Calcium and wound healing in *Xenopus* early embryos

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SUMMARY

The role of calcium in the healing of wounds made in the ectoderm of *Xenopus* neurulae has been studied. Embryos have been wounded in the presence of calcium inhibitors, and the effects on wound healing observed by scanning electron microscopy. In addition, unwounded embryos have been exposed to a local application of ionophore A23187 to simulate the possible calcium fluxes following wounding. Lanthanum, which competes for calcium channels, inhibits wound healing. EDTA, which binds divalent cations, also inhibits wound healing, but its effect can be reversed by the addition of excess calcium. Local application of ionophore A23187, which promotes transport of calcium across biological membranes, results in a local change in cell shapes. These observations lend support to the hypothesis that wound healing in amphibian early embryos, which is effected by changes in cell shapes similar to those seen in certain examples of normal morphogenesis, is initiated by a local influx of calcium into cells.

INTRODUCTION

Although it has been known for many years that amphibian embryos show a remarkable ability to heal following experimental manipulation, only recently has the process of wound healing *per se* been studied. Scanning electron microscopy of wounds made in the ectoderm of *Xenopus* neurulae has shown that immediately following incision the wound gapes (Stanisstreet, Wakely & England, 1980), presumably because the ectoderm is under lateral tension at this stage (Jacobson & Gordon, 1976). Within 5 min, however, the ectoderm cells surrounding the wound have become tapered towards the cut edge and the wound has started to close, suggesting that coordinated changes in cell shape might be partly responsible for wound closure (Stanisstreet, *et al.*, 1980). The effects of inhibitors on this process imply that microfilaments are involved in these cell shape changes, since wound healing is inhibited by cytochalasin-B but not by colchicine (Stanisstreet & Panayi, 1980). In other systems the contraction of microfilaments is activated by an increase in the level of intracellular free calcium, and so calcium is implicated in wound healing in *Xenopus* embryos.

One model which has been proposed for the mechanism of wound healing in *Xenopus* embryonic ectoderm, consistent with these observations, would be that

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wounding allows a local influx of calcium ions into ectoderm cells (Stanisstreet & Panayi, 1980). An influx of calcium ions through the lateral membranes, newly exposed during wounding, could lead to a local elevation of intracellular free-calcium levels resulting in a contraction of microfilaments causing the changes in cell shape observed by scanning electron microscopy. The lateral cell membranes might be naturally more permeable to calcium ions than the normally exposed apical membranes, or they might be made more permeable by damage during wounding.

The present experiments are designed to test this hypothesis by determining the importance of calcium ions to wound healing in *Xenopus* early embryos. First the effect on wound healing of lanthanum ions, which compete for calcium channels (Weiss, 1974), was assessed. Then the effects of removal of calcium and/or magnesium ions from the medium were studied. Finally, unwounded embryos have been exposed to a local application of the ionophore A23187, which promotes transport of divalent cations, especially calcium, across biological membranes (Reed & Lardy, 1972), in an attempt to simulate the proposed influx of calcium at the wound. So that the shapes of the ectoderm cells surrounding the wound could be observed the embryos were studied, as previously (Stanisstreet, *et al.*, 1980), by scanning electron microscopy.

**MATERIALS AND METHODS**

*Xenopus* embryos were obtained by injecting pairs of adult *Xenopus laevis* with chorionic gonadotrophin (‘Chorulon’, Intervet Ltd.) and were staged according to Nieuwkoop & Faber (1956). The jelly coats were removed chemically by a modification of the method of Dawid (1965); embryos were placed in 2% cysteine hydrochloride in 10% Steinberg saline (Steinberg, 1957) brought to pH 7-8 with 2 M-NaOH. The embryos were washed and subsequently cultured in 10% Steinberg saline, pH 7-8. When the embryos had reached the blastula stage (stage 8½) the vitelline membranes were removed using watchmakers’ forceps. The embryos were cultured overnight over 1% ionagar in 10% Steinberg saline, pH 7-3, until they had reached the neurula stage (stage 15-16).

For each concentration of each medium at least 20 embryos from at least 2 batches of embryos were used and observed microscopically. At least 8 embryos were examined by scanning electron microscopy for every condition, with the exception of the lanthanum experiments where fewer embryos were examined by electron microscopy. Observations for each condition were consistent unless stated in the Results section.

**Effects of lanthanum or EDTA on wound healing**

Embryos were transferred to the test medium over 1% ionagar in either 10% Steinberg saline (for the lanthanum experiment) or EDTA (for the EDTA experiments) for various times before wounding; in the lanthanum experiments embryos
Calcium and wound healing

were exposed for 15 min before wounding, in the EDTA experiments embryos were exposed for 1 min before wounding. The embryos were then wounded in the test medium using an electrolytically sharpened tungsten needle. A longitudinal incision approximately 0.45 mm long was made in the lateral ectoderm. Embryos were fixed for scanning electron microscopy 15 min after wounding. Each experiment was accompanied by controls wounded in 10% Steinberg saline.

The composition of the standard 10% Steinberg saline was: NaCl 5.8 x 10^{-3} M, KCl 6.7 x 10^{-5} M, Ca(NO₃)₂ 3.4 x 10^{-5} M, MgSO₄ 8.3 x 10^{-5} M, Tris 4.6 x 10^{-4} M. The pH was corrected to 7.3 with HCl. For the wounding experiments lanthanum was used at 10^{-2}, 3 x 10^{-3}, 10^{-3}, 3 x 10^{-4} or 10^{-4} M in 10% Steinberg saline. For the EDTA experiments 10% Steinberg saline was prepared as above but without calcium or magnesium. To this calcium- and magnesium-free saline were added 10^{-3}, 5 x 10^{-4} or 2.5 x 10^{-4} M EDTA. 10^{-3} M EDTA was found to prevent wound healing consistently, and to determine which ion, calcium or magnesium, was essential to wound healing embryos were wounded in calcium- and magnesium-free saline with 10^{-3} M EDTA to which had been added 2.5 x 10^{-2} M-CaCl₂, 2.5 x 10^{-2} M-MgCl₂ or both.

Ionophore simulation of wounding

Local ionophore application was accomplished by coating a fine round-ended glass rod drawn from glass capillary tubing (Griffin Ltd.) with ionophore. The tip of the rod was dipped into a solution of 1 mg/ml ionophore A23187 in 100% ethanol, removed and allowed to dry. The process was repeated so that the tip of the rod received five coatings. For the controls, rods were dipped five times into 100% ethanol. Intact neurulae were laid in small depressions in 1% ionagar in standard 10% Steinberg saline and the rod was positioned and held using a micromanipulator so that the tip lay against the lateral ectoderm. After 15 min the embryos were fixed and prepared for scanning electron microscopy.

Scanning electron microscopy

Embryos were fixed overnight in 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2 (modified from Karnovsky, 1965). They were washed in changes of cacodylate buffer and then dehydrated in a graded acetone series. The absolute acetone was replaced with liquid CO₂ and the embryos dried using the critical-point method. The embryos were fixed to stubs with ‘Durofix’ adhesive (Rawplug Ltd.), coated with gold-palladium and observed and photographed using a Phillips 501B scanning electron microscope.

RESULTS

Controls

Microscopical examination of embryos before fixation showed that in standard 10% Steinberg saline 15 min after incision the wound had contracted. The
wound had become filled with loose cells and cellular debris, and this was gently removed with a soft hair loop before fixation. Scanning electron microscopy of unwounded control embryos confirmed previous observations (Smith, Osborn & Stanisstreet, 1976; Stanisstreet, et al. 1980); ectoderm cells were polygonal with slightly sunken margins and the cells had discrete borders with some pits (Fig. 1). Scanning electron microscopical observations of embryos 15 min after wounding in standard 10% Steinberg saline were also consistent with previous observations (Stanisstreet, et al., 1980); the wound was no longer gaping and was starting to close. The cobble-stone effect characteristic of cells around the wound immediately following incision was no longer present (Fig. 2). Cells at the margin of the wound were elongated and tapered towards the wound (Fig. 3).

Effect of lanthanum on wound healing

Scanning electron microscopy of embryos wounded in 10^{-4} M lanthanum confirmed preliminary microscopical observations that wounds were healing normally (Fig. 4); the ectoderm surrounding the wound was flat and not cobble-stoned and the cells at the wound edge were tapered radial to the wound (Fig. 5). Embryos in 10^{-2} or 3 \times 10^{-3} M lanthanum were not healing (Fig. 6); the cells surrounding the wound showed a pronounced cobble-stoned effect and the cells at the edge of the wound were not tapered (Fig. 7). The wound appeared similar to a wound immediately after incision in standard Steinberg saline. Embryos in 10^{-3} M lanthanum showed partial healing, some of the cells at the edge of the wound had become tapered (Fig. 8). Embryos in 3 \times 10^{-4} M lanthanum showed a variable response, some were partially healed, others were not. Thus lanthanum at certain concentrations prevents the change in cell shape associated with normal wound closure.

Effect of EDTA on wound healing

Scanning electron microscopy of embryos wounded in 2.5 \times 10^{-4} M EDTA in calcium- and magnesium-free saline confirmed the preliminary microscopical observations that almost all of the embryos were healing normally (Fig. 9); the

**Figures 1-6**

Fig. 1. Scanning electron micrograph of unwounded ectoderm of *Xenopus* neurula showing regular polygonal cells. × 1100.

Fig. 2. Wound in *Xenopus* neurula ectoderm 15 min after incision in 10% standard Steinberg saline showing wound closure. × 150.

Fig. 3. End of wound in *Xenopus* neurula ectoderm 15 min after incision in 10% standard Steinberg saline showing radially elongated cells at wound margin. × 500.

Fig. 4. Wound in *Xenopus* neurula ectoderm 15 min after incision in 10^{-4} M lanthanum showing wound closing normally. × 150.

Fig. 5. End of wound in *Xenopus* neurula ectoderm 15 min after incision in 10^{-4} M lanthanum showing radially elongated cells at wound margin. × 500.

Fig. 6. Wound in *Xenopus* neurula ectoderm 15 min after incision in 10^{-3} M lanthanum showing wound has not closed. × 150.
Calcium and wound healing
Calcium and wound healing

The ectoderm surrounding the wound was not cobble-stoned and the cells at the edge of the wound were tapered (Fig. 10). At $5 \times 10^{-4} \text{ M EDTA}$ produced more variable results; the wounds of some embryos healed normally, in some cases wound healing was inhibited and in some cases the wound not only failed to close but gaping widely. EDTA at a concentration of $10^{-3} \text{ M}$ completely inhibited wound healing (Fig. 11); the wound gaped widely and had become circular, and cells within the wound were dissociating. The ectoderm cells at the wound edge were stretched tangentially to the wound (Fig. 12). Thus it appears that EDTA inhibits the cell shape changes associated with normal wound healing.

Embryos wounded in $10^{-3} \text{ M EDTA}$ plus $2-5 \times 10^{-2} \text{ M calcium}$ healed normally and the cells showed the changes in shape associated with normal wound healing (Fig. 13). In contrast, embryos wounded in $10^{-3} \text{ M EDTA}$ plus $2-5 \times 10^{-2} \text{ M magnesium}$ appeared like those in $10^{-3} \text{ M EDTA}$ alone; the wound gaped and the cells at the wound edge were stretched tangentially (Fig. 14). Thus calcium appears to be the divalent cation essential for wound healing and the concomitant cell shape changes. Embryos in $10^{-3} \text{ M EDTA}$ plus both $2-5 \times 10^{-2} \text{ M calcium}$ and $2-5 \times 10^{-2} \text{ M magnesium}$ healed normally showing that high magnesium ion concentration does not inhibit wound healing.

Ionophore simulation of wounding

Preliminary microscopical examination of embryos exposed to a local application of ionophore A23187 showed what appeared to be a concentration of pigment at the point of application (Osborn, Duncan and Smith, 1979). Control embryos showed no such pigment alterations. Scanning electron microscopy confirmed that the ectoderm of control embryos appeared normal. Thus the slight pressure of the application rod or any residual substances from the ethanol did not effect the appearance of the ectoderm. Scanning electron microscopy of embryos exposed to ionophore showed that a 15 min exposure had affected the ectoderm. Cells had taken on a more rounded appearance; the ectoderm surface

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**Figures 7-12**

Fig. 7. Margin of wound in *Xenopus* neurula ectoderm 15 min after incision in $10^{-2} \text{ M lanthanum}$ showing cells at wound margin are not radially elongated. $\times$ 500.

Fig. 8. Wound in *Xenopus* neurula ectoderm 15 min after incision in $10^{-3} \text{ M lanthanum}$ showing that wound has partially closed. $\times$ 150.

Fig. 9. Wound in *Xenopus* neurula ectoderm 15 min after incision in $2-5 \times 10^{-4} \text{ M EDTA}$ showing wound is closing normally. $\times$ 150.

Fig. 10. End of wound in *Xenopus* neurula ectoderm 15 min after incision in $2-5 \times 10^{-4} \text{ M EDTA}$ showing radially elongated cells at wound margin. $\times$ 500.

Fig. 11. Wound in *Xenopus* neurula ectoderm 15 min after incision in $10^{-3} \text{ M EDTA}$ showing wound gaping widely. $\times$ 80.

Fig. 12. Margin of wound in *Xenopus* neurula ectoderm 15 min after incision in $10^{-3} \text{ M EDTA}$ showing cells stretched tangentially to wound. $\times$ 275.
Fig. 13. Wound in *Xenopus* neurula ectoderm 15 min after incision in $10^{-3}$ M EDTA plus $2.5 \times 10^{-2}$ M calcium showing wound closing normally. $\times 275$.

Fig. 14. Wound in *Xenopus* neurula ectoderm 15 min after incision in $10^{-3}$ M EDTA plus $2.5 \times 10^{-2}$ M magnesium showing wound gaping widely. $\times 90$.

Fig. 15. Unwounded *Xenopus* neurula ectoderm 15 min after local exposure to ionophore A23187 showing cell shape changes. $\times 150$.

Fig. 16. Part of *Xenopus* unwounded neurula ectoderm 15 min after local exposure of ionophore A23187 showing cells with smooth surfaces and other cells with microvilli. $\times 1100$. 
Calcium and wound healing

in the area of application was irregular and somewhat reminiscent of the
cobble-stoned effect observed lateral to incisions (Fig. 15). Cells within the
effectected area were not uniform; some cells were smooth whereas others were
covered with many microvilli (Fig. 16). Thus exposure to ionophore A23187 had
induced cell shape changes within 15 min.

DISCUSSION

The present results suggest that the changes in the shapes of the cells at the
margins of wounds in *Xenopus* neurula ectoderm are correlated with the degree
of wound healing (radially tapered cells in healing wounds, tangentially stretched
cells in gaping wounds). This observation is consistent with the hypothesis that
wound closure is affected, at least in part, by coordinated changes in the shape
of the cells at the wound edge. In addition since these changes in cell shape are
prevented by removal of calcium from the external medium or by addition of a
calcium competitor, and since changes in cell shape can be induced by an agent
which facilitates calcium fluxes, it is probable that calcium acts as the immediate
trigger for these changes in local cell contractility.

The concentration of calcium in the standard saline used in these experiments
was $3.4 \times 10^{-5}$ M. Indirect estimates of the levels of calcium in *Xenopus* early
embryos made by observing the morphogenetic effects of calcium-EGTA buffers
injected into embryos suggested that the level of calcium in early cleavage stages
of *Xenopus* is of the order of $3 \times 10^{-6}$ M (Baker & Warner, 1972). More recent
direct measurements using ion-selective microelectrodes suggest that the con-
centration of intracellular free calcium in *Xenopus* embryos is even lower at
$2.5 \times 10^{-7}$ M (Rink, Tsien & Warner, 1980). If intracellular free calcium is main-
tained at this low level until the neurula stage, then extracellular calcium is
higher than intracellular calcium concentrations and ionophore treatment, or
the hypothetical increase in membrane permeability following wounding, would
result in a calcium influx. Damage to cells during wounding might release stored
calcium and result in an elevation of extracellular calcium local to the wound,
and ionophore might also penetrate the membrane and act intracellularly
releasing stores of calcium. Thus for a number of reasons it is probable that
application of ionophore will result in an increase in the level of intracellular
free calcium. Conversely lanthanum might prevent wound healing by competing
for calcium channels on the newly exposed lateral membranes and thus inhibiting
calcium influx (Weiss, 1974). EDTA presumably acts by chelating free extra-
cellular calcium, as demonstrated by the reversal of its action in the presence of
excess calcium ions.

It is becoming apparent that calcium is of major importance to a number of
events in early development (Osborn, *et al.*, 1979). Calcium appears to be the
intermediary trigger in the initiation of the changes associated with egg activation
at fertilization (Gilkey, Jaffe, Ridgway & Reynolds, 1978) and in cell cleavage
following fertilization (Schroeder & Strickland, 1974). Morphogenetic movements such as neurulation, which is effected by changes in the shapes of cells from cuboidal to tapered (Schroeder, 1970), may also be controlled by changes in calcium fluxes. In both amphibian (Moran, 1976) and chick (Lee & Nagele, 1979) embryos neurulation is inhibited by papaverine, which inhibits calcium fluxes, and the inhibitory effect of papaverine can be ameliorated by ionophore A23187 (Moran & Rice, 1976). Similarly treatment of early neurula stages of chick embryos with ionophore A23187 precipitates premature fusion of the neural folds (Lee, Nagele & Karasanyi, 1977). There is also some preliminary evidence that calcium fluxes may have a role in gastrulation in amphibian embryos, since treatment of amphibian gastrulae with ionophore A23187 causes the cells of the yolk plug to constrict and the plug to withdraw (Moran & Rice, 1977).

In both of these major morphogenetic movements, as well as in the closure of wounds in the ectoderm of amphibian early embryos, co-ordinated changes in cell shape occur to form tapered or “bottle-shaped” cells. If the hypothesis, supported by the present observations, that cell shape changes during wounding are controlled by local calcium influx is correct, it may be possible to extend this idea to other morphogenetic systems. Thus major morphogenetic movements might be initiated by rather simple factors, such as a local change in membrane ionic permeability.

REFERENCES

Calcium and wound healing


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