Monitoring of Intracellular Ca^{2+} Elevation in a Single Neural Cell Using a Fluorescence Microscope/Video-Camera System

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Abstract—For monitoring the changes in intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$), we developed a simple system combining a fluorescence microscope, an image intensifier, a video-camera, a cathode ray tube display and a photodiode, employing quin2 as a Ca^{2+} indicator. We recorded increases of the fluorescence intensity due to $[\text{Ca}^{2+}]_i$ rises, when high K^+ medium, neurotransmitter and Ca^{2+} ionophore were applied to the single cells of nervous system origin in culture. The present system is capable of simultaneous detection of the $[\text{Ca}^{2+}]_i$ changes from multiple separate cells.

It is known that various cellular activities are regulated by cytosolic free calcium ion concentration ($[\text{Ca}^{2+}]_i$) (1). In the nervous system, especially in neurons, release of neurotransmitter depends on the Ca^{2+} fluxes from the environment into nerve terminals. Hence, when the degree of Ca^{2+} influx is affected by a certain factor, the magnitude of $[\text{Ca}^{2+}]_i$ rise and thereby the signal transmission efficiency are altered. Elementary learning behavior in invertebrates (2, 3) is one of the best analyzed examples, and presynaptic inhibition by enkephalin in mammalian spinal neuron is thought to share the equivalent mechanism (4). Neuronal $[\text{Ca}^{2+}]_i$ is postulated to play a critical role in modulating the transmission efficiency in the postsynaptic component as well (5).

The glial cell, another member of the nervous system, has been recently reported to possess Ca^{2+} entry systems (6, 7), whose roles in the regulation of glial functions are debated.

In analyses of these phenomena, it is essential to directly examine the $[\text{Ca}^{2+}]_i$ changes. Among various Ca^{2+} indicators developed for this purpose, a fluorescent Ca^{2+} chelator quin2 (8) is being extensively used,

since this dye is incorporated by the cell without using a microinjection procedure and thus is applicable to small cells like mammalian lymphocytes (9). Since the signal-to-noise ratio of the Ca^{2+} fluorometry using quin2 is low when compared with the methods using light-emissive Ca^{2+} -binding proteins, quin2 was generally applied to cell suspensions, where the signal is an averaged response from tens of thousands of cells.

However, it is impossible to obtain a homogeneous cell suspension from the brain, which is a collection of heterogeneous populations of neuron and glia. So it is desirable to develop a method which can monitor the signals of $[\text{Ca}^{2+}]_i$ from a single individual cell. A few methods of this have so far been reported (10–13), where the total emitted light from the visual field of a fluorescence microscope (whether or not limited by an inserted diaphragm) was detected by a photomultiplier.

We describe here a new technique with a relatively simple combination of all commercially available devices that enables $[\text{Ca}^{2+}]_i$ monitoring from multiple desired loci (multiple cells, or, in principle, multiple sites within a cell).

Materials and Methods

Cells: In the present experiment, cells of mouse neuroblastoma $\times$ rat glioma hybrid NG108-15 line (14, 15) and rat glioma C6BU-1 line (14, 16) were used, both of which are widely used cell lines expressing mammalian neuronal and astroglial properties, respectively (17). The cells were maintained in 95% Dulbecco's modified Eagle's medium (Gibco)/5% precolostrum newborn calf serum (Mitsubishi Chem. Ind.) as described previously (7, 18). Two to three days prior to the experiment, the cells were plated at a low density (around 10^4 cells/cm²) on a thin glass coverslip (Matsunami Glass, 40 mm $\times$ 50 mm, thickness #1) enclosed with a silicon-rubber wall (Heraeus; Flexiperm with bottom sheet removed).

Quin2 loading: After replacement of culture medium with a basal salt solution (BSS, composed of 130 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgCl₂, 5.5 mM glucose and 20 mM HEPES-10 mM NaOH-HCl pH 7.3, 37°C), 50 μ M quin2/acetoxymethyl ester (Dojin Chem., stored in 50 mmol/l dimethylsulfoxide) dispersed in BSS was introduced. Thirty minutes (37°C) later, the cells were washed twice with BSS not containing quin2 and allowed to lie still at 37°C for more than 30 min (during this period, quin2/acetoxymethyl ester was hydrolyzed to quin2 free acid by the cell's esterase). Then the coverslip carrying the quin2-loaded cells was mounted on a Nikon TMD inverted fluorescence microscope. With these loading conditions, intracellular quin2 concentration was estimated to be 0.8~2.5 mM as determined by fluorescence titration (9).

Microscope/video-camera system: The recording setup is diagrammatically shown in Fig. 1. Ultraviolet light from a xenon lamp (Ushio Elec., Inc.; 500W) was filtered by an interference filter (Hoya Corp.; 37% transmittance at 340 nm, half value width $\pm$ 10 nm) and conducted to the specimen through a dichroic mirror and an epifluorescence objective (Nikon Fluor $\times$ 20). After filtration of the emitted light by a Nikon sharp-cut filter ($<$ 450 nm), the image formed was displayed on an 11-inch cathode ray tube (CRT,

Fig. 1. Diagrammatic presentation of the present system. a, xenon lamp; b, shutter; c, filter for excitation light; d, dichroic mirror; e, filter for emitted light; f, specimen; g, objective lens; h, image-intensifier; i, video-camera; j, cathode ray tube display; k, optical fiber(s); l, video-tape recorder; m, photodiodes; n, integrator; o, pen recorder.

Hamamatsu Photonics Co., C-1846) screen by an image-intensifier (H.P.C., C-1525) and a video-camera (H.P.C., C-1000). Viewing the display on the CRT, a 1 mm ϕ plastic optical fiber (Mitsubishi Rayon Ind., Super Eska SH-4001) was placed vertically to the image of a cell ($\sim$ 5 mm ϕ) on the CRT screen by the aid of a magnet. Then the CRT was covered with a dark box to eliminate stray light. To the other end of the optical fiber, a photodiode (H.P.C., S-874) was connected, whose output current was converted to voltage. Recording was done by a pen-recorder (Yokogawa Elec., Model 3047) coupled with an integration circuit (this was needed to sustain the photodiode output signals which were delivered discretely at 1/60 sec intervals). The contrast control of the video-camera was set so that the photodiode output was null when the light cable was placed at a cell-free area (background subtraction). The gain of the image-intensifier was fixed so that the photodiode output did not saturate at the maximal brightness. Occasionally, the CRT image was recorded by a video-tape recorder (Toshiba Elec. Ind., V-D4).

To minimize bleaching of quin2, a shutter installed in front of the excitation filter was kept closed during the preparation pro-

cedures. In case of a long-duration recording, the shutter was opened periodically (e.g., for 1 sec after 9 sec closure), where an envelope of the intermittent signals gave the $[Ca^{2+}]_i$ signal.

Drug application: In the chamber carrying the specimen, two polyethylene capillary tubings were placed through which the BSS including stimulants was perfused. Stimulants used here were 50 mM KCl (osmotically counterbalanced by reduction of NaCl), 1 μ M serotonin creatinine sulfate (Sigma) and 1 μ M ionomycin (Calbiochem., stored in 1 mmol/l dimethylsulfoxide).

$[Ca^{2+}]_i$ recording in cell suspension: The same clonal cells plated and grown on 100 mm plastic dishes (Corning) were suspended in BSS. Loading of quin2 and $[Ca^{2+}]_i$ measurement in a spectrofluorometer were done as we described perviously (7).

Results

A typical trace of the photodiode output is shown in Fig. 2a, where a single NG108–

Fig. 2. Fluorescence enhancement in NG108–15 neuronal cells exposed to K^+ -elevated (50 mM) medium, recorded in the present single-cell system (a) and in the conventional cell-suspension system (b). In a, the dot indicates the beginning of perfusion of the high K^+ medium. The cell was continuously subjected to excitation light. Upward deflection of the trace corresponds to a fluorescence increase (F). In b, the dot indicates the addition of 40 μ l of 2.25 M KCl in the cuvette containing 2.0 ml of cell suspension (in the suspension recording, osmotic compensation was not included). A transient fluorescence surge at the moment of KCl addition is an artifact due to a contamination of stray light. Cytosolic free Ca^{2+} levels (nM) calculated are shown at vertical calibration. Time calibration is valid for a and b.

15 cell was stimulated by an elevation of extracellular K^+ . When the specimen was continuously exposed to the excitation light, a decay of the photodiode output signal was prominent. However, superposed on the decay, a clear increment of the output signal was recognized. This increment closely paralleled the rise of fluorescence intensity detected by the conventional quin2 fluorometry using a cell suspension (Fig. 2b). So we concluded that the present system faithfully transmitted the change in the original fluorescence signal that resulted from the $[Ca^{2+}]_i$ alteration. When the perfusing medium was deprived of Ca^{2+} , the fluorescence increase was abolished (not shown here). A short delay between onsets of the high K^+ perfusion and the signal increment might be due to a late arrival of the

Fig. 3. Fluorescence enhancement in C6BU-1 glial cells exposed to serotonin (1 μ M)-containing medium, recorded in the single-cell system (a) and in the cell-suspension system (b). In a, excitation ultraviolet light was given intermittently (1 sec illumination after 9 sec pause); therefore an envelope of the signal peaks expresses the fluorescence alteration. The dot indicates the beginning of stimulant perfusion. In b, the dot indicates the addition of 20 μ l of 0.1 mM serotonin in the cuvette containing 2.0 ml of cell suspension. Calculated values of $[Ca^{2+}]_i$ are shown at the side (nM).

Fig. 4. Recording from multiple cells by means of video-tape reproduction. C6BU-1 cells were stimulated once by a perfusion of serotonin ($1 \mu\text{M}$)-containing medium. Digits 1–6 beside the cells in a phase-contrast photomicrograph (a) correspond to six fluorescence traces shown in b.

stimulant, since the tubings for perfusion was placed rather distantly to minimize the noise caused by a solution-surface turbulence.

For monitoring of a longer time span, an intermittent excitation was adopted to reduce the rate of quin2 bleaching. Figure 3a showed the fluorescence signal from a single C6BU-1 glial cell during a 10 min exposure to $1 \mu\text{M}$ serotonin. As reported previously, this glial cell line responds to applied serotonin by an $[\text{Ca}^{2+}]_i$ elevation due to an activation of serotonin-specific receptors (7). This response was fully reproduced in the single-cell fluorometry (Fig. 3b). The elevation was totally abolished by $0.01 \mu\text{M}$ metergoline (not shown here).

Recording of the $[\text{Ca}^{2+}]_i$ signals from multiple cells was done either by reproducing the cell images repeatedly from the video-tape (Fig. 4) or by increasing the number of photodiodes placed on the CRT screen (Fig. 5). The $[\text{Ca}^{2+}]_i$ behavior of six C6BU-1 cells exposed to $1 \mu\text{M}$ serotonin was shown together with a photomicrograph of the corresponding cells in Fig. 4. Interestingly, each cell responded slightly differently (rates of fluorescence increase and decrease), though the cells were placed closely and thus stimulated identically.

Traces a and b in Fig. 5 are the fluorescence intensity changes recorded from two single NG108–15 cells identified on the CRT screen and exposed to the BSS containing $1 \mu\text{M}$ ionomycin, a known Ca^{2+} ionophore (19).

Fig. 5. Fluorescence enhancement in NG108–15 neuronal cells exposed to ionomycin ($1 \mu\text{M}$)-containing medium, recorded simultaneously from two single cells in the present system (a, b) and in the suspension system (c). In a and b, the intermittent excitation was done as described in the legend of Fig. 3. Perfusion of ionomycin-containing medium was begun at the dots, and MnCl_2 (final concentration 3 mM) was added at the triangles. In c, an addition of ionomycin was done as explained in Fig. 3. Calculated values of $[\text{Ca}^{2+}]_i$ are shown on the side (nM).

The drug caused a steady Ca^{2+} influx into NG108-15 cells. In this case, two cells located at a distance of about 200 μm from each other showed simultaneous elevations of the $[\text{Ca}^{2+}]_i$ level. When 3 mM Mn^{2+} was added to the medium, the fluorescence was quenched presumably due to penetration of Mn^{2+} and to formation of Mn^{2+} /quin2 complex. The equivalent fluorescence rise upon the exposure to ionomycin was recorded in the conventional suspension recording (Fig. 5c). In the single-cell recording, there occasionally occurred a sudden decrease of the fluorescence signal several minutes after the signal reached its peak (not shown here). Since this was never observed in the suspension recording, we supposed that the signal fall was due to dispersion of quin2 upon the cell's puncture by an excess Ca^{2+} entry. In the suspension system, the puncture of the cells did not affect the signal, since the number of fluoroprobe molecules within a light path does not change upon cell puncture.

Discussion

Our present system is capable of monitoring the changes of $[\text{Ca}^{2+}]_i$ in single cells. The primary advantage of our system in comparison with those of previous authors is multiplicity of the recording site. We demonstrated here a simultaneous recording of the fluorescence signals from two separate cells. Reproduction of the CRT image recorded on a magnetic tape will allow a multiple-site monitoring equivalent to the real-time one, as demonstrated in Fig. 4. Such a recording can hardly be done by the method which directly measures the emitted light intensity by a photomultiplier, since the choice of the recording site was usually done by placing the specimen in the center of the visual field and by closing of a field-diaphragm. If the emitted fluorescence is sufficiently strong and steady, our system enables the recording from multiple loci within a cell.

The second advantage is the freedom of choice of the recording site. This is much more limited in the direct photomultiplier method. Actually, some of the previous authors have adopted the videocamera/CRT display in single-cell fluorometry (11-13). However, in these methods the CRT's were

used supplementarily to give a wide view of the visual field and were not connected to photometrical devices.

The third advantage of the present system is its relative ease to subtract the background level of fluorescence and to amplify the signal after the subtraction by electronic modulation circuitries of the image-intensifier and the video-camera.

On the other hand, we should point out problems in our system that remain to be improved. The first is its low time-resolution. From the video-camera's scanning nature, the sampling interval cannot be shorter than 1/60 sec (i.e., 60 pictures are delivered in 1 sec). However, in many instances, the $[\text{Ca}^{2+}]_i$ alterations take place relatively slowly so that the time-resolution of our system should be good enough to follow them (e.g., in a skeletal muscle twitch, the $[\text{Ca}^{2+}]_i$ peaks around 100 msec after the action potential ignites (20)).

The second problem is that the present system monitors the $[\text{Ca}^{2+}]_i$ changes only qualitatively. To know the absolute value of $[\text{Ca}^{2+}]_i$ in the conventional cell suspension system, detergent is added to disrupt the cells to release the trapped quin2 to the environment (9). This calibration is based on the nature that the number of quin2 molecules in the light path does not depend on whether the dye is trapped in the cell or not. This does not hold true in the single-cell system. An alternative way to estimate the absolute $[\text{Ca}^{2+}]_i$ is to measure the emission intensities under excitation light of two different wavelengths (e.g., 340 nm and 360 nm). The ratio, irrespective of the amount of the dye, indicates the $[\text{Ca}^{2+}]_i$ level (11). This is applicable to the single-cell system, and we are presently developing a 2-wave excitation device.

The third problem is a reverse aspect of the third advantage described above. In case the electronic modulations are applied to the primary fluorescence signal, the final output becomes unproportional to the actual emitted fluorescence. This might be critical in a quantitative measurement, where the absolute values of fluorescence intensity are needed. However, in a preliminary examination, the correction to parallelize the

photodiode outputs to the original fluorescence intensities caused no difficulty.

Quite recently, a new fluorescent Ca^{2+} indicator with high and stable fluorescence performance (fura-2) became available (21, 22). We have confirmed the advantage of fura-2 in a preliminary examination (not shown).

At present, our system allows the qualitative monitoring of $[\text{Ca}^{2+}]_i$ changes. Although remodeling of the system for a quantitative measurement is under way, the qualitative recording is already of substantial value in the study of neural cells to which the suspension-style measurement is not applicable. The present system can be coupled with other types of measurements such as a micro-electrode examination of membrane potential or a cytosolic pH determination with fluorescent pH indicators (10).

During preparation of this manuscript, a sophisticated method for single-cell quin2 fluorometry employing a video-camera and a digital image computation was published (23). Though the principle of the fluorometry was the same as ours, ours is still superior to that one in the aspects of applicability to small cells, ease in choosing the measuring sites, and its relative inexpensiveness.

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