CONFOCAL MICROSCOPIC IMAGING OF [Ca^{2+}]$_i$ IN CULTURED RAT HIPPOCAMPAL NEURONS FOLLOWING EXPOSURE TO N-METHYL-D-ASPARTATE

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SUMMARY

1. The confocal laser scanning microscope (CLSM) was used in conjunction with the calcium indicator dye Fluo-3 to record changes in free intracellular calcium concentration ([Ca^{2+}]$_i$) in cultured hippocampal neurons in response to superfusion of N-methyl D-aspartate (NMDA).

2. NMDA caused a rapid rise in [Ca^{2+}]$_i$ in all parts of the neuron. The rise in [Ca^{2+}]$_i$ was dependent on activation of an NMDA receptor, was enhanced by the removal of Mg^{2+} and addition of glycine to the superfusion medium, and was dependent on normal [Ca^{2+}]$_o$.

3. The rise of [Ca^{2+}]$_i$ was seen first near the membrane. A wave of elevated [Ca^{2+}]$_i$ moved centripetally at a rate of 117 μm/s.

4. Dantrolene pre-incubation caused a significant reduction in the efficacy of the NMDA-induced rise in [Ca^{2+}]$_i$, indicating that at least part of the rise is caused by intracellular release of calcium.

5. The replacement of calcium by barium caused a reduction in the response to NMDA, but a significant response was still present in these cells, supporting the assumption that NMDA causes release of calcium from intracellular stores.

6. The removal of sodium from the superfusion medium prolonged the [Ca^{2+}]$_i$ rise in response to NMDA indicating that the Na–Ca antiporter is instrumental in reducing [Ca^{2+}]$_i$.

7. These studies demonstrate the multiplicity of regulating mechanisms of [Ca^{2+}]$_i$ following activation of NMDA receptors.

INTRODUCTION

Activation of an acidic amino acid receptor has been reported to result in influx of calcium into central neurons (MacDermott, Mayer, Westbrook, Smith & Barker, 1986). This response has been associated selectively with the N-methyl-D-aspartate (NMDA) receptor subtype (see Collingridge & Lester, 1989; Mayer & Westbrook, 1987 for review) and has been implicated in neuronal plasticity (Collingridge & Bliss 1987). Earlier studies, employing Fura-2 imaging of [Ca^{2+}]$_i$, have demonstrated that not only NMDA but also ligands which activate other receptor types, kainate and quisqualate also causes a reversible increase in [Ca^{2+}]$_i$ (Murphy & Miller, 1988). It has been suggested that quisqualate activates a metabotrophic receptor which causes

release of calcium from intracellular stores, and thus this response is independent of $[Ca^{2+}]_o$, whereas the NMDA response is dependent on $[Ca^{2+}]_o$ and not associated with release of calcium from intracellular stores (Furuya, Ohmori, Shigemoto & Sugiyama, 1989). While there is evidence that $[Ca^{2+}]_o$ is necessary for the NMDA-induced rise in $[Ca^{2+}]_i$, it is not clear that it is sufficient to cause this rise, i.e. that NMDA-induced rise in $[Ca^{2+}]_i$ is independent of changes in calcium stores.

The recently evolved confocal laser scanning microscope (CLSM) allows optical sectioning of the examined tissue at a desired thickness and a subsequent 3-D reconstruction of the imaged cell. We have used Fluo-3 imaging of $[Ca^{2+}]_i$ (Cornell-Bell, Finkbeiner, Cooper & Smith 1990; Hernandez-Cruz, Sala & Adams 1990; Kao, Harootunian & Tsien 1989; Wahl, Schousboe, Honore & Drejer, 1989) in the CLSM to measure changes in $[Ca^{2+}]_i$ associated with a response to NMDA. Specifically, we studied the origin, time course and fate of elevated $[Ca^{2+}]_i$ in cultured hippocampal neurons. We confirmed earlier observations but added that a certain proportion of elevated $[Ca^{2+}]_i$ is derived from release of calcium from internal stores, and that the rise of $[Ca^{2+}]_i$ is most prominent in the nucleus.

METHODS

Cell culture

Pregnant Wistar rats were killed by rapid cervical dislocation and their embryos were removed and placed in sterile, ice-cold Leibowitz L15 medium. Embryonic (day 18–19) rat hippocampi were dissected out in the medium, dissociated mechanically and plated on 12 mm diameter glass cover-slips coated with L-polylysine and placed in Nunc twenty-four-well plates. Cultures were plated at a density of 150000 cells/well in a minimum essential medium containing 10% horse serum and 10% fetal calf serum. At 3–4 days after plating the medium was replaced by a medium containing 10% horse serum. Most of the neurons died within 2–3 weeks after initial plating under these conditions. The same glass cover-slips were then reused for plating a new dissociated cell culture on the remaining glial substratum. The new culture grew faster and the cells appeared larger within 1–2 days of plating. This culture was treated with FUDR to block glial proliferation (Segal, 1983). Occasionally, the glial substrate was resuspended before replating to assure the absence of older neurons in the culture dish. The cultures were used 1–30 days after plating.

On the day of experiment, the glass cover-slips were removed from the twenty-four-well plates, washed in 10 mM-HEPES-buffered, balance salt solution containing (mM): $CaCl_2$, 2; $MgCl_2$, 1; NaCl, 126; KCl, 4; and glucose, 4·2. Sucrose was added to bring osmolarity to 320 mosmol, at pH = 7·4. In experiments where low $[Na^+]_o$ was tested, NaCl was replaced by N-methyl-D-glucamine (NMG). In experiments with low $[Ca^{2+}]_o$, calcium was removed from the medium, and 0·2 mM-EGTA was added. Tetrodotoxin (TTX, 0·5 μM) was added before incubation. The 1 ml incubation medium contained 10 μM-Fluo-3 acetoxymethyl ester (AM) (Molecular Probes Inc., USA) and the non-ionic detergent pluronic F127 (Molecular Probes, 0·5 mg/ml).

Incubation was carried out in a gently shaking bath at room temperature for 2 h. The cultures were washed and introduced into the CLSM. Cultures were used within 5 h after incubation with the dye. In longer-waiting cultures there was compartmentation of the dye which interfered with measurements of $[Ca^{2+}]_i$.

The CLSM

The CLSM was a Wild–Leitz system (Heidelberg, Germany) composed of an upright microscope equipped with a single argon-ion laser beam emmitting light at 488 nm. The light intensity was reduced with neutral density filtering to 10% of nominal intensity to prevent photodynamic damage and dye bleaching. These were marginal under the conditions used, as judged by the reproducibility of the results across periods of testing.

Images of 512×512, 256×256 or 128×128 pixels were acquired at maximal rats of 1·3, 3·7 and 6·6 frames/s, respectively. Single-line scans lasted 0·44 ms ($\pm 5\%$) and the delay between lines was

0·94 ms. The glass cover-clip was glued upside-down to the top of a small volume (0·15 ml) chamber and perfused at a rate of 1–3 ml/min. Cells were routinely visualized with a 50 × water immersion objective. A pinhole of 100 μM allowed optical sections thinner than 1 μM. The optical resolution was in fact, better than 0·5 μm, as judged by cutting serial optical sections, in steps of 0·5 μm and observing cellular organelles in individual sections only. In most experiments images were taken at steady states (before, 30–60 s after onset of drug perfusion, and 2–5 min after removal of drugs, when recovery was completed). This was done routinely to minimize exposure of the cells to the light and allow repeated tests. However, when time course of drug effects were studied, images were taken at a rapid rate during the perfusion with the tested drugs.

Some experiments were conducted with glass cover-slips glued right-side-up in the perfusion chamber. In these cases the cultured cells faced the objective lens which was a water immersion one. In these experiments a glass pipette with tip diameter of 1–2 μm containing NMDA was inserted between the objective lens and the cell. It was aimed at the cell and a puff of drug was synchronized with the scan. This allowed precise timing of drug application, averaging of successive scans to the same stimulation, and detection of small changes in $[Ca^{2+}]_i$.

Calibration

Fluo-3 is a single-wavelength chromophore. As such, its measured fluorescence is a function of the concentration of calcium as well as the dye concentration (Kao *et al.* 1989). The unique feature of the CLSM, allowing it to slice a consistent, thin optical section through the tissue, reduces problems associated with the volume of the cells and interference of the tissue with the light path of excitation. Since loading of the cultures was done with the Fluo-3-AM compound and the formation of the active dye in the cell depends on a number of factors (Tsien 1980; Lipscombe, Madison, Poenie, Reuter, Tsien & Tsien, 1988), we do not know the actual concentration of the dye in the cells. Moreover, the presence, in the cell, of unknown concentrations of buffers, different from Fluo-3, compounds any estimate of $[Ca^{2+}]_i$, based on Fluo-3. Thus, the estimate of $[Ca^{2+}]_i$ can only be approximate. We have used the calibration method proposed by Kao *et al.* (1989) as follows. At the end of an experiment, where effects of NMDA were measured, the culture was superfused with ionomycin (5 μM, Fig. 1A). When fluorescence reached maximum, the culture was superfused with a medium containing 5 mM-Mn^{2+}, replacing Ca^{2+}, until fluorescence reached a new stable level (about 5 min). At that point the cells were treated with digitonin (40 μM) and the rapid disappearance of fluorescence was monitored after cell lysis. The predicted fluorescence F_{max} would be

$$(F_{Mn} - F_{bkg})/0·2 + F_{bkg}.$$

The camera signal F_{min} should be

$$(F_{max} - F_{bkg})/40 + F_{bkg}.$$

F_{bkg} is the fluorescence in digitonin-treated cells, and corresponds to the dark noise level. $[Ca^{2+}]_i$ could then be estimated according to the following equation:

$$[Ca^{2+}]_i = K_d(F - F_{min})/(F_{max} - F),$$

where the dissociation constant, K_d, was estimated at 400 nM for Fluo-3 at vertebrate ionic strength (see Kao *et al.* 1989, for further discussion on the methodology). Using this calibration procedure in seven different experiments, we calculated basal $[Ca^{2+}]_i$ to be 74 ± 5·6 nM (mean ± S.E.M., here and throughout; $n = 10$) (equivalent to 41 fluorescence intensity units on a scale of 0–255, measured with the CLSM, see Fig. 1B). The calculated response to 10 μM-NMDA (in the absence of Mg^{2+}) amounted to 266 ± 27 nM $[Ca^{2+}]_i$ in these same cells. Since most experiments were conducted with similar CLSM parameters and dye-loading conditions, variations in fluorescence intensity among cells can be due to inherent variations in dye loading and sequestration, which were not large in normal cells under identical conditions. We can estimate the basal levels and the changes induced by the drugs in a consistent manner. Nonetheless, we prefer to present our results in fluorescence intensity units since calibration was not conducted routinely in all experiments.

Drugs

The following drugs were used: SF-6847 (3,4-ditert, butyl-4-hydroxylbenzylidenemalononitrile), a gift from Dr Y. Shahak, The Weizmann Institute, Israel. All other chemicals were purchased from Sigma, Tocris Neuramin Inc. (UK) and Molecular Probes Inc. Drug solutions were prepared from frozen stocks on the day of experiment.
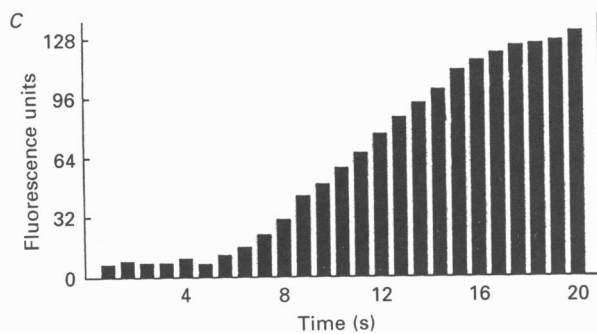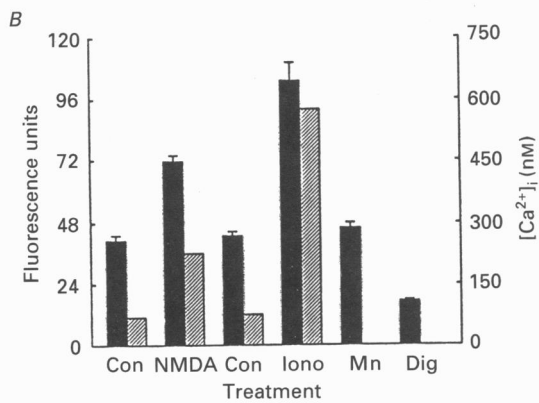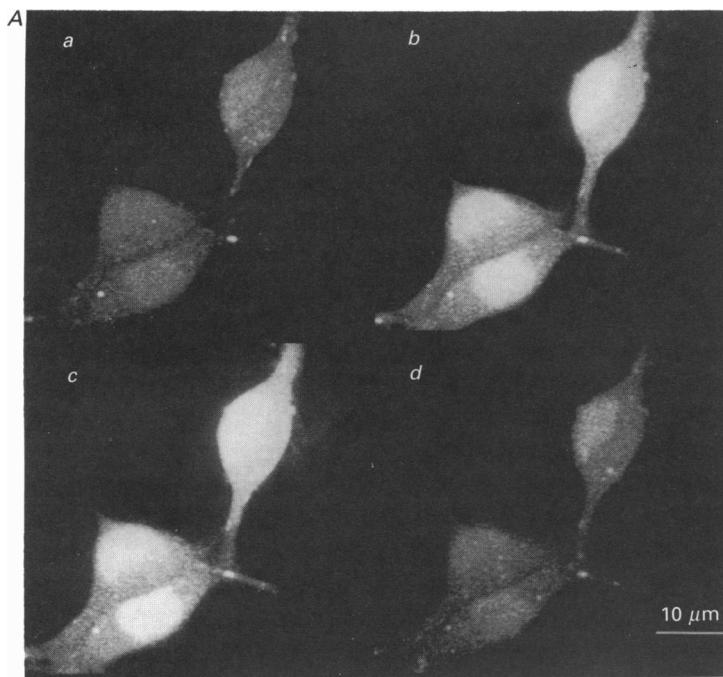
Fig. 1. For legend see facing page.

RESULTS

Basal levels of $[Ca^{2+}]_i$

Basal levels of Fluo-3 fluorescence varied among cells. This variation is partly due to dye loading and sequestration. However, cells with relatively high fluorescence levels were not responsive to drug applications and were considered unhealthy. Most of the reported cells had relatively low basal fluorescence, hence low $[Ca^{2+}]_i$. There were more cells with high $[Ca^{2+}]_i$ in older cultures (more than 14 days in culture (DIC). Within the cell, $[Ca^{2+}]_i$ was heterogeneously distributed, with higher concentrations in perinuclear somatic regions. The nucleus had a consistently lower $[Ca^{2+}]_i$ level than the rest of the cell, except for the nucleoli, whenever visualized. This might be due to dye exclusion by DNA in the nucleus.

Clusters of high-calcium-containing organelles were seen in the soma, outside the nucleus, especially at the base of the main (apical?) dendrite. These organelles were more labelled in older cells than in young ones and were more labelled the longer the time after incubation with the dye. The fluorescence of these organelles did not change much in response to NMDA. Because the variability associated with measurements of $[Ca^{2+}]_i$ in perinuclear regions, where these organelles are abundant, we restricted measurements of $[Ca^{2+}]_i$, in the pharmacological experiments reported below, to the nucleus.

Spontaneous activity

In normal, undrugged cultures there were occasional sudden rises in $[Ca^{2+}]_i$ in clusters of neurons, probably associated with spontaneous bursts seen in these neurons in the culture (Segal, 1983). These bursts lasted several seconds and a return to baseline $[Ca^{2+}]_i$ was usually seen. Interestingly, not all cells in a cluster tended to express a rise in $[Ca^{2+}]_i$. The source of this heterogeneity is unknown. Blockade of Na^+ spikes with TTX, or replacing Na^+ with NMG blocked these spontaneous fluctuations and kept $[Ca^{2+}]_i$ at a low, stable level. For this reason, all drug experiments were conducted in the presence of TTX.

Effects of NMDA

Superfusion of hippocampal cultures with NMDA resulted in a rapid rise of $[Ca^{2+}]_i$ in most cells studied (Fig. 2). The amount of rise varied among cells in a manner that was not predictable from the basal level within a cell cluster, or the morphology of

Fig. 1. Calibration procedure for hippocampal culture. A, successive images taken at rest (a), during exposure to 10 mM-NMDA (b, 10 μM 1 min after beginning of superfusion with the drug), during exposure to 10 mM-ionomycin (c, 1 min exposure to the drug) and during exposure to 5 mM-Mn²⁺ (d, 5 min exposure). Following this, the culture was perfused with digitonin which virtually eliminated the fluorescence. B, fluorescence intensity, (filled columns), averaged for the three cells depicted in A, and calculated $[Ca^{2+}]_i$ (hatched columns) using equations listed in Methods. Abbreviations are Con, control; Iono, ionomycin; Dig, digitonin, C, time course of rise in $[Ca^{2+}]_i$ during perfusion with ionomycin. Images were taken at a rate of 2·4 frames/s, and two successive images were averaged. Reading of fluorescence was taken from the nucleus. Perfusion of ionomycin was made at a rate of 3 ml/min.

the cells. The maximal rise of $[Ca^{2+}]_i$ was estimated to be over 600 nM following NMDA. There were always cells within a cluster that did not respond to NMDA. Some of them could respond to kainic acid (KA) with a rise in $[Ca^{2+}]_i$ as other cells did. Their proportion varied in the different fields of view and different experiments.
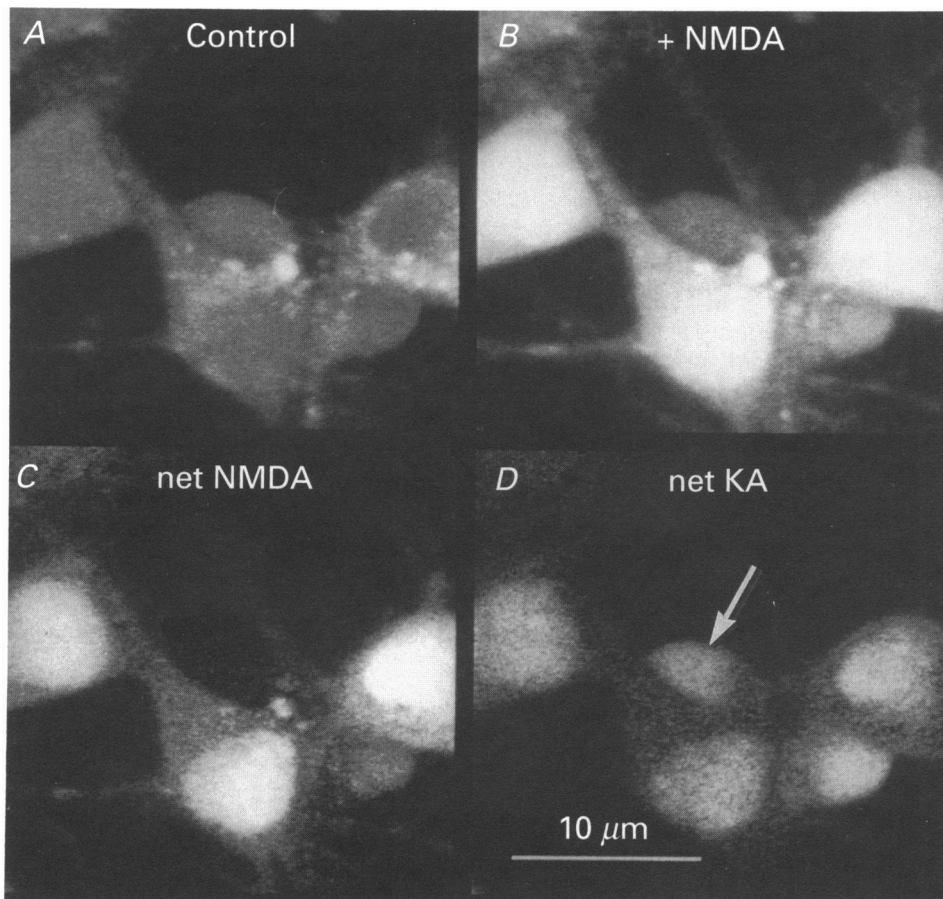

Fig. 2. Effects of NMDA and kainate (KA) on $[Ca^{2+}]_i$ in a cluster of cultured hippocampal neurons. *A*, before; *B*, after exposure to 100 μM-NMDA in normal medium. *C*, subtraction of image in *A* from image in *B* to yield the net increase in $[Ca^{2+}]_i$ due to NMDA. *D*, a similar subtracted image depicting the response to 100 μM-KA; 12 DIC. Note the large increase in $[Ca^{2+}]_i$ in the nucleus in all cells. Note that KA causes a smaller rise in $[Ca^{2+}]_i$ than NMDA in the same cells, but it causes a rise in a cell not affected by NMDA (arrow). NMDA and KA images were taken right after perfusion of 0·75 ml of drug solution, 30–60 s after onset of drug perfusion. Random frames were taken during the perfusion to assure that drug effects reached maximum. Each image is an average of five successive 256 × 256 pixel frames taken at a rate of one frame/1·5 s.

All cells responded to a rise in $[K^+]_o$ or to ionomycin in a relatively more uniform manner (Fig. 1). Dose–response curves were constructed by random applications of varying concentrations of NMDA. ED_{50} were in the range of 50–100 μM-NMDA. In
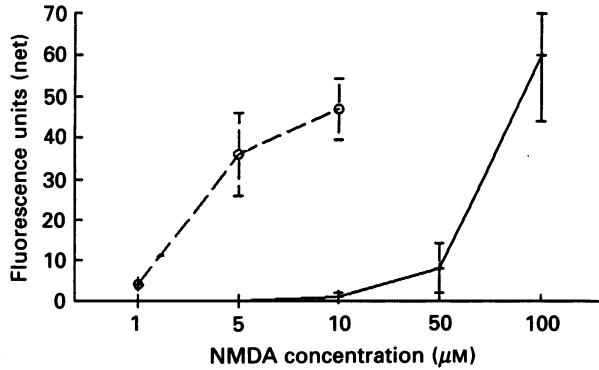
Fig. 3. Dose–response curves constructed in response to different concentrations of NMDA in presence (+) and absence (O) of magnesium (and addition of 10 μM-glycine). Ordinate, mean fluorescence intensity with background subtracted; n = 2–6 experiments, with each tested in at least two concentrations, in the two conditions. The cultures were washed after each tested dose.
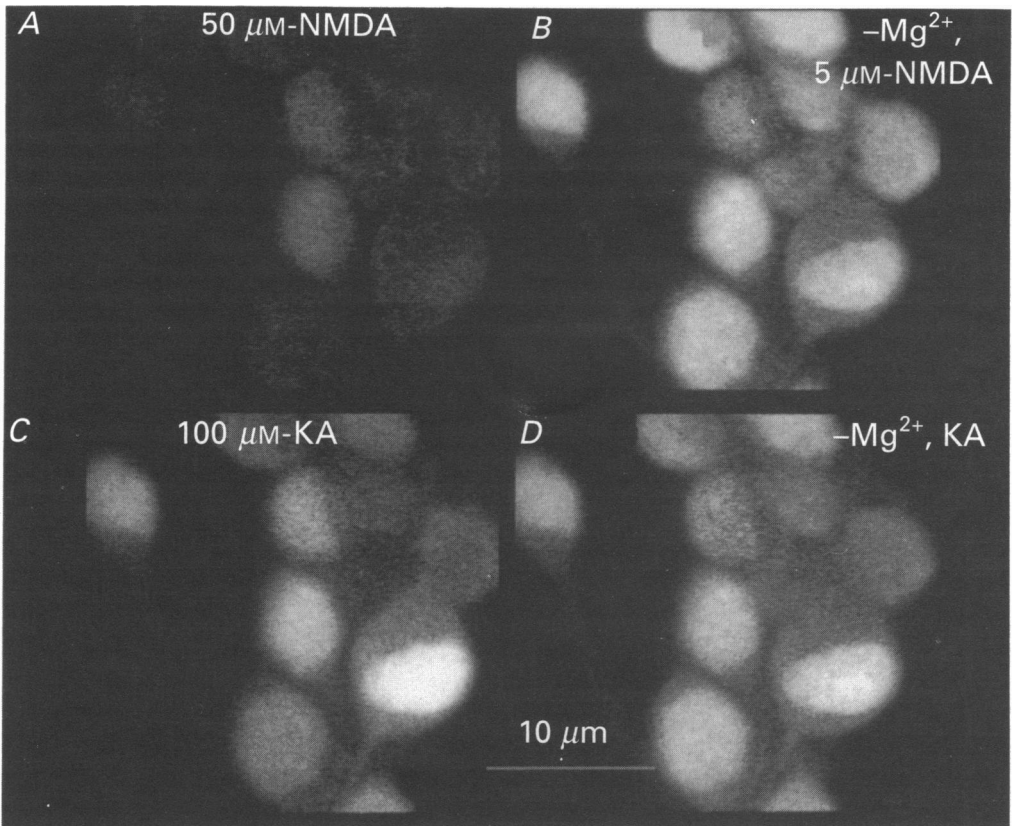

Fig. 4. Net effects of NMDA (top row) and KA (bottom row) in presence (left) and absence (right) of added magnesium, in the same culture. While removal of magnesium has a drastic effect on response to NMDA, such that a 10-fold lower concentration of NMDA (5 μM) could now produce a nearly maximal elevation of [Ca]$_i$ (B), it has no effect on responses to KA. 15 DIC. Same recording conditions as in Fig. 2.

many experiments a saturating dose of 100–200 μM was used. Removal of Mg^{2+} from the extracellular medium and the addition of 10 μM-glycine caused over a 10-fold increase in the efficacy of NMDA; a dose of 10 μM could evoke a change of $[Ca^{2+}]_i$ equivalent to that produced by 100 μM of the drug in normal (1 mM) magnesium-
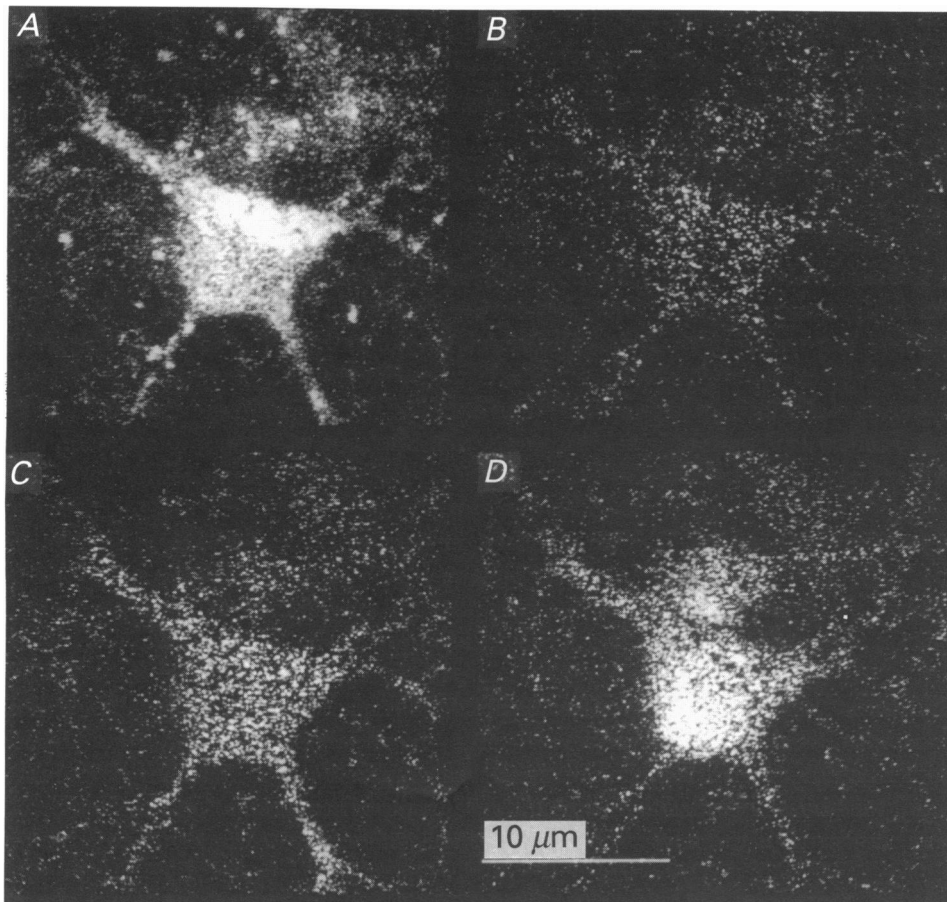

Fig. 5. Fractional rise in $[Ca]_i$ during perfusion with 100 μM-NMDA. A, image taken before start of perfusion. B, C and D, single frames, subtracted each from the previous image during the perfusion with NMDA. The first detected change is in the dendrites and perinuclear upper region of the cell (B). In the next net image, C, there is a homogenous rise in $[Ca]_i$ in all parts of the cell, whereas a more restricted rise is seen in the nucleus in the following image (D). Images were taken at a rate of 3·75 frames/s with a perfusion rate of 3 ml/min. 9 DIC.

containing medium (Fig. 3). The removal of Mg^{2+} did not greatly affect responses of these same neurons to kainic acid ($n = 9$ cells, Fig. 4). Since NMDA could evoke a consistent and reversible rise in $[Ca^{2+}]_i$ in normal, Mg^{2+}-containing medium, we conducted most of the subsequent experiments in such medium.

There was an initial increase in dendrites and perinuclear regions of the cell (Fig. 5). Calcium levels rose to maximal values within 4–6 s of perfusion with a saturating
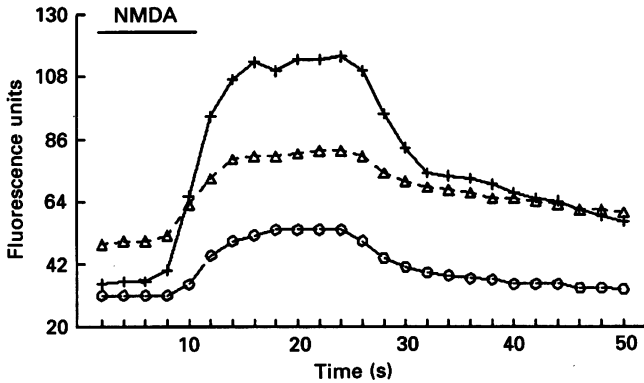
Fig. 6. Changes in [Ca]$_i$ in the nucleus (+), soma ($\triangle$) and dendrite ($\bigcirc$) of the cell during perfusion with 10 μM-NMDA (in the absence of added Mg$^+$, and with 10 μM-glycine) 15 DIC. Frames were taken at a rate of 0·5/s, the regions of interest analysed for each frame and plotted. Ordinate is the fluorescence intensity measured in each region, unsubtracted. The largest change is seen in the nucleus, which does not recover back to rest level within 1 min recording time. [Ca^{2+}] in the dendrite does recover to approximately pre-drug level.
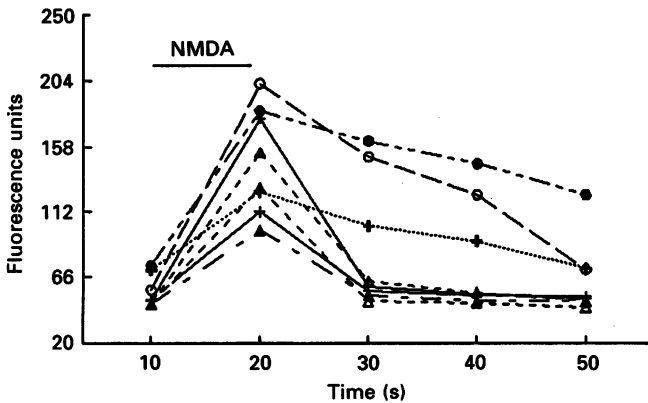

Fig. 7. Heterogeneity of responses to NMDA in both the initial magnitude and the rate of recovery, as seen in a single cluster of neurons (illustrated also in Fig. 4), 100 μM-NMDA in normal medium. Note that a large response to NMDA is not necessarily associated with a slow recovery and vice versa. Images are taken at 10 s/intervals before, during and after perfusion with the drug. Symbols represent different neurons.

concentration of NMDA, in soma as well as the primary dendrites. Upon continuous perfusion, dendritic [Ca^{2+}] regulated at lower level than that of somatic [Ca^{2+}]. Within the soma, nuclear [Ca^{2+}] kept increasing above perinuclear values and reached the highest absolute values among all parts of the cell (Fig. 6). There was a distinct demarcation line between the nucleus and the rest of the cell. During recovery, dendritic [Ca^{2+}] was first to recover, somatic [Ca^{2+}] second, and nuclear [Ca^{2+}] last. In many cases, nuclear [Ca^{2+}] did not recover to the level lower than that

of perinuclear one and remained elevated throughout the experiment. The rate of recovery was heterogenous in different cells within a cluster, and was not directly related to the initial rise (Fig. 7). Older cells had a slower rate of recovery than younger ones, indicating that they have slower clearing mechanisms of excess $[Ca^{2+}]$ than younger cells (Fig. 8).
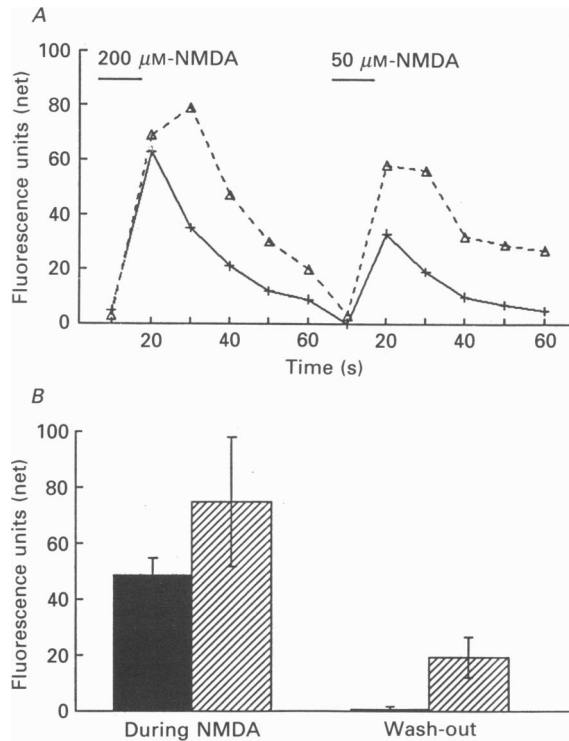

Fig. 8. Age-dependent clearing of excess $[Ca^{2+}]_i$ from nuclei following NMDA. A, comparison of two cell clusters, measured in the same experimental conditions, ages 4 DIC ($+$) and 16 DIC ($\triangle$), following superfusion with NMDA. The drug was applied twice, once at a concentration of 200 μM in normal medium (left), and once with NMDA at a concentration of 50 μM, in absence of added Mg^{2+} (right). The older cell cluster expresses a larger response but recovers slower than the younger cell cluster, and in fact, does not recover within the 1 min recording period. Averaged images were taken at a rate of 1/10 s. Fluorescence intensity, with subtracted background was measured in the nuclear region of three and four cells in the older and young cultures, respectively. B, a summary of several experiments, comparing young (1–7 DIC, filled column, $n = 18$) and adult (14–28 DIC, hatched column, $n = 11$), changes in $[Ca^{2+}]_i$ during (left) and approximately 1 min after exposure to NMDA. There was no significant difference in reactivity to NMDA in the adult cultures, but there was a significant elevated $[Ca^{2+}]_i$ after wash-out of NMDA in the mature cultures. Ordinate, averaged fluorescence intensity with background substracted.

Fast line scan

The presence of a calcium wave, from the membrane into the cell has been seen elsewhere as a result of activation of voltage-gated calcium channels (Hernandez-Cruz, *et al.* 1990). If indeed $[Ca^{2+}]_o$ is the source of elevated $[Ca^{2+}]_i$, one can expect

to find a calcium wave into the cell as a result of activation of NMDA receptors. We have scanned a line through the centre of neurons following puff applications of NMDA from a nearby pipette (Fig. 9A). A wave of elevated calcium could be seen, approaching the centre from both ends of the cell at a rate of $116\cdot9\pm12\cdot6\ \mu m/s$ ($n = 10$). This was conspicuous when the scan was made through the centre of the cell (approximately 3–4 μm from the surface of the cell which is attached to the glass). A scan at a level further from the centre did not yield a clear wave of [Ca^{2+}]$_i$ (Fig. 9B). The level of [Ca^{2+}]$_i$ in the nucleus increased continuously, while the level near the membrane regulated at a relatively low level.

The receptor

The NMDA-dependent increase in [Ca^{2+}]$_i$ is mediated by activation of an NMDA receptor, as the response was reversibly blocked by a concommitant perfusion with 100 μM of the antagonist 2-amino-5-phosphonovalerate (2-APV) (Fig. 10). The non-NMDA antagonist 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) (Collingridge & Lester, 1989) was not effective in blocking the response to NMDA (data not shown).

The extracellular calcium dependence of the response to NMDA was examined by removing calcium from the perfusion medium. In three experiments (eleven cells), the rise in [Ca^{2+}]$_i$ was reversibly reduced to 22 % of control values in nominally calcium-free medium (data not shown). In another series of experiments we examined the effects of calcium antagonists on the NMDA-dependent change in [Ca^{2+}]$_i$. While nifedipine at 10 μM concentration did not affect responses to NMDA (three experiments, twelve cells measured, Fig. 11), cadmium (at 0·1 mM, six experiments, eighteen cells) and lanthanum (at 0·1 mM, two experiments, eight cells) totally eliminated responses to NMDA.

Selectivity

NMDA was not the only ligand to evoke a rise in [Ca^{2+}]$_i$. Other ligands tested, kainate and quisqualate also evoked this response. Interestingly, the subpopulations of cells responding to NMDA were not identical to those excited by kainate and quisqualate (e.g. Fig. 1), indicating that they do not share the same receptor subtypes but have the ability to raise [Ca^{2+}]$_i$. It has been suggested that quisqualate responses are independent of [Ca^{2+}]$_o$ and that the drug causes release of calcium from intracellular stores (Murphy & Miller, 1988; Furuya *et al.* 1989). We found, however, that in twenty-two cells examined in nine experiments the response to quisqualate was dependent on [Ca^{2+}]$_o$; on average, the responses amounted to $10\cdot4\pm4$ % ($n = 18$) of control values in the absence of [Ca^{2+}]$_o$ (Fig. 12). The lack of response to quisqualate was not due to our inability to detect the presence of a transient response, as successive sampling of images at high rate did not reveal such responses (four cells, Fig. 12B). The lack of response to quisqualate in absence of [Ca^{2+}]$_o$ does not indicate that these cells are unable to express an elevation of [Ca^{2+}]$_i$; the proton uncoupler SF-6847 could still cause an increase in [Ca^{2+}]$_i$ in the absence of [Ca^{2+}]$_o$. The response to quisqualate was not dependent on activation of an NMDA receptor; 2-APV, at a dose that blocked NMDA, did not affect responses to quisqualate (Fig. 10).
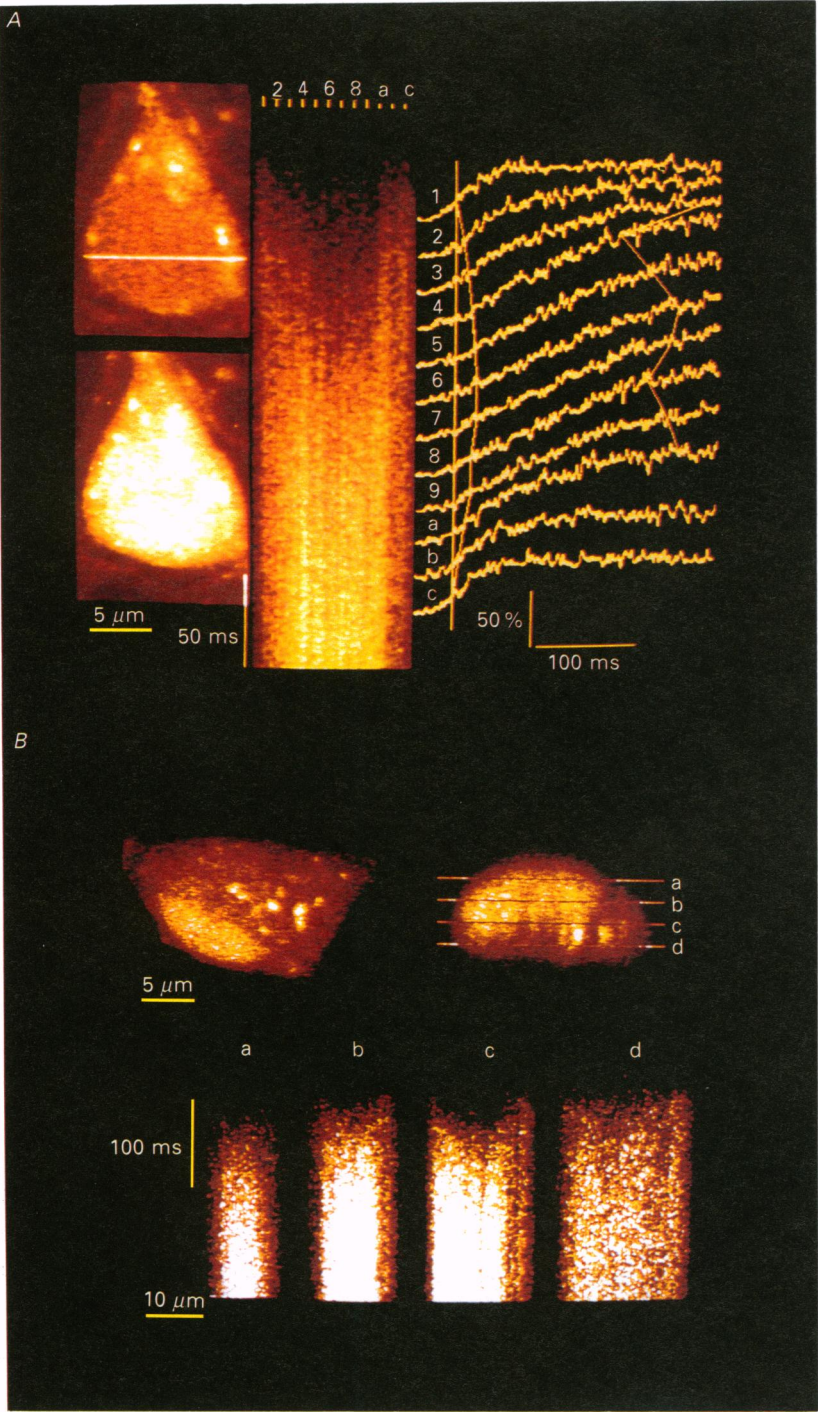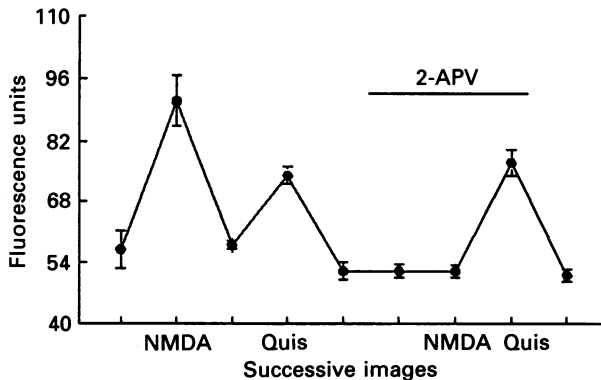
Fig. 9. For legend see facing page.

Fig. 10. Selective blockade of responses to NMDA by the antagonist 2-amino-5-phoshono-valerate (2-APV). The response to NMDA was totally blocked by the drug, while the responses to quisqualate (Quis) remained unaltered. A cluster of four cells, 6 DIC. Abscissa, successive images taken at steady states before during and after each drug superfusion test, same protocol as in Fig. 2. Ordinate, averaged fluorescence intensity, uncorrected for background. Means ± S.E.M.

The possibility that quisqualate or kainate cause a rise in $[Ca^{2+}]_i$ indirectly, by depolarizing the cell which will, in turn, activate voltage-gated calcium channels, was examined by removing sodium from the superfusion medium. Replacing Na^+ by NMG shifts the reversal potential of NMDA in the hyperpolarizing direction and prevents the depolarization-induced activation of voltage-gated calcium channels (Iino, Ozawa & Tsuzuki, 1990). Under these conditions, quisqualate and kainate (eight of seventeen cells) could still evoke a rise in $[Ca^{2+}]_i$ (Fig. 13). Interestingly, the clear lack of response to kainate in some cells indicates that kainate may activate two distinct types of receptors. These experiments indicate that, at least in some cells quisqualate or kainate act at a non-NMDA receptor to cause a comparable rise in $[Ca^{2+}]_i$ by allowing an inflow of calcium from the extracellular space.

Fig. 9. A, fast line scans illustrate the rapid rise of $[Ca^{2+}]_i$ in response to NMDA. Left, the cell before (top) and after (bottom), exposure to NMDA (puff from a pipette located near the soma). Centre, a scan taken at the line illustrated at top left during exposure to NMDA. A puff of drug was applied at onset of scan. A clear initiation of the rise in $[Ca^{2+}]_i$ is obvious on both sides of the cell. Right, twelve fluorescence intensity curves summarizing the intensity changes detected at various distances along the axis depicted in the marked positions on top of the line scan. A fast rise to a rather low level is seen near the periphery, whereas a slow rise to a higher level of $[Ca^{2+}]_i$ is seen in the centre of the cell. B, line scans through different depths of the cell show different patterns of rise of $[Ca^{2+}]_i$. Top left, a typical image of the cell in the x–y plane. Top right, the same cell in the x–z plane, cut at different scan depths (a–d) shown below. When the scan is near the surface, the rise of $[Ca^{2+}]_i$ is simultaneous across the front of the recording, indicating that calcium goes into the cell directly from above or below the plane of recording. Scans through the centre of the cell yield the typical pattern of rise in $[Ca^{2+}]_i$.
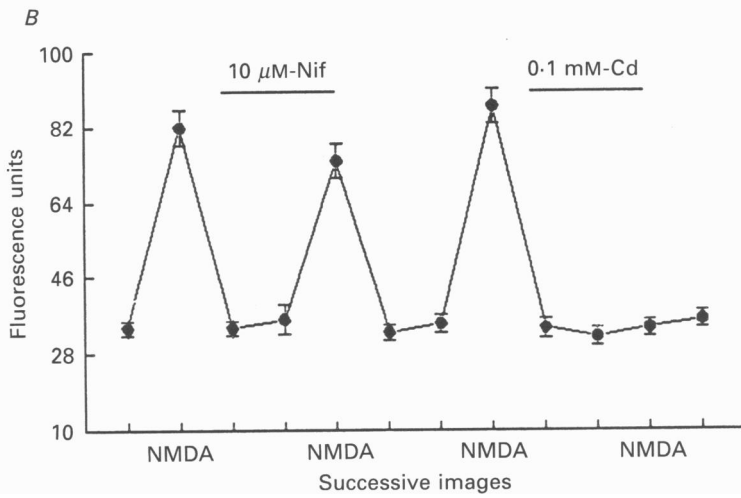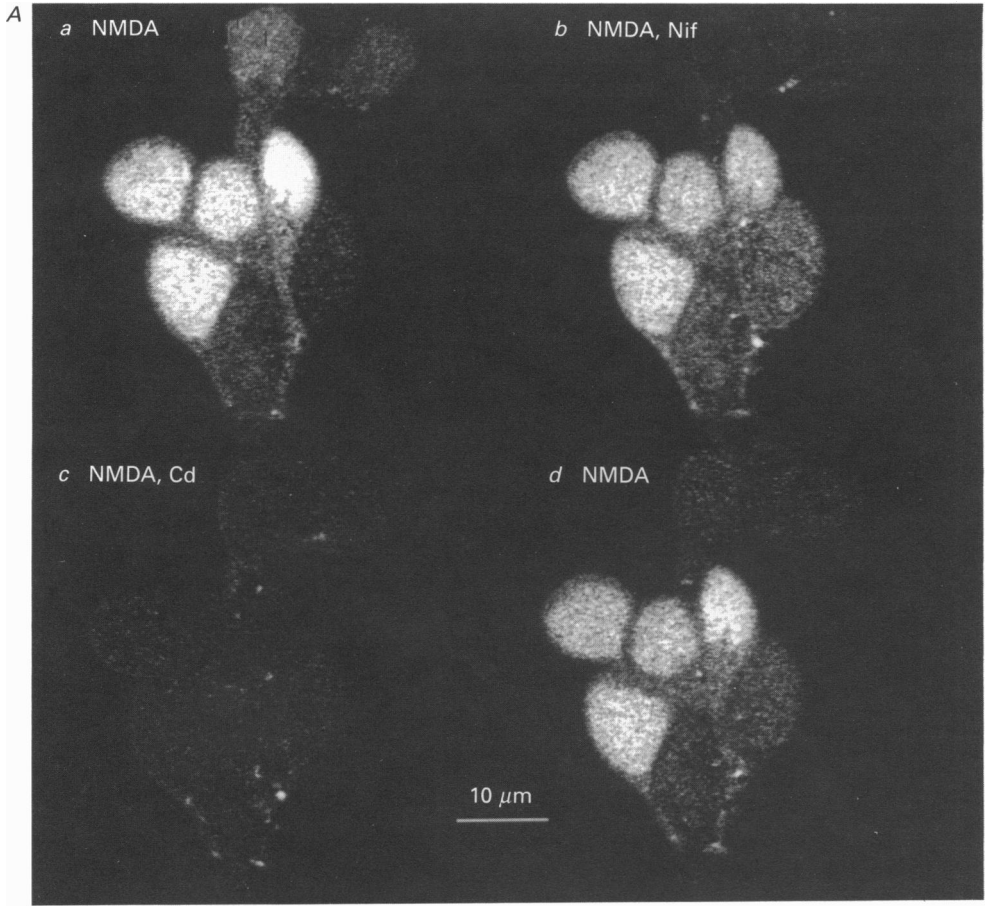
Fig. 11. For legend see facing page.

Sources of the elevated [Ca^{2+}]$_i$: *effects of dantrolene*

The lack of NMDA-induced change in [Ca^{2+}]$_i$ in the absence of [Ca^{2+}]$_o$ indicates that calcium influx triggers the change in [Ca^{2+}]$_i$ but it is not necessarily its only source. It is quite possible that influx of calcium causes the release of calcium from intracellular stores, as seen elsewhere (see Berridge & Irvine, 1989 for review). Several experiments examined this possibility. We incubated cultures for 10–20 min with 10 μM-Na-dantrolene, which blocks the release of calcium from endoplasmic reticulum (Carafoli, 1987; Blaustein, 1988). Dantrolene had two effects: it reduced basal levels of [Ca^{2+}]$_i$ in fifteen of twenty-two cells by about 11 %, but it reversibly reduced the effects of NMDA on [Ca^{2+}]$_i$ to 33 ± 11 % of control. A recovery from the effects of dantrolene was seen within 15–20 min after washing out the drug (Fig. 14).

Effects of barium

Barium ions can flow into neurons via calcium channels, but constitute only poor substrate for Fluo-3 fluorescence; in a calibration experiment done in recording medium containing 8 mM-bis(O-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), a 10 mM solution of Ba^{2+} yielded the same Fluo-3 fluorescence intensity as that produced by 1 mM-calcium, while 5 mM-Ba^{2+} produced half as much fluorescence as was produced by 1 mM-Ca^{2+}. By comparison, 5 mM-Ca^{2+} reached saturation of the A–D converter under these conditions tested. Nonetheless, when Ba^{2+} replaced [Ca^{2+}]$_o$, it caused a rise in [Ca^{2+}]$_i$ which, on average, amounted to 28 ± 6 % of the response produced in the same neurons in the presence of normal [Ca^{2+}]$_o$ (five experiments, twenty-four cells, Fig. 15A). This indicates that entry of barium through calcium channels caused a rise in intracellular calcium, most likely to be released from intracellular stores.

Fate of elevated [Ca^{2+}]$_i$: Na^+–Ca^{2+} *exchanger*

We have examined the possible role of the Na^+–Ca^{2+} antiporter in calcium homeostasis. This mechanism exchanges calcium with sodium in an energy-independent mechanism (Blaustein, 1988). The exchanger depends on a sodium gradient across the cell membrane, being normally high outside the cell. We replaced Na^+ with NMG, hence interfering with the normal functioning of the exchanger. In this medium NMDA (as quisqualate, see above) was still able to cause a normal rise in [Ca^{2+}]$_i$ (five experiments, Fig. 13). However, there was no recovery to normal levels of [Ca^{2+}]$_i$ after removal of NMDA. In fact, in five experiments examined, the rise in [Ca^{2+}]$_i$ continued after removal of the drug. Only extensive washing with control solution restored normal [Ca^{2+}]$_i$ in these cells (Fig. 15B).

Fig. 11. Effects of calcium antagonists nifedipine (Nif) and cadmium (Cd) on the NMDA-induced rise in [Ca^{2+}]$_i$. A, images are subtracted from background, depicting net increase in [Ca^{2+}]$_i$ in control (a), in the presence of nifedipine (b), in presence of cadmium (c), and in recovered control condition (d). Note that cadmium, but not nifedipine, blocked the effect of NMDA. 5 DIC. B, analysed data of the experiment illustrated in A. Abscissa, successive images taken at steady states before, during and after drug applications. Ordinate, fluorescence intensity, unsubtracted, measured from the four responding cells seen in Fig. 11A. Same recording protocol as in Fig. 2.

DISCUSSION

The present results demonstrate that acidic amino acids NMDA kainate and quisqualate cause a rise in $[Ca^{2+}]_i$ in cultured hippocampal neurons. These observations confirm and extend earlier studies using Fura-2 and other calcium
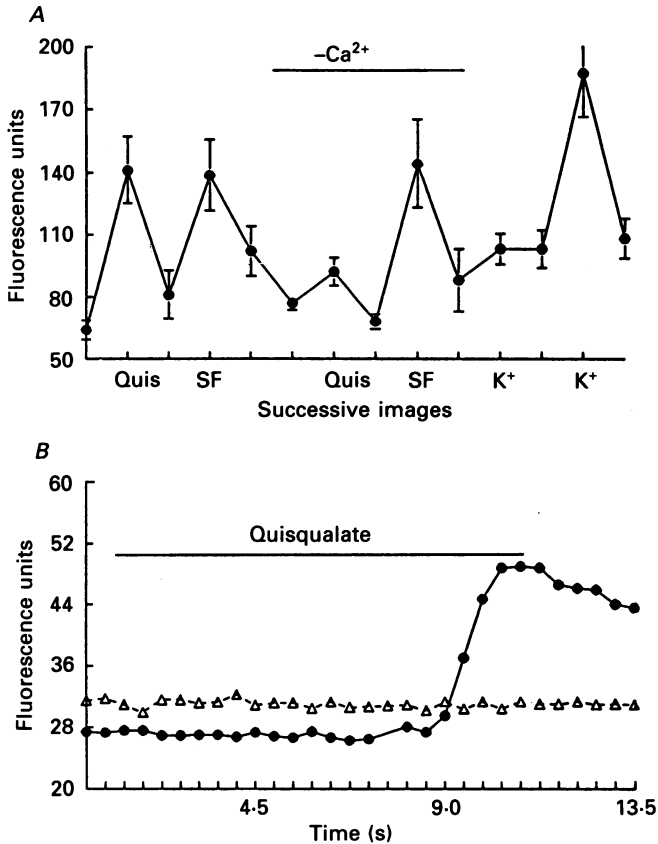

Fig. 12. Effects of removal of extracellular calcium on responses to quisqualate (Quis). *A*, Quis, as well as SF-6847 (SF, 10 μM) caused a reversible rise in $[Ca^{2+}]_i$ in normal medium. Upon removal of Ca^{2+} from the bathing medium, quisqualate is no longer able to cause a rise in $[Ca^{2+}]_i$, whereas SF-6847 still causes this rise. High potassium (K^+, 50 mM) is unable to raise $[Ca^{2+}]_i$ in the absence of normal $[Ca^{2+}]_o$, but is able to do this upon returning to normal medium. Abscissa, successive images taken at steady state, before, during and after drug perfusion. Ordinate, averaged fluorescence intensity, measured in a cluster of four cells, 6 DIC. *B*, time course of effect of quisqualate on $[Ca^{2+}]_i$ in normal medium (●) and in the absence of calcium in the bathing medium (△). Images were taken at a rate of 2·2 frames/s and perfusions was at a rate of 3 ml/min.

indicators in hippocampal neurons (Murphy & Miller, 1988; Furuya *et al.* 1989; Garthwaite & Garthwaite, 1989; Reynold & Miller, 1989; Glaum, Scholz & Miller, 1990; Oliver, Shacklock, Kessler, Lynch & Baimbridge, 1990; Regehr & Tank, 1990). One of the striking features in the neurons seen here but also elsewhere (e.g. Furuya *et al.* 1989) is the heterogeneity of reactivity of the cultured neurons to the

drugs; some cells expressed a large rise in $[Ca^{2+}]_i$ whereas others were not affected at all. By the same token, the rate of recovery was different among different cells in the same field of view. Such heterogeneity has not been reported for electrophysiological effects of glutamate in these neurons (see Mayer & Westbrook, 1987 for review).
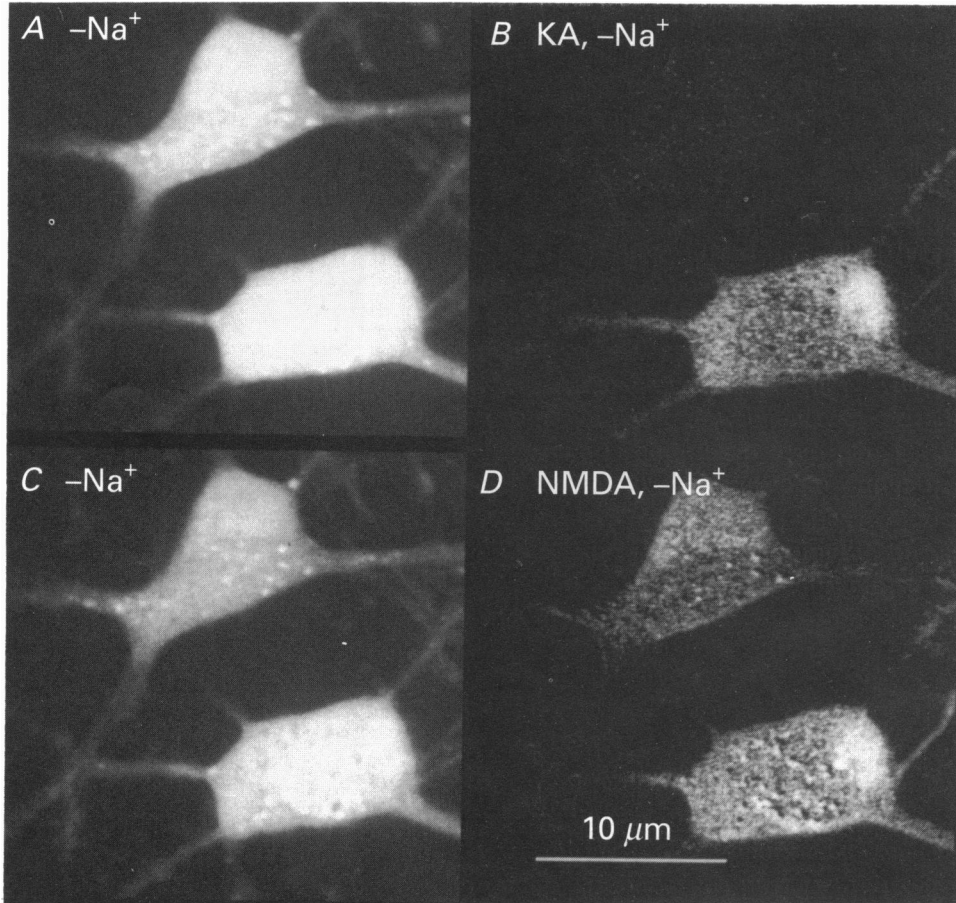

Fig. 13. Effects of replacement of extracellular Na^+ by NMG on reactivity of neurons to NMDA and KA. *A*, control image, before drug treatment, in the presence of NMG. *B*, net effect of KA in the presence of NMG. Note that only one of two cells responded to KA. *C*, control. *D*, effects of NMDA in the presence of NMG. Both cells seen in this image respond to NMDA by an increase in $[Ca]_i$.

There was no morphological distinction between the responsive and non-responsive cells. Furthermore, the fact that there was no overlap between the efficacy of NMDA, kainate and quisqualate indicates that the difference between the responding and non-responding cells is not due simply to lack of ability to mobilize calcium. This is also supported by the more uniform reactivity of these neurons to a rise in $[K^+]_o$ or to ionomycin (data not shown). The cultured hippocampus contains a large

proportion of γ-aminobutyric acid (GABA)ergic neurons, and these have been shown to express *in vivo* reactivity to antibodies to calcium-binding proteins (Kosaka, Katsumaru, Hama & Henzmann, 1987). These calcium-binding proteins are likely to be instrumental in removing excess calcium and thus may not allow us to detect
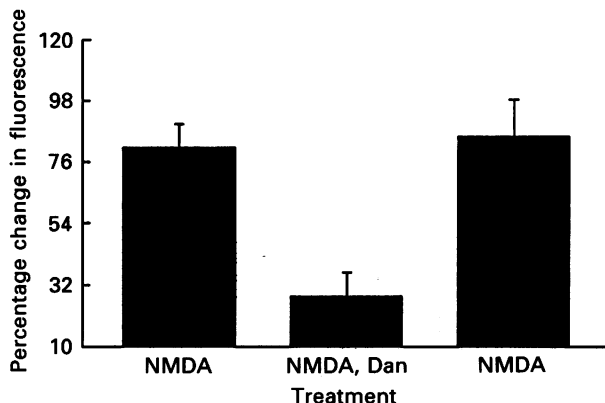

Fig. 14. Dantrolene reduces the NMDA-mediated increase in $[Ca^{2+}]_i$. Average of eight cells in four different experiments. To standardize the effect, the change produced by NMDA was expressed a percentage change above background fluorescence. Dantrolene (Dan) produced a significant reduction in the efficacy of NMDA, which recovered, following extensive wash-out.

changes due to activation of NMDA receptors. The possibility that GABA-containing neurons vary in reactivity to NMDA needs to be addressed in a double-labelling experiment.

Most of the present results were obtained in the presence of magnesium ions in the intracellular medium, a condition shown to retard electrophysiological reactivity of neurons to NMDA. While we were able to confirm the action of low Mg^{2+} on reactivity to NMDA, we preferred to conduct most of these experiments in the presence of physiological concentrations of Mg^{2+}. This condition could evoke stable responses to NMDA, if the drug was applied at higher concentration than that seen in the absence of Mg^{2+}.

A common cited distinction between NMDA, kainate and quisqualate is that the former is associated with a rise in $[Ca^{2+}]_i$, whereas the latter are not (see Collingridge & Bliss, 1987; Mayer & Westbrook, 1987, for reviews). We found that kainate and quisqualate are also capable of causing a rise in $[Ca^{2+}]_i$, and not necessarily because quisqualate is associated with a metabotropic receptor, as suggested earlier (Murphy & Miller, 1988; Furuya *et al.* 1989). It is true that, both kainate and quisqualate caused a rise in $[Ca^{2+}]_i$ in fewer cells when $[Na^+]_o$ is replaced with NMG, and at least quisqualate is able to cause a rise in $[Ca^{2+}]_i$ in some cells in the nominal absence of $[Ca^{2+}]_o$, unlike NMDA. Yet, a large enough proportion of cells elevate $[Ca^{2+}]_i$ in response to kainate and quisqualate, to justify a modification of the common conception associating a rise in $[Ca^{2+}]_i$ only with the NMDA receptor. This is actually supported by a recent electrophysiological study which measured inward current in

cultured hippocampal cells (Iino *et al.* 1990), and found that in about half of the cells tested, kainate caused inward current (carried by calcium) in the near absence of [Na^+]$_o$, indicating that kainate may activate calcium flow into these cells directly and not just by activating voltage-gated calcium channels. These same authors did
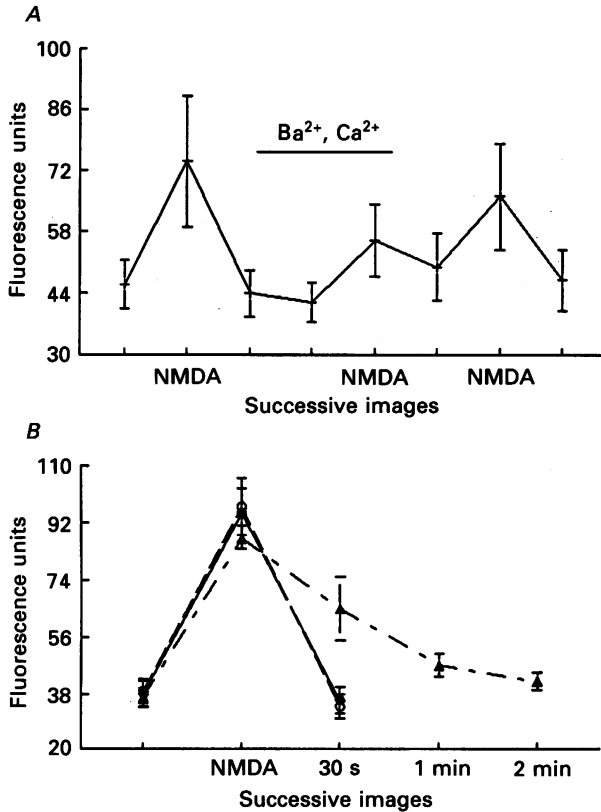

Fig. 15. *A*, replacement of [Ca^{2+}]$_o$ by equimolar [Ba^{2+}]$_o$ reduces but does not eliminate reactivity to NMDA. A cluster of six cells, 8 DIC. Barium causes a slight reduction in the rest level of [Ca^{2+}]$_i$ but in the presence of barium, NMDA could still cause a rise in all six cells in the clusters, by about 25 % of control level. Same recording conditions as in Fig. 2. *B*, absence of sodium in the bathing medium slows down the rate of recovery from the effects of NMDA. A cluster of four cells, 8 DIC, was exposed three times to NMDA, twice in normal medium ●,○) and once in low-Na^+, NMG-containing medium (△). The three tests are overlapped to illustrate the slow time course of recovery in the presence of NMG.

not find a similar effect of quisqualate, in the absence of normal [Na^+]$_o$, unlike our results. This may be due to the blockade of the Na^+–Ca^{2+} antiporter by the removal of [Na^+]$_o$, which will lead to accumulation of calcium inside the cell, and thus may interfere with calcium gradient and calcium-related currents. This will be particularly pronounced with long incubation periods with low-[Na^+]$_o$ solutions.

The difference between these results and previous ones may be due to the culture conditions, the age of the culture (see below), or the sensitivity of the method used, employing Fluo-3 in conjunction with the confocal microscope. Also, the culture

contains a mixture of CA1, CA3 and dentate cells, which may be different in their sensitivity to kainate and possible quisqualate. Clearly, the removal of Mg^{2+}, and addition of glycine sensitizes only the responses to NMDA (Nowak, Bregestovski, Ascher, Herbet & Prochiantz, 1984), and thus, when used with low agonist concentration, only NMDA will activate a rise in $[Ca^{2+}]_i$.

The responses to acidic amino acids are developmentally regulated; a 2-fold increase in reactivity to glutamate in hippocampal cultures between 1–3 DIC and 9 DIC has been reported (Koller, Seibler, Schmalenbach & Muller, 1990). We find that hippocampal cells are able to respond to NMDA by a rise in $[Ca^{2+}]_i$ even within the first day in culture, which will be equivalent to prenatal day 20. If there is a significant difference between young and more mature cultures, it is in the ability of the former to dispose faster than the latter of excess $[Ca^{2+}]_i$. A similar observation was made with cerebral cortical cells in culture by Wahl *et al.* (1989). This might be due to the difference in the size of these neurons, allowing the nucleus of the smaller cells a shorter path to the plasma membrane. However, the difference in the size of these cells between 7 and 21 DIC is not large enough to account for this difference. It is likely that the younger cells possess more efficient mechanisms for removal of excess calcium, such that they can regulate calcium-dependent plastic mechanisms more efficiently than the older ones.

The large increase in $[Ca^{2+}]_i$ in the nucleus, compared with that of dendritic $[Ca^{2+}]$ is unique; rest levels of $[Ca^{2+}]$ are lower in the nucleus than in perinuclear regions, yet in response to NMDA, $[Ca^{2+}]_i$ is higher in the nucleus than in perinuclear regions. This can be due to genuine low $[Ca^{2+}]$ levels in the nucleus, to an interaction of the negative charge of the nuclear DNA with the dye–Ca^{2+} in the nucleus, to a preferential exclusion of Fluo-3 from the nucleus, or to an artifact of the binding of Fluo-3 to calcium-containing organelles outside the nucleus. These will not respond to changes in free $[Ca^{2+}]_i$ and thus the nuclear fluorescence will rise above that of the perinucleus. This is not likely since the nuclear–perinuclear distinction was seen even in cases where measurements were made right after incubation, and there was little detected compartmentation of the dye. The dye compartmentation may however contribute to the reduction in reactivity of perinuclear regions, since we did detect a reduction in reactivity to NMDA, with longer delays between the incubation and the measurements.

It is not likely that there is a preferential exclusion of Fluo-3 from the nucleus since $[Ca^{2+}]_i$ rises in the nucleus (nearly) as fast as it does in the rest of the soma, and this is not likely to occur as a result of diffusion of Fluo-3 into the nucleus during the action of NMDA. Even if it does, why should $[Ca^{2+}]_i$ be higher in the nucleus after NMDA than in the rest of the cell? Thus it is likely that the nucleus maintains active mechanism(s) for the removal of excess calcium at rest and these are deactivated during the action of NMDA. Some of these mechanisms may involve regulation of gene expression, as suggested before (Berridge & Irvine, 1989). The differential presence of calcium buffers with different affinities to calcium in the nucleus and perinuclear regions can also underly the different responses to NMDA in the two compartments. This possibility has to be analysed in future experiments.

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