat application of KA. The ratio increased from 0.66 to 2.2. Cells were clamped at -65 mV. *C* shows the KA-induced membrane currents during the procedure in *B*. Calibration, 0.2 nA.

up arrow) and after (second up arrow) blocking the voltage-sensitive channels, and Figure 1*C* shows the KA-induced membrane current for each case. Complete block of the voltage-sensitive Ca^{2+} channel altered neither the change in $[Ca^{2+}]_i$, as reflected by the identical changes in fluorescence shown in Figure 1*B*, nor the size of the KA-induced membrane current. This type of experiment clearly demonstrates that the Ca^{2+} increase mediated by the agonist is due either to permeation through the

Table 1. Concentration–response values that correspond to the curves generated in Figure 2

Agonist	$[Ca^{2+}]_{max}$ (nM)	EC_{50} (μ M)	Slope
Quisqualate	325	1.7	3.3
Glutamate	537	15	3.5
NMDA	1011	79	3.8
Kainate	3400	33	3.9

$[Ca^{2+}]_{max}$, maximum Ca^{2+} response produced by agonist. EC_{50} , concentration of agonist that produced half-maximal responses. Slope is the Hill coefficient.

KA-gated channel and/or to an indirect mechanism involving Ca^{2+} mobilization.

Figure 2 shows the concentration–response curves in which $[Ca^{2+}]_i$ was measured during applications of Glu, NMDA, KA, and QA applied to cells under concentration- and voltage-clamp conditions. $[Ca^{2+}]_i$ concentrations were calculated according to the equation described in Materials and Methods. Each agonist, applied under these conditions, produced a sustained increase in intracellular Ca^{2+} that returned to baseline (≈ 100 nM) when washed out with normal or Mg^{2+} -free catfish saline. All experiments with Glu included normal Mg^{2+} in the solution. Table 1 summarizes the values that correspond to the parameters of the curves fitted to the data points using the Hill equation. The order of potency for the Ca^{2+} increase is QA > Glu > KA > NMDA. This is nearly the same order of potency obtained from measurements from synaptoneurosome in the presence of Ca^{2+} channel blockers (Benavides et al., 1988). The agonist EC_{50} values follow the same order of affinities in these cells as those measured previously from concentration–response curves where the membrane current was the response measured (O'Dell and Christensen, 1989). The maximum Ca^{2+} response occurs in the presence of KA, followed by NMDA, Glu, and QA, respectively. The Hill coefficient ranged from 3.3 to 3.9.

The Hill coefficients obtained when membrane current is measured as a function of agonist concentration was near 1 for NMDA and 1.5 for non-NMDA agonists (O'Dell and Christensen, 1989). If the agonist-induced increase in $[Ca^{2+}]_i$ depends solely on permeation through the agonist-gated channel, then it would be expected that the Hill coefficients measured from the data in Figure 2 would be similar to those reported previously when current was measured as the independent variable. This assumes that the increase in $[Ca^{2+}]_i$ is directly proportional to membrane current. One interpretation, although not the only one, of the larger Hill coefficients obtained for the concentration–response curves shown in Figure 2 could be amplification of the Ca^{2+} response by a Ca^{2+} -stimulated mobilization from an intracellular store. Further support for calcium-induced calcium release (CICR) is provided below.

Permeation of Ca^{2+} through agonist-gated channels

Figure 3 provides further evidence that the increase in $[Ca^{2+}]_i$ is due at least in part to permeation through the agonist-gated channels. The right column in each histogram illustrates the mean increase in fluorescence ratio recorded when glutamate agonists (50 μ M NMDA, 20 μ M KA, and 10 μ M QA) are applied to the horizontal cells. The left column in each histogram represents the change in fluorescence ratio when extracellular Ca^{2+} is lowered from 3 mM to 0.3 mM. Under these conditions, the glutamate agonists elicit little or no change in intracellular Ca^{2+} . The center column in each histogram illustrates the effects of

Figure 2. Dose-response curves obtained from isolated horizontal cells that have been loaded with fura-2 ($2 \mu\text{M}$) for 30 min. NMDA (*A*), KA (*B*), L-glutamate (*C*), or QA (*D*) was applied to cells under concentration-clamp conditions while voltage clamped at -65 mV . Data points were curve fit using the Hill equation $[\text{Ca}^{2+}] = ([\text{Ca}^{2+}]_{\text{max}} \times ([A]^n / \text{EC}_{50}^n + [A]^n))$, where A is the agonist concentration; EC_{50} is the concentration of agonist producing half-maximal response, and n is the Hill coefficient. Vertical bars represent SDs; the data points are means from 3–10 cells.

the three agonists when competitive antagonists are used to block binding at the receptor. Ninety percent of the KA- and QA-induced Ca^{2+} response is blocked with $2 \mu\text{M}$ 6-cyano-7-nitroquinoxaline-2,3-dione, and the NMDA-induced Ca^{2+} response is completely blocked by $100 \mu\text{M}$ 2-amino-5-phosphonopentanoate. These results confirm that part of the increase in $[\text{Ca}^{2+}]_i$ is a result of permeation through the ligand-gated channels. The lack of a response in reduced Ca^{2+} and in the presence of competitive antagonists implies that none of these agonists can mobilize Ca^{2+} from intracellular stores by binding to a metabotropic receptor. Trans-1-amino-1,3-cyclopentanedicarboxylic acid ($10\text{--}200 \mu\text{M}$) also failed to increase $[\text{Ca}^{2+}]_i$. Thus, there appears to be no QA-like metabotropic receptor on catfish cone horizontal cells.

Evidence for a caffeine-sensitive intracellular Ca^{2+} store

Two intracellular Ca^{2+} pools have been identified in cells. One is sensitive to IP_3 (for review, see Berridge and Irvine, 1989), whereas the second pool is sensitive to Ca^{2+} and drugs such as caffeine and ryanodine (Fill and Coronado, 1988). We have tested for subsequent release of Ca^{2+} from this IP_3 -insensitive Ca^{2+} pool by applying caffeine (10 mM) to voltage-clamped horizontal cells.

Application of caffeine to catfish horizontal cells causes a rapid transient rise in intracellular Ca^{2+} with no associated membrane current, indicating that caffeine acts directly on intracellular Ca^{2+} stores. Figure 4*A* shows the change in $[\text{Ca}^{2+}]_i$ following repetitive caffeine applications (10 mM). The intracellular Ca^{2+} levels rise from 100 nM to an average of 1229 nM (SD, ± 248 ; $n = 8$) and subsequently return to resting levels within 20 sec even though the caffeine concentration remains unchanged. This same Ca^{2+} response can be repeated many times if the caffeine applications are repeated at intervals longer than 1 min. Each application of

caffeine must be followed by return to caffeine-free solution before a subsequent Ca^{2+} release can be evoked. Applications of caffeine at intervals progressively shorter than 1 min result in progressively smaller increases in $[\text{Ca}^{2+}]_i$. We estimate from repeated caffeine applications like those shown in Figure 4*A* that, under physiological conditions, it takes about 1 min to refill the caffeine-sensitive Ca^{2+} store.

Figure 4*B* shows the effect of superimposing a second dose of caffeine on a cell already bathed in caffeine and demonstrates that the single Ca^{2+} response is likely a result either of pool depletion or desensitization of the Ca^{2+} release mechanism. No further rise in intracellular Ca^{2+} occurs. Increasing the extracellular Ca^{2+} concentration during continuous application of caffeine also has no effect on the caffeine response, as shown in Figure 4*C*. When the external Ca^{2+} concentration is increased from 1 to 10 mM during a caffeine exposure, there is no further change in intracellular Ca^{2+} levels.

The data shown in Figures 3 and 4 provide evidence supporting the idea that the gating of non-NMDA and NMDA receptors that are linked to ion channels permits Ca^{2+} influx and that these cells have an intracellular Ca^{2+} store sensitive to caffeine. Figure 5, *A* and *B*, provides further evidence that Ca^{2+} permeation through the KA-activated channel and release of Ca^{2+} by caffeine from intracellular stores are separate processes. In Figure 5*A*, the caffeine-induced Ca^{2+} release is superimposed on a $15 \mu\text{M}$ KA response. The addition of caffeine further increases the intracellular Ca^{2+} levels in the presence of KA for 20 sec and then declines to a new sustained level that is smaller than the increase produced in KA alone. The decrease in the Ca^{2+} response is likely to be due primarily to the decrease in membrane current by about 50% (Fig. 5*C*) because caffeine acts as an inhibitor to the KA-induced membrane current. However, we cannot rule out a contribution to the reduction in the Ca^{2+}

Figure 3. Shows the change in fluorescence ratio in the presence of agonist, reduced extracellular Ca^{2+} , and specific competitive antagonists. The *left column* in each histogram represents the response that occurs when extracellular Ca^{2+} is reduced. Under these conditions, all three agonists (20 μM KA, 10 μM QA, and 50 μM NMDA) elicit little or no response. The *center column* in each histogram illustrates the effect of the three agonists when competitive antagonists are used to block receptor binding. Again, in all three cases, little or no Ca^{2+} response is observed. However, when the agonists are used in normal or Mg^{2+} -free saline, each elicits a large ratio change corresponding to a significant increase in intracellular Ca^{2+} (*right columns* in each histogram). *Vertical bars* indicate SDs. Each cell was subjected to all three conditions. Data for each agonist are from three to five cells.

Figure 4. Existence of caffeine-sensitive intracellular stores. *A* illustrates the effect of three applications of 10 mM caffeine. Following each response to caffeine, the cell is rinsed with NCFS for at least 1 min before a subsequent dose is applied. Similar responses were observed in four other cells. *B* demonstrates the effect of two repetitive caffeine applications. The response to the first caffeine dose is illustrated at 50 sec as a large increase in intracellular Ca^{2+} . The second dose was applied at 100 sec and elicits no response. This experiment was repeated on two other cells. *C* demonstrates the effect of increasing extracellular Ca^{2+} on the caffeine response. Caffeine (10 mM) in NCFS (containing 3 mM Ca^{2+}) was applied at 40 sec and elicited a large increase in intracellular Ca^{2+} . A second dose of caffeine in saline containing 20 mM Ca^{2+} was applied at 100 sec and elicited no response. Similar results were observed in two other cells.

Figure 5. Demonstration that permeation of Ca^{2+} through the KA channel and release of Ca^{2+} from intracellular stores are two separate events. *A*, Caffeine (10 mM) is applied (at 100 sec) during a 10 μM KA response (from 60 to 150 sec). The caffeine produces a Ca^{2+} release superimposed on the KA-induced Ca^{2+} response. The lower trace (*C*) illustrates the corresponding membrane current changes that occur during drug application. The KA-induced membrane current is reduced by 50% in the presence of 10 mM caffeine. *B* shows the initial caffeine-induced Ca^{2+} release followed by an application of 20 μM KA (at 120 sec). The lower trace (*C*) illustrates the corresponding current changes that occur during drug application. Caffeine itself produces no membrane current. Calibration, 0.5 nA.

response resulting from the depletion and/or desensitization of the intracellular store and removal of CICR. In either case, the remaining Ca^{2+} signal is likely to be primarily the result of Ca^{2+} influx through the ligand-gated channel.

Figure 5*B* demonstrates the reverse situation where caffeine (10 mM) is applied to deplete the intracellular store. Following washout of the caffeine, KA is applied and a second increase is observed. As illustrated in Figure 5*A*, under these conditions, the KA-induced Ca^{2+} response is due primarily to Ca^{2+} influx.

Figure 6, *A* and *B*, provides evidence for agonist-initiated CICR. In Figure 6*A*, an initial control caffeine response is recorded. This is followed by an application of 10 μM KA, which produces a small change in $[\text{Ca}^{2+}]$. A subsequent test caffeine application reproduces the original control Ca^{2+} response. In Figure 6*B*, from a different cell, following the control caffeine response, a larger concentration of KA is applied, resulting in a larger Ca^{2+} response. The subsequent test application of caffeine produces only a small response, suggesting that the original influx of Ca^{2+} through the agonist-gated channel resulted in release of Ca^{2+} from the intracellular store.

To support the hypothesis that the KA-induced Ca^{2+} response is composed of both influx and Ca^{2+} mobilization, Figure 7 illustrates the reduction in the KA-induced Ca^{2+} response following exposure to caffeine. Initially a control KA response is recorded. The KA-induced membrane current is shown below the $[\text{Ca}^{2+}]$ changes. This is followed by a response to caffeine that is then washed out, and KA is then reapplied. The second KA-induced Ca^{2+} response is reduced compared to control. The reduction in the KA-induced Ca^{2+} response following caffeine-induced depletion is consistent with the hypothesis that Ca^{2+} influx through the agonist-gated channel is capable of CICR. There is no change in the KA-induced membrane current as demonstrated in the traces in Figure 7*B* following the caffeine-induced Ca^{2+} release. The inset in Figure 7*A* demonstrates that repeated applications of KA produce nearly the same change in $[\text{Ca}^{2+}]$; therefore, the reduced response following caffeine-induced Ca^{2+} depletion cannot be attributable to KA response variability.

Discussion

Permeation of calcium ions through glutamate channels

In the present study, we have measured significant increases in intracellular Ca^{2+} due to influx through NMDA- as well as non-

NMDA-gated channels using the Ca^{2+} -sensitive indicator fura-2. Influx through voltage-gated Ca^{2+} channels was prevented by controlling membrane potential of individual cells with a voltage clamp.

Ca^{2+} permeation through NMDA channels has been well documented in the literature. These studies have been based on ion substitution experiments (Iino et al., 1990), on shifts in reversal potentials that occur when extracellular Ca^{2+} concentrations are raised and on the measured changes in $[\text{Ca}^{2+}]$, using the Ca^{2+} -sensitive dye arsenazo III (MacDermott et al., 1986; Mayer and Westbrook, 1987; Mayer et al., 1987), on single-channel currents (Ascher and Nowak, 1988), and more recently on results using fura-2 (Connor et al., 1988). The shift in reversal potential due to non-NMDA receptor activation in high external Ca^{2+} is only 3% of the shift seen when NMDA receptors are activated. Ca^{2+} permeation through non-NMDA-activated channels based on these studies does not appear to be significant. Other studies using arsenazo III suggest that responses to QA and KA are accompanied by minimal Ca^{2+} signals (Mayer et al., 1987).

Although the evidence for the influx of Ca^{2+} through non-NMDA channels is not well established, recent articles have reported some instances in which Ca^{2+} can permeate non-NMDA activated channels. Off-center bipolar cells in salamander retina do not contain NMDA receptors but respond to Glu, KA, and QA (Gilbertson et al., 1991). There was a positive shift in agonist reversal potential when extracellular Ca^{2+} was increased. Iino et al. (1990) described significant shifts in the KA reversal potential when changing $[\text{Ca}^{2+}]_o$ in a solution in which the impermeant cation *N*-methylglucamine was substituted for sodium and potassium in cultured hippocampal cells. They described two types of KA responses, only one (type II response) of which produced a change in intracellular Ca^{2+} . This second type of KA response had a current-voltage relationship with a predominant inward rectification. There was no Ca^{2+} influx through the QA-gated channel. Their results differ from ours in that QA was effective in increasing intracellular Ca^{2+} in horizontal cells and the KA-activated channel in horizontal cells does not show inward rectification. These differences can best be explained by differences that are reflected in the protein structure for these excitatory amino acid channels, suggesting the existence of a family of non-NMDA receptor-channel proteins (Boulter et al., 1990), and provide further evidence for the reported differences in agonist selectivity depending on whether studies use binding

Figure 6. Evidence for CICR. *A*, An initial control caffeine response is recorded followed by an application of 10 μ M KA (at 80 sec). A subsequent test caffeine response is identical to the original control Ca^{2+} release. These results were observed in six cells. *B*, A larger dose of KA (30 μ M at 125 sec) was applied to the cell following the initial caffeine control response. The subsequent test application of caffeine (at 220 sec) produced a much reduced Ca^{2+} release. This is interpreted as a reduction in the Ca^{2+} store due to CICR by influx of Ca^{2+} during the KA application. This experiment was repeated in three cells.

or physiological assays. In a recent report Hollman et al. (1991) used molecular cloning to demonstrate that the selective permeability of glutamate channels depends on the specific combination of subunits.

Ogura et al. (1990), also using cultured hippocampal neurons and fura-2, demonstrated that both KA and QA permit significant Ca^{2+} entry. Our studies support this finding. Activation of NMDA and KA/QA receptors significantly increased intracellular Ca^{2+} levels while the cells were voltage clamped at -65 mV. This Ca^{2+} increase was blocked in low extracellular Ca^{2+} saline by at least 90% and was blocked when competitive antagonists were used to bind to the agonist receptors. These results provide compelling evidence for a role for both NMDA and non-NMDA receptors in intracellular Ca^{2+} regulation that does not involve a metabotropic receptor. Our inability to identify

Figure 7. *A*, A control Ca^{2+} response to 10 μ M KA in normal catfish saline is recorded and rinsed out at 50 sec. A 10 mM caffeine application (at 140 sec) depletes the Ca^{2+} store. A second application of KA results in a response that is reduced by 60% (at 160 sec). CICR has been reduced due to the depletion of the Ca^{2+} store following caffeine. The inset in *A* shows the change in $[Ca^{2+}]_i$ to three separate applications of 10 μ M KA. *B* illustrates that the KA-induced current does not change following depletion of intracellular Ca^{2+} stores. The left side of the trace illustrates the inward current from 10 μ M KA in NCFS. The right side of the trace shows the current from 10 μ M KA in NCFS after 10 mM caffeine had been applied to the cell. There is no significant difference between the two currents. Calibration: *A*, inset, 200 nM Ca^{2+} , 50 sec; *B*, 0.2 nA.

a metabotropic receptor could be due to washout of an important factor during the recording period by dialysis with the patch electrode solution. We believe this to be unlikely, since the dopamine-activated cAMP system responsible for gap junction uncoupling remains intact under similar recording conditions (B. N. Christensen, unpublished observations).

The maximal Ca^{2+} changes occur when KA is applied to the cells, followed by NMDA and QA. We believe that this reflects that (1) there are relatively more KA/QA than NMDA receptors, (2) QA acts as a partial agonist, and (3) there is subsequent CICR from intracellular stores that amplifies the increase in $[Ca^{2+}]_i$ entering through the ligand-gated channels.

Measurements from macroscopic currents recorded from catfish cone horizontal cells suggest that the number of KA receptors is significantly greater than NMDA receptors (O'Dell and Christensen, 1989) and would therefore be a factor in the maximal Ca^{2+} response observed in KA. Furthermore, agonist and antagonist studies of these same cells indicate that catfish horizontal cells may contain only one type of glutamate non-NMDA receptor that is activated by both KA and QA (O'Dell and Christensen, 1989). Although QA has the highest affinity for the receptor, it is only a partial agonist and is thus not as effective in gating the channel as KA.

The results presented in this article also provide some information on the mechanisms of Ca^{2+} store refilling. Following release of Ca^{2+} from intracellular stores, the cytoplasmic $[Ca^{2+}]_i$ rapidly returns to baseline. This is observed both in the contin-

ued presence of caffeine and during Ca^{2+} oscillations that occur occasionally. What happens to the released Ca^{2+} ? Presumably its concentration is controlled by buffering and active pumps that remove Ca^{2+} from the cell or return it to the store. It seems unlikely that the decrease in $[\text{Ca}^{2+}]_i$ observed during a caffeine challenge is due only to a rapid refilling of the store directly from the cytoplasm. Unless there is a refractory period that prevents release, a second challenge with caffeine should produce a full Ca^{2+} spike. A full Ca^{2+} spike, however, cannot be evoked until about 1 min following removal of the caffeine. We conclude that refilling occurs by a mechanism in which Ca^{2+} bound to the buffering system is in equilibrium with free cytoplasmic Ca^{2+} . The equilibrium is shifted toward free Ca^{2+} during store refilling. As free Ca^{2+} becomes available, it is immediately returned to the store, thus driving the system in this direction until the store is refilled. We have not yet determined if extracellular Ca^{2+} is necessary for refilling.

Can influx through the agonist-gated channels account for all of the change in intracellular Ca^{2+} ? Because little is known about either the kinetics, binding constants, or the magnitude of Ca^{2+} buffering in the cell cytoplasm, this is a difficult question to answer quantitatively. However, using the data shown in Figure 7, calculations were made using both the changes in $[\text{Ca}^{2+}]_i$ and the measured membrane current in the presence of KA. We chose to calculate the rate of change in $[\text{Ca}^{2+}]_i$ over a 15 sec time period, that is, until almost steady state is reached, rather than make calculations from steady-state measurements because at long times, Ca^{2+} buffering and removal become more significant. We accounted for fura-2 buffering by assuming that equilibrium was established between the outside and inside concentrations of fura-2, that is, that intracellular fura-2 reaches 2 μM and conversion to the acid form is complete. In addition, we assumed that over the 15 sec time period, Ca^{2+} buffering and removal by other mechanisms were minimal. In addition, to measure the charge carried by Ca^{2+} from the current trace, we assumed a permeability ratio of 0.07 (Gu and Huang, 1991). With these assumptions in mind, the rate of Ca^{2+} entry measured from the rate of change in $[\text{Ca}^{2+}]_i$ and current was 4.09 and 4.54 $\times 10^{-14}$ coulombs/sec, respectively. Therefore, following depletion, the rate of Ca^{2+} entry measured from the change in Ca^{2+} can be entirely accounted for by the measured membrane current. Since the response measured before depletion in Figure 7A is 2.5 $\times$ larger than that measured after depletion, and the membrane current is unchanged, some CICR must occur to account for the large increase in $[\text{Ca}^{2+}]_i$.

References

- Akaike N, Inoue M, Krishtal OA (1986) "Concentration-clamp" study of gamma-aminobutyric-acid-induced chloride current kinetics in frog sensory neurons. *J Physiol (Lond)* 379:171-185.
- Ascher P, Nowak L (1988) The role of divalent cations in the *N*-methyl-D-aspartate responses of mouse central neurones in culture. *J Physiol (Lond)* 399:247-366.
- Benavides J, Claustre Y, Scatton B (1988) L-Glutamate increases internal free calcium levels in synaptosomes from immature rat brain via quisqualate receptors. *J Neurosci* 8:3607-3615.
- Berridge MJ, Irvine RF (1989) Inositol phosphates and cell signalling. *Nature* 341:197-205.
- Boulter J, Hollmann M, O'Shea-Greenfield A, Hartley M, Deneris E, Maron C, Heinemann S (1990) Molecular cloning and functional expression of glutamate receptor subunit genes. *Science* 249:1033-1037.
- Burgoyne RD, Cheek TR, Morgan A, et al. (1989) Distribution of two distinct Ca^{2+} -ATPase-like proteins and their relationships to the agonist-sensitive calcium store in adrenal chromaffin cells. *Nature* 342:72-74.
- Chad JE, Eckert R (1986) An enzymatic mechanism for calcium current inactivation in dialysed *Helix* neurones. *J Physiol (Lond)* 378:31-51.
- Chadwick CC, Saito A, Fleischer S (1990) Isolation and characterization of the inositol trisphosphate receptor from smooth muscle. *Proc Natl Acad Sci USA* 87:2132-2136.
- Cheek TR, O'Sullivan AJ, Moreton RB, Berridge MJ, Burgoyne RD (1990) The caffeine-sensitive Ca^{2+} store in bovine adrenal chromaffin cells: an examination of its role in triggering secretion and Ca^{2+} homeostasis. *FEBS Lett* 266:91-95.
- Choi DW, Rothman SM (1990) The role of glutamate neurotoxicity in hypoxic-ischemic neuronal death. *Annu Rev Neurosci* 13:171-182.
- Collingridge GL, Lester RAJ (1989) Excitatory amino acid receptors in the vertebrate central nervous system. *Pharmacol Rev* 41:143-210.
- Connor JA, Wadman WJ, Hockberger PE, Wong RKS (1988) Sustained dendritic gradients of Ca^{2+} induced by excitatory amino acids in CA1 hippocampal neurons. *Science* 240:649-653.
- Ehrlich BE, Watras J (1988) Inositol 1,4,5-trisphosphate activates a channel from smooth muscle sarcoplasmic reticulum. *Nature* 336:583-586.
- Fill M, Coronado R (1988) Ryanodine receptor channel of sarcoplasmic reticulum. *Trends Neurosci* 11:453-457.
- Finch EA, Turner TJ, Goldin SM (1991) Calcium as a coagonist of inositol 1,4,5-trisphosphate-induced calcium release. *Science* 252:443-446.
- Furuichi T, Yoshikawa S, Miyawaki A, Wada K, Maeda N, Mikoshiba K (1989) Primary structure and functional expression of the inositol 1,4,5-trisphosphate binding protein P_{400} . *Nature* 342:32-38.
- Furuya S, Ohmori H, Shigemoto T, Sugiyama H (1989) Intracellular calcium mobilization triggered by a glutamate receptor in rat cultured hippocampal cells. *J Physiol (Lond)* 414:539-548.
- Gilbertson TA, Scobey R, Wilson M (1991) Permeation of calcium ions through non-NMDA glutamate channels in retinal bipolar cells. *Science* 251:1613-1615.
- Grynkiewicz G, Poenie M, Tsien RY (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 260:3440-3450.
- Gu Y, Huang L-YM (1991) Block of kainate receptor channels by Ca^{2+} in isolated spinal trigeminal neurons of rat. *Neuron* 6:777-784.
- Hals G, Christensen BN, O'Dell T, Christensen M, Shingai R (1986) Voltage-clamp analysis of currents produced by glutamate and some glutamate analogues on horizontal cells isolated from the catfish retina. *J Neurophysiol* 56:19-31.
- Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch* 391:85-100.
- Hollmann M, Hartley M, Heinemann S (1991) Ca^{2+} permeability of KA-AMPA-gated glutamate receptor channels depends on subunit composition. *Science* 252:851-853.
- Holopainen I, Enkvist MOK, Åkerman KEO (1989) Glutamate receptor agonists increase intracellular Ca^{2+} independently of voltage-gated Ca^{2+} channels in rat cerebellar granule cells. *Neurosci Lett* 98:57-62.
- Holopainen I, Louve M, Enkvist MOK, Åkerman KEO (1990) Coupling of glutamatergic receptors to changes in intracellular Ca^{2+} in rat cerebellar granule cells in primary culture. *J Neurosci Res* 25:187-193.
- Iino M, Ozawa S, Tsuzuki K (1990) Permeation of calcium through excitatory amino acid receptor channels in cultured rat hippocampal neurones. *J Physiol (Lond)* 424:151-165.
- Linn CL, Christensen BN (1990) Excitatory amino acid induced intracellular changes in voltage clamped isolated horizontal cells. *Soc Neurosci Abstr*.
- Lipscombe D, Madison DV, Poenie M, Reuter H, Tsien RW, Tsien RY (1988) Imaging cytosolic Ca^{2+} transients arising from Ca^{2+} channels in sympathetic neurons. *Neuron* 1:355-365.
- MacDermott AB, Mayer ML, Westbrook GL, Smith SJ, Barker JL (1986) NMDA-receptor activation increases cytoplasmic calcium concentration in cultured spinal cord neurones. *Nature* 321:519-522.
- Malgaroli A, Fesce R, Meldolesi J (1990) Spontaneous $[\text{Ca}^{2+}]_i$ fluctuations

- tuations in rat chromaffin cells don't require 1,4,5-trisphosphate elevations but are generated by a caffeine- and ryanodine-sensitive intracellular Ca^{2+} store. *J Biol Chem* 265:3005–3008.
- Manzoni O, Fagni L, Pin J-P, Rassendren F, Poultz F, Sladeczek F, Beckaert J (1990) (*trans*)-1-Amino-cyclopentyl-1,3-dicarboxylate stimulates quisqualate phosphoinositide-coupled receptors but not ionotropic glutamate receptors in striatal neurons and *Xenopus* oocytes. *Mol Pharmacol* 38:1–6.
- Mayer ML, Westbrook GL (1987) Permeation and block of *N*-methyl-D-aspartic acid receptor channels by divalent cations in mouse cultured central neurones. *J Physiol (Lond)* 394:501–527.
- Mayer ML, MacDermott AB, Westbrook GL, Smith SJ, Barker JL (1987) Agonist- and voltage-gated calcium entry in cultured mouse spinal cord neurons under voltage clamp measured using arsenazo III. *J Neurosci* 7:3230–3244.
- Miyawaki A, Furuichi T, Maeda N, Mikoshiba K (1990) Expressed cerebellar-type inositol 1,4,5-trisphosphate receptor, P_{400} , has calcium release activity in a fibroblast L cell line. *Neuron* 5:11–18.
- Murphy SN, Miller RJ (1988) A glutamate receptor regulates Ca^{2+} mobilization in hippocampal neurons. *Proc Natl Acad Sci USA* 85:8737–8741.
- Murphy SN, Miller RJ (1989) Two distinct quisqualate receptors regulate Ca^{2+} homeostasis in hippocampal neurons *in vitro*. *Mol Pharmacol* 35:671–680.
- Murphy SN, Thayer SA, Miller RJ (1987) The effects of excitatory amino acids on intracellular calcium in single mouse striatal neurons *in vitro*. *J Neurosci* 7:4145–4158.
- Nicoletti F, Wroblewski JT, Novelli A, Guidotti A, Costa E (1986) The activation of inositol phospholipid metabolism as a signal-transducing system for excitatory amino acids in primary cultures of cerebellar granule cells. *J Neurosci* 6:1905–1911.
- Nicoll RA, Kauer JA, Malenka RC (1988) The current excitement in long-term potentiation. *Neuron* 1:97–103.
- Norden JJ, Lettes A, Costello B, Lin L-H, Wonters B, Bock S, Freeman JA (1991) Possible role of GAP-43 in calcium regulation/neurotransmitter release. *Ann NY Acad Sci* 627:75–93.
- O'Dell T, Christensen BN (1986) *N*-methyl-D-aspartate receptors co-exist with kainate and quisqualate receptors on single isolated catfish horizontal cells. *Brain Res* 381:359–362.
- O'Dell TJ, Christensen BN (1989) Horizontal cells isolated from catfish retina contain two types of excitatory amino acid receptors. *J Neurophysiol* 61:1097–1109.
- Ogura A, Akita K, Kudo Y (1990) Non-NMDA receptor mediates cytoplasmic Ca^{2+} elevation in cultured hippocampal neurones. *Neurosci Res* 9:103–113.
- Palade P, Dettbarn C, Alderson B, Volpe P (1989) Pharmacologic differentiation between inositol-1,4,5-trisphosphate-induced Ca^{2+} release and Ca^{2+} - or caffeine-induced Ca^{2+} release from intracellular membrane systems. *Mol Pharmacol* 36:673–680.
- Schoepp DD, Johnson BG (1988) Excitatory amino acid agonist-antagonist interactions at 2-amino-4-phosphonobutyric acid-sensitive quisqualate receptors coupled to phosphoinositide hydrolysis in slices of rat hippocampus. *J Neurochem* 50:1605–1613.
- Shingai R, Christensen BN (1986) Excitable properties and voltage-sensitive ion conductances of horizontal cells isolated from catfish (*Ictalurus punctatus*) retina. *J Neurophysiol* 56:32–49.
- Sugiyama H, Ito I, Hirono C (1987) A new type of glutamate receptor linked to inositol phospholipid metabolism. *Nature* 325:531–533.
- Supattapone S, Worley PF, Baraban JM, Snyder SH (1988) Solubilization, purification, and characterization of an inositol trisphosphate receptor. *J Biol Chem* 263:1530–1534.
- Thayer SA, Hirning LD, Miller RJ (1988a) The role of caffeine-sensitive calcium stores in the regulation of the intracellular free calcium concentration in rat sympathetic neurons *in vitro*. *Mol Pharmacol* 34:664–673.
- Thayer SA, Perney TM, Miller RJ (1988b) Regulation of calcium homeostasis in sensory neurons by bradykinin. *J Neurosci* 8:4089–4097.