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USE OF A VIBRATING ELECTRODE TO MEASURE CHANGES IN CALCIUM FLUXES ACROSS THE CELL MEMBRANES OF OXIDATIVELY CHALLENGED *APLYSIA* NERVE CELLS

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A self-referencing and non-invasive Ca^{2+}-sensitive vibrating electrode was used to assess the effects of hydrogen peroxide-induced oxidative challenges on the efflux and influx of calcium across the plasma membrane of single nerve cells cultured from abdominal ganglion of *Aplysia californica*. A reduced net efflux of Ca^{2+} from the cell soma occurred immediately after the addition of hydrogen peroxide (0.0025 mM, 0.005 mM or 0.01 mM) to the culture medium, indicating damage to the cell membrane or Ca^{2+} transport mechanism. There then followed a marked efflux, the extent and duration of which was related to the concentration of hydrogen peroxide used and which may reflect compensatory activity by the Ca^{2+} regulatory mechanisms in the plasmalemma. No morphological changes were observed in cells challenged with 0.0025 mM hydrogen peroxide and the enhanced rate of Ca^{2+} efflux rapidly decreased to pre-exposure values. Sustained and enhanced Ca^{2+} effluxes from those cells exposed to 0.005 mM or 0.01 mM hydrogen peroxide were also consistent with regulatory pumping of Ca^{2+} out of the cell although contraction and blebbing of neurites and swelling of the soma may indicate that a proportion of the efflux arose from release of Ca^{2+} from disrupted intracellular stores. The vibrating electrode is a useful additional technique for the study of the pathogenesis of neurological conditions, as ionic fluxes across single nerve cells exposed to physiologically-relevant concentrations of free radicals can be monitored non-invasively for prolonged periods.

KEY WORDS: Calcium flux, nerve cell, hydrogen peroxide, vibrating probe, *Aplysia californica*.

INTRODUCTION

Reactive oxygen species (ROS) can disrupt cellular calcium (Ca^{2+}) homeostasis by inactivating regulatory mechanisms such as Ca^{2+}-ATPases, Na^{+}-Ca^{2+} exchange mechanisms and the voltage-sensitive ryanodine receptor.[1] The resulting loss of control of the asymmetric movement of Ca^{2+} across the plasma membrane can precipitate cell death by activation of potentially destructive biochemical pathways involving phospholipase A_2, neutral proteases and lysosomal acid hydrolases.[2] Nerve cells may be particularly susceptible to such ROS-mediated damage as when, for example, neuronal activity involving essential amino acid receptors leads to pro-oxidant conditions. Such pro-oxidants may then contribute to the development of a wide range of disorders of the nervous system including Alzheimer's disease and Parkinson's disease.[3]

To date, electrophysiological studies of transmembrane Ca^{2+} transport have been inhibited by the inherent noise and instability of the electrode preventing the accurate measurement of the weak voltage field associated with the movement of ions across

cell membranes.[4] Recently, however, these problems have been overcome by the development of a non-invasive and self-referencing ion-selective probe which has markedly increased sensitivity compared with conventional techniques and can measure electrically neutral ion transport.[4] Consequently, the aim of this study was to assess whether this ion-selective vibrating probe could be used to study the effects of oxidative challenges on the transport of Ca^{2+} across the plasmalemma of single nerve cells.

MATERIALS AND METHODS

Animals and cell culture *Aplysia californica* (200–400 g) were maintained in seawater aquaria at 6°C for 2 weeks. The abdominal ganglion was then removed under sterile conditions and incubated at 22°C for 18h in artificial seawater (mM: NaCl, 460; KCl, 10.4; $MgCl_2$, 27; $MgSO_4$, 28; $CaCl_2$, 11; Dextrose, 5.5; HEPES, 15) containing 1.5% neutral protease. The bag cell cluster was then dissected out and nerve cells triturated into standard 50 mm diameter glass culture dishes where they were maintained in 3 ml artificial seawater for a further 24 h. Immediately prior to measurement of transmembrane potentials, the culture dishes were rinsed 5 times with artificial seawater of low Ca^{2+} concentration (0.1 mM Ca^{2+} concentration, final volume 3 ml). Under these conditions cells grow well and remain healthy for a week. Resting potentials can be measured between -32 mV and -51 mV with cells exhibiting spontaneous and induced action potentials on current injection.

Detection of calcium efflux and influx The principles and use of the vibrating Ca^{2+}-selective electrode are described elsewhere.[4] In brief, silanised micropipettes pulled from 1.5 mm outer diameter borosilicate glass and containing a filtered solution of 100 mmol/l $CaCl_2$ in 0.5% Agar gel were front filled with FLUKA Calcium Ionophore I-cocktail A. All electrodes used demonstrated Nernstian properties when calibrated in different solutions of known calcium concentration. The DC-potential associated with background calcium concentration was removed by the high pass filter in the amplifier.[4] The steady state calcium gradient around the cell, measured as a ΔV (change in voltage over the vibrational amplitude of 10 μM) could be converted into a flux value but only approximately. An electrode could vary from the ideal by up to 50% (see ref. 4). The negative microvolt values found in this study approximate to effluxes in the order of 1–5 pmol.cm^{-1}. A net influx would give positive microvolt values.

The electrode was positioned to within 5 μm of the soma of single nerve cells in the culture dish using an othogonal array of stepper motors which also control vibrational angle and length in X, Y and Z planes. The vibration distance was 10 μm. Data was collected via a FET operational amplifier located in the head stage of the vibrating probe. Signals from the amplifier, representing the voltages measured at each extreme of vibration were digitalised (Data translations, DT 2800 Series) for continuous computation of a running average of the microvolt changes. After 10–20 min. aliquots (5, 10 or 20 μl) of hydrogen peroxide (final concentrations, 0.0025 mM, 0.005 mM or 0.01 mM) were carefully added to the culture dish containing the cell under study. Changes in soma diameter were measured with the graticular eyepeice and $\times 20$ objective lenses of a binocular Axiovort (Zeiss, Germany).

Our initial experiments to generate ROS by the addition of solutions to the culture dishes to give final concentrations of 6 μMFe, 2 mMADP and 0.05 mM ascorbate resulted in the formation of a brown precipitate which may have resulted from the

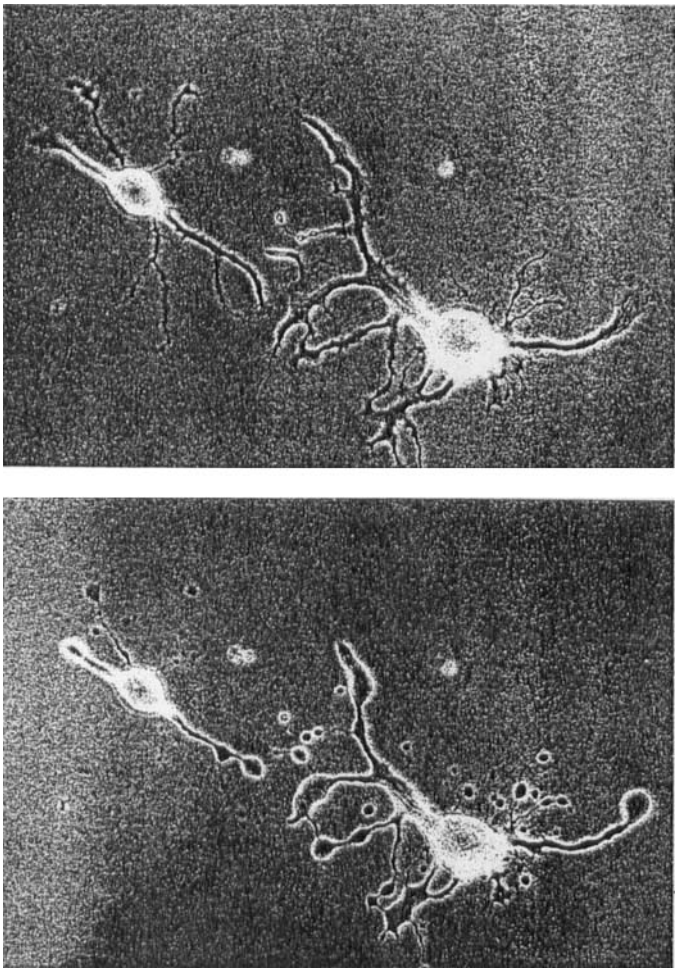

PLATE 1 The upper print shows a neuron prior to the addition of an oxidative load. The lower print shows the swelling of the soma and "blebbing" of dendrites which were observed 45 min after exposure to 0.01 mM hydrogen peroxide.

chelation of iron with the carbon dioxide and carbonic acid present in the seawater. The uncertainty as to how this unexpected reaction would affect the Fenton chemistry and subsequent generation of OH-type radicals lead us to add H_2O_2 to the culture dishes. We are thus unable to accurately quantify the production of ROS which will rely in the main on the concentrations of Fe^{2+} present as contaminants in the reagents used. The iron content of the artificial seawater was estimated to be approximately 1 μM.

RESULTS

For at least 2 h, single nerve cells consistently exhibited a steady Ca^{2+} efflux across the plasmalemma as indicated by a negative microvolt reading with a variation

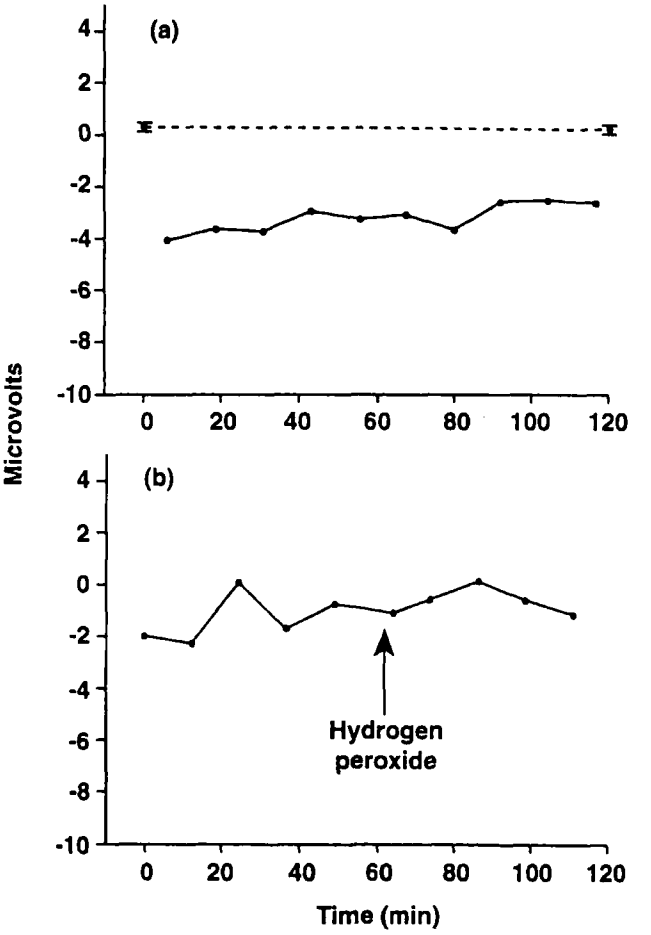

FIGURE 1 (a) The relatively constant efflux of Ca^{2+} from a single neuron of *Aplysia californica* as measured by the vibrating electrode and (b) the effect of the addition of 0.01 mM hydrogen peroxide on the stability of the electrode in cell free culture medium. The dotted line represents the microvolt change ascribed to background noise.

in electrical field of less than $\pm 2\,\mu$volts (Fig. 1a). Addition of hydrogen peroxide (final concentration, 0.01 mM) to cell-free culture medium caused no significant change in background voltage confirming that the specifications of the vibrating Ca^{2+}-sensitive electrode were unaffected by the oxidative agent (Fig. 1b).

A reduced net efflux of Ca^{2+} from the cell soma occurred immediately following the addition of hydrogen peroxide (Fig. 2). The magnitude of this change was related to the concentration of hydrogen peroxide to which the cells were exposed (increase of 3 mVolts, 8 mVolts and 17 mVolts for 0.0025 mM, 0.005 mM and 0.01 mM, respectively for the examples in Fig. 2). There then followed a marked increase in the Ca^{2+} efflux from the cells. Neurons exposed to the mildest oxidative load rapidly re-achieved a steady efflux similar to that observed before the addition of hydrogen peroxide whereas the efflux persisted and increased for approximately 40–60 min from cells subjected to 0.005 mM and 0.01 mM hydrogen peroxide before

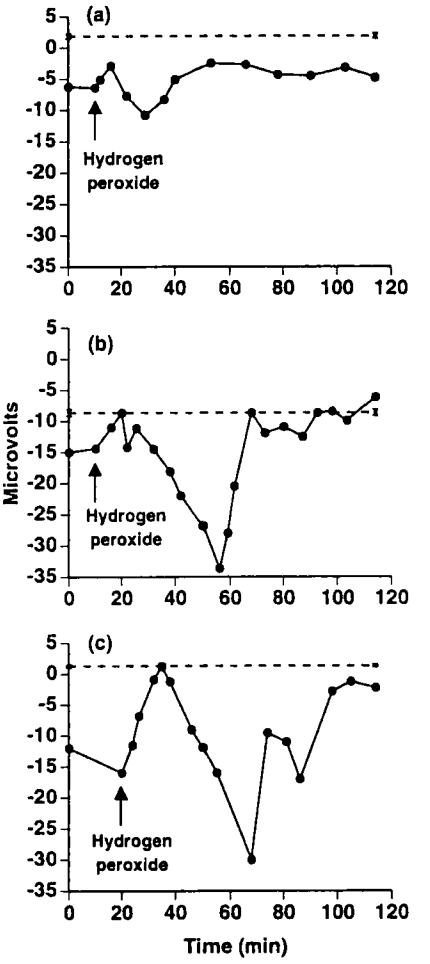

FIGURE 2 Examples of the effect of the addition of hydrogen peroxide (a) 0.0025 mM, (b) 0.005 mM and (c) 0.01 mM on Ca^{2+} fluxes across the plasmalemma of single *Aplysia* neurons. The dotted lines denote the microvolt change ascribed to background noise.

values near to background were attained. This perturbed ability to regulate Ca^{2+} movement across the plasmalemma was reflected by a pronounced swelling of the soma and blebbing of the dendrites of the cell (Plate 1), phenomena which were less marked in those cells exposed to the lower concentrations of hydrogen peroxide (Fig. 3).

DISCUSSION

The cytosolic free Ca^{2+} concentration of the nerve cells of *Aplysia californica* is several orders of magnitude less than that of the experimental medium.[5] This concentration gradient indicates that the steady efflux of Ca^{2+} detected from the soma

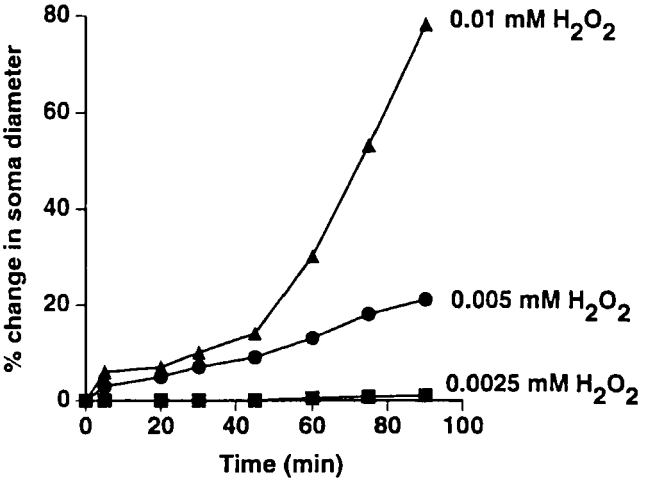

FIGURE 3 Percentage change of soma diameter with time following exposure to 0.0025 mM, 0.005 mM or 0.01 mM hydrogen peroxide. Data represents the mean of 4 cells per treatment.

of the nerve cells is an active process involving Ca^{2+} transport pumps. The reduced net efflux of Ca^{2+} from the cell soma immediately following exposure to 0.0025 mM hydrogen peroxide may therefore reflect a free radical-mediated loss of cell membrane integrity allowing Ca^{2+} to enter the cytosol from the external medium. The subsequent enhanced efflux may represent rapid compensatory upregulation in the activity of the Ca^{2+}-pumps, enabling the cells challenged with 0.0025 mM hydrogen peroxide to rapidly re-established a steady Ca^{2+} efflux similar to that observed pre-exposure. Moreover, the lack of detectable morphological changes in those cells following exposure to 0.0025 mM hydrogen peroxide also indicates that they are able to adapt to the imposed oxidative load at least for the period of observation.

The greater efflux of Ca^{2+} from those cells exposed to the higher concentrations of hydrogen peroxide may also indicate a similar but more pronounced compensatory regulation. However, the marked swelling of the soma and contraction of the dendrites strongly suggests that the oxidative-load had exceeded the capacity of the antioxidant defence system of the neuron. A proportion of the sustained efflux may arise from the release of Ca^{2+} from disrupted intracellular stores, thus raising the concentration of free Ca^{2+} in the cytosol. Ultimately, it appears that the Ca^{2+} homeostatic regulation fails. Whether such failure of cellular Ca^{2+} regulation occurs *in vivo* is unclear although numerous neurological syndromes have been associated with pro-oxidant conditions.[6]

This report indicates that the self-referencing Ca^{2+}-sensitive vibrating electrode is a useful additional technique for the study of the effects of physiologically-relevant oxidative stress on ionic fluxes across nerve cell membranes. When used in conjuction with Ca^{2+}-sensitive fluorescent dyes and measures of indices of lipid peroxidation, it may offer invaluable insights into the processes involved in the development of major neurological conditions and in the assessment of the protective efficacy of therapeutic and putative antioxidants. The technique could also be used to assess the toxicological effects of many xenobiotics on a range of cell types.

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