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Selective fura-2 loading of presynaptic terminals and nerve cell processes by local perfusion in mammalian brain slice

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We describe a method for filling presynaptic terminals and cell dendrites in adult brain slices with the fluorescent calcium indicator fura-2 by localized perfusion of the acetoxymethyl (AM) ester derivative. The method provides labeling selectivity, similar to that produced by intracellular microinjection of fura-2, with the simplicity of bath application of membrane-permeant AM esters. Application of the method to mossy fiber tracts in hippocampal region CA3 and parallel fiber tracts in cerebellum resulted in distant presynaptic terminals well labelled with fura-2 without concomitant postsynaptic labelling, allowing optical measurements of calcium concentration in individual presynaptic terminals. Application of the method to CA1 pyramidal cells produced intracellular loading of apical dendrites with fura-2. Dendritic calcium changes produced by afferent fiber stimulation were similar to those determined from cells filled with fura-2 by intracellular microinjection. The method appears to be general, and should provide a means to fill projecting axons and dendritic processes in many areas of the brain with fluorescent indicators, allowing optical measurements of ion concentration dynamics to be performed in brain slice that were previously impractical.

Introduction

Optical imaging of fluorescent indicators like fura-2 (Grynkiewicz et al., 1985) is a powerful method for measuring the spatio-temporal dynamics of intracellular ion concentrations. (For a review of fluorescent indicators see Tsien (1989).) Important questions on the role of intracellular ions in cellular physiology, synaptic plasticity, and neural circuit properties can be answered by the application of optical imaging of fluorescent indicators to intact neural systems such as mammalian brain slice.

To apply indicator imaging to mammalian brain slice the indicator molecule must be loaded into

the neurons or glial cells of interest. Two methods have been explored: (1) bulk loading of cells with bath-applied membrane permeant derivatives of the indicator, such as fura-2 AM, and (2) microinjection of the indicator molecule into the cell(s). These two methods differ both in their technical simplicity and the degree to which they provide optical specificity by labelling only the cells or cell processes of interest.

Bulk loading of fura-2 AM is simple in concept and has been widely applied to cultured cells (Connor, 1986; Kudo and Ogura, 1986; Lipscombe et al., 1988; Miller, 1988). In this method fura-2 AM is dispersed in saline using an organic solvent/detergent mixture such as DMSO/Pluronic acid and the labelling solution is bath applied to the cell preparation. The indicator molecule is trapped intracellularly when de-esterification of the membrane permeant derivative by intracellular esterases occurs. Although conceptu-

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ally simple it has been difficult to apply this method to load cells in adult brain slice. For example, although good intracellular loading of cortical pyramidal cells can be accomplished in neonatal and early postnatal rat (Yuste and Katz, 1989), neuronal cell bodies and dendrites label very poorly after P7.

Intracellular microinjection of indicator has been successfully applied to mammalian brain slice but is technically demanding and often impractical. Examples include the study of dendritic calcium accumulations in Purkinje cell dendrites (Ross and Werman, 1987; Tank et al., 1988) and hippocampal pyramidal cells (Regehr and Tank, 1990) following intrasomatic injection of the acid form of fura-2 in brain slice preparations. A similar approach has been taken with squid (Charlton et al., 1982) and crayfish preparations (Delaney et al., 1989) where intra-axonal microinjection of fura-2 acid labels the entire axonal arbor, including synaptic boutons and branches, allowing measurement of calcium accumulations during synaptic transmission. Although an extremely useful method, injection requires delicate intracellular microelectrode techniques that are in some cases impractical. For example, in order to study calcium changes in presynaptic terminals, individual presynaptic cells must be injected with fura-2, the entire length of axon must be located in the plane of the brain slice, and the labelled terminals must rise close enough to the slice surface to be imaged without scattering distortion. Because of the low probability of success in attaining all three conditions experiments like this are impractical.

In addition to the differences of technical simplicity and successful application to mammalian brain slice, an extremely important distinction between bath application and microinjection of indicator molecules is the degree to which they provide labelling selectivity of only the cells or cell processes of interest. Selective labelling allows unambiguous attribution of fluorescence to specific cell structures. In general bath applying fura-2 AM non-selectively labels the entire population of cells. In intact tissue, with all cellular elements labelled, it would be extremely difficult to interpret fluorescent signals and to attribute them to a

particular cell type or substructure. Microinjection implicitly provides selectivity because only the cell of interest is filled with indicator molecule. Although non-selective labelling may be preferable in some experiments, for example determining the spatial geometry of cells that respond to a particular neurotransmitter, there are many cases where specificity is necessary.

We demonstrate here a labelling method that combines the simplicity of bulk labelling methods with aspects of the optical specificity previously obtained only by single cell microinjection. The method is based upon providing a local continuous stream of extracellular labelling solution containing fura-2 AM by micro-perfusion methods. Nerve cell processes near the perfusion site fill with fura-2, overcoming a barrier to intracellular labelling with fura-2 AM that exists in adult brain slice incubated with bath applied label. Intracellularly trapped fura-2 then diffuses, filling cellular processes distant from the perfusion site.

Materials and methods

Slice preparation and maintenance followed standard procedures (Regehr et al., 1989; Schwartzkroin, 1975). For labelling, slices were bathed in a submersion chamber, maintained at 30–32°C, and perfused (1 ml/min) with artificial cerebral spinal fluid (ACSF) equilibrated with 95% O₂ and 5% CO₂. For experiments involving mossy fiber and parallel fiber presynaptic terminals ACSF contained (in mM): 124 NaCl, 3 KCl, 4 CaCl₂, 4 MgCl₂, 26 NaHCO₃, and 10 D-glucose, while for experiments measuring dendritic calcium accumulations in CA1 pyramidal cells ACSF contained (in mM): 124 NaCl, 5 KCl, 2 CaCl₂, 1.6 MgCl₂, 26 NaHCO₃, and 10 D-glucose. Small areas of the slice were labelled with a local perfusion method consisting of a delivery pipette containing labelling solution and a suction pipette as described below. The labelling solution was prepared as follows: (1) 50 µg of fura-2 AM (Molecular Probes, Eugene, OR) was dissolved in a solution of 10 µl 75% DMSO/25% pluronic acid, (2) 240–990 µl of ACSF and 10 µl of 1% Fast Green was added to this solution, (3) this mixture was

vortexed for 1 min, sonicated for 1 min (Laboratory Supply, NY), and then filtered ($0.2\ \mu\text{m}$, Gelman #4192 or $0.2\ \mu\text{m}$ Rainin #38-159mm). The prefiltered solution contained 1–4% DMSO/pluronic acid, and 5–20 μM fura-2 AM. Labelling solution was prepared each day and used within 4 h.

Fura-2 measurements of intracellular calcium concentration were performed using the ratio method (Grynkiewicz et al., 1985) on an inverted epifluorescence microscope (Zeiss IM35) with a computer controlled (MacIIx) cooled CCD camera (CH220, Photometrics, Tucson, AZ) as described previously (Regehr et al., 1989). In order to view the spread of fluorescent label during local perfusion, the normal transmitted light illumination system on the inverted microscope was replaced with a dissecting microscope. Perfusion was performed on the top surface of the slice as described in the text. By illuminating the brain slice from below using the standard epi-fluorescence system of the inverted microscope, but observing the fluorescence through the dissecting microscope, the progress of the local labelling could be followed. For imaging experiments the slices were then turned over and viewed through the inverted microscope. In some cases fiber tracts ran obliquely through the slice and labelled presynaptic terminals following perfusion were best observed on the slice surface opposite to that used for local perfusion.

Results

Localized perfusion of fura-2 AM

Our local perfusion method is schematically illustrated in Fig. 1. The brain slice is contained in a submersion chamber and a delivery pipette with a tip diameter of about $10\ \mu\text{m}$ is positioned on the slice surface in the region to be intracellularly labelled. A larger tip-diameter suction pipette is positioned nearby. The delivery pipette, which contains labelling solution, is first used to clean the surface of the slice by alternating mouth-applied pressure and suction (Edwards et al., 1989). A pressure head of about 15 cm of solution is then applied to the delivery pipette, providing a steady thin stream of labelling solution. Under a dissect-

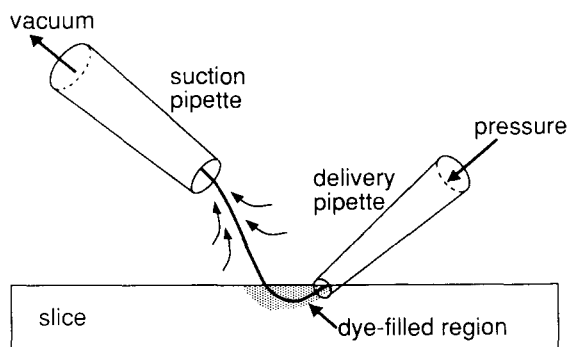


Fig. 1. Localized perfusion of fura-2 in brain slice. A steady stream of labelling solution is pressure ejected from the tip of a delivery pipette positioned at the slice surface in the region to be labelled. This stream flows into the orifice of a larger suction pipette positioned nearby.

ing microscope the position of the suction pipette and its vacuum are adjusted so that the stream of labelling solution (visualized using bright field illumination by the addition of fast green dye, or using fura-2 AM fluorescence) hits the slice surface but flows as a continuous stream into the orifice of the suction pipette. Since most of the dye solution is immediately washed out of the bath, perfusion of the brain slice with fura-2 only occurs locally.

Loading presynaptic terminals by localized perfusion

Application of this method to label presynaptic mossy fiber terminals in region CA3 of the adult guinea pig hippocampus is illustrated in Fig. 2. The mossy fibers are a distinct band of efferents from dentate granule cells that synapse on proximal apical dendrites of CA3 pyramidal cells (Fig. 2b). Large en passant synaptic terminals (approx. $1\text{--}5\ \mu\text{m}$ diameter) are present along the lengths of the mossy fibers (Amaral, 1979; Claiborne et al., 1986).

When the delivery pipette was positioned over the proximal apical dendrites of a group of CA3 pyramidal cells (Fig. 2b), local perfusion of fura-2 AM (30 min at 32°C) labelled both CA3 pyramidal neurons and a band of mossy fibers. Under low magnification ($10\times$ objective, Fig. 2c) fluorescent regions that extended away from the perfusion site were produced in a direction parallel to the apical dendrite and also in a direction parallel to the cell body layer. Although we did not de-

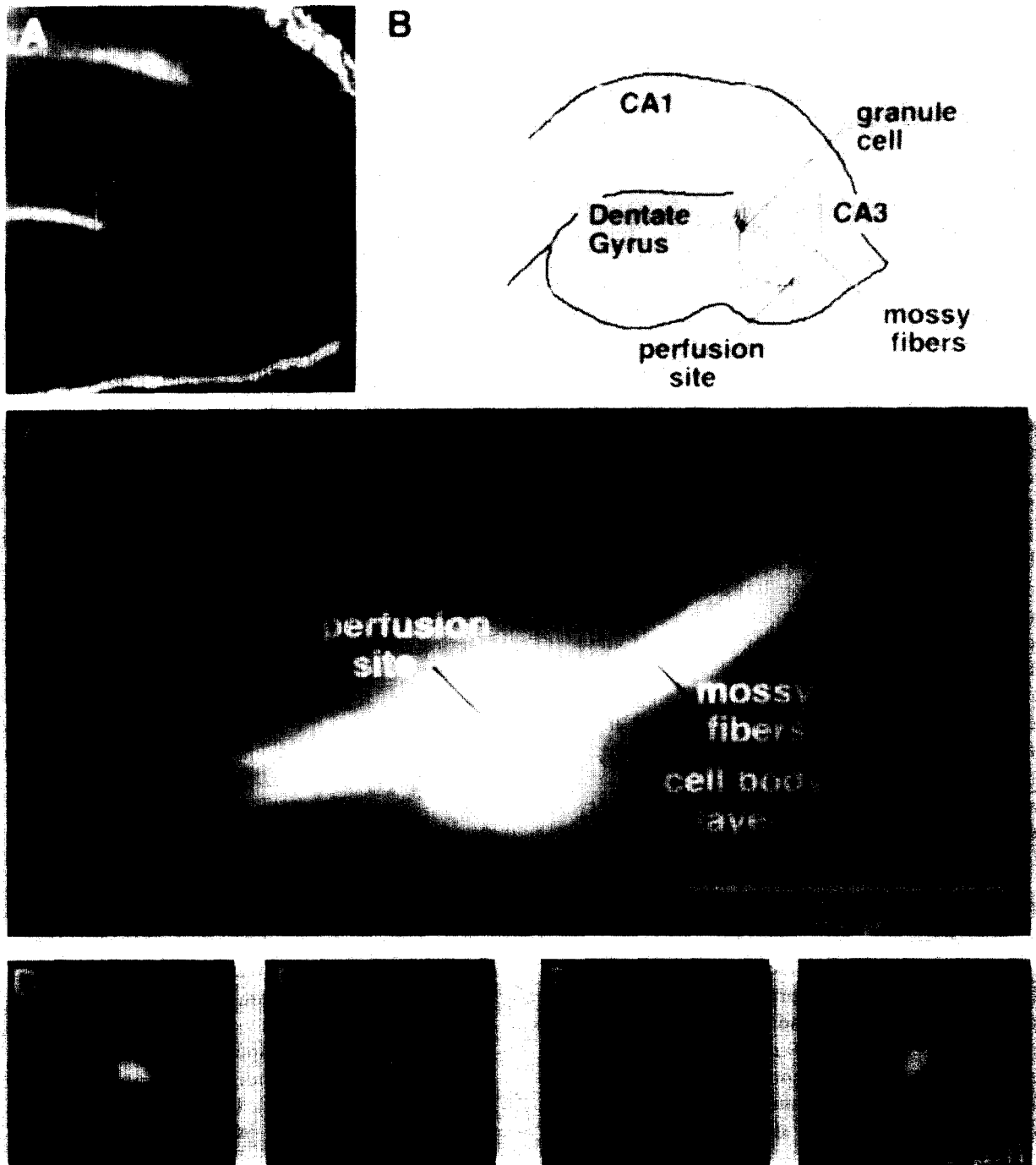


Fig. 2. Fura-2 loading of mossy fibers by localized perfusion. a: low power transmitted light image of hippocampal slice. The box outlines the area shown in (c). b: schematic diagram illustrating the site of localized perfusion in the mossy fiber tract in region CA3. c: low-power fluorescence image montage (380 nm excitation) following localized perfusion of fura-2. The band of fluorescence parallel to the cell body layer is produced by labelled mossy fibers. At high magnification, individual mossy fiber presynaptic terminals are observed (d, e) at a site distant from the localized perfusion area where background fluorescence is minimized and no postsynaptic cell processes are labelled. The morphology of these terminals is similar to that observed (f, g) in individual mossy fiber presynaptic terminals loaded by iontophoretic microinjection of the pentapotassium salt of fura-2 into dentate granule cells.

termine the origin of the fluorescent labelling extending parallel to the apical dendrite, by analogy to experiments performed on CA1 pyramidal cell dendrites (see below) it is likely that it was produced by intracellular diffusion of fura-2 within CA3 cell apical dendrites.

The fluorescent band that extended in the direction parallel to the cell body layer is produced by labelled projections of nerve cell fibers. When

observed under higher power magnification ($40\times$ objective) at a site distant from the perfusion site, the fibers had the characteristic appearance of mossy fibers. As shown in Fig. 2d,e the labelled fibers had large boutons $1-5\ \mu\text{m}$ in diameter that were located en passant along the fibers. To directly demonstrate the morphology of mossy fibers in our slice preparations, dentate granule cells were filled by intracellular iontophoresis of fura-2

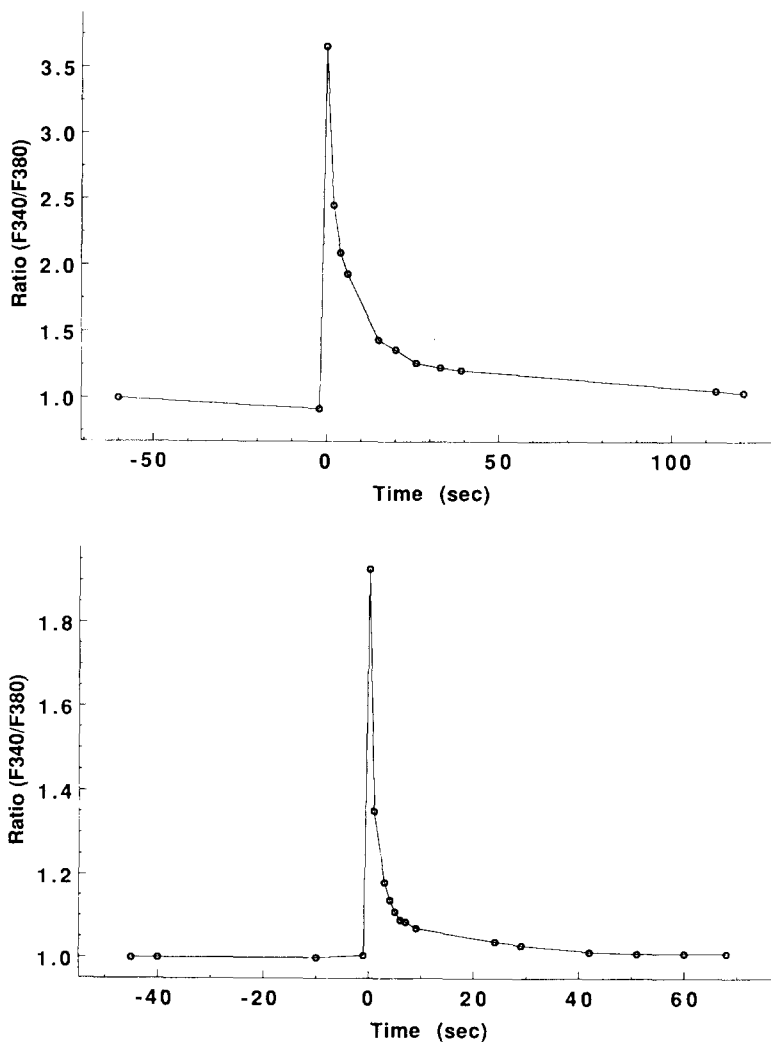


Fig. 3. Fluorescence ratio changes produced by brief tetanic stimulation in fura-2 filled mossy fiber and parallel fiber presynaptic terminals. a: time course of ratio of 340 nm excited fluorescence to 380 nm excited fluorescence measured in individual mossy fiber presynaptic terminals produced by 50 Hz 2 sec electrical stimulation. b: time course of ratio change (340 nm/380 nm) in a population of cerebellar parallel fibers produced by 50 Hz 2 sec electrical stimulation. In both the mossy fibers and the parallel fibers, the decay of intracellular calcium is composed of at least two characteristic decay times and well approximated by a sum of two exponentials.

acid using previously described methods (Regehr and Tank 1990). Although axons of filled dentate cells frequently left the slice or were not located near enough to the slice surface to image in region CA3, in several cases we were able to directly image mossy fiber terminals, labelled by anterograde movement of fura-2 in the granule cell axon. These known mossy fiber terminals were

located in the same dendritic region as those labelled by the local perfusion method and had a similar morphology (Fig. 2f,g). The characteristic morphology and defined anatomical position of mossy fibers, and the similarity between perfusion labelled and iontophoretically filled fibers, together demonstrate that the fibers labelled by local perfusion are mossy fibers.

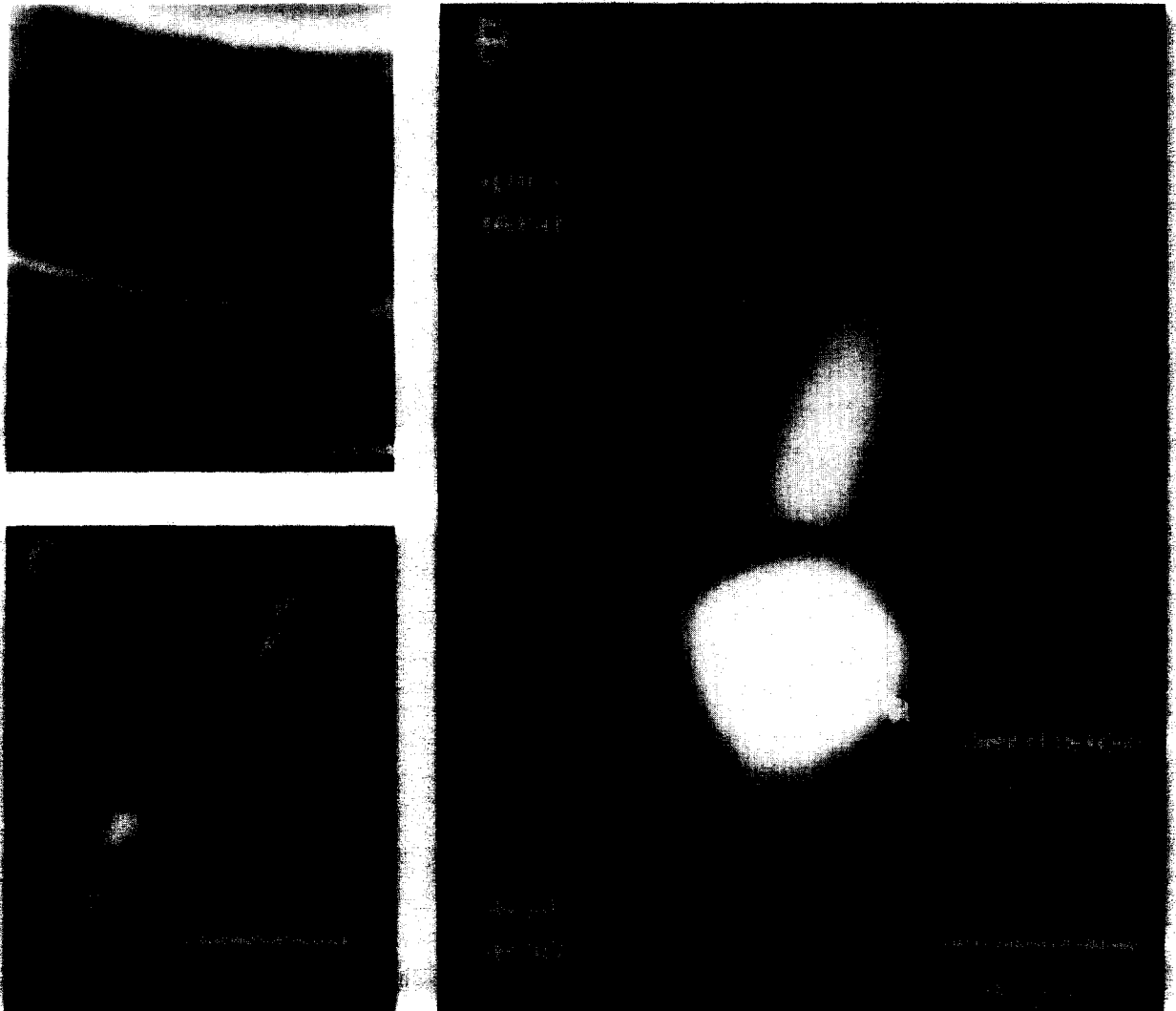


Fig. 4. Fura-2 loading of CA1 pyramidal cell dendrites by local perfusion. A: low power transmitted light image of hippocampal slice. The box outlines the area shown in (B), and the circle indicates the site of local perfusion (s.m., stratum moleculare; s.r., stratum radiatum; s.p., stratum pyramidale; s.o., stratum oriens). B: low power fluorescence image (380 nm excitation) of hippocampal region CA1 following localized perfusion of fura-2 AM in the cell body layer. An expanding sector of fluorescence is observed in the region of the apical dendrites. This fluorescence is produced by CA1 dendrites filled with fura-2. Apical dendrites that rise to the surface of the slice can be individually observed (C) under high magnification.

Calcium measurements determined from fura-2 fluorescence excited at 340 nm and 380 nm by the ratio method were performed on microperfusion labelled and microinjection labelled mossy fibers. Unstimulated terminals had resting calcium levels in the range of 40–80 nM, similar to resting levels measured in CA1 pyramidal cell bodies and dendrites (Regehr et al., 1989). Fibers were stimulated by bipolar tungsten electrodes positioned in the mossy fiber band 400–500 μm proximal to the imaged terminals. For local perfusion labelling, this corresponded to the perfusion site. For both labelling methods tetanic stimulation (50 Hz, 2 sec; 100 Hz, 1 sec) produced a rapid increase in the ratio of fura-2 fluorescence excited at 340 nm to that excited at 380 nm measured in individual terminals. This is illustrated in Fig. 3a for local perfusion labelled terminals. This ratio increase indicates an increase of intracellular free calcium in the presynaptic terminals. The elevated intracellular calcium level decayed following stimulus train termination, with the kinetics of terminals filled by both local perfusion and intracellular injection being indistinguishable. The kinetics, pharmacology, and dependence on PTP/LTP induction will be described elsewhere (Regehr, Delaney and Tank, in preparation). The qualitative characteristics of this calcium accumulation are similar to those previously measured in presynaptic terminals at the crayfish neuromuscular junction (Delaney et al., 1989). The results demonstrate that the local-perfusion-labelled terminals respond to electrical stimulation qualitatively in the manner expected, and that no alteration in calcium dynamics is observed in local perfusion labelled vs microinjected terminals.

Local perfusion also successfully labelled parallel fibers in cerebellar slice. Adult guinea pig cerebellum was sectioned in the coronal plane so that parallel fiber orientation was parallel to the brain slice surface. A band of parallel fibers was selected for labelling by placing the delivery pipette in the Purkinje cell dendrite region, about 100 μm from the cell body layer. Following 30 min of local perfusion and 1 h of post-perfusion incubation, a narrow band of fluorescence was visible perpendicular to the axis of the Purkinje cell dendrites. Under high magnification (40 \times objective),

this band was observed to consist of extremely thin fibers with en passant synapses similar to those reported for parallel fibers (Palay and Chan-Palay, 1974). Electrical stimulation using a bipolar electrode positioned near the perfusion site produced increases in intracellular calcium in fibers several hundred μm from the local perfusion site. In response to brief tetanic stimulation (50 Hz, 2 sec), the fura-2 fluorescence ratio (Fig. 3b) quickly rose and subsequently decayed following termination of stimulation. As observed with mossy fiber stimulation, the decay phase of the ratio was characterized by at least two time constants. Although individual terminals are not imaged in these experiments, the measured fluorescence changes primarily reflect calcium changes in presynaptic terminals, since the intracellular volume of presynaptic terminals comprises about 90% of the volume of parallel fibers (Palay and Chan-Palay, 1974).

Loading dendrites by localized perfusion

Local perfusion also successfully labelled CA1 pyramidal cell dendrites. Following local perfusion of fura-2 AM from a delivery pipette positioned in the cell body layer in region CA1 of adult guinea pig hippocampus (Fig. 4a), an expan-

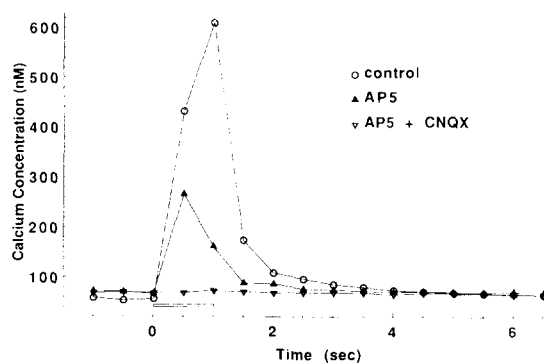


Fig. 5. Fura-2 measurements of intracellular calcium concentration in CA1 cell dendrites loaded by localized perfusion of fura-2 AM in the cell body layer. Following tetanic stimulation (100 Hz, 1 sec) of the Schaffer collateral fibers, an accumulation and decay of intracellular calcium is measured in distal apical dendrites. The accumulation is partially blocked by AP5 and completely eliminated by AP5 + CNQX, demonstrating that it is produced by postsynaptic calcium accumulations. The accumulations are similar to those measured by intracellular injection of fura-2 acid in CA1 pyramidal cell somata (Regehr and Tank, 1990)

ding sector of fluorescence was observed in the apical dendritic region (Fig 4b). Under high power magnification, individual dendrites could be observed (Fig. 4c) if the dendrites came close enough to the slice surface to be optically resolved. The response of dendritic intracellular calcium to afferent fiber stimulation was measured and compared to those previously measured following intrasomatic injection of the pentapotassium salt of fura-2 (Regehr et al., 1989; Regehr and Tank, 1990). Synaptic activation of Schaffer collateral fibers with a brief tetanus (100 Hz, 1 sec) produced a rapid accumulation of intracellular calcium in dendritic areas postsynaptic to stimulated afferents (Fig. 5). In saline containing the NMDA-receptor antagonist AP5 (100 μ M), the response was greatly diminished. In saline containing both AP5 (100 μ M) and the non-NMDA glutamate receptor antagonist CNQX (20 μ M) no changes in intracellular calcium were observed. These results are similar to those previously measured by intracellular microinjection of fura-2 (cf. Fig. 2b, Regehr et al., 1989; Fig. 4Ba, Regehr and Tank, 1990). They demonstrate that the fura-2 fluorescence is produced by indicator located in postsynaptic CA1 pyramidal cell processes, and that localized perfusion loading does not alter the calcium dynamics of labelled cell dendrites relative to those measured by microinjection of indicator.

Discussion

We have described a fura-2 labelling procedure which provides selective intracellular labelling of nerve cell processes distant from a local perfusion site. The technique relies upon the ability to perfuse localized regions of brain slice using an inflow-outflow dual pipette perfusion system. Simple incubation of adult slice in ACSF containing fura-2 AM does not label cell body somata and dendrites (Yuste and Katz, 1989). Since application of the local perfusion method to cell body layers does label, we hypothesize that the stream of solution makes physical access of the solvent dispersed dye molecules to the cell membrane possible. This may be analogous to the methods used to "clean" soma surface membranes of corti-

cal neurons in brain slice in preparation for patch electrode seal formation (Edwards et al., 1989). Following de-esterification-induced entrapment, intracellular diffusion leads to areas of dendrite or axon (synaptic terminals) loaded with indicator in tissue regions containing no other labelled structures, similar to that obtained with intracellular injections.

The response properties of fura-2 loaded presynaptic terminals and CA1 pyramidal cell dendrites to electrical stimulation suggest that the local perfusion method does not damage the labelled neurons. Preliminary experiments do indicate, however, that fura-2 fluorescence ratios for cells at rest are slightly elevated at the cell soma compared to levels measured by fura-2 acid microinjection. Whether or not this difference is due to loading of internal organelles by AM fura-2 that are not labelled by fura-2 acid injection, background errors due to extracellularly bound fura-2 molecules, or physical injury to the cell somas produced by the perfusion procedure is unknown at present. At dendritic and axonal sites distant from the perfusion sites, we have no evidence for altered cell physiology.

The application of the local perfusion methods will make it possible to measure intracellular calcium concentrations in presynaptic terminals in brain slice that cannot be selectively labelled by other methods, or that can only be selectively labelled with great difficulty. For example, although granule cell axons (mossy fibers) originating from the dentate gyrus can be loaded by intracellular injection of fura-2, the method is often impractical because the success rate of labelling terminals in the mossy fiber layer of CA3 that are amenable to high resolution imaging (i.e., within about 50 μ m of the slice surface) is quite low. The local perfusion method, however, makes experiments such as that illustrated in Fig. 3 quite easy. Our ability to also label cerebellar parallel fibers suggests that this method is general, and that it will provide a practical method of labelling projecting axons in many areas of the brain.

The loading method we have described could potentially be adapted for other uses. In principle the method is not restricted to use with fura-2 and could be used with other membrane-permeant

chemically-trapped molecules that are difficult to load in adult neurons by simple microinjection. It may also be possible to extend this method to label neurons in vivo.

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