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Fluorophore toxicity in mouse eggs and zygotes

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Summary

Ion-sensitive fluorophores are commonly used for quantitative measurements of intracellular ion concentrations. However, both the method of intracellular loading – which for many fluorophores involves endogenous esterase-mediated removal of hydrophobic groups such as acetoxymethyl esters (AM) – and fluorescence excitation of fluorophores in the cell, can produce toxic metabolites and reactive species. Techniques used to measure intracellular ion concentrations in mammalian eggs and embryos are being increasingly employed, yet little information is available about any detrimental effects of the use of fluorophores. We have therefore used *in vitro* fertilisation (IVF) to assess potential fluorophore toxicity in mouse eggs, and whole cell patch-clamp recordings to detect fluorophore-associated membrane damage in zygotes. Four fluorophores were examined: SNARF-1 and BCECF (pH indicators), Fura-2 (Ca^{2+}) and MQAE (Cl^-). Cleavage of AM groups alone had no effect either on the success of IVF or on membrane electrical properties of mouse zygotes. Intracellularly loaded BCECF, SNARF-1 and Fura-2 followed by fluorescence excitation were not cell-toxic under the conditions examined. In contrast, MQAE demonstrated significant toxicity both alone and in combination with fluorescence excitation.

Keywords: Eggs, Fluorophore, pH, Toxicity, Zygotes

Introduction

The development of ion-sensitive fluorophores that can be loaded into cells has proved invaluable in the study of mammalian egg and embryo physiology. Since few eggs or embryos can be obtained from mammals, such fluorescent indicators are often the only practical means of investigating processes such as Ca^{2+} signalling or intracellular pH (pH_i) regulation in these cells. For measurements to be physiologically relevant, the techniques must not be excessively damaging to the cell. In most cases, there has been no obvious damage to eggs or embryos due to the use of ion-sensitive fluorophores. In our experience, embryo morphology does not change during experiments using pH-sensitive fluorophores for 2 h, nor has overt damage been reported by others. In addition, the apparently normal function of processes such as the transient increase in intracellular Ca^{2+} at fertilisation, the Ca^{2+} oscillations that follow (Kline & Kline, 1994; Jones *et al.*, 1995*a*, *b*) and pH_i regulatory systems in embryos (Baltz *et al.*, 1991; Zhao *et al.*, 1995; Zhao & Baltz, 1996; Phillips & Baltz, 1996) is an indication that fluorophore-loaded embryos are not severely compromised during the course of the measurements.

Many fluorophores are loaded as membrane-permeant esters that cross the cell membrane and are hydrolysed by intracellular esterases, resulting in accumulation of membrane-impermeant fluorophores in the cytoplasm. Chemically, these ester groups consist of acetate derivatives of aromatic alcohols and acetoxymethyl (AM) esters of carboxylic acids. Cleavage of these ester groups may result in acute, short-term toxicity to the embryo due to the production of formaldehyde

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and acetic acid (Haugland, 1996). In addition, the use of fluorophores necessitates exposure to excitation illumination repeatedly during an experiment. There is a chance that each absorbed photon will break intramolecular bonds within the fluorophore, rather than exciting fluorescence. The products of such photolysis can also be toxic and damage the cell.

There is some evidence to suggest that fluorophore toxicity can occur under normal experimental conditions. Mouse 2-cell embryos, loaded with the pH_i indicator BCECF and exposed to excitation illumination, exhibit decreased development to the blastocyst stage. In contrast, SNARF-1, another pH_i indicator, demonstrated no significant effect on embryo development, either alone or in combination with excitation illumination (Baltz *et al.*, 1990, Zhao & Baltz, 1996; J.M. Baltz, unpublished).

The use of techniques based on ion-sensitive fluorophores with mammalian eggs and embryos has become common, but there is very little data regarding any possible toxic effects and none systematically assessing toxicity during or immediately after exposure. We have therefore performed experiments to determine whether any of several fluorescent indicators are detrimental under the same conditions normally used in eggs and embryos for intracellular ion measurements.

Materials and methods

Chemicals and media

KSOM embryo culture medium (Lawitts & Biggers, 1993) contains (in mM, except as noted) 95 NaCl, 2.5 KCl, 0.35 KH_2PO_4, 0.2 $MgSO_4$, 10 sodium lactate, 0.2 glucose, 0.2 sodium pyruvate, 25 $NaHCO_3$, 1.7 $CaCl_2$, 1.0 glutamine, 0.01 tetrasodium EDTA, 0.03 streptomycin sulphate, 0.16 K penicillin G and 1.0 mg/ml fatty-acid-free bovine serum albumin (FAF-BSA) (all from Sigma, St Louis, MO, embryo culture tested or cell culture tested), and was equilibrated with 5% CO_2/air (pH 7.35). KSOM was modified for sperm capacitation and *in vitro* fertilisation (IVF) with 5.56 mM glucose and 30 mg/ml FAF-BSA (Summers *et al.*, 1995). Hepes-KSOM medium (Lawitts & Biggers, 1993), wherein 21 mM of the $NaHCO_3$ in KSOM was replaced by equimolar Hepes (pH adjusted with NaOH to 7.4 at room temperature), was used for obtaining and handling eggs and zygotes. For intracellular ion measurement using fluorophores on the quantitative imaging fluorescence videomicroscopy system (see below), KSOM and Hepes-KSOM were modified slightly to include (in mM) 104 NaCl, 1.0 sodium lactate, and no BSA. Patch-clamp measurements were made in Hepes-KSOM (BSA-free) at room temperature. The pipette solution consisted of (in mM): 100 KCl, 1 NaEGTA, 10 NaHepes, 2.5 Na_2ATP and 3.5 $MgCl_2$ (pH 7.2, room temperature). To facilitate the formation of gigaohm seals, $CaCl_2$ was increased to 10 mM and NaCl reduced to 85 mM during seal formation.

Egg and embryo collection

Female CF1 mice (5–6 weeks old; Charles River, Canada) were superovulated using 5 IU pregnant mare serum gonadotropin (PMSG; Sigma) followed 48 h later by 5 IU human chorionic gonadotropin (hCG; Sigma), both administered intraperitoneally. For unfertilised eggs, oviducts were removed from 5- to 6-week-old unmated females 13–15 h post-hCG. Cumulus masses were released into 300 µg/ml hyaluronidase for 6–8 min until eggs could be removed from cumulus cells (Hogan *et al.*, 1986). Eggs were washed and, if appropriate, immediately loaded with one of the fluorophores tested (see below). For zygotes, female mice were caged with BDF males immediately following the administration of hCG and zygotes were collected 18–20 h later. Fertilisation was confirmed by the appearance of two pronuclei. Cumulus cells were removed using hyaluronidase (as above) and zygotes were then loaded with one of the fluorophores, if appropriate.

Sperm collection

CD-1 male mice (age 12–15 weeks) were killed by cervical dislocation immediately prior to egg collection (13.5 h post-hCG; females). Sperm in epididymal fluid were released from each cauda epididymis and placed into 1 ml modified KSOM (high glucose, BSA) in separate Eppendorf tubes. Sperm were capacitated for 1 h at 37 °C, 5% CO_2, after which the top 500 µl of the sperm suspension from each Eppendorf tube was combined in a third tube.

In vitro fertilisation

Immediately following fluorophore loading (Hepes-KSOM incubation for controls) and, where appropriate, exposure to excitation illumination (Fig. 1), 75 µl of motile, capacitated sperm was added to each 25 µl drop of modified KSOM (high glucose, BSA) containing treated eggs (see below). Eggs and sperm were co-incubated for 1 h (37 °C, 5% CO_2) after which eggs were washed free of unbound sperm in microdrops of KSOM under oil and cultured. Fertilisation was confirmed by the presence of two pronuclei at 6–8 h post-IVF or cleavage to the 2-cell stage 24 h post-IVF. In some instances, cleavage did not occur 24 h post-IVF, although two pronuclei were present. Thus, when fertilisation was scored at 24 h post-IVF it includes both zygotes (with 2 pronuclei) and 2-cell embryos.

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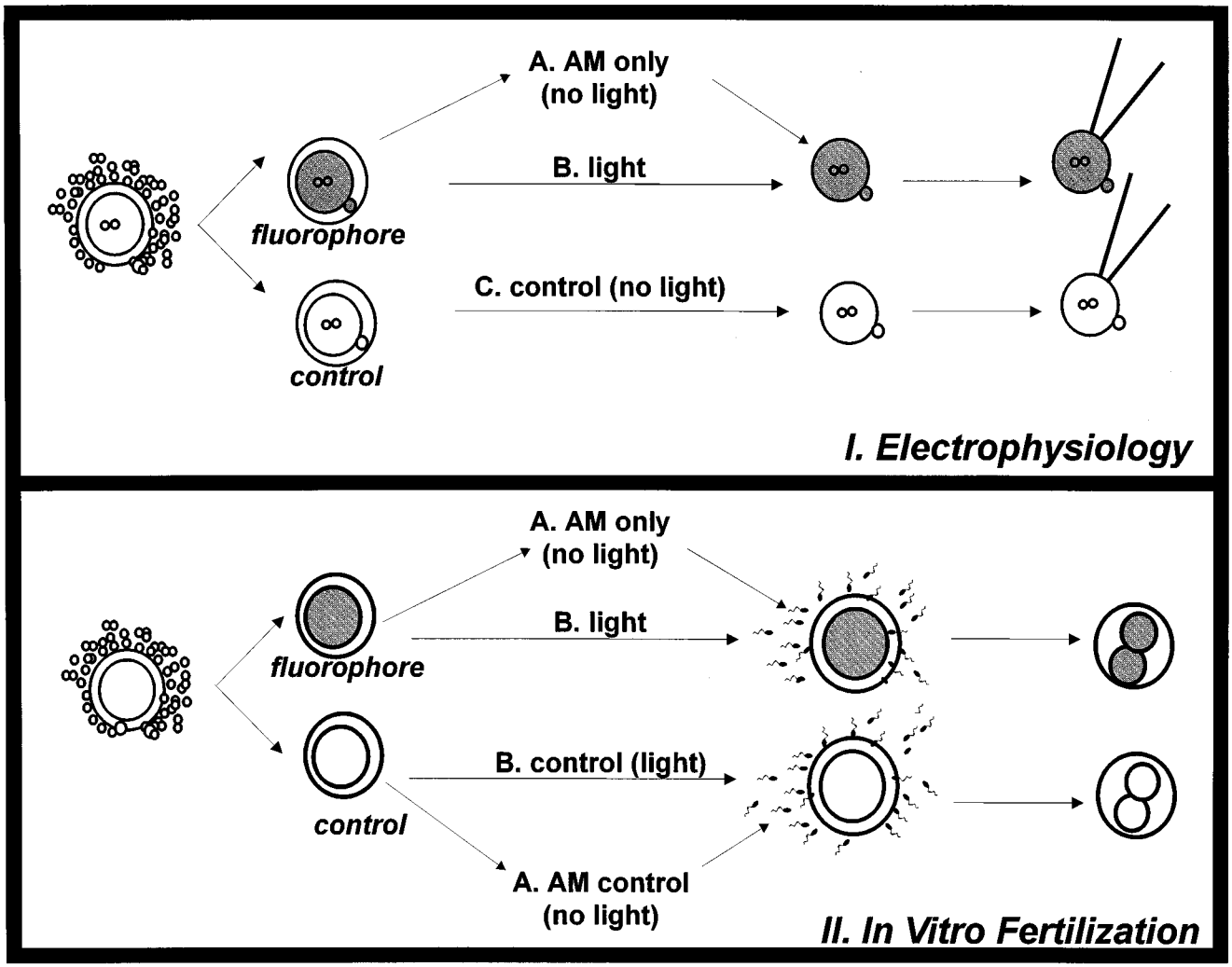


Figure 1 Experiment protocols. *I. Electrophysiology*. A. Zygotes were loaded with SNARF-1-AM and subsequently patch-clamped to determine the effect of AM loading alone on the electrophysiological properties of zygotes. B. Zygotes were loaded with one of four fluorophores, exposed to appropriate excitation illumination and then used for electrophysiological measurements using the whole-cell patch-clamp technique. C. Zygotes not loaded with fluorophore or exposed to excitation illumination were used as controls. *II. In vitro fertilisation*. A. Eggs were loaded with SNARF-1-AM (control – no fluorophore) and immediately used for IVF to determine the effect of AM loading alone on developmental potential. B. Eggs were loaded with one of four fluorophores, exposed to appropriate excitation illumination (controls – no fluorophore) and then used for IVF.

Whole-cell patch-clamp recording

Following exposure to excitation illumination in a temperature-controlled chamber (Biophysica, Baltimore, MD), zonae pellucidae were removed and the embryo surface cleaned using 0.75 mg / ml pronase for 10 min at 37 °C (Fig. 1). Embryos were then returned to the chamber in Hepes-KSOM (BSA-free). The bath solution was changed to modified Hepes-KSOM (high CA^{2+}) at room temperature to facilitate formation of gigaohm seals. Solutions were exchanged using a perfusion system (BPS-4, Adams & List, Westbury, NY) and a vacuum pump (Barnant, Barrington, IL). After formation of whole-cell recording configuration, zygotes were maintained in a bath solution of Hepes-KSOM (room temperature) under constant perfusion (0.5 ml / min) for the duration of the experiment.

Patch pipettes, made from borosilicate glass (Garner Glass, Claremont, CA), were pulled in a two-stage vertical pipette puller (List-Medical, Westbury, NY). They were coated with Sylgard 184 (Dow Corning, Midland, MI) and fire polished for several seconds after coating. When filled with pipette solution and immersed in Hepes-KSOM, the pipettes exhibited a resistance of 5–10 MΩ. Whole-cell current recordings were obtained using the whole-cell patch-clamp technique (Hamill *et al.*, 1981) with a patch-clamp amplifier (Axopatch-200B, Axon Instruments, Forster City, CA). Briefly, a gigaohm seal was formed on the membrane surface of the zygote. Membrane potential was determined at the

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beginning of each experiment upon initial breakthrough of the membrane patch to attain the whole-cell configuration (in current-clamp mode with $I = 0$). Command voltages were applied and data collected with a DigaData 1200 (Axon Instruments) using pClamp6 version 6.02 software. Whole-cell currents were recorded at a holding potential of –20 mV and a voltage ramp of –60 mV to 40 mV repeated every 100 s (the total duration of each ramp was about 1 s). The whole-cell current data reported here were measured at 40 mV. Current–voltage (*I–V*) relationships were generated for each embryo and membrane conductance (at 30 mV) was determined by fitting a linear regression (SigmaPlot, Jandel Scientific, San Rafael, CA) between 20 mV and 40 mV.

At the end of each experiment, seal resistance was assessed by withdrawing the patch pipette to obtain an outside-out patch. If the resistance was found to be less than 1 GΩ, the recording was rejected, enabling data from experiments where seals had deteriorated to be excluded. The liquid-junction potential was corrected after each experiment according to Fenwick *et al.* (1982).

Fluorophore loading and light exposure

Cumulus-denuded eggs were loaded with one of four fluorophores (using loading protocols described below) and transferred to the temperature-controlled chamber (Biophysica) that was modified to allow solution changes and control of the atmosphere. The bath solution was immediately changed to pre-equilibrated (5% CO_2, 37 °C) KSOM for eggs or Hepes-KSOM (37 °C) for zygotes.

The methods used in pH_i and Ca^{2+} measurements have been described in detail previously (Baltz *et al.*, 1990; Zhao *et al.*, 1995, 1997; Phillips & Baltz, 1996; Seguin & Baltz, 1997). Briefly, fluorescence was detected using a quantitative imaging fluorescence videomicroscopy system (Inovision, Durham, NC) with a Zeiss Axiovert inverted epifluorescence microscope and grating-type monochrometer (Photon Technologies International, New Brunswick, NJ) and a Geniisys image itensifier (DAGE-MTI, Michigan City, IN). SNARF-1 fluorescence (see below) was detected using a variable imaging monochrometer (VariSpec model VISI5, Cambridge Research Instruments, Cambridge, MA). Fluorophore-loaded eggs and their respective unloaded controls, or zygotes, were exposed to excitation illumination (5 min, 1 exposure every 30 s) at the wavelength or pair of wavelengths appropriate for each fluorescent indicator. Each exposure was 1 s (single wavelength) or 2 s (1 s at each excitation wavelength) and thus the total exposure of SNARF-1- and MAQE-treated eggs and embryos (single wavelengths, see below) was 10 s while BCECF- and Fura-2 treated eggs and embryos (two wavelengths) were exposed for a total of 20 s under this protocol. Following light exposure, eggs and zygotes were retained for either IVF (eggs) or electrophysiological measurements (zygotes).

SNARF-1. Eggs or zygotes were transferred to Hepes-KSOM containing 5.0 µM SNARF-1-AM (carboxyseminaphthorhodafluor-1-acetoxymethyl ester; Molecular Probes, Eugene OR) and incubated at 37 °C for a period of 30 min (House, 1994; Phillips & Baltz, 1996). SNARF-1-loaded eggs or zygotes were washed with Hepes-KSOM. Excitation wavelength for SNARF-1 was 565 nm.

BCECF. Eggs or zygotes were transferred to Hepes-KSOM containing 1.2 µM BCECF-AM (2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester; Molecular Probes) and incubated at 37 °C for a period of 10 min (Baltz *et al.*, 1990). BCECF-loaded eggs or zygotes were washed with Hepes-KSOM. Dual excitation wavelengths were used: 490 nm and 450 nm.

Fura-2. Eggs or zygotes were transferred to Hepes-KSOM containing 2.0 µM Fura-2-AM (5-oxazolecarboxylic acid, 2-(6-(bis(2-((acetyloxy)methoxy)-2-oxoethyl)amino)-5-(2-2-(bis(2-((acetoxyloxy)methoxy)-2-oxoethyl)amino)-5-methylphenoxy)ethoxy)-2-benzofuranyl)-(acetyloxy)methyl ester; Molecular Probes) and incubated at 37 °C for a period of 30 min (Seguin & Baltz, 1997). Fura-2-loaded eggs or zygotes were washed with Hepes-KSOM. Dual excitation wavelengths were used: 380 nm and 350 nm.

MQAE. Eggs or zygotes were transferred to Hepes-KSOM containing 20 mM MQAE (*N*-6-methoxyquinolyl)acetoethyl ester; Molecular Probes) and incubated at 37 °C for a period of 30 min (Zhao *et al.*, 1997). MQAE-2-loaded eggs or zygotes were washed with Hepes-KSOM. The excitation wavelength for MQAE was 365 nm.

Photobleached embryos

To purposely induce phototoxicity, zygotes loaded with BCECF (as above) were exposed to continuous excitation illumination (450 nm) for 28 min. Following light exposure, zygotes were retained for electrophysiological measurements as described above.

Statistical analysis

For electrophysiology data, groups were compared using ANOVA followed by the Tukey–Kramer Multiple Comparison Test (InStat, GraphPad, San Diego,

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CA). For IVF results, where each group consisted of a paired control and tested fluorophore, chi-square analysis or Fisher's Exact Test was used to determine significance (Instat).

Results

Effect of fluorophores on the electrophysiological properties of zygotes

Effect of AM hydrolysis alone

SNARF-1-loaded zygotes, not exposed to excitation illumination, were used to investigate the effect of AM hydrolysis alone on zygotes. Resting membrane potential was determined as described, upon initial breakthrough of the membrane patch to attain the whole-cell configuration (Fig. 2). The mean resting membrane potential (± SEM) for SNARF-1-loaded zygotes was –24.9 ± 0.1 mV, which was not significantly different from control (–25.6 ± 1.0 mV). The *I–V* (current–voltage) curve for SNARF-1-loaded embryos typically shows an outwardly rectifying current with a negative reversal potential that was similar to the *I–V* relationship for the control group (Fig. 3). The membrane conductance at 30 mV was determined by linear regression to the *I–V* relationship between 20 and 40 mV (Fig. 4). The whole-cell conductance for SNARF-1-loaded embryos was 25.4 ± 1.7 nS, which was not significantly different from control (31.5 ± 3.0 nS). Finally, the whole-cell current, determined at a command voltage of 40 mV, was 1.2 ± 0.1 nA for SNARF-1-loaded zygotes, which was not significantly different from control (1.4 + 0.1 nA; Fig. 4). Together, these results indicate that AM hydrolysis alone has no effect on the electrophysiological properties of zygotes.

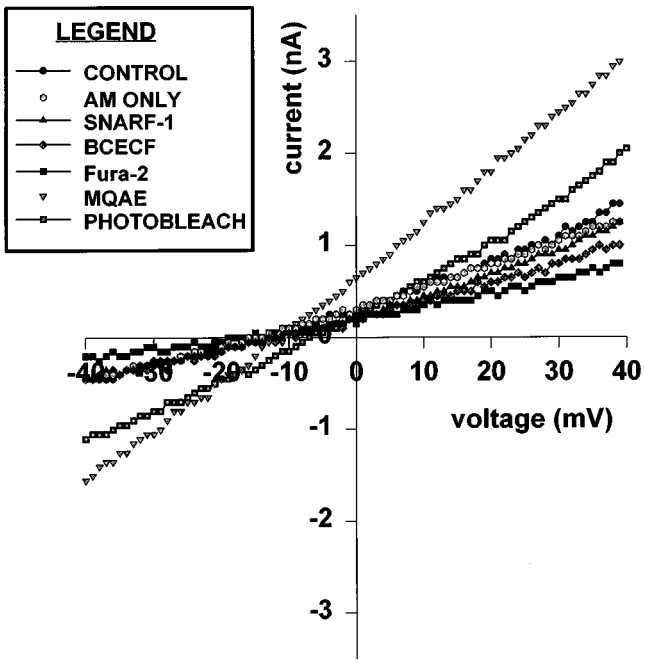


Figure 3 Representative current–voltage (*I–V*) curves obtained from fluorophore-loaded zygotes after exposure to excitation illumination (except AM loading). Whole-cell currents were recorded while zygotes were at a holding potential of –20 mV and a voltage ramp of –60 mV to 40 mV repeated every 100 s.

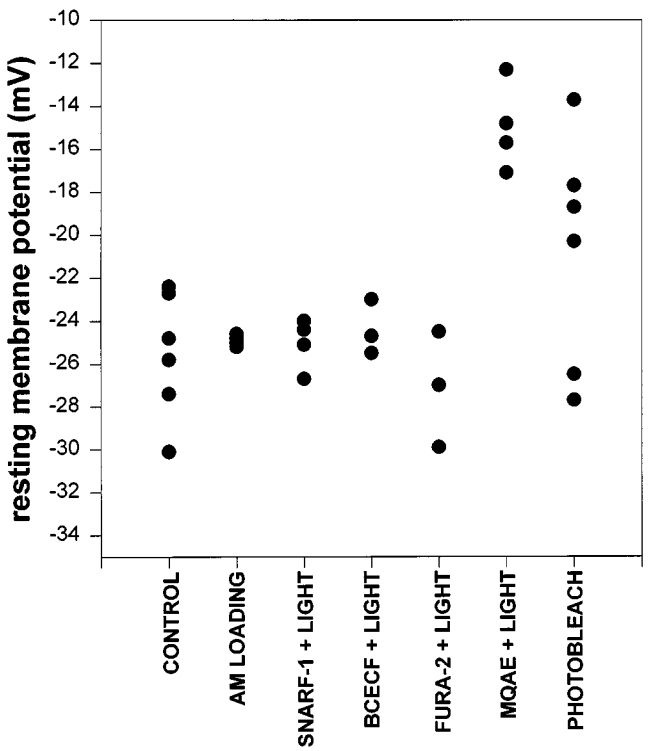


Figure 2 Resting membrane potentials in fluorophore-loaded zygotes. Mean resting membrane potentials (± SEM) are as follows (in mV): control ($n = 6$), –25.6 ± 1.0; AM loading (no excitation light; $n = 4$), –24.9 ± 0.1; SNARF-1 + light ($n = 4$), –25.1 ± 0.6; BCECF ($n = 4$), –24.7 ± 0.6; Fura-2 ($n = 3$), –27.1 ± 1.6; MQAE ($n = 4$), –14.9 ± 1.0; photobleach ($n = 6$), –20.7 ± 2.2. MQAE was significantly different from control ($p < 0.001$; ANOVA; Tukey–Kramer Multiple Comparison Test). *n represents both the number of zygotes and the number of experiments; 1 zygote per experiment, for all electrophysiological parameters measured, i.e. Figs. 2–5.*

Effect of fluorophores with excitation illumination

SNARF-1. SNARF-1 loaded embryos were exposed to excitation illumination at 535 nm as described above. The mean resting membrane potential (± SEM) after exposure for SNARF-1 loaded zygotes (Fig. 2) was –25.1 ± 0.6 mV, which was not significantly different from control (–25.6 ± 1.0 mV). The *I–V* curve with SNARF-1 (Fig. 3) is similar to the *I–V* relationship for the control example. The whole-cell conductance (Fig. 4) with SNARF-1 was 35.2 ± 4.2 nS, which was not significantly

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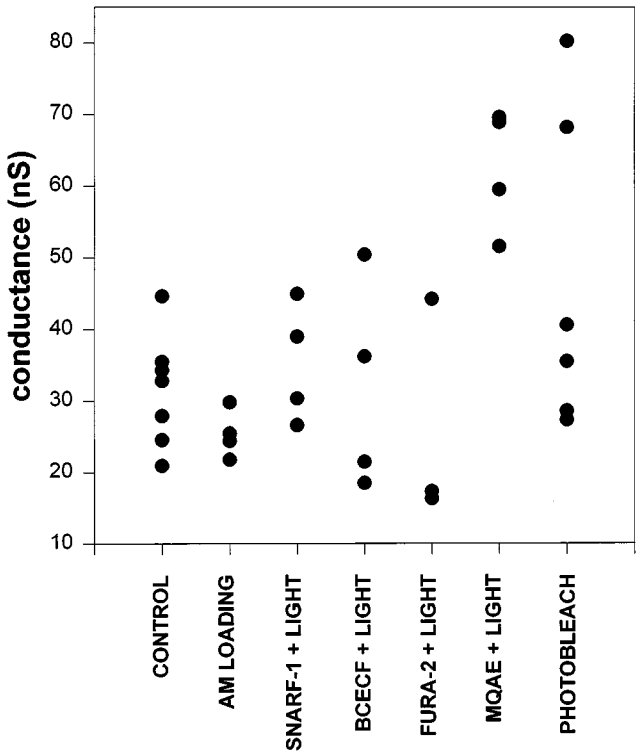


Figure 4 Whole-cell conductance of fluorophore-loaded zygotes. Whole-cell conductances were calculated from the *I–V* curves at 30 mV of command voltage for each experiment. The mean conductance (± SEM) for each group is as follows (in nS): control, 31.5 ± 3.0; SNARF-1, 35.2 ± 4.2; BCECF, 31.6 ± 7.4; Fura-2, 26.0 ± 9.1. There was no significant effect of SNARF-1, BCECF or Fura-2 on the membrane conductance in zygotes. The conductance of the photobleached group was variable and there was no significant difference between the mean conductance of the group (46.7 ± 4.3 nS) and that of the control. However, in MQAE-loaded zygotes the whole-cell conductance increased to 62.4 ± 4.3 nS, which was significantly different from control ($p < 0.05$; ANOVA; Tukey–Kramer Multiple Comparison Test).

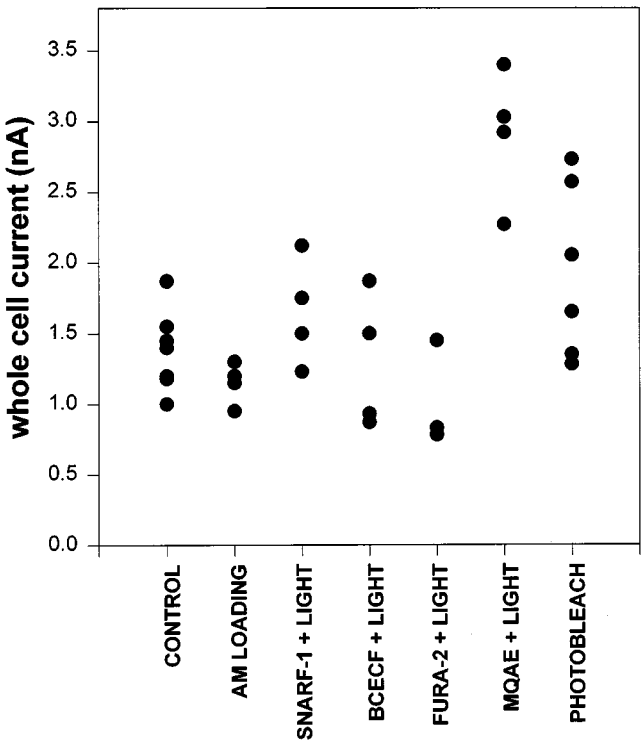


Figure 5 Whole-cell currents of fluorophore-loaded zygotes. The mean whole-cell current (± SEM) for each group is as follows (in nA): control, 1.4 ± 0.1; SNARF-1, 1.7 ± 0.2; BCECF, 1.3 ± 0.2; Fura-2, 1.0 ± 0.2; MQAE, 2.9 ± 0.2; photobleach, 1.9 ± 0.3. The whole-cell current in MQAE-loaded zygotes was significantly different compared with control ($p < 0.001$; ANOVA; Tukey–Kramer Multiple Comparison Test). There was no significant effect of any of the other fluorophores tested, including the photobleached group.

different from control (31.5 ± 3.0 nS). The whole-cell current at 40 mV for SNARF-1 was 1.7 ± 0.2 nA (Fig. 5), which was not significantly different from control (1.4 ± 0.1 nA).

BCECF. BCECF-loaded embryos were exposed to excitation illumination at 490 nm and 450 nm as described above. The mean resting membrane potential (± SEM) after exposure for BECEF-loaded zygotes (Fig. 2) was –24.7 ± 0.6 mV, which was not significantly different from control (–25.6 ± 1.0 mV). The *I–V* curve was BCECF (Fig. 3) is similar to the *I–V* relationship for the control example. The whole-cell conductance (Fig. 4) with BCECF was 31.6 ± 7.4 nS, which was not significantly different from control (31.5 ± 3.0 nS). The whole-cell current at 40 mV for BCECF was 1.3 ± 0.2 nA (Fig. 5), which was not significantly different from control (1.4 ± 0.1 nA).

Fura-2. Fura-2-loaded embryos were exposed to excitation illumination at 380 nm and 350 nm (dual excitation illumination) as described above. The mean resting membrane potential (± SEM) after exposure for Fura-2-loaded zygotes (Fig. 2) was –27.1 ± 1.6 mV, which was not significantly different from control (–25.6 ± 1.0 mV). The *I–V* curve with Fura-2 (Fig. 3) is similar to the *I–V* relationship for the control example. The whole-cell conductance (Fig. 4) for Fura-2 was 26.0 ± 9.1 nS, which was not significantly different from control (31.5 ± 3.0 nS). The whole-cell current at 40 mV for Fura-2 was 1.0 ± 0.2 nA (Fig. 5), which was not significantly different from control (1.4 ± 0.1 nA).

MQAE. MQAE-loaded embryos were exposed to excitation illumination at 365 nm as described above. The mean resting membrane potential (± SEM) after exposure for MQAE-loaded zygotes (Fig. 2) was –15.0 ± 1.0 mV, which was very significantly different from control (–25.6 ± 1.0 mV; $p < 0.001$; ANOVA; Tukey–Kramer Multiple Comparison Test). The *I–V* curve with MQAE (Fig. 3) was linear rather than rectified and

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had a much larger slope than control curves. The whole-cell conductance (Fig. 4) with MQAE was 62.4 ± 4.3 nS, which was significantly different from control (31.5 ± 3.0 nS ($p < 0.05$; ANOVA; Tukey–Kramer Multiple Comparison Test). The whole-cell current at 40 mV for MQAE was 2.9 ± 0.2 nA (Fig. 5), which was very significantly different from control (1.4 ± 0.1 nA; $p < 0.001$; ANOVA; Tukey–Kramer Multiple Comparisons Test).

Effect of photobleaching on the electrophysiological properties of zygotes

To obtain measurements of the membrane properties of zygotes that had been purposely photodamaged, zygotes were loaded with 1.2 µM BCECF and subsequently exposed to continuous excitation illumination (450 nm) for 28 min followed immediately by electrophysiological recordings. Many of these 'photobleached' zygotes were resistant to the formation of gigaohm seals and thus recordings were impossible. Data are thus presented for a selected population on which recordings were possible.

Zygotes that were successfully patched had a mean resting membrane potential (± SEM) of –20.8 ± 2.2 mV, which was not significantly different from control (–25.6 ± 1.0 mV; Fig. 2). The *I–V* curve for bleached zygotes was relatively linear when compared with the control trace (Fig. 3). The whole-cell conductance (Fig. 4) for photobleached embryos was 46.7 ± 4.3 nS, which was not significantly different from control (31.5 ± 3.0 nS). The whole-cell current (Fig. 5) at 40 mV for photobleached zygotes was 1.9 ± 0.3 nA, which was not significantly different from control (1.4 ± 0.1 nA). However, examination of the data shows a bimodal distribution, with some zygotes having 'normal' parameters while others have distinctly 'abnormal' values.

Effect of fluorophores on subsequent IVF

Effect of AM hydrolysis alone

Following SNARF-1 loading, SNARF-1-loaded eggs and controls (incubation in Hepes-KSOM; see above), not exposed to excitation illumination, were subsequently used for IVF to determine the effect of AM hydrolysis alone on cleavage rates. Cleavage was assessed at 24 h post-IVF. There was no significant difference between the rate of 2-cell cleavage in SNARF-1-loaded eggs (69.2%) and control eggs (67.1%; $p = 0.93$, chi-square test; Fig. 6).

Effect of AM fluorophores with excitation illumination

The effects of the three AM fluorophores – SNARF-1, BCECF and Fura-2 – were examined following excitation illumination, appropriate for each fluorophore (Fig. 7*A–C*), after which treated eggs were used for IVF. There was no significant difference between the rates of 2-cell cleavage in these AM fluorophores and their respective controls (Fisher's Exact Test; SNARF-1, $p = 0.19$, BCECF, $p = 0.61$; Fura-2, $p = 0.23$).

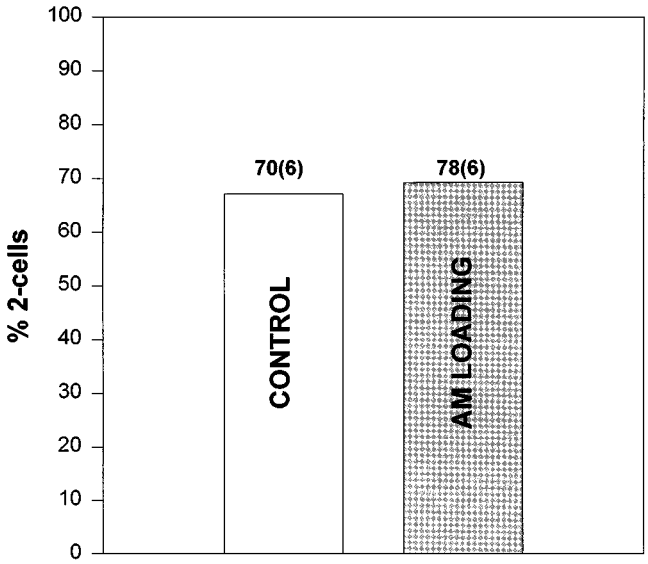


Figure 6 Effect of AM hydrolysis on 2-cell cleavage in SNARF-1 loaded eggs fertilised *in vitro*. SNARF-1-AM loaded eggs, not exposed to excitation illumination, were fertilised *in vitro* and assessed for embryo development 24 h post-IVF. The cleavage rate for AM loading only was 69.2%, which was not significantly different from control (67.1%; chi-square test). *n(N); where n represents number of embryos and 'N' represents number of experiments.*

MQAE

MQAE-loaded eggs and control eggs were exposed to an excitation wavelength of 365 nm (Fig. 7*D*). Following light exposure, MQAE-loaded eggs and controls were immediately used for IVF and assessed 24 h post-IVF. Of the MQAE-loaded eggs, 14.7% cleaved to the 2-cell stage, which was significantly different from control (60.4%; $p < 0.001$; Fisher's Exact Test). MQAE, without excitation illumination, was found to significantly slow the rate of pronuclear development (Fig. 8*A*) 6–8 h post-IVF (5.8%; control 64.2%; $p < 0.001$; Fisher's Exact Test). However, by 24 h post-IVF, 61.5% of embryos had developed to the 2 pronuclei stage, demonstrating that MQAE-loaded eggs had been fertilised *in vitro* at a rate nearly equal to control (83.0%; $p = 0.017$; Fisher's Exact Test). Cleavage of these fertilised eggs was reduced, however, as only 17.3% of MQAE-loaded eggs cleaved to the 2-cell stage compared with 62.3% of the control ($p < 0.001$; Fisher's Exact Test; Fig. 8*B*).

Since MQAE was found to reduce development of fluorophore-loaded eggs fertilised *in vitro*, zygotes resulting from natural matings were loaded with MQAE to assess their viability. A significantly lower number of MQAE-loaded zygotes (Fig. 9) exposed to excitation illumination cleaved to the 2-cell stage 24 h

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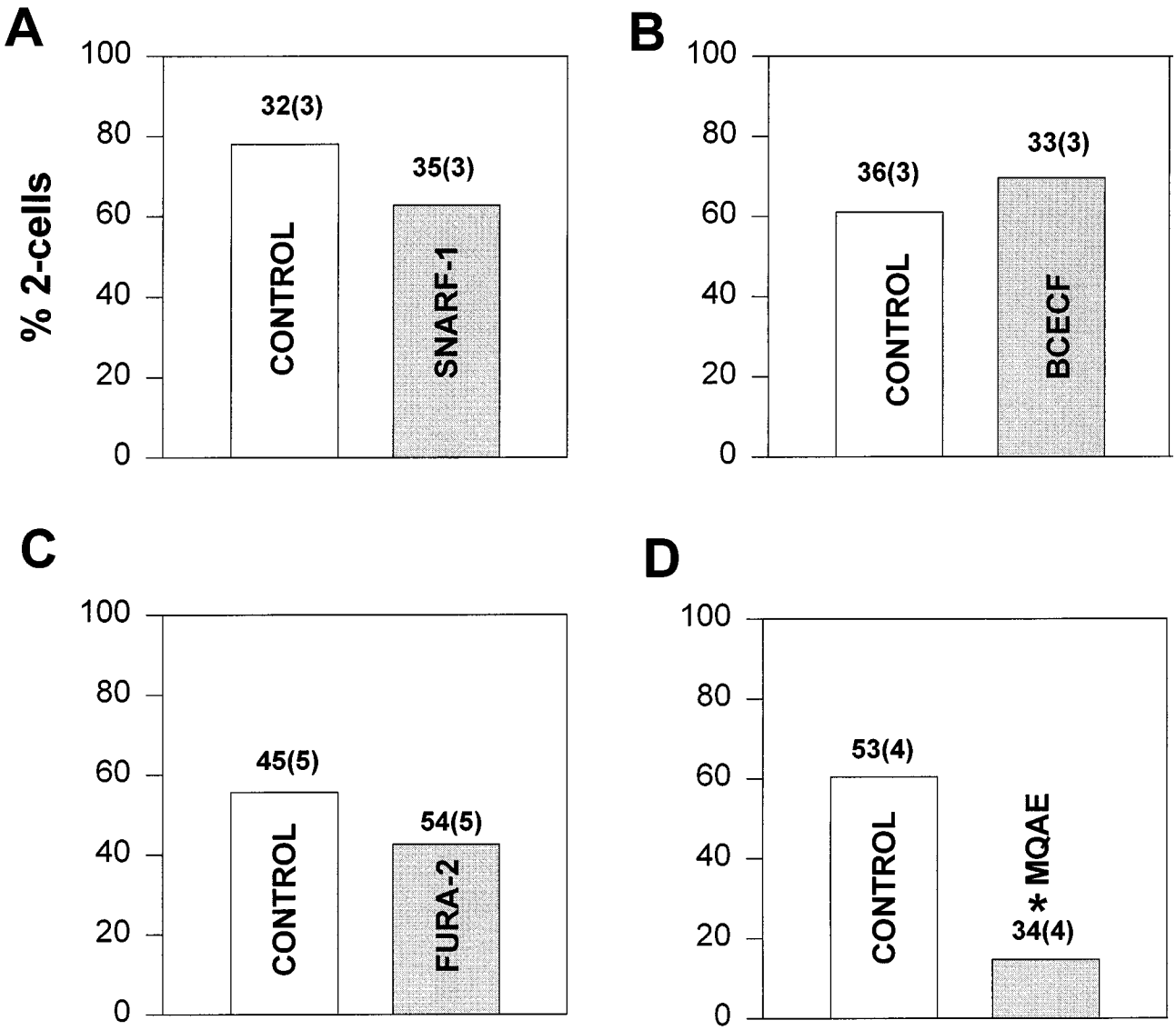


Figure 7 Effect of fluorophore loading and exposure to excitation illumination on preimplantation development. Immediately following light exposure, fluorophore-loaded eggs and controls were fertilised *in vitro* and assessed 24 h later to determine whether embryos had cleaved to the 2-cell stage. (*A*) SNARF-1. Excitation wavelength: 535 nm. There was no significant difference between the rate of 2-cell cleavage in SNARF-1-loaded eggs (69.2%) and control (78.1%). (*B*) BCECF. Excitation wavelengths: 450, 490 nm. There was no significant difference between the rate of cleavage in BCECF-loaded eggs (69.7%) and control (61.1%). (*C*) Fura-2. Excitation wavelengths: 350, 380 nm. There was no significant difference between the rate of cleavage in FURA-2-loaded eggs (42.6%) and control (55.6%). (*D*) MQAE. Excitation wavelength 365 nm. There was a significant difference between the rate of cleavage in MQAE-loaded eggs (17.2%) and control (60.4%; Fisher's Exact Test). *n(N); where 'n' represents number of embryos and 'N' represents number of experiments.*

post-illumination compared with control (24% vs 87.9%; $p < 0.001$; Fisher's Exact Test).

Discussion

Toxicity could result either from fluorophore-loading (AM hydrolysis or exposure to high levels of fluorophore, as in MQAE), the fluorophore itself, or exposure of the fluorophore to excitation illumination. The effect of AM hydrolysis alone was examined by using SNARF-1-AM-loaded eggs and zygotes that were not exposed to excitation illumination. SNARF-1-AM was chosen as it was loaded at the highest concentration of the AM-loaded fluorophores used. It is clear from both the electrophysiological measurements (membrane potential, conductance, whole-cell current) and the cleavage rates for SNARF-1-loaded eggs subsequently used for IVF, that cleavage of AM groups does not have a significant effect on egg or embryo viability.

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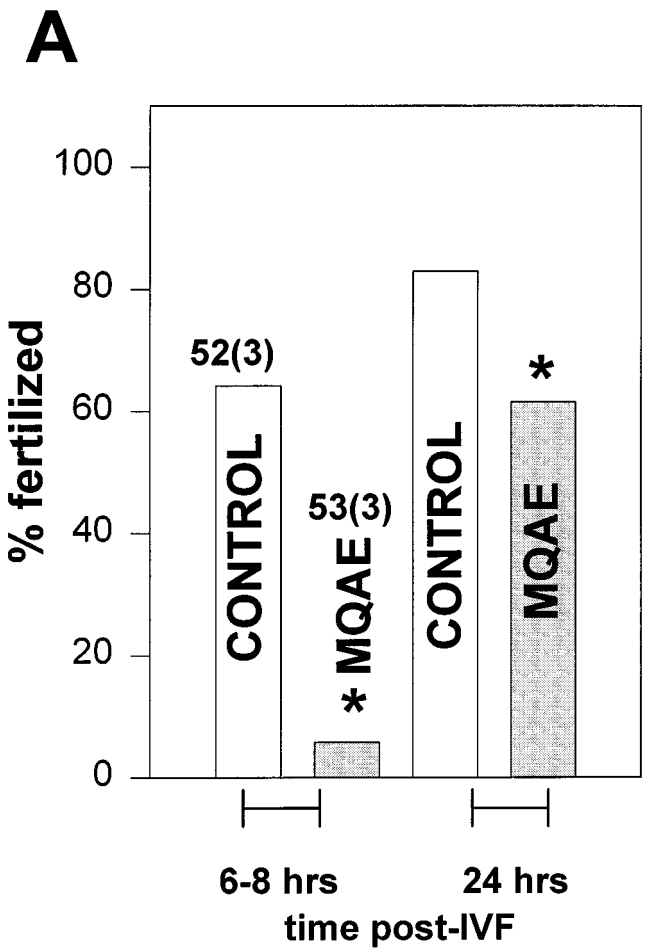


Figure 8 Effect of MQAE alone on fertilisation and preimplantation development. (*A*) MQAE-loaded eggs and controls were fertilised *in vitro* and assessed 6–8 h and 24 h post-IVF for pronuclear development. At 24 h post-IVF, zygotes with 2 pronuclei and 2-cell embryos were scored as fertilised. There was a significant difference in the rate of pronuclear development between MQAE-loaded eggs control at both 6–8 h post-IVF ($p < 0.001$) and 24 h ($p = 0.017$; Fisher's Exact Test). The same group of embryos was assessed 24 h post-IVF to determine whether embryos had cleaved to the 2-cell stage. There was a significant difference between the rate of cleavage in MQAE-loaded eggs and control ($p < 0.001$; Fisher's Exact Test). *n(N); where 'n' represents number of embryos and 'N' represents number of experiments.*

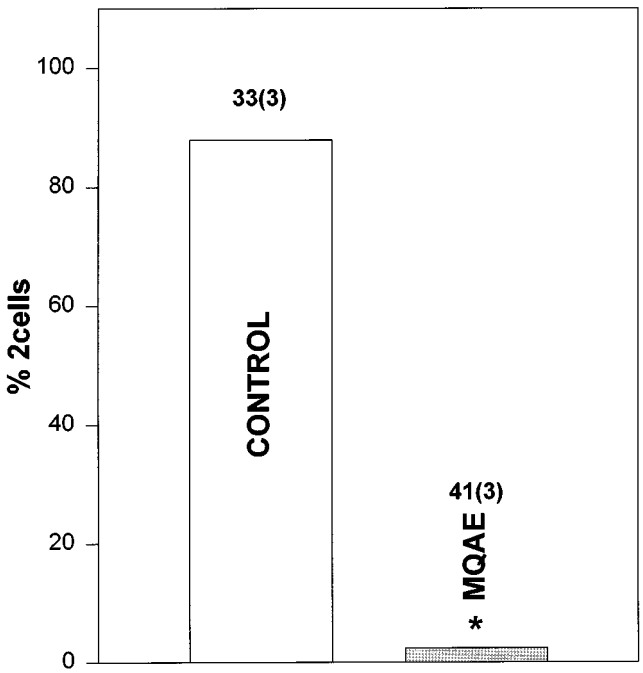
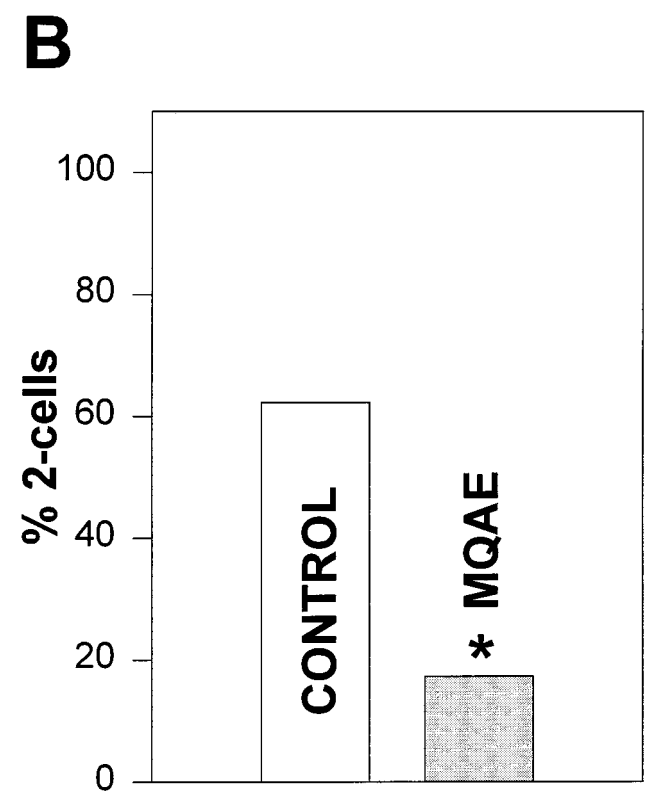


Figure 9 Effect of MQAE and excitation illumination on preimplantation development of *in vivo* produced zygotes. Following light exposure, MQAE-loaded zygotes and controls were cultured and assessed 24 h post-exposure to determine whether embryos had cleaved to the 2-cell stage. There was a significant difference between the rate of cleavage in MQAE-loaded zygotes and control ($p < 0.001$; Fisher's Exact Text). *n(N); where 'n' represents number of embryos and 'N' represents number of experiments.*



Possible damage due to exposure of fluorophore-loaded eggs or zygotes to excitation illumination was examined using SNARF-1, BCECF, Fura-2 and MQAE. The resting membrane potential (Fig. 2) appeared to be the most sensitive measure of damage along the electrophysiological parameters determined. SNARF-1, BCECF and Fura-2 had no significant effect on the resting membrane potential, indicating that their use in a short-term experimental protocol does not significantly

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compromise the integrity of the membrane or its permeability properties. This is further supported by the high rates of cleavage obtained with fluorophore-loaded eggs exposed to excitation illumination and subsequently fertilised *in vitro* (Fig. 2). However, the number of embryos that developed to the blastocyst stage was very low (10–30% in fluorophore-loaded eggs exposed to excitation illumination compared with standard IVF blastocyst rates, not shown), indicating that there is, in fact, some long-term damage and loss of developmental potential resulting from phototoxicity.

The chloride-sensitive fluorophore, MQAE, is not available as an AM or similar form; instead, exposure to 20 mM MQAE was used to load eggs and zygotes. MQAE-loaded eggs not exposed to excitation illumination and subsequently used for IVF, exhibited slow rates of pronuclear formation and decreased cleavage rates compared with control (Fig. 8*B*) indicating that it is MQAE itself, rather than fluorescence excitation, which was cytotoxic.

The acute effects of MQAE on the electrophysiological properties of zygotes were examined using whole-cell patch-clamp techniques. The resting membrane potential became depolarised compared with control following exposure to excitation illumination in MQAE-loaded embryos. Whole-cell membrane conductance and current were also significantly increased by MQAE. These results are strongly indicative of membrane damage. MQAE-loaded eggs exposed to excitation illumination and subsequently fertilised *in vitro* displayed a significant decrease in development to the 2-cell stage (Fig. 7*D*). This reduction in developmental potential was mirrored in MQAE-loaded zygotes, cultured following exposure to excitation illumination (Fig. 9). These results strongly suggest that care should be taken while using MQAE, as cell toxicity occurs both as a result of fluorophore loading and during exposure to excitation illumination.

Zygotes loaded with very high levels of BCECF or exposed to long periods of continuous excitation illumination, do not develop (Baltz *et al.*, 1990; Y. Zhao and J.M. Baltz, unpublished). It has also been reported that electrophysiological recordings revealed damage to leech glial cells caused by high levels of BCECF and excitation illumination (Nett & Deitmer, 1996). We purposely photobleached BCECF-loaded zygotes by exposing them to continuous excitation illumination for 28 min. Although no significant difference was found between the photobleached zygotes and control, the photobleached group seemed to have two populations: one group apparently damaged and the other appearing normal. This is, in part, due to our inability to patch-clamp what were probably the most severely photodamaged zygotes, since gigaohm seals could not be obtained. Some individual photobleached zygotes showed clear depolarisation of resting membrane potential, and increases in membrane conductance and whole-cell current compared with control, although the variation within the group yielded no significant difference. It was evident that some zygotes were damaged by the photobleaching protocol, emphasising the importance of minimising light exposure during experiments.

Even the most damaging fluorophore examined, MQAE, has been used successfully to measure chloride efflux in mouse zygotes upon removal of extracellular chloride. The chloride efflux measured using MQAE was appropriately inhibited by H_2DIDS and IAA-94, indicating that specific Cl^- transporters were functioning (K.P. Phillips and J.M. Baltz, unpublished). MQAE has also been used to study specific chloride transport during blastocoele formation in mouse blastocysts (Zhao *et al.*, 1997). Blastocysts expanded normally in medium containing 20 mM MQAE, indicating low toxicity over the several hours of incubation (Y. Zhao and J.M. Baltz, unpublished). In addition, although fertilisation was delayed, MQAE-loaded eggs, fertilised *in vitro*, still had fairly high rates of pronuclear formation 24 h post-IVF, although subsequent cleavage was significantly reduced. Thus it is still possible to obtain physiologically relevant, consistent data using MQAE.

Conclusion

The use of SNARF-1, BCECF and Fura-2 for intracellular ion measurements did not result in excessive damage to eggs and embryos since we detected no membrane damage and developmental potential was not affected. The damage caused by MQAE was most probably due to the high concentration of the fluorophore required to obtain sufficient intracellular loading for ion measurements.

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