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# Glutamate-induced increase in intracellular $Ca^{2+}$ concentration in isolated hippocampal neurones

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**1** A system for real-time quantitative monitoring of intracellular free calcium ion concentration ($[Ca^{2+}]_i$) on a single cell basis was developed by the combination of a fluorescent $Ca^{2+}$ indicator fura-2, a fluorescence microscope, a video-camera and photometrical devices. It was applied to rat individual hippocampal neurones under culture for detection of L-glutamate-induced alterations in the $[Ca^{2+}]_i$ level.

**2** L-Glutamate (0.01–100 μM) induced a dose-dependent elevation of the $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ in the rat hippocampal neurone was found to be around 30 nM in the resting state, and was increased up to 500 nM by the application of 100 μM L-glutamate. *N*-methyl-D-aspartate, kainate and quisqualate in a concentration of 10 μM also increased the $[Ca^{2+}]_i$ level in the same single neurone, but their efficacy varied between individual cells.

**3** The L-glutamate-induced $[Ca^{2+}]_i$ elevation was abolished after removal of extracellular $Ca^{2+}$ and was much reduced by $Mg^{2+}$ (3 mM). The increase was, however, still observed in a $Na^+$-free medium.

**4** The L-glutamate-induced $[Ca^{2+}]_i$ elevation was not affected substantially after treatment with nitrendipine (10 μM) which blocked the increase in $[Ca^{2+}]_i$ induced by an isotonic high KCl-medium (50 mM).

**5** The present results suggest that the L-glutamate-induced $[Ca^{2+}]i$ elevation in the hippocampal neurone is due to an influx of $Ca^{2+}$ through both L-glutamate receptor-coupled and voltage-sensitive ionic channels.

## Introduction

The hippocampal cortex provides a promising clue for the understanding of the cellular mechanism of memory. After an intense excitation had been evoked in hippocampal neurones, a subsequent stimulus provoked a markedly enhanced response (Bliss & Lømo, 1973). This phenomenon, called long-term potentiation (LTP), has been explained either by facilitated release of an excitatory neurotransmitter (Bliss & Dolphin, 1982; Dolphin *et al.*, 1982; Lynch *et al.*, 1985), presumably L-glutamate, or by an increased postsynaptic sensitivity. The latter explanation led to an asumption of the following sequence of events in the activated hippocampal neurone (Baudry & Lynch, 1980; Lynch *et al.*, 1983; Siman *et al.*, 1985): activation of L-glutamate receptor, elevation of the intracellular free calcium ion concentration ($[Ca^{2+}]_i$), activation of a $Ca^{2+}$-dependent protease (calpain I), breakdown of a cytoskeletal protein (fodrin), increase in the number of functional L-glutamate receptors. But the key step in this sequence, the L-glutamate-induced $[Ca^{2+}]_i$ rise, has been suggested by circumstantial evidence such as a blockade of long-term potentiation by an injection of a $Ca^{2+}$-chelator (Lynch *et al.*, 1983) or a decrease in extracellular $Ca^{2+}$ activity induced by intense electrical stimulation (Heinemann *et al.*, 1984). These indirect approaches, although reflecting $Ca^{2+}$ entry into the hippocampal neurones, do not specify the pathway of the $Ca^{2+}$ entry, i.e. whether the L-glutamate receptor-coupled ion channel or the voltage-sensitive $Ca^{2+}$ channel is involved. Moreover, no quantitative estimate of the extent to which the $[Ca^{2+}]_i$ rises has been made. Hence, we developed a method for monitoring the $[Ca^{2+}]_i$ in a single small cell loaded with a fluorescent $Ca^{2+}$ indicator fura-2 (Grynkiewicz *et al.*, 1985). The system consisted of a fluorescence microscope, a video-camera equipped with an image-intensifier, and a photodiode to measure the brightness of the image of a neurone displayed on a cathode ray tube. This relatively inexpensive combination of all commercially available devices is as sensitive as any system previously developed for single cell fluorometry (Perney *et al.*, 1984; Kruskal *et al.*, 1984;

Tsien *et al.*, 1985). We applied this method to cultured hippocampal cells of the rat to examine the L-glutamate-induced elevation of $[Ca^{2+}]_i$.

## Methods

### *Primary culture of rat hippocampal neurones*

Hippocampal neurones were isolated following the protocol of Banker & Cowan (1977). Briefly, hippocampal cortices were dissected out from 16–20 day embryonic cerebra of Wistar rats with a scalpel. After removal of meninges and treatment with 0.25% trypsin (Difco)/0.02% deoxyribonuclease I (Sigma) for 20 min at 37°C, cells were dispersed and plated on a poly-D-lysine (Sigma) coated glass coverslip (40 × 50 × 0.13 mm) with a silicon rubber wall (Heraeus, Flexiperm) at a density of $2-6 \times 10^5$ cells per well (15 mm in diameter). From the same embryos, hippocampal tissue pieces were prepared and one placed in each well (i.e. cell-tissue co-culture). The culture was maintained for 3–10 days with Dulbecco's modified Eagle's medium (Gibco; containing no L-glutamic acid) supplemented with 10% precolostrum newborn calf serum (Mitsubishi Chemical Ind.), 1 mM sodium pyruvate, 50 u $ml^{-1}$ penicillin and 0.01% streptomycin in a humidified atmosphere of 90% air plus 10% $CO_2$. In the case of maintenance for long periods (> 5 days), the culture was treated with 10 μM cytosine arabinofuranoside (Sigma) for 24 h on the 5th or 7th day in order to suppress any growth of the glial cells.

### *Microfluorometry of the fura-2 loaded single neurone*

The culture medium and the tissue preparation were removed and a basal salt solution (BSS; composition, mM: NaCl 130, glucose 5.5, KCl 5.4, $CaCl_2$ 1.8, tetrodotoxin 0.001, HEPES-NaOH 20, pH 7.3) containing 1.5 μM fura-2 acetoxymethylester (Molecular Probes Inc.) was introduced in the well. After 90 min incubation (37°C), the cells were washed with BSS and the coverslip carrying the cells was mounted on a Nikon TMD inverted microscope. Neurones were identified (Figure 1a) by their large distinct cell body, phase brightness, presence of long neurites, survival from the cytosine arabinofuranoside treatment and their responsiveness to a high $K^+$ depolarizing stimulus at the end of each experiment (see Figure 2d).

For epifluorescence, the light source was switched to a 500 W xenon lamp equipped with an interference filter (Hoya Corp., 340 ± 10 nm). The microscope image (Figure 1b, objective lens was a Nikon Fluor × 20) was filtered sharply (cut off < 450 nm) and projected on to a 12 inch cathode ray tube (Hamamatsu Photonics Co. (HPC), C-1846) with the aid of an image-intensifier (HPC, V-1329) and a video-camera (HPC, C-1000) as shown in Figure 1c. In practice, only the neurones showed bright fluorescence, presumably due to the thickness of the cell (see a scanning electron micrograph of Figure 1d).

On the image of the neuronal soma identified on the CRT screen (around 5 mm in diameter), a light conducting fibre (Mitsubishi Rayon, SH-4001, 1 mm in diameter) was vertically fixed with the aid of a magnet. The other end of the fibre was connected to a photodiode (HPC, S-874), the output of which was amplified, integrated (integration was necessary, because the light from the CRT was delivered intermittently due to the scanning nature of the video camera), and recorded with a d.c. pen writer. The electronic controls of all photometrical devices were adjusted so that the fluorescence intensity of the neurone was linearly amplified to the final output. On most occasions, the excitation light was given at intervals by a shutter (e.g. 1 s opening after 9 s closure) so as to minimize the bleaching of the fluoroprobe. A more detailed description of the system will be published elsewhere. All recordings were done at room temperature (26 ± 2°C).

### *Dual beam microfluorometry*

The $[Ca^{2+}]_i$ was quantitatively estimated by a dual beam excitation method. A rotatory disc carrying two interference filters (340 ± 10 and 360 ± 10 nm, Hoya Corp.) was installed in front of the xenon lamp to illuminate specimens with two excitation beams alternately. Ratios of emitted fluorescence (fluorescence intensity under excitation light of 340 nm wavelength ($F_{340}$) divided by that under 360 nm ($F_{360}$)) give the absolute $Ca^{2+}$ concentration: (Grynkiewicz *et al.*, 1985) after a proper calibration. For that, small droplets of 20 mM Pipes-NaOH buffer (pH 6.8) containing EGTA and $CaCl_2$ in calculated ratios (Harafuji & Ogawa, 1980) and 10 μM fura-2 pentasodium salt (Molecular Probes Inc.) were suspended in liquid paraffin and illuminated by the 340 and 360 nm beams alternately.

### *Drugs*

Excitatory amino acid agonists and antagonists, such as sodium L-aspartate (Wako Pure Chem.), sodium L-glutamate (Wako Pure Chem.), kainic acid (Sigma), *N*-methyl-D-aspartic acid (NMDA, Sigma), quisqualic acid (Sigma), DL-α-aminoadipic acid (Tokyo Kasei), D(−)-2-amino-5-phosphonovaleric acid (Tocris), kynurenic acid (Sigma) and chemicals such as $MgCl_2$ and nitrendipine (Bayer) were included in the perfusing medium. A high KCl medium was prepared by adding 50 mM KCl to BSS and withdrawing 50 mM of the NaCl. A $Ca^{2+}$-free medium was made

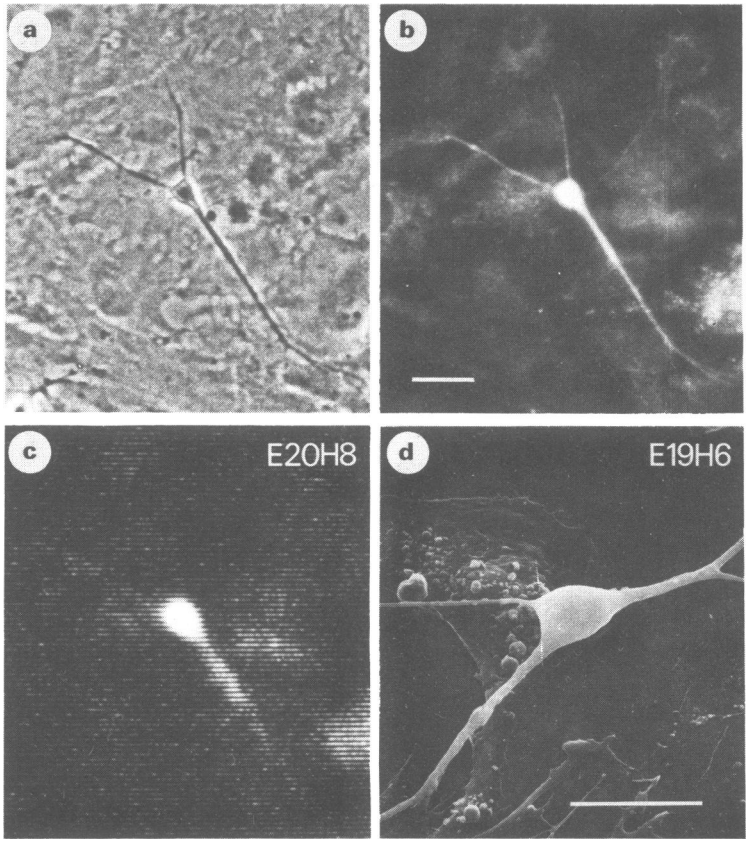


**Figure 1** Hippocampal neurone loaded with fura-2 viewed through phase contrast optics (a), through epifluorescence optics (b) and through an image-intensifier/video-camera system (c). Images (a–c) are from the same specimen. A scanning electron micrograph taken from a similar culture is shown in (d). Calibration bars indicate 20 μm. Descriptions such as 'E20H8' in this and subsequent figures represents a neurone isolated from embryonic day 20 hippocampus and cultured for 8 days before the experiment.

by omitting $CaCl_2$ from BSS and adding 0.1 mM EGTA (Dojin Chem). A $Na^+$-free medium was prepared by replacing NaCl with isotonic tetramethylammonium chloride (Tokyo Kasei) and buffering the HEPES with Tris.

## Results

### *Effects of L-glutamate and its analogues on the $[Ca^{2+}]_i$ in cultured single hippocampal neurones*

When a hippocampal neurone loaded with fura-2 was exposed to L-glutamate, a considerable increase in fluorescence intensity, indicating a rise in $[Ca^{2+}]_i$ (Figure 2a), was observed in 161 out of the 167 neurones examined. To monitor the $[Ca^{2+}]_i$ over a longer period, we used intermittent excitation to minimize the bleaching of the fluoroprobe (Figure 2b). All L-glutamate analogues examined (including NMDA, quisqualate, aspartate and kainate) induced the $[Ca^{2+}]_i$ elevation (Figure 2b, Table 1). This confirmed the concomitant existence of multiple subtypes of receptors for acidic amino acids in a single neurone (Evans & Watkins, 1981; Yamamoto & Sato, 1985). However, the magnitudes of the responses varied between individual cells. For example, in 11 out of 21 cells NMDA evoked larger $[Ca^{2+}]_i$ elevation than quisqualate, while in 10 other cells quisqualate was more effective than NMDA. It was unlikely that the excitatory amino acids induced the release of (an)other types of excitatory neurotransmitter which evoked the $[Ca^{2+}]_i$ elevation in the neurones under observation, since we chose cells having no apparent contact (either

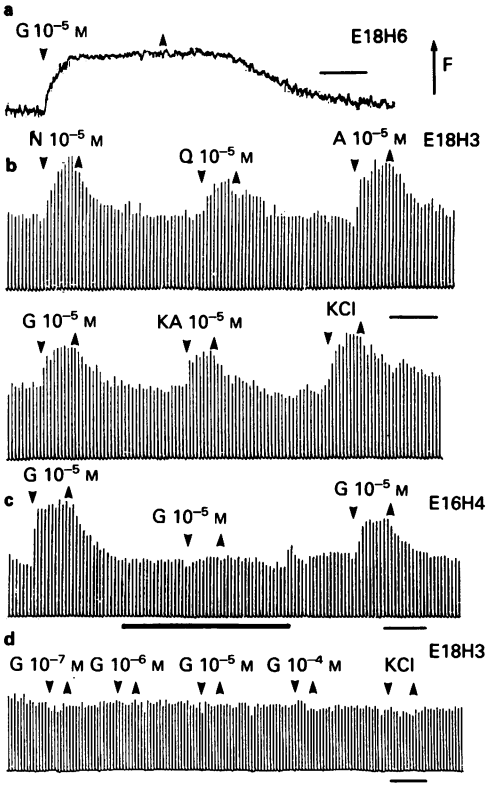


**Figure 2** Elevation of $[Ca^{2+}]_i$ induced by L-glutamate in individual hippocampal neurones (a–c) and non-neuronal cell. (d). (a) Short duration recording with continuous exposure to excitation light. (b–d) Long duration recording with intermittent excitation. Envelopes of fluorescence signals indicate the alteration in $[Ca^{2+}]_i$. Fluorescence (F) increase is represented by upward deflection. Calibration bars are 20 s in (a), 2 min in (b–d). At downward arrowheads, perfusions with medium containing 10 μM of the indicated L-glutamate analogue or 50 mM KCl were begun. G, N, Q, A and KA stand for L-glutamate, *N*-methyl-D-aspartate, quisqualic acid, L-aspartate and kainic acid, respectively. At downward arrowheads, perfusions with medium containing no stimulant were begun. In (c), the perfusing medium was free of $Ca^{2+}$ during the period indicated by the thick bar. In (d), L-glutamate of increasing concentration was applied.

by cell bodies or by processes) with other neurones and a high concentration of tetrodotoxin was included in the perfusing medium.

Unexpectedly, drugs known as organic L-glutamate antagonists including α-aminoadipic, 2-aminophosphonovaleric and kynurenic acids caused a gradual increase in the $[Ca^{2+}]_i$ *per se* (Table 1).

If $Ca^{2+}$ was removed from the medium, L-glutamate reversibly failed to cause the increase in fluorescence (Figure 2c, Table 2) indicating that the $[Ca^{2+}]_i$ rise was caused by an influx of $Ca^{2+}$ from the external medium. When the same fluorometry was applied to a non-neuronal flat cell chosen intentionally, neither L-glutamate nor KCl induced the fluorescence increase (Figure 2d). It has recently been found that astroglial cells under culture exhibit a L-glutamate receptor (Bowman & Kimelberg, 1984) and voltage-dependent $Ca^{2+}$ channels (MacVicar, 1984). However, as far as we could see, they failed to show the $[Ca^{2+}]_i$ elevation upon exposure to L-glutamate or to KCl (they may have responded with a magnitude so small, compared with that of the neurone, that our system could not detect it).

### *Examination of the mechanisms responsible for the elevation of the $[Ca^{2+}]_i$ induced by L-glutamate*

To determine if the increase in $[Ca^{2+}]_i$ was caused directly by drug activation of the L-glutamate receptor-coupled ion channel or by opening of the voltage-sensitive $Ca^{2+}$ channel, we performed the following experiments. First, $Mg^{2+}$ was employed to block the L-glutamate receptor (Ault *et al.*, 1980; Nowak *et al.*, 1984). Addition of 3 mM $MgCl_2$ to the medium reversibly suppressed the L-glutamate-induced response whilst having a lesser effect on the KCl-induced response (Figure 3a, Table 2). This suggested that $Ca^{2+}$ entry through the channels operated by membrane depolarization is capable of elevating the $[Ca^{2+}]_i$. In view of this result, we routinely used a medium free of $Mg^{2+}$, though the presence of 0.8 mM $MgCl_2$ had little effect on the L-glutamate-induced response. In the next experiment, nitrendipine (10 μM) was applied to block the voltage-sensitive $Ca^{2+}$ channels. As expected, the drug depressed the KCl-induced response with little effect on the L-glutamate-induced response (Figure 3b, Table 2). Finally we removed $Na^+$ from the medium. L-Glutamate elevated the $[Ca^{2+}]_i$ to about the same extent as in the $Na^+$-containing medium (Figure 3c, Table 2). This indicates that $Ca^{2+}$ acts as a charge carrier cation in the L-glutamate receptor-coupled ion channel (otherwise, L-glutamate should have failed to cause the $Ca^{2+}$ influx in the $Na^+$-free medium). At the same time, this result excludes the possibility that the $[Ca^{2+}]_i$ elevation resulted from an attenuated $Na^+/Ca^{2+}$ exchange due to the activation of L-glutamate uptake machinery coupled with $Na^+$ entry. The KCl-induced $[Ca^{2+}]_i$ elevation was unaffected by the removal of external $Na^+$.

### *Quantitative estimation of the $[Ca^{2+}]_i$ within the neurone*

To estimate the $[Ca^{2+}]_i$ in the hippocampal neurones, we adopted a dual beam excitation method (Kruskal *et*

**Table 1** Increases of flurescence intensity induced by various agonists and antagonists for the L-glutamate receptor in hippocampal neurones

| *Drugs* | n | *Conc.* (M) | $F(\%) \pm$ s.d. | $\Delta[Ca^{2+}]_i$ (nM) |
|---|---|---|---|---|
| *Agonists* | | | | |
| L-Glutamate | 19 | $10^{-5}$ | $207 \pm 43$ | 303 |
| L-Aspartate | 13 | $10^{-5}$ | $191 \pm 15$ | 229 |
| NMDA | 21 | $10^{-5}$ | $151 \pm 26$ | 68 |
| QA | 21 | $10^{-5}$ | $146 \pm 21$ | 64 |
| KA | 21 | $10^{-5}$ | $127 \pm 24$ | 28 |
| *Antagonists* | | | | |
| α-AA | 14 | $10^{-4}$ | $149 \pm 59$ | 182 |
| 2-APV | 10 | $10^{-5}$ | $130 \pm 58$ | 90 |
| Kyn | 6 | $10^{-3}$ | $227 \pm 87$ | 267 |

Abbreviations used: NMDA, *N*-metyl-D-aspartic acid; QA, quisqualic acid; KA, kainic acid; α-AA, α-aminoadipic acid; 2-APV, 2-aminophosphonovaleric acid; Kyn, kynurenic acid. *n*: number of neurones examined. F: relative fluorescence intensity at peak of the response (intensity prior to the perfusion is expressed as 100%). $\Delta[Ca^{2+}]_i$: mean magnitude of $[Ca^{2+}]_i$ increase estimated from the value of fluorescence increase. In this series of experiments, a single-beam excitation (at 340 nm) was employed. So, conversion from fluorescence intensity to absolute value of $[Ca^{2+}]_i$ in the true sense is not possible. However, examination of the results from dual-beam excitation (Figure 4) revealed the resting $[Ca^{2+}]_i$ to be $36 \pm 2$ (means $\pm$ s.e.) nM ($F_{340}/F_{360} = 0.62 \pm 0.02$). Using this value, we could reasonably reconstruct the level of $F_{360}$ in the single-beam recording as well. Thus the peak $[Ca^{2+}]_i$ induced by each drug was estimated and presented.

*al.*, 1984; Tsien *et al.*, 1985). Since fura-2 fluorescence is independent of the $[Ca^{2+}]_i$ at an excitation wavelength of 360 nm (Grynkiewicz *et al.*, 1985), the ratio of fluorescence intensities at 340 nm and 360 nm ($F_{340}/F_{360}$) should be independent of the amount of fluoroprobe loaded in the cell. For calibration, we illuminated droplets of buffers containing fura-2 and the determined concentration of free $Ca^{2+}$ (Figure 4a). As expected, the $F_{340}/F_{360}$ ratio was independent of the thickness of the droplets (i.e. amounts of fura-2). Next we illuminated the hippocampal neurones during exposure to L-glutamate and KCl (cf. Figure 4b, c). Note that the $F_{340}$ surpassed the $F_{360}$ during the stimulant exposure. By applying L-glutamate at various concentrations to several neurones, we obtained a relationship between the applied dose of L-glutamate and the peak $[Ca^{2+}]_i$ (Figure 4d). L-Glutamate, as low as 10 nM induced a detectable elevation of $[Ca^{2+}]_i$, revealing a high sensitivity of the present microfluorometrical method. The peak $[Ca^{2+}]_i$ obtained with 100 μM L-glutamate was around 500 nM.

## Discussion

There have been several hypotheses for the mechanism of hippocampal LTP. In both presynaptic and postsynaptic hypotheses, as grossly classified, the $[Ca^{2+}]_i$ level is assumed to play a key role.

**Table 2** Effects of various modifications of the perfusing medium on the fluorescence increases induced by L-glutamate and KCl in hippocampal neurones

| *Modification* | *L-Glutamate*[a] | *KCl*[b] |
|---|---|---|
| $-Ca^{2+}$ | $3 \pm 2 (n = 4)$ | $0 \pm 0 (n = 4)$ |
| $+Mg^{2+}$ | $38 \pm 10 (n = 5)$ | $92 \pm 5 (n = 4)$ |
| $-Na^{+}$ | $51 \pm 9 (n = 5)$ | $108 \pm 22 (n = 4)$ |
| + Nitrendipine | $73 \pm 5 (n = 4)$ | $35 \pm 14 (n = 4)$ |

Fluorescence intensity increase ($F_{340}$ at peak/$F_{340}$ at rest) induced by a perfusion of 10 μM L-glutamate was calculated separately before and after the modification of the medium. Then, the ratio between these increases (after/before) was calculated and presented as % ($\pm$ s.e.) of response remaining after modification[a]. Thus 0% and 100% mean complete and no suppression, respectively. The effects of the medium modification on the KCl-induced response were calculated identically and the results presented similarly[b]. The modified medium containing no L-glutamate (or no elevated KCl) was perfused at least 5 min before the application of L-glutamate (or elevation of KCl). The resting fluorescence level was not significantly altered during this period. $-Ca^{2+}$: addition of 0.1 mM EGTA without addition of $CaCl_2$ to the medium. $+Mg^{2+}$: addition of 3.0 mM $MgCl_2$. $-Na^{+}$: replacement of NaCl with tetramethylammonium chloride. + Nitrendipine: addition of 10 μM nitrendipine to the perfusing medium.

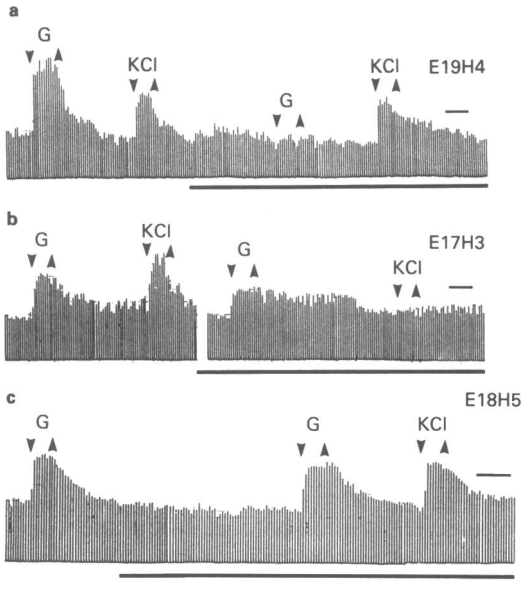


**Figure 3** Determination of $Ca^{2+}$ entry pathway. (a) Effect of $Mg^{2+}$. During the period indicated by a thick bar, the perfusing medium contained 3 mM $MgCl_2$. (b) Effect of nitrendipine (10 μM). The illumination was interrupted for 10 min before the application of 10 μM L-glutamate (G) or KCl (50 mM) to reduce the decomposition of nitrendipine by ultraviolet light. (c) Effect of removal of $Na^+$. The perfusing medium was free of $Na^+$ during the period marked by the bar. Downward and upward arrowheads show beginnings of perfusions with the stimulant-containing and normal medium respectively. Time calibrations indicate 2 min.

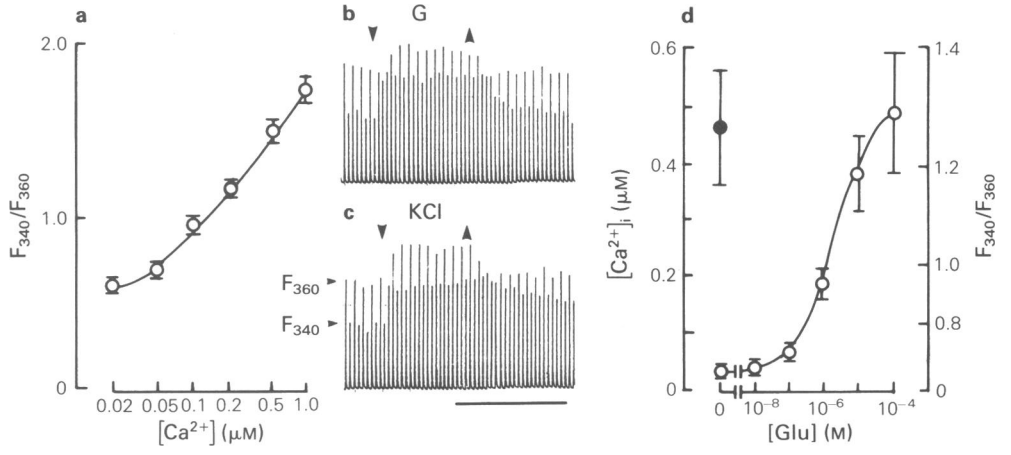


**Figure 4** Quantitative estimation of the $[Ca^{2+}]_i$ elevation induced by L-glutamate and high $K^+$ stimuli. (a) Calibration with fura-2 containing droplets suspended in liquid paraffin. The droplets were illuminated by 340 nm beam and 360 nm beam alternately, and the ratios of the corresponding fluorescence intensities were obtained. Mean values from 5 droplets of different sizes (10–50 μm in diameter) are given with s.e.mean shown by vertical lines. (b) Representative dual beam recording on a single hippocampal neurone. Note that $F_{360}$ was little affected by 10 μM L-glutamate (G), while $F_{340}$ changed greatly and surpassed $F_{360}$. (c) $[Ca^{2+}]_i$ increase induced by 50 mM KCl in the same neurone as that in (b). Upward and downward arrowheads demarcate beginning of perfusions with the stimulant-containing and non-containing BSS, respectively. (d) $[Ca^{2+}]_i$ peak levels (○) as a function of applied dose of L-glutamate (Glu). At zero L-glutamate concentration (○) represents the resting $[Ca^{2+}]_i$ level; (●) shows $[Ca^{2+}]_i$ peak induced by 50 mM KCl. Mean values from 4 neurones (E18H5, E19H7) are given with s.e.mean shown by vertical lines.

In the calpain-fodrin hypothesis for LTP by Lynch and colleagues (see above), the L-glutamate activated $Ca^{2+}$ entry into the postsynaptic neurone was assumed to occur as a first key step. This step was directly and quantitatively verified by the present results. Application of 100 μM L-glutamate increased the $[Ca^{2+}]_i$ of the neuronal soma to 500 nM as an average of whole volume. Although the measurement of $[Ca^{2+}]_i$ in a more limited volume is desired for more detailed analysis (e.g. by the aid of an objective lens of higher magnification and a CRT of larger screen size), it is reasonable to assume a $[Ca^{2+}]_i$ several times higher in the dendritic and submembraneous compartments, which exceeds a threshold $[Ca^{2+}]_i$ for the activation of the $Ca^{2+}$-dependent enzymes including calpain I.

All of the L-glutamate analogues examined induced the $[Ca^{2+}]_i$ elevation. It is noteworthy that NMDA was active since the NMDA-responsive subtype of L-glutamate-receptor is supposed to be mostly responsible for the establishment of LTP (Collingridge *et al.*, 1983; Foster & Fagg, 1984). We applied α-aminoadipic acid and 2-aminophosphonovaleric acid, both known as antagonists of the NMDA-subtype of L-glutamate receptor, and kynurenic acid, a known non-specific antagonist of all subtypes. However, as mentioned above (Table 1), the 'antagonists' *per se* gradually elevated the $[Ca^{2+}]_i$. The mechanism for the 'antagonist'-induced increase in $[Ca^{2+}]_i$ is not known.

The present $Ca^{2+}$ microfluorometry will be more informative when it is combined with the techniques for monitoring other cellular activities such as membrane potential and currents. However, when we inserted a glass microelectrode (filled with 4 M potassium acetate, having a tip resistance of more than 80 MΩ) into a cell loaded with fura-2, the $[Ca^{2+}]_i$ rose to more than 1 μM and showed little recovery in the following 15 min. Therefore, membrane potential should be monitored or controlled by different techniques such as those using voltage-sensitive dyes.

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