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Stable, resealable pores formed in sea urchin eggs by electric discharge (electroporation) permit substrate loading for assay of enzymes in vivo

Robert R. Swezey and David Epel
Hopkins Marine Station of Stanford University
Pacific Grove, California 93950

We describe a simple electroporation procedure for loading suspensions of unfertilized sea urchin eggs with impermeant small molecules under conditions that allow close to 90% successful fertilization and development. Poration is carried out in a low-Ca^{2+} medium that mimicks the intracellular milieu. The induced pores remain open for several minutes in this medium, allowing loading of the cells; resealing is achieved by adding back millimolar calcium ions to the medium. While the pores are open, an influx of exogenous molecules and efflux of endogenous metabolites takes place, and the eggs can lose up to 40% of their ATP content and still survive. Introduced metabolites are utilized by the cells, e.g., introduced ^{3}H-thymidine is incorporated into DNA. This procedure will be useful for loading impermeant substrates into eggs, permitting in vivo assessment of metabolism, and also for introducing other interesting impermeant molecules, such as inhibitors, fluorescent indicators, etc. Though the details may differ, the principle of electroporation in an intracellular-like medium may prove to be useful for loading other cell types with minimal loss of viability.

Introduction

In vivo studies of cell metabolism often require the introduction of substrates or inhibitors into cells. Membrane transport may not suffice, since transporters can be absent or make unwanted modifications to the substance during transport. Microinjection circumvents this problem for cell physiological studies monitored microscopically but is inappropriate for biochemical investigations where hundreds of cells need to be injected. A procedure for introducing substances into bulk populations of cells would, therefore, be useful.

Fusion of cells with liposomal vesicles containing a molecule of interest has been used to load some cells (Fraley *et al.,* 1980; Schaefer-Ridder *et al.,* 1982; Uchida, 1988), but not all cell types are fusible. Alternatively, cell membranes can be reversibly permeabilized by the use of mechanical (McNeil *et al.,* 1984; McNeil and Warder, 1987), chemical (Kucera and Paulus, 1982; Heppel *et al.,* 1985), enzymatic (Lemons *et al.,* 1988; Otero and Carrasco, 1988), or electrical (Kinosita and Tsong, 1977; Kinosita and Tsong, 1979; Sokoloski *et al.,* 1986; Jastreboff *et al.,* 1987) treatments. Electroporation, in particular, has been used to transfect cells with foreign DNA (Potter, 1988), but few studies have documented the permeability status of such cells after exposure to high voltages [but cf. Kinosita and Tsong, 1977; Kinosita *et al.,* 1988; Rols and Teissie, 1989).

Our interest is to introduce radiolabeled substrates into unfertilized sea urchin eggs to compare the rates at which the substrates are metabolized both before and after fertilization (Swezey and Epel, 1986; Swezey and Epel, 1988). Here we show that a mild electrical treatment of eggs suspended in a Ca^{2+}-free, intracellular-like medium enhances the permeability to low molecular weight compounds with minimal loss of egg viability. The resulting permeabilized state can be sustained for at least 20 min, allowing the uptake of extracellular labeled molecules (and the loss of intracellular metabolites). Permeabilized eggs can then be closed by adding millimolar Ca^{2+} to the medium, thus sealing in the labeled molecules. On transfer back to seawater, these eggs can be fertilized and undergo normal embryonic development. This loading procedure should permit an unambiguous determination of enzyme activity changes in vivo brought about by fertilization. The principle of loading cells in a low-Ca^{2+}, intracellular-like medium should also be applicable to other cell types (Hughes and Crawford, 1989).

Results

Our basic observation is that subjecting unfertilized sea urchin eggs to electrical discharge abolishes the permeability barrier to low-molecular-weight compounds, if the permeabilization

medium (PM) is free of calcium ions. In this open state, compounds added to the PM diffuse into the egg cytoplasm; these can then be later sealed into the cells by reclosing the plasma membrane by adding 1 mM Ca^{2+} to the medium. Higher concentrations of Ca^{2+} (such as 11 mM found in seawater) were found to cause a partial activation of up to 40% of the treated eggs, as judged by the appearance of small patches of elevated fertilization membranes similar to that which follows fertilization. Low-calcium seawater (LCaSW) apparently contains sufficient calcium (~1.1 mM) to reseal the eggs before enough calcium enters to cause this partial activation of the fertilization membrane elevation. Wangh (1989) has similarly found that healing of microinjected *Xenopus* oocytes is optimal at Ca^{2+} concentrations of 1–2 μM, which facilitates hole closure without allowing an influx of Ca^{2+} ions that causes oocyte activation.

In initial experiments, penetration of an impermeant fluorescent dye [either 4,6 diamidino-2-phenylindole (DAPI) or 6-carboxyfluorescein] was used to assess permeabilization. We observed that two distinct populations of cells existed after resealing. One group was highly fluorescent and unfertilizable, while the other group was less fluorescent (but well above background) and maintained >90% fertilizability. The highly fluorescent cells always showed partial activation (patches of elevated fertilization membrane) on transfer back to seawater (SW), whereas the less fluorescent cells rarely did. Our current protocol typically yields 85–90% of the treated eggs to be of the less-fluorescent, viable, and unactivated type.

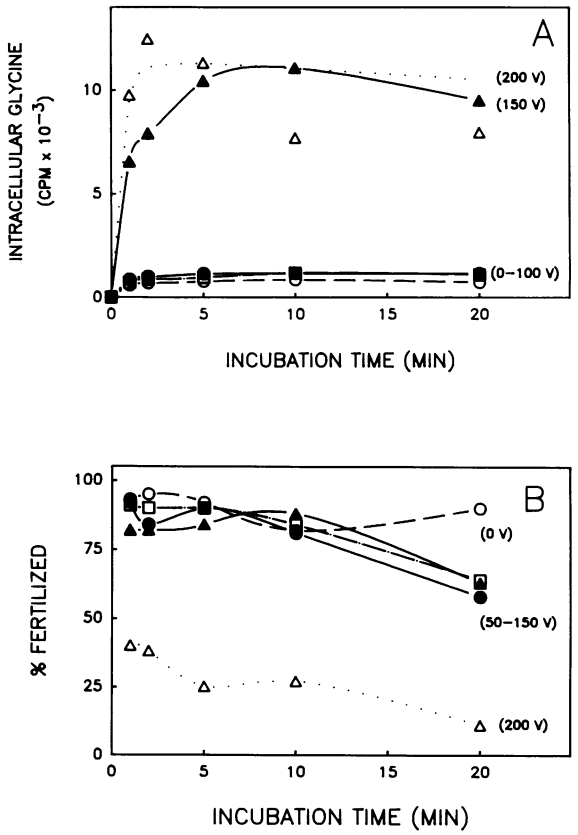

Figure 1. **Voltage dependence of sea urchin egg permeabilization.** A suspension (40% v/v) of unfertilized *S. purpuratus* eggs in a PM containing 5 μCi/ml ^{3}H-glycine was pulsed 5 times at 10-s intervals at 0 V (○), 50 V (●), 100 V (□), 150 V (▲), or 200 V (△). Samples were taken at the indicated times after the final pulse, washed into LCaSW (twice) then SW (twice), and assayed for incorporation of radiolabel (A) or ability to fertilize (B).

Voltage threshold/optimal conditions for permeabilization

To enhance permeability, the voltage applied must exceed a threshold value that varies inversely with cell size (Knight and Baker, 1982). As is seen in Figure 1, the threshold voltage in *Strongylocentrotus purpuratus* for enhancing permeability to glycine is between 100 and 150 V. The threshold voltage for *Lytechinus pictus* is between 120 and 130 V (data not shown). These voltages correspond to an applied transmembrane potential of 0.9 V for *S. purpuratus* and 1.1 V for *L. pictus* (Knight and Baker, 1982). Above the threshold voltage, permeability increases, but viability (here measured as fertilizability) is lost (Figure 1).

The permeability of cells is also affected by the number of suprathreshold pulses applied. Figure 2 shows that the rate of glycine influx increases with the number of pulses applied, such that at 15 pulses the equilibrium value for glycine entry is reached within 1 min. However, as also seen in Figure 2, fertilizability is sacrificed as the eggs are subjected to greater than five pulses. The data in Figures 1 and 2 demonstrate that there is a tradeoff between enhanced permeability and egg viability. In our hands the optimal protocols for permeabilization are five pulses at 125 V for *L. pictus* or 150 V for *S. purpuratus.*

Stability of the permeabilized state

The kinetics of uptake (Figures 1A and 2A) show that apparent equilibration of labeled glycine requires several minutes, indicating that the eggs are not resealing immediately. We wondered whether the progressive decrease in uptake rate represented 1) a slow resealing of the cells in the

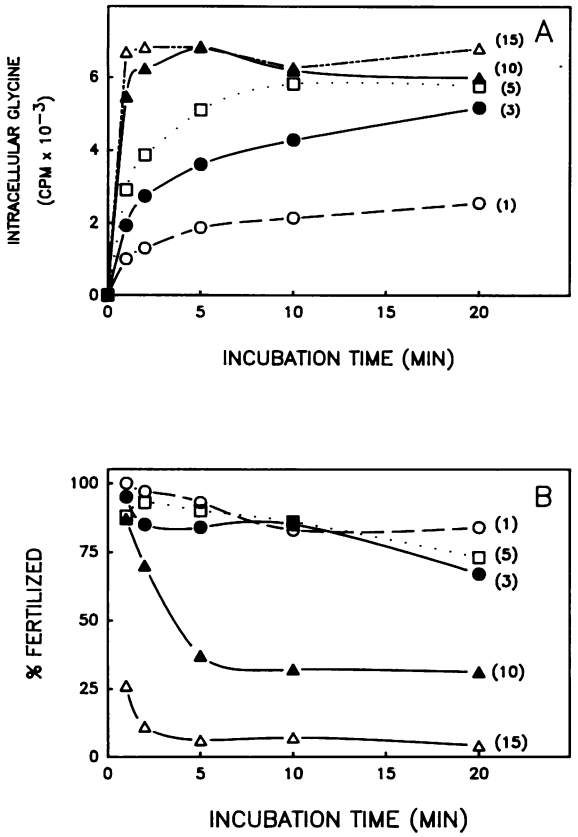

Figure 2. **Dependence of sea urchin egg permeability on pulse number.** A suspension (40% v/v) of unfertilized *S. purpuratus* eggs in a PM containing 5 μCi/ml ^{3}H-glycine was pulsed (with 10-s intervals between all pulses) 1 time (○), 3 times (●), 5 times (□), 10 times (▲), or 15 times (△) at 150 V. Samples were taken at the indicated times and assayed for incorporation of radiolabel (A) or ability to fertilize (B).

PM, or 2) a true equilibration of radiolabel between the ooplasm and the external PM. To examine this, we permeabilized eggs in the absence of a radiolabel, and then we added ^{3}H-glycine to the treated cell suspensions at different times post-permeabilization. Ten minutes after the radiolabel was added, the eggs were washed into LCaSW and then SW to reseal the cells (see Methods) and the radioactivity retained in the cells then determined. If the eggs reseal in the PM, then progressively less labeled glycine should enter the cells the longer the interval between permeabilization and addition of label. The results (Figure 3A) show that label influx is independent of the time of its addition postpermeabilization for at least 20 min. Therefore the cells are not resealing in PM, and time courses for uptake (Figures 1A and 2A) represent true times for these molecules to equilibrate across the permeabilized cell membranes.

We tested whether Ca^{2+} might be the resealing agent in SW by adding millimolar Ca^{2+} to a suspension of treated eggs in PM and then determining whether they remained open to external glycine. The experiment was carried out as described in the previous paragraph, except that at t = 7.5 min an equal volume of PM either containing or lacking millimolar Ca^{2+} was added, and the extent of ^{3}H-glycine incorporated after a 10-min incubation was determined. The results (Figure 3B) showed that permeability to glycine is abolished at millimolar Ca^{2+} concentrations.

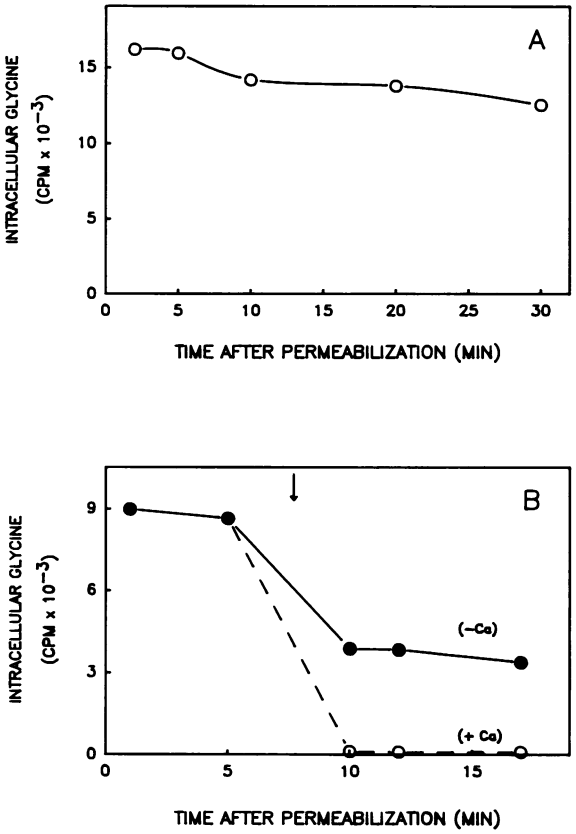

Figure 3. **Stability of the permeabilized state and resealing by Ca^{2+}.** A. Stability. A suspension (40% v/v) of unfertilized *S. purpuratus* eggs in PM (no radiolabel) was pulsed 5 times at 150 V, and at the indicated times samples were transferred to tubes containing ^{3}H-glycine (final concentration = 5 μCi/ml). Ten minutes after adding samples to label, the cells were washed into LCaSW (twice) and SW (twice) and assayed for incorporation of radiolabel into cells. B. Closure by Ca^{2+}. The procedure given for part A was followed, except that at 7.5 min one-half of the remaining permeabilized cells were mixed with an equal volume of PM containing 26 mM $CaCl_2$ (+Ca), and the other one-half mixed with an equal volume of PM (−Ca). Note that these additions dilute the cell concentrations of t > 7.5 min by one-half relative to earlier time points, resulting in an ~50% decrease in amount of glycine sedimented per sample.

Permeability to charged species

The above experiments show that electrically treated eggs are permeable to glycine, a neutral molecule at physiological pH. Other compounds were examined to determine whether there is any intrinsic charge discrimination in the permeabilized state. The results (Table 1) are expressed as percent exchange, by which we mean the percentage of label that could equilibrate into the eggs if they were hollow spheres lacking binding sites for these molecules. For example, if eggs occupy 40% of the volume in a suspension, then they would be expected to accumulate maximally 40% of the total label at full equilibration (=100% exchange).

As is seen in Table 1, both negatively and positively charged molecules can enter treated eggs, albeit more slowly than neutral species. Other workers (Escande-Gerand *et al.*, 1988) have reported similar findings with electrical permeabilization of mammalian cells in culture. The ease of loading decreases with charge of the molecule (e.g., compare glucose-6-phosphate with 6-phosphogluconate), yet even compounds as highly charged as thymidine triphosphate can be loaded.

Molecular size must in part also determine permeability since we found that extracellular proteins cannot penetrate the treated cells. For example, exogenously supplied lactate dehydrogenase (chosen because this enzyme is not present in sea urchin eggs) will not enter electrically pulsed eggs. Furthermore, after permeabilization, eggs lose <2% of their protein after 10 min (data not shown).

Efflux of endogenous metabolites

Since electrically treated eggs permit entry of extracellular small molecules, we wondered whether this permeability is bidirectional, i.e., are these cells leached of their metabolites as they incubate in the PM? To examine this, we measured the rate at which two abundant intracellular compounds, glycine (in *S. purpuratus* and *L. pictus*) and ATP (in *L. pictus*), accumulate in the PM surrounding electrically treated cells.

The results in Figure 4 show that intracellular molecules do leak from pulsed eggs, to the extent that by 20 min postpermeabilization 40–70% (depending on the species and applied voltages) of the measured intracellular metabolites escape. Nonetheless, these depleted cells are still capable of fertilization and development at least to gastrulae. Figure 4 for *S. purpuratus* shows an example where permeabilization had a significantly detrimental effect on fertilizability; this has been seen in <10% of the batches of eggs that we have used (see below). At higher voltages (200 V, *S. purpuratus*) the total depletion of intracellular glycine occurs rapidly (<1 min), and these cells could not be fertilized.

Table 1. Compounds introduced into sea urchin eggs by electroporation

Compound	% Exchanged after 2 min
Glycine	16.2 (10.1)
Thymidine	9.4 (11.9)
Thymidine-5′-Monophosphate	8.0 (22.8)
Glucose	7.7 (3.9)
Glucose-6-Phosphate	5.2 (4.7)
Lysine	6.9 (3.6)
Thymidine-5′-Triphosphate	3.7 (2.7)
6-Phosphogluconate	1.1 (6.6)

100% exchange is defined as the percentage of total added radiolabel which could theoretically equilibrate into permeabilized cells, assuming them to be hollow spheres with no binding sites for the labeled molecules. Values given here show the percentage of this theoretical limit that is actually attained after 2 min of incubation with these labeled compounds. Numbers in parentheses show the -fold increase in radiolabel incorporated over that of unpulsed controls handled identically.

DNA synthesis

If ^{3}H-thymidine loaded into eggs by electrical permeabilization (Table 1) enters a metabolically useful compartment, then it should participate in DNA synthesis after fertilization. Figure 5 shows that this is the case, which also indicates that the cells incorporating radiolabel are viable, since they replicate DNA at approximately the expected time.

Initial experiments had used a PM containing 2 mM EGTA to ensure submicromolar calcium concentrations, but this chelator was subsequently shown to be unnecessary for sustained permeability. Furthermore, we found that pulsing in an EGTA-containing PM had the undesirable side effect of retarding the timing of the cell division cycle of treated, then fertilized, eggs by as much as 12 min. Such an effect of EGTA injected into developing sea urchin embryos has been described (Steinhardt and Alderton, 1988), which suggests that the chelator in the PM got into the unfertilized eggs and affected their Ca^{2+} levels after fertilization. In contrast, the cell cycles of eggs pulsed in an EGTA-free PM were retarded by only 0–5 min relative to untreated controls (for each treatment, 100 cells were counted at 5-min intervals, starting at 55 min postinsemination).

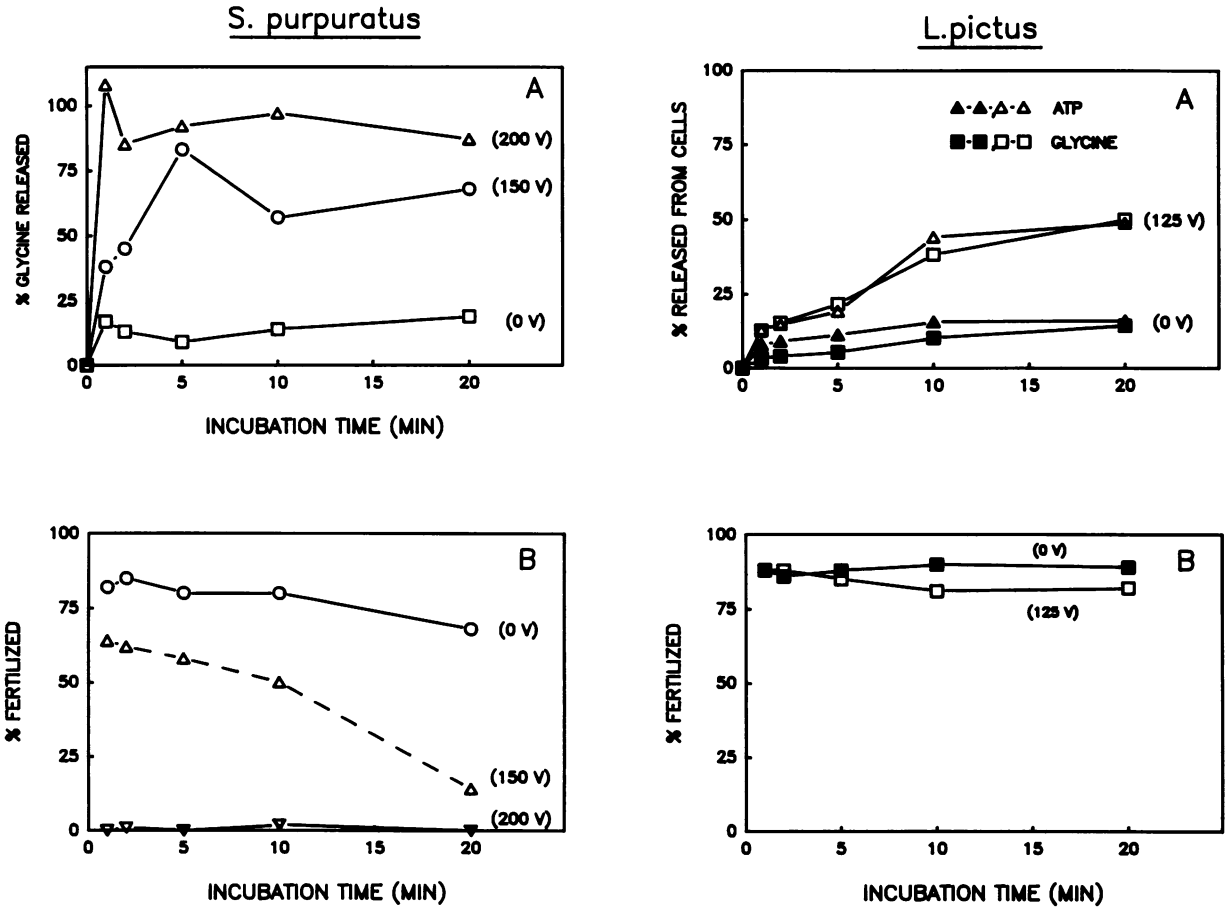

Figure 4. **Efflux of endogenous metabolites from permeabilized sea urchin eggs.** Suspensions (40% v/v) of unfertilized *S. purpuratus* (left panels) or *L. pictus* (right panels) in PM were pulsed 5 times at the indicated voltages, and then samples were centrifuged at the indicated times after the final pulse. The supernatants were withdrawn, and 100% TCA added to make the final concentration of TCA 10%. After incubation for 2 h at 0°C, the samples were centrifuged, neutralized, and then analyzed for glycine (both species), or ATP (*L. pictus*) (A). The 100% values are derived from the levels of these compounds found in centrifuged sonicates of the pulsed cells and treated as above. Assay methods are given in Material and Methods section. The eggs were also analyzed for ability to be fertilized after the pulses (B).

Discussion

Probing the metabolism of unfertilized sea urchin eggs has been hindered severely by the impermeability of their plasma membranes to substrate molecules. In this paper, we show that these permeability barriers can be abolished reversibly to permit introduction of metabolites of interest without sacrificing the egg's competence to fertilize and develop. Bulk loading of cells is achieved by altering their plasma membrane structure with brief exposures to moderate-strength electric fields. In our low-Ca^{2+}, intracellular-like medium, this treatment then permits a sustained bidirectional exchange of low-molecular-weight species across the cell membrane until the cells are resealed by the addition of Ca^{2+} ions to the medium.

Previous workers have shown that electric fields affect sea urchin egg plasma membranes. Using a very different external medium (low ionic strength + 7 mM Ca^{2+}), Millonig (1969) found that sea urchin eggs partially raise a fertilization membrane when exposed for several seconds to voltages that would be subthreshold for permeabilization under our conditions. The longer exposure period (several seconds vs. 70 μs) to the electric fields used by Millonig must enhance permeability of these eggs to the point that the extracellular Ca^{2+} can enter, causing partial cortical granule exocytosis. More recently, Kinosita *et al.* (1988) have presented evidence for the formation of localized pores, assayed as the breakdown of membrane potential, in sea urchin eggs exposed for 25 μs to voltages similar to those used in our

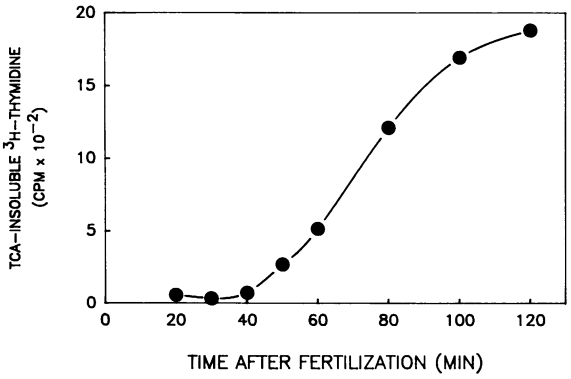

Figure 5. **Incorporation into DNA of ^{3}H-thymidine loaded into sea urchin eggs by electrical permeabilization.** A suspension (40% v/v) of unfertilized *S. purpuratus* eggs in a PM containing 100 μCi/ml ^{3}H-thymidine was pulsed 5 times at 150 V, incubated 2 min in this medium, and washed into LCaSW (twice) then SW (twice). The eggs were brought to 10 ml in SW and fertilized at time zero. Nonfertilized eggs were removed by the use of the protamine-coated petri dish technique described in the Materials and Methods section. At the indicated times 1-ml samples were withdrawn, centrifuged, and the cell pellets extracted with 10% TCA (4°C). The extracted pellets were washed with TCA (3×), methanol (1×), and solubilized for determining radioactivity incorporated into DNA. Unpulsed controls had no ^{3}H-Thy counts above background in the TCA precipitates of fertilized samples.

experiments. The breakdown of the membrane potential recovered in <1 s after turning off the electric field, which they interpreted as a resealing of the pores. Their experiments were conducted at 20°C, a temperature that others have shown to foster resealing of electrically generated membrane pores. It is thus surprising that the sea urchin eggs we used remained open for at least 20 min at 16°C (Figure 3A).

A key difference between our approach and that of others is in the nature of the permeabilization medium used. Our medium is designed to mimic the intracellular milieu to minimize perturbations during metabolite exchange, whereas other workers have typically pulsed cells in isotonic saline (Kinosita and Tsong, 1977; Kinosita and Tsong, 1979), cell culture medium (Mir *et al.*, 1988; Bazile *et al.*, 1989), or low-ionic-strength media made isotonic with sugars (Mehrle *et al.*, 1985; Stopper *et al.*, 1987; Escande-Gerand *et al.*, 1988; Lopez *et al.*, 1988; Mehrle *et al.*, 1989; Rols and Teissie, 1989). It is therefore likely that egg resealing at 16°C is impeded by the PM we use. Since the external medium influences the membrane potential, it also affects whether one (Millonig, 1969; Mehrle *et al.*, 1985; Mehrle *et al.*, 1989) or two (Knight and Baker, 1982; Kinosita *et al.*, 1988) pores form in the membrane per pulse. Our high-potassium medium should depolarize the egg membrane potential to nearly 0 mV, which should cause two pores to form per pulse.

Properties of the pores

In erythrocytes the size of electrically generated pores increases with the strength and duration of the applied electric field and decreases with ionic strength of the medium (Kinosita and Tsong, 1977). Whereas we have not carried out a careful discrimination of the size of the putative pores in sea urchin eggs, a few characteristics of the permeabilization are evident:

1) No apparent molecular binding event, as to a transporter, is involved with uptake of external substances. ^{3}H-glycine influx occurs in the presence of 0.3 M unlabeled glycine. Furthermore, we have found that influx of ^{3}H-thymidine is not reduced by the addition of millimolar quantities of unlabeled thymidine (data not shown). Since there is no competition between labeled and unlabeled molecules, this enhanced permeability shows the characteristics expected of true pores in the plasma membrane, as opposed to those of a saturable carrier.

2) The pores do not discriminate between the charge of various compounds and are large enough to accommodate substances of molecular weight at least 600 (e.g., ATP). In a mammalian cell line, the upper size limit observed for permeant molecules was 1500 for cells that survived electrical pulsing (Escande-Gerand *et al.*, 1988).

3) This treatment does not appear to permit the passage of macromolecules, such as globular proteins, across the egg's cell membrane. This contrasts with the findings of others working with cultured cell lines (Stopper *et al.*, 1987; Mir *et al.*, 1988).

4) The pore radius is probably larger than that of ion channels. From the data in Figure 4A, the rate constant for glycine efflux is 2.5×10^{-3} s^{-1}. With an initial intracellular concentration of glycine of 236 mM [our measurements, consistent with other workers (Fry and Gross, 1970)], and assuming two pores per pulse, one can calculate that, immediately on perforation, 1.6×10^{-14} moles glycine/s exits through each pore. This exit is faster that the movement of ions through the most conductive channels [$\sim 10^{-16}$ moles ions/s, assuming 100 pS conductivity and 100 mV membrane potentials (Hille, 1984)] suggesting that these pores may be larger than naturally occurring ion channels.

Properties of the treated cells

A most striking, though expected, finding is the inverse relationship between permeability and viability in eggs. Increasing the pulse voltage or pulse number quickens both influx and efflux of compounds, but fewer cells survive the treatment. The observation that the nonfertilizable eggs were those shown to be most permeable to fluorescent dyes reinforces this notion. This narrow window of useful electric field strengths has also been seen in mammalian cells (Mir *et al.,* 1988; Bazile *et al.,* 1989).

Since eggs can be depleted of much of their metabolite contents and still fertilize and develop (see Figure 4), it would appear that harsher treatments leave the eggs with insufficient quantities of these compounds to recover. Alternatively, the rapid loss of metabolites may be indicative of internal damage to cell structure, possibly to the extent that they have been made permeable to species of higher molecular weight (e.g., proteins) critical for survival. Notice that solute exit is more rapid for *S. purpuratus* than for *L. pictus* (Figure 4). This could have resulted either from an inherent species difference in susceptibility to permeabilization or from the unequal permeabilization conditions used (i.e., 150 V for *S. purpuratus,* 125 V for *L. pictus*).

Our results also indicate that radiolabeled substrates are not preferentially being taken into those cells that are rendered nonviable by permeabilization. First, by eliminating nonfertilizable eggs by the protamine-coated petri dish procedure, we find that radiolabel is contained in the viable cells. More importantly, the ^{3}H-thymidine loaded into these eggs will be incorporated into DNA after fertilization.

It should be noted that reversibly permeabilized eggs never appear to come to complete equilibrium with respect to influx or efflux of molecules. For example, the maximal glycine incorporation in Figures 1 and 2 is ~20% that expected if the concentrations inside and outside the cells were equal. Also, viably permeabilized cells lose up to 60%, not 100%, of their ATP contents. We suggest that this apparent disequilibrium between internal and external concentrations of molecules in the perforated cells results from the erroneous assumption that the entire cell volume is available to these molecules; given the density of organelles and other intracellular vesiculated compartments seen in electron micrographs of sea urchin eggs, it is clear that much of the egg volume is exclusive of the cytoplasm. However, it is also possible that this apparent disequilibrium of molecules reflects an enhanced capacity of permeabilized cell water, relative to the bulk external water, to dissolve metabolites.

Limitations

While this approach is generally useful for introducing impermeant small molecules, some inherent limitations should be mentioned. Some female sea urchins (<10%) yield eggs that are nonviable after this treatment. One should always make a preliminary permeabilization/resealing test of each batch of eggs to ensure that they survive the treatment, as this will spare precious experimental materials (e.g., radioisotopes, pharmacologically active compounds, etc.).

Second, and curiously, we find that this procedure does not enhance the permeability of fertilized eggs unless the voltages are increased to lethal values; as a loading procedure, therefore, this procedure is limited to unfertilized eggs. Possibly this difference is related to the extensive reformation of the cell surface attending fertilization (Epel and Johnson, 1976). Recently it has been reported (Grigoryev and Shmukler, 1986) that fertilized sea urchin eggs suspended in 0.5 M KCl could be permeabilized to fluorescent dyes by electric shocks, and thus loading metabolites into developing embryos may be possible by the use of a different permeabilization medium.

Suggested loading protocol

For routine loading of sea urchin eggs with impermeant molecules we recommend the following procedure. First, transfer the eggs to PM as outlined in Materials and Methods. The final concentration of eggs in the suspension to be pulsed can be as high as 40% v/v, but viability does decrease somewhat above 10% v/v. This suspension, containing the substance to be introduced, is then pulsed five times at a field strength of 150 V/cm for *S. purpuratus* or 125 v/cm for *L. pictus,* and the suspension is incubated for 2–5 min beyond the final electrical pulse. This permits ≥75% of maximal uptake.

At the end of this incubation, 10–20 volumes of LCaSW are added gently to reseal the eggs, and then the subsequent washings of the eggs back to SW are carried out as described in the Materials and Methods section, with great care being taken to use low centrifugal forces to sediment the eggs. (After permeabilization eggs are fragile and will fragment when centrifuged too rapidly). Once the preloaded eggs are returned to SW, they can be fertilized or artificially activated, and the fate of the introduced compound

followed. If necessary, unfertilized eggs can be removed using the protamine-coated petri dish method outlined in the DNA synthesis portion of the Materials and Methods section.

Applicability to other cell types

This procedure introduces small pores that remain open in a low-Ca^{2+} medium for several minutes (at least 20 min; see Figure 3) and that permit partial equilibration of exogenous small molecules. The key to a successful loading may be achieving a balance between sufficient influx of the desired molecule before there is excessive loss of essential small molecules from the cell. Poration in an intracellular-like medium may prevent extensive irreversible alteration of the intracellular milieu.

This principle of electroporating in an intracellular-like medium has been used with success for permeabilizing rat and human platelets (Hughes and Crawford, 1989), and may be generally applicable to other cell types. The permeabilized platelets were resealed by raising the temperature to 37°C (Hughes and Crawford, 1989), but, for some cells, a temperature increase may be harmful to viability. We suggest that addition of Ca^{2+} ions to the medium is a useful alternative means for resealing perforated cells, and, indeed, divalent cations have been shown to reseal cells permeabilized by pore-forming agents such as melittin and *Staphylococcus aureus* α-toxin (Bashford *et al.*, 1988), and to facilitate healing of microinjected *Xenopus* oocytes (Wangh, 1989). Electroporation may permit finer control over permeabilization than is possible by chemical means; electroporation in an intracellular-like medium may improve the chance of obtaining viable cells that are loaded with the desired substance.

Material and Methods

Materials

Strongylocentrotus purpuratus were collected in the intertidal zone at Point Arena, CA. *Lytechinus pictus* were purchased from Alacrity Marine Biological Services (Redondo Beach, CA). Gametes from these organisms were collected and processed as described previously (Swezey and Epel, 1986), except that in vitro fertilization was carried out at a final sperm dilution of 1:30 000. All biological experiments were carried out at 16°C.

All chemicals were obtained from Sigma Chemical Corp. (St. Louis, MO), except for ^{3}H-thymidine monophosphate (TMP) and ^{3}H-glycine (Amersham), ^{3}H-lysine (ICN), and fluoraldehyde reagent (Pierce).

Permeabilization conditions

Permeabilization medium. Eggs in SW were freed of extracellular calcium with two washes (i.e., centrifugation and resuspension) of calcium-free artificial seawater (CaFSW, MBL formula) and then washed three times in the PM which contained (in mM) 300 glycine, 225 K-gluconate, 185 mannitol, 20 NaCl, 10 spermidine, 5 $MgCl_2$, 4 ATP, 2 $NaHCO_3$, and 1.6 reduced glutathione. This medium was prepared fresh daily, and the pH of this medium was adjusted to 6.8 with HCl just before cell permeabilizations; with time (~30–60 min), this weakly buffered solution tended to drift to a more alkaline pH (7.2–7.3).

Permeabilization apparatus and protocol. The permeabilization chamber was similar to that described previously (Swezey and Epel, 1988), except that the volume capacity was 0.5 ml, and the stainless steel plates were separated by 1 cm. When the chamber contained 0.5 ml of a suspension of cells in PM, it had an electrical resistance of 140 Ω. Permeabilization was achieved by discharging a 0.5-μF capacitor across the plates, and under these conditions the electric field decays by 1/e every 70 μs. Unless otherwise specified, five pulses were applied at 10-s intervals, and postpermeabilization times marked in the figures refer to time after the final pulse.

After incubating the permeabilized cells in PM for the desired periods, they were transferred to a LCaSW, which consisted of 9:1 (v/v) CaFSW:SW, washed once more in LCaSW, then washed twice in filtered SW.

Influx of radiolabeled substances. Eggs were pulsed in the presence of the labeled compound, and then, at various times, postpermeabilization aliquots were removed and transferred back to SW as described above. At the final suspension in SW, a small sample of the eggs was removed to assay fertilization and development, and the remainder was centrifuged and dissolved (≥3 h) in 0.5 M NaOH/5% Triton X-100 and radioactivity quantified by liquid scintillation counting. The samples were also assayed for protein concentration (Esen, 1978) to correct for any differences in the concentration of cells that occurred during sampling.

Efflux of endogenous metabolites. To assay efflux of endogenous glycine and/or ATP, the eggs were pulsed as described above, except that the PM contained 300 mM proline substituted for glycine and it lacked ATP. At various times, postpermeabilization aliquots were withdrawn and centrifuged. Trichloroacetic acid (TCA) was added to the supernatants to 10% final concentration, and the samples were incubated at 0°C for 2 h. This solution was then centrifuged (15 000*g*, 5 min), and the supernatant was neutralized with 6 M NaOH/0.5 M Tris and stored at −20°C until assayed for ATP by a firefly luciferase assay with the use of a liquid scintillation counter (Karl and LaRock, 1975) or for glycine by high-performance liquid chromatography (Jones and Gilligan, 1983). The total content of these metabolites was determined from sonicates of the eggs in PM, which were then acidified with TCA and treated as above.

DNA synthesis measurements. Unfertilized eggs in PM were pulsed in the presence of 100 μCi/ml ^{3}H-thymidine, incubated for 2 min, and then resealed with LCaSW and SW washes. Treated eggs were fertilized at time zero (within 5 min of resealing).

[In early experiments only 60–70% of treated eggs fertilized, and thus the following procedure was developed to remove the nonfertilized eggs. At 3 min postinsemination, the egg suspension was poured onto a plastic petri dish previously coated with protamine (1 mg/ml), allowed to settle for 1.5 min, and then gently shaken and permitted to resettle. Only zygotes with well-formed fertilization membranes remain unbound, whereas all unfertilized eggs will stick to the petri dish. At 6 min postinsemination, those zygotes were poured into a fresh

uncoated petri dish, thus separating them from the eggs rendered unfertilizable by the permeabilization procedure.]

Beginning at 20 min postinsemination, 1-ml samples were removed, centrifuged, and the cell pellets washed three times with SW lacking label. The cell pellet from the final wash was resuspended in 1 ml 10% TCA, and the cells were extracted in TCA for ≥4 h at 4°C. After centrifugation, the cell pellets were dissolved in 0.5 N NaOH/5% Triton X-100 and counted for radioactivity incorporated into TCA insoluble (i.e., DNA) material.

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