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SHORT COMMUNICATION

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Real-time measurements of calcium dynamics in neurons developing in situ within zebrafish embryos

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Abstract We have developed a non-invasive technique to measure intracellular calcium ($[Ca^{2+}]_i$) in neurons growing within intact embryos of the zebrafish (*Danio rerio*). A single blastomere was injected with a calcium-sensitive fluorescent dye (Calcium Green dextran) between the 32- and 128-cell stage and the embryo imaged between 16 h and 20 h postfertilisation using laser scanning confocal microscopy. Labelled nerve cells from embryos preinjected with dye and dissociated at 16 h showed a fluorescence increase (66±22%; n=11) in response to depolarisation with KCl confirming that the dye remained intracellular and was sensitive to calcium. In addition, fluorescence changes in activated muscle cells of intact embryos showed that the dye was capable of responding to $[Ca^{2+}]_i$ changes in vivo. Imaging of dye loaded cells over 30-min periods in embryos between 16 and 20 h revealed that the majority of neurons within the brain and spinal cord did not show spontaneous fluorescence changes distinguishable from noise. However, a subset of neurons within the ventral spinal cord exhibited spontaneous, repetitive $[Ca^{2+}]_i$ oscillations which may have a functional significance during neuronal development.

Key words Calcium oscillations · Development · In situ · Intracellular calcium ($[Ca^{2+}]_i$) measurement · Neurons · Zebrafish

Introduction

One of the major morphological changes that occurs during neuronal development is neurite extension, and calcium ions play a central role as an intracellular signal during this outgrowth [10]. However, the multiple effects of $[Ca^{2+}]_i$ on neuronal motility in vitro makes it difficult to form hypotheses about which of these mechanisms are important during nervous system development within an intact embryo. Only calcium measurements from neurons developing in situ can resolve this question.

To date there have been relatively few studies that have explored calcium activity in nerve cells growing in situ. Bentley and colleagues injected Ti1 pioneer neurons within grasshopper embryonic limb buds with Fura-2 and measured $[Ca^{2+}]_i$ as they underwent axogenesis. Ti1 neurons in the early stages of axogenesis exhibited a small spatial calcium gradient, highest at the growth cone; however, when the growth cone contacted a guidepost cell this pattern changed [1]. Changes in $[Ca^{2+}]_i$ have also been recorded in neurons in isolated embryonic *Xenopus* spinal cord incubated in Fluo-3 AM. The spontaneous $[Ca^{2+}]_i$ transients observed could be divided into two patterns, termed "spikes" and "waves", which were proposed to be involved in neuronal differentiation and neurite extension respectively [4]. In addition, fluorescence imaging of Fura-2 loaded rat neonatal cortex slices revealed spontaneous $[Ca^{2+}]_i$ rises in groups of coactive neurons [12]. Similarly, neurons in isolated chick spinal cord loaded either with Fura-2 AM or retrogradely filled with calcium-sensitive dextran-conjugated dyes showed synchronous, rhythmic fluorescence signals [8]. Although the role of these $[Ca^{2+}]_i$ transients is unknown the importance of simultaneous activity in strengthening synapses in the developing and adult brain suggests that these phenomena may be important in establishing the pattern of connectivity in the spinal cord and neocortex.

The studies described above imply that $[Ca^{2+}]_i$ does indeed play a role in neuronal development in vivo, and that $[Ca^{2+}]_i$ dynamics under normal physiological conditions may be very different from the situation in the simple models studied in culture. To address this question further we have developed a non-invasive technique to observe $[Ca^{2+}]_i$ changes during the outgrowth of identified neurons within intact embryos of the zebrafish (*Danio rerio*). These embryos make a good model because they have the advantage of being transparent, their

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anatomy has been extremely well characterised, and it is easy to label single cells without disturbing their gross development [5, 6].

Materials and methods

Fish maintenance

Zebrafish (*Danio rerio*) were purchased from a local pet store and maintained as described in Westerfield [11]. Twenty-five fish (males and females) were kept in 45 l tanks containing de-ionised water with added sea salts (60 mg/l) and held on a 14 h light and 10 h dark cycle. Eggs were collected at 10 a.m. from each tank at the beginning of the light cycle and washed in embryo medium composed of: 13.7 mM NaCl, 0.54 mM KCl, 25.2 μM Na_2HPO_4, 44.1 μM KH_2PO_4, 1.3 mM $CaCl_2$, 1 mM $MgSO_4$, 10 mM HEPES, 4–5 drops Methylene Blue, pH 7.2. All subsequent manipulations were performed in embryo medium unless stated otherwise. Staging of the embryos was performed as described by Kimmel and colleagues and are given in the text as standard developmental time (hours) [6, 11].

Dye injection of embryos

The eggs were transferred to dishes with a 1% agar bottom and dechorionated between the 2- and 8-cell stage using two pairs of fine forceps under a dissecting microscope (30×, Vickers Instruments, UK). The embryos were transferred with a fire polished Pasteur pipette to an agar (1%) injection dish on a microscope [Nikon upright Labophot 2 Microscope with Plan 10× objective (NA 0.3) fitted with a 100 W UV lamp and fluorescein filter set]. Pipettes were drawn from thin-walled borosilicate glass micropipettes (Clark Electromedical) to give a resistance in the range of 100 MΩ, and were backfilled at the tip with approximately 0.2 μl prefiltered (Ultrafree-MC filter units, 0.22 μm, Millipore) dye, either 20 mM Calcium Green-1 dextran or fluorescein dextran (mol. wt. 10,000, Molecular Probes), in 130 mM KCl. To inject the blastomere the pipette was positioned on the cell surface and a short hyperpolarising current applied, which resulted in penetration of the cell. One to three short puffs of dye were injected at a pressure just below 2 bar until the outline of the cell was evident and then the pipette was rapidly retracted. The embryos were visually checked 15 min later to ensure that the dye had been retained and that the cell had survived.

To obtain embryos containing isolated labelled daughter cells dye was injected into a single blastomere between the 32- and 128-cell stage when the majority of blastomeres are completely cleaved (Fig. 1b) [6]. During the gastrula period (5–10 h) and later cells undergo extensive migration and re-arrangement so that labelled cells are typically found throughout the resulting embryo (Fig. 1c–e). A slight modification was used to label all the cells of an embryo for subsequent dissociation and cell culture. A single blastomere was injected before the 8-cell stage, when the cells remain interconnected via cytoplasmic bridges allowing equilibration of dye throughout the embryo. Successfully injected embryos were transferred to multiwell plates with a 1% agar bottom containing embryo medium with 100,000 U/l penicillin, 0.25 mg/l amphotericin and 100 mg/l streptomycin, pH 7.2 and incubated in the dark at 28°C until the desired developmental stage [6].

In vivo calcium imaging using confocal microscopy

To image intracellular fluorescence embryos were embedded in 1% agar (low melting point, Sigma) in a Petriperm dish (Heraeus, UK) and orientated horizontally so that the brain and spinal cord could be viewed laterally. After 16 h the zebrafish exhibit their first muscular contractions, thus to prevent gross movements during imaging embryos were anaesthetised in 2 mM Tricaine in embryo medium. The dishes were then placed on the stage of a confocal laser scanning microscope (CLSM, Leica, Germany). Images were acquired using the 488 nm line of an argon laser for excitation and viewed through a Nikon 20× objective (NA 0.75). Time series measurements were made with the emission pinhole fully open. Under these conditions 90% of the measured signal originated in a 3 μm thick slice of the embryo, as estimated by reflectance from a plane glass surface. Each time point comprised a series of images from different focal planes which, unless stated otherwise, was five images at 3 μm separation. After ensuring that the stack of images contained the cell and its axon completely it was summed to produce an "extended focus" image. Under this protocol large movements of the cell relative to the stack were detected as a failure of the cell to lie within the stack and the data set was discarded; small movements of the cell will alter the relative contribution of individual images to the extended focus image but will not affect its final intensity. Images were further analysed using Global Lab Image (Data Translation, Marlboro, Mass., USA) and custom software.

In vitro cell dissociation experiments

Embryos, labelled with Calcium Green dextran before the 8-cell stage and allowed to develop to 16 h, were transferred to calcium-free dissociation medium and washed several times, before being left for 15 min at room temperature. The embryos were dissociated by gentle milling between a glass slide and coverslip and the cell suspension was centrifuged at 300 *g* for 7 min. The cell pellet was resuspended in growth medium comprising L-15 Medium Leibovitz supplemented with 3% fetal calf serum and 100,000 U/l penicillin, 100 mg/l streptomycin, 0.25 mg/l amphotericin before being centrifuged again. The pellet was resuspended in growth medium to make a final concentration of 15 fish/ml and the cells plated on to polylysine-coated (0.1 mg/ml) Petriperm dishes. The dishes were incubated for 2–4 h at 28°C before being transferred to the stage of a confocal microscope as described above. Experimental solutions perfused using a diamond-shaped perfusion chamber (RC-26, Warner Instruments, Conn., USA) sealed onto the dish with silicone grease. The cells were viewed through a 100× Nikon objective (glycerol, NA 1.3) and scanned every 320 ms. For the experiment, a number of control images were acquired and then the cells were depolarised with a solution containing 130 mM KCl, 2 mM $CaCl_2$, and 5 mM HEPES in a 10% dilution of the growth medium.

Results

Confirmation of the technique

Examination of embryos between 16 and 24 h revealed Calcium Green dextran labelling randomly distributed in all regions, including notochord, muscle, skin and spinal cord, indicating that the developmental choices available to the injected cell and daughters were not grossly curtailed by injection of dye into blastomeres at an earlier stage, as is consistent with previous reports [5].

To test that the Calcium Green dextran within neurons was still functioning as an intracellular calcium-sensitive dye after incubation, embryos preinjected with Calcium Green dextran were dissociated at 16 h and the cells allowed to differentiate in culture for 2–4 h. Nerve cells displaying neurites and growth cones were identified in culture and fluorescence changes before and after depolarisation with KCl were monitored. After KCl addition the fluorescence increased by 66% (±22%, n=11), confirming that the dye was still responsive to changes in $[Ca^{2+}]_i$ (Fig. 1g).

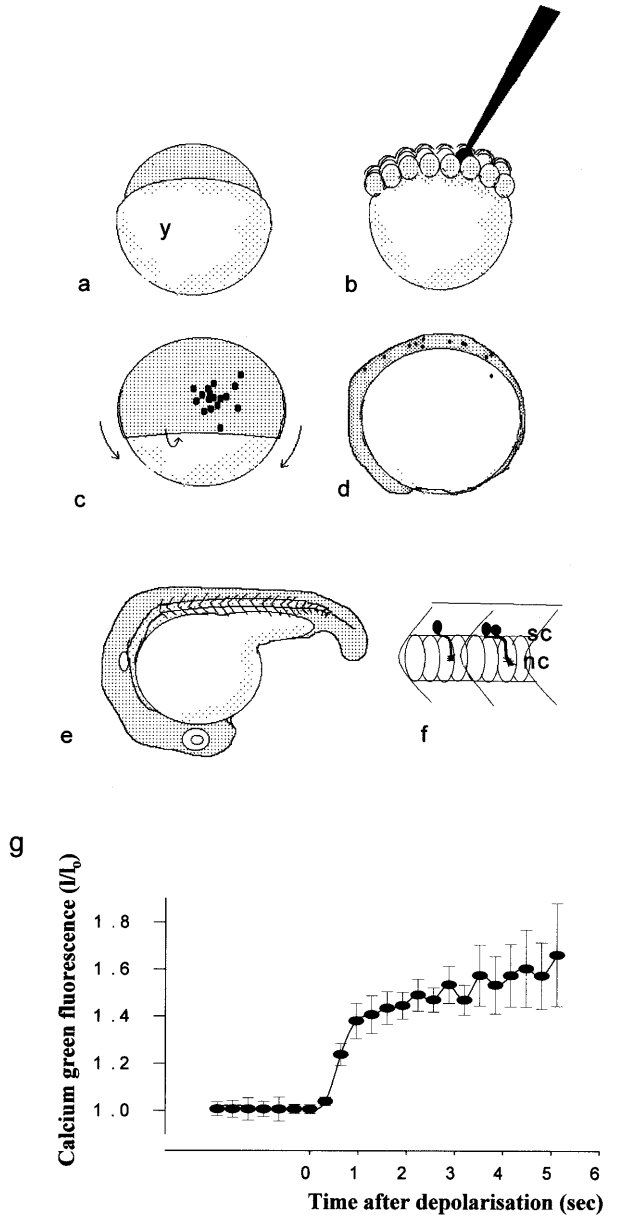

Fig. 1a–f Key stages in early zebrafish development. **a** The one cell stage (0 h) (*y* Yolk). **b** The 32-cell stage (1.75 h). Calcium Green dextran is injected into a central blastomere. **c** 70% epibole (approx. 7 h). During development the blastomere cells get smaller as they divide and migrate over the yolk cell. During epiboly and involution cells of one clone are widely scattered and contribute to both germ layers. **d** Bud stage (10 h). **e** At the 20-somite stage (19 h) the primary sensory (trigeminal ganglion neurons and Rohon-Beard neurons) and motoneurons (**f**) have started growing axons. (*sc* Spinal cord, *nc* notochord.) **g** Fluorescence changes in Calcium Green dextran-labelled neurons depolarised with KCl. Neurons, cultured from dissociated zebrafish embryos (16 h) pre-injected with Calcium Green dextran, were depolarised with a bathing medium containing 130 mM KCl, 2 mM $CaCl_2$, and 5 mM HEPES in 10% growth medium. Images were acquired every 320 ms (n=11±SEM)

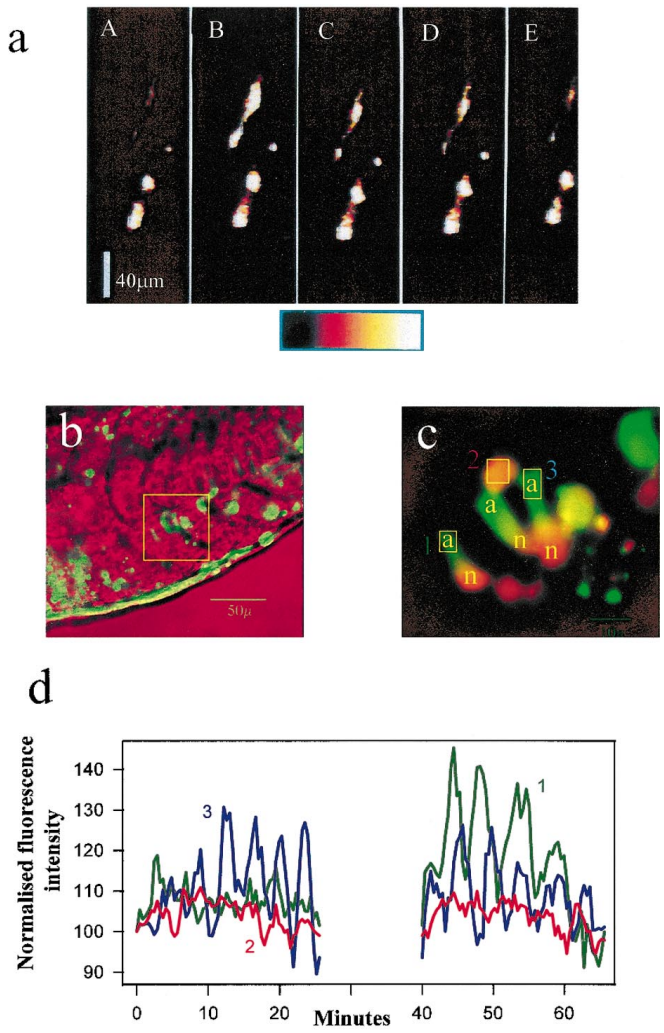

Fig. 2 a Confocal fluorescence images of Calcium Green dextran-labelled muscle cells in an embryo (20 h). Muscle contraction associated with escape behaviour was triggered by gently warming the bathing medium. The *images A–E* are taken at 180 ms intervals. Movement of the body is visible as a downward movement of a small centrally labelled cell. Immediately before this movement at a time when $[Ca^{2+}]_i$ would be expected to be increasing, the fluorescence of the upper muscle cell increased dramatically. In order to achieve high resolution in this measurement we did not acquire image stacks, rather the microscope was focused such that the intensity from the upper muscle was maximal in the resting embryo. Under these conditions movement of the embryo could produce an artefactual fall of fluorescence as the cell moved away from the plane of focus, but an increase of fluorescence must represent a true increase of dye signal. Calibration bar is 40 µm. The *bar* shows the pseudocolour scale used: *black* represents an intensity of 0 through to *white* which represents the maximum intensity of 255. **b–d** Localised fluorescence oscillations in neurons developing within the ventral spinal cord. **b** Low-power transmitted light image of part of an embryo with dye-labelled cells indicated in *green*. The dorsal surface of the embryo is at the *bottom*. The *box* indicates a region of the ventral spinal cord shown in close-up in **c**. **c** Higher power fluorescence images. *Red* and *green* indicate two focal planes. (*n* Neuronal somata, *a* axons extending to the ventral spinal cord.) *Boxes* indicate the regions whose fluorescence is plotted in **d**. **d** Calcium Green dextran fluorescence as a function of time in the three regions boxed in **c**. No measurements were made during the 26–40 min period. The changes in $[Ca^{2+}]_i$ can be viewed as a movie at the web site address, http://www.geribolsover.physiol.ucl.ac.uk/Personal/Rachel/zebrafish1.html

In order to ensure that the Calcium Green dextran remained sensitive to changes in $[Ca^{2+}]_i$ during the course of our recordings in vivo, changes in fluorescence were recorded from muscle fibres during escape behaviour in embryos at 20 h (Fig. 2a). Escape behaviour was triggered by increasing the temperature of bathing medium and fluorescence changes were measured over several seconds. During the contractions the fluorescence rose to a maximum of 2.7 times baseline intensity (range 2.3–3.1, *n*=3) which is comparable to reported values for the dynamic range of Calcium Green dextran in vivo [9].

Oscillations in cells in the ventral spinal cord

In zebrafish embryos at 18–20 h the central nervous system is still relatively simple, for instance the spinal cord contains only five classes of well characterised neurons that have begun axogenesis [7]. Individual nerve cells labelled with Calcium Green dextran were located within the spinal cord and brain of intact embryos between 16 and 20 h and fluorescence intensities monitored by acquiring stacks of five images containing the whole cell every 30 s over a 30-min period. A total of 113 neurons from 26 embryos were identified and the fluorescence was analysed by plotting the intensity within defined regions of the nerve cell body and axon as a function of time (Fig. 2b–d). None of the nerve cells in the brain (*n*=26), nor in the dorsal spinal cord (*n*=22) showed spontaneous fluorescence changes distinguishable from noise; however, a subset of neurons located within the ventral spinal cord of the embryo showed spontaneous, transient fluorescence changes (*n*=9/48, recorded from 16 different embryos). The intensity variations in region 2 (Fig. 2c,d) are typical of changes which were classed as indistinguishable from noise, whereas region 3 (Fig. 2c,d) showed changes typical of oscillations observed in nerve cells within the ventral spinal cord. Region 1 defines an axon that was quiescent during the first observation period, but exhibited clear fluorescence transients during the second time period. Fluorescence transients were observed in either the nerve soma but not in the axon (*n*=2) or vice versa (*n*=6) and in a single instance in both regions (*n*=1). In the cell soma the oscillations had an average amplitude of 27.9% (±10.5, *n*=3; as calculated as a % of minimum after background correction) and averaged 11.8 per hour (±3.5, *n*=3), whereas in axons the transients had an average amplitude of 45.7% (±9.6, *n*=7) and an average frequency of 10.4 per hour (±2.6, *n*=6). These values are comparable to, but significantly smaller than, the 66% fluorescence increase seen when neurons in vitro were depolarised with 130 mM KCl.

As Calcium Green dextran is a non-ratiometric calcium probe it could not be ruled out that the oscillations are due not only to $[Ca^{2+}]_i$ changes but other effects, such as shape changes, within the cells. Therefore fluorescence changes in neurons within the ventral spinal cord of embryos injected with fluorescein-dextran, as a control, were also analysed. Fluorescein-dextran-loaded neurons within the ventral spinal cord of embryos between 16 and 20 h did not show any fluorescence changes that were distinguishable from noise (*n*=32 from seven embryos). Comparison of the number of neurons exhibiting fluorescence oscillations within the ventral spinal cord of embryos labelled with Calcium Green dextran compared to those labelled with fluorescein dextran confirmed that the observed changes were significant (Fishers Exact Test for 2×2 tables, $P<5\%$). Therefore it seems likely that the fluorescence transients are indeed oscillations in $[Ca^{2+}]_i$.

Discussion

Work in vitro has provided an abundance of information about the effect of modulating $[Ca^{2+}]_i$ on the developing nervous system; however, which of these $[Ca^{2+}]_i$ changes are relevant to neurons growing in vivo within their natural environment is still undetermined. This study has developed a method to monitor changes in $[Ca^{2+}]_i$ in nerve cells developing in an intact embryonic zebrafish and describes transient $[Ca^{2+}]_i$ changes which were confined to a group of neurons localised in the ventral spinal cord.

The use of Calcium Green dextran as an indicator of $[Ca^{2+}]_i$ in neurons of post-hatched zebrafish has been described previously [2]. In the present study $[Ca^{2+}]_i$ was recorded from individually labelled neurons of zebrafish embryos during the first 16–20 h of development, when neuronal differentiation has begun and primary neurons begin extending their axons [7]. Injection of blastomeres with dye at an early stage did not appear to disrupt the subsequent development of the embryo but allowed random labelling of isolated cells. Depolarisation of nerve cells from dye-injected embryos dissociated in vitro showed that the Calcium Green dextran was retained in the cytoplasm and had remained responsive to calcium. Furthermore, fluorescence changes in activated muscle cells of intact embryos confirmed that the dye was capable of reporting $[Ca^{2+}]_i$ changes in vivo.

Several studies have characterised calcium transients in embryonic neurons and proposed that they have various functions during neuronal development [3, 4]. Gu and colleagues have characterised two patterns of spontaneous $[Ca^{2+}]_i$ transients within embryonic *Xenopus* spinal neurons; namely “spikes” which are large, rapid and usually global $[Ca^{2+}]_i$ rises and “waves” that appear slower, occurring from seconds to minutes, and more localised. In addition, the occurrence of rapid $[Ca^{2+}]_i$ events appeared to be developmentally regulated whereas waves continued over an extended period, suggesting different roles in the maturation of the nervous system. Studies of neurons in culture showed that spikes promote neurotransmitter expression and channel maturation, whereas wave activity regulates neurite extension.

In the present study the majority of nerve cells examined both in the brain and spinal cord of embryonic zebrafish showed no changes in fluorescence; however, a

small percentage of neurons located in the ventral spinal cord displayed localised, spontaneous and repetitive rises of $[Ca^{2+}]_i$. The calcium transients described in this paper were comparable to the slower calcium waves observed in developing *Xenopus* embryonic neurons, in that they were fairly localised and lasted over a period of several minutes, with a frequency of 10–11/h. Various authors have proposed that the spatial and temporal restriction of the calcium signal is a mechanism which enables the cell to activate specific cellular responses without activating all $[Ca^{2+}]_i$-dependent processes at once. In this context, the localisation and timing of the $[Ca^{2+}]_i$ changes within the neurons of the ventral spinal cord of the zebrafish embryo may be selectively activating local calcium responsive elements important at this stage of development and we are in the process of characterising this further.

The development of a non-invasive technique for recording $[Ca^{2+}]_i$ in an embryonic nerve cell as it matures within its native environment will provide important insights into the calcium changes that occur in vivo. We have observed that a small number of neurons located in the ventral spinal cord of embryonic zebrafish display spontaneous $[Ca^{2+}]_i$ transients and it is likely that these changes have a functional significance during neuronal development.

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