Proc. Nat. Acad. Sci. USA
Vol. 71, No. 5, pp. 1915–1919, May 1974

Activation of Sea-Urchin Eggs by a Calcium Ionophore

(fertilization/membrane conductance/respiration/protein synthesis/DNA synthesis)

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Communicated by Daniel Mazia, February 13, 1974

ABSTRACT **Micromolar amounts of the divalent ionophore A23187 can activate echinoderm eggs. The activations by ionophore A23187 were examined in terms of membrane elevation, the program of membrane conductance changes, the respiratory burst, and the increases in protein and DNA synthesis which normally accompany activation by sperm. In all these respects activation by the ionophore was fairly normal although subsequent cleavage and embryonic development was limited.**

Ionophore A23187 activations of the cortex of *Lytechinus pictus* and *Strongylocentrotus purpuratus* eggs were compared in various ionic media and were found to be completely independent of the ionic composition of the external solution. Respiration and protein synthesis of *L. pictus* eggs in singly substituted ionic media also indicated that these activations were independent of external sodium, calcium, or magnesium. These results suggest that the ionophore acts by releasing intracellular Ca^{++}. Consistent with this interpretation is the finding that eggs preloaded with ^{45}Ca show a 20-fold increase in ^{45}Ca-efflux when activated by ionophore A23187 or fertilization. Measurements of the "free" and "bound" calcium and magnesium in homogenates of the unfertilized eggs show that most of the Mg^{++} is already available in the soluble form, whereas Ca^{++} is sequestered but available for release.

We propose that both normal fertilization and ionophore activation affect the metabolism of the egg by releasing Ca^{++} sequestered in intracellular stores.

An increase of the intracellular concentrations of calcium ion at fertilization may play a key role in the promotion of cell synthesis which follows the activation of eggs. Mazia (1) observed an increase in Ca^{++} in the ultrafiltrates of homogenates of *Arbacia* eggs which had just been fertilized. Similar estimates of increases in intracellular "free" Ca^{++} have since been made following the activation of other species of echinoderm egg (2) and by other methods (3).

If intracellular calcium activity is central to activation, then we should be able to parthenogenetically activate eggs with divalent cation-transporting ionophorous antibiotics. These compounds promote transport of divalent cations through lipid barriers (4, 5). We found that micromolar amounts of the ionophores X-537A (Hoffman-LaRoche) and A23187 (Lilly) could activate *Lytechinus pictus* eggs and cause the development of new potassium conductance just as in normal fertilization. We extended our investigations of the ionophore activation, comparing the changes of membrane conductance, respiration, protein synthesis, and DNA synthesis with those resulting from normal fertilization. For these experiments we confined ourselves to A23187 which is reportedly specific for divalent cation transport (5) as opposed to X-537A which transports monovalent cations to some extent (6, 7).

A23187 activations of *L. pictus* and *Strongylocentrotus purpuratus* eggs were compared in various ionic media and surprisingly showed complete independence from the ionic composition of the external solution. For these tests the cortical reaction proved the most conclusive since it could be done in the absence of any divalent cations. Measurements of respiration and protein synthesis in singly substituted ionic media also indicated that these activations were independent of external Na^+, Ca^{++}, and Mg^{++}.

Since the activation of eggs by A23187 did not depend on external ions, the ionophore may act by releasing divalent ions, most probably Ca^{++}, sequestered in intracellular stores. Preloaded eggs in fact were found to release large quantities of ^{45}Ca as a consequence of ionophore activation or normal fertilization.

Determinations of the "free" and "bound" calcium and magnesium were made from homogenates of the unfertilized egg. These showed that most of the calcium is bound while most of the magnesium is already freely available, suggesting that the ionophore activates by its effect on calcium stores.

MATERIALS AND METHODS

Handling of Gametes. The sheddings of *L. pictus* and *S. purpuratus* gametes were induced by intracoelomic injection of 0.5 M KCl. Jelly was removed mechanically by passing the eggs once through a silk mesh and washing. Experiments were done at 16–18° using filtered seawater (SW), except in iron-substitution experiments, where artificial SW was used (see below).

Ionophores. A23187 was obtained from R. L. Hamill, Eli Lilly Co., Indianapolis, Ind. For use with *L. pictus* embryos, the 5 mM stock solution was prepared in one part *N,N*-dimethyl formamide (DMF) to three parts ethanol. DMF–ethanol and dimethyl sulfoxide (Me_2SO) had no activating effects at the concentrations used. For use with *S. purpuratus* embryos A23187 was dissolved in Me_2SO since these eggs will lyse if activated or fertilized in the presence of ethanol. When diluting in seawater, the solutions were continuously stirred to insure good mixing, since the ionophore is very insoluble

Abbreviations: SW, seawater; TCA, trichloroacetic acid; DMF, *N,N*-dimethyl formamide; Me_2SO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; 0 Na^+ SW, artificial SW with glycerol substituted for NaCl; 0 Mg^{++} SW, artificial SW with NaCl substituted for $MgCl_2$; 0 Ca^{++} SW, artificial SW with NaCl substituted for $CaCl_2$, + 2 mM ethyleneglycol-bis(β-aminoethylether)*N,N'*-tetraacetic acid.

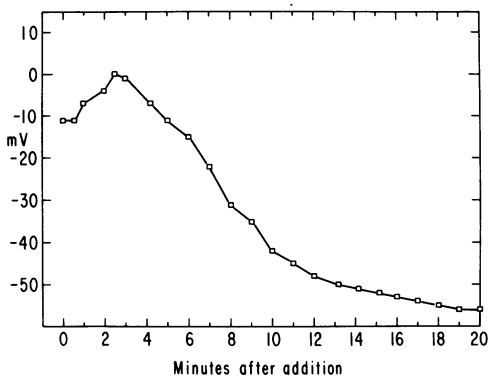

FIG. 1. Membrane potential of *L. pictus* eggs following activation with 18 μM ionophore A23187. In this and all subsequent figures, time 0 refers to addition of A23187 or sperm.

in water and there is some precipitation. Final concentrations given in the text are therefore maximum estimates. A23187 should be kept in the dark, since we observed loss of activity when exposed to light.

Artificial Sea Waters. Artificial seawater had the composition: 460 mM NaCl, 55 mM $MgCl_2$, 11 mM $CaCl_2$, 10 mM KCl, and 2.5 mM $NaHCO_3$, adjusted to pH 8.0 with NaOH. 0 Na^+ SW had 920 mM glycerol substituted for the NaCl. 0 Mg^{++} SW had NaCl substituted for the $MgCl_2$. 0 Ca^{++} SW had NaCl substituted for $CaCl_2$ plus 2 mM ethyleneglycol-bis(β-aminoethylether)*N,N'*-tetraacetic acid (EGTA). Isotonic KCl contained 2 mM ethylenediaminetetraacetic acid (EDTA).

Membrane Conductance. Electrophysiological measurements were made by the methods of Steinhardt, Lundin, and Mazia (8).

Respiration. Respiration was measured polarographically using a Yellow Springs model 5331 oxygen electrode inserted into a 1.3-ml water-jacketed chamber maintained at 18°. The rate of O_2 consumption of the 10% egg suspension was calculated from the slope of the recorder tracing, using a value of 5.6 ml of O_2 per ml for O_2-saturated seawater at 18°.

Protein Synthesis. Transport and incorporation into protein of labeled amino acid during 5-min pulses were measured as described earlier (9), using 0.2% egg suspensions. Incorporation into protein is expressed as percent incorporation, using the ratio "dpm in trichloroacetic acid (TCA) insoluble per dpm in TCA soluble + TCA-insoluble."

DNA Synthesis. A 0.1% suspension of *L. pictus* eggs was incubated in 0.2 μCi/ml of [^{3}H]thymidine (methyl label, 72 Ci/mM, Cal Atomic). Following activation by ionophore or sperm, 2-ml samples were collected at the indicated times, washed free of exogenous isotope with two sea-water washes, and then extracted in 5 ml of 10% TCA. The TCA extracts were heated at 60° for 10 min, the pellets washed twice with TCA, dissolved in 1 ml of NCS, and counted in a toluene-based solution by liquid scintillation counting as above.

Measurement of Ca^{++} and Mg^{++}. Eggs were extracted as described in the text, and ion content was analyzed on a Perkin-Elmer 403 Atomic Absorption Spectrometer using a Ca^{++}-Zn^{++} or Mg^{++}-hollow cathode lamp.

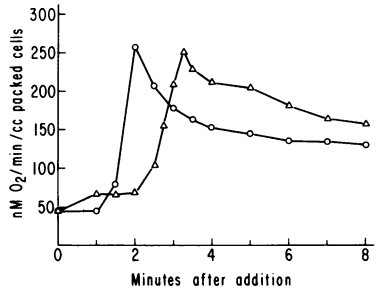

FIG. 2. Changes in respiration following ionophore A23187 activation or fertilization of 10% suspension of *L. pictus* eggs. O——O, fertilized; Δ——Δ, 7.5 μM A23187.

RESULTS

Physiological and biochemical activation

Cortical Reactions. Exposure of *L. pictus* or *S. purpuratus* eggs to micromolar amounts of A23187 resulted in rapid discharge of the cortical granules and elevation of fertilization membranes. Using *L. pictus* eggs (less than 1% suspension), the time from the addition of the ionophore to the beginning of elevation of the fertilization membranes (latency period) depended on the concentration of A23187. The latency period with sperm is 25–30 sec. We found that 0.5 μM A23187 did not activate more than 30% of the eggs, the first elevation occuring after a 4-min latency period. Membrane elevation began at 30 sec in 5 μM and at 10–15 sec in 50 μM concentration. At the higher concentrations of ionophore, membrane elevations were synchronous and proceeded simultaneously at all points on the egg surface instead of starting at one point and propagating as is the case with sperm activation. Similar results were obtained with *S. purpuratus* eggs. Using these eggs, we found that the activation by A23187 also depended on egg concentration, suggesting that the drug is sequestered by the eggs. Using initiation of membrane elevation in 50% of the eggs as a criterion, the latency period in 2 μM A23187 was 20 sec in 0.04% suspension, 45 sec in 0.2% suspension, and 120 sec in 0.8% suspension.

Membrane Conductance. The A23187 activation of the program of membrane conductance changes appeared to be nearly identical with normal fertilization (Fig. 1). First a transient Na^+-dependent depolarization develops, followed shortly by a permanent K^+-sensitive hyperpolarization which is accounted for by new potassium conductance (8). The initial depolarization is absent in 0 Na^+ SW. The final inside negative values of membrane voltages were sensitive to external K^+ and fell off in the expected manner, going below 12 mV at concentrations of K^+ above 120 mM.

Respiration. The effect of A23187 on O_2 uptake was measured polarographically, testing over the range 0.75–75 μM with 10% suspensions of *L. pictus* eggs. At this higher concentration of eggs, no activation was seen at 0.75 μM, but at 1.5 μM A12387 the peak O_2 uptake during the respiratory burst was nearly at the maximal rate. As with the cortical reaction, however, the latency period depended on the concentration of ionophore. The respiratory burst in normal fertilization occurs within 2 min of sperm addition. With 25–75 μM A23187, this peak was at 3 min; at 7.5 μM, 4 min; at 1.5 μM, 7 min; with no activation of respiration occurring at lower concen-

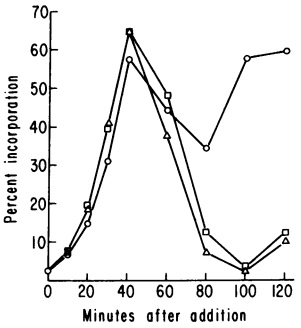

FIG. 3. Changes in protein synthesis following ionophore A23187 activation or fertilization of 0.2% suspension of *L. pictus* eggs. O——O, fertilized; □——□, 0.5 μM A23187; Δ——Δ, 5 μM A23187.

trations. Fig. 2 shows the similar patterns of O_2 uptake during A23187 activation and fertilization.

Protein Synthesis. The effect of A23187 on protein synthesis was measured by [^{14}C]leucine incorporation during 5-min pulses over a 2-hr period. Fig. 3 shows that up until the time of entry into the first prophase following 0.5 μM or 5 μM A23187 activation, the rate of protein synthesis was as high as that resulting from normal fertilization. After this period, however, the rate of protein synthesis in ionophore activated eggs fell behind fertilized eggs.

DNA Synthesis. DNA synthesis during the first cell cycle was determined by measuring [^{3}H]thymidine incorporation into hot TCA-insoluble material. The ionophore-activated DNA synthesis during the first hour was, as expected, close to 50% of the diploid fertilized controls (Fig. 4). [^{3}H]Thymidine transport into eggs is increased 10-fold by 30 min from the addition of sperm or 5 μM A23187.

Cytological Observations. Microscopic observations of living eggs during the first few hours following A23187 activation revealed nuclear membrane breakdown and formation of clear areas suggestive of asters or mitotic apparatus. Around the time of first cleavage of the fertilized egg controls, surface changes resembling cleavage were seen in some ionophore-treated cells. Furrowing was generally unsuccessful but some 2-celled "embryos," containing one nucleus per cell, were seen. No further cleavage activity was observed.

More detailed observations of mitotic activity were made with cells fixed in Carnoy's fluid and examined by phase contrast microscopy. *L. pictus* eggs were activated with 0.5 or 5 μM A23187 and fixed between 80 and 180 min. Events were similar in both concentrations. In general, mitotic events were asynchronous and lagged behind fertilized control embryos. For example, control eggs were in anaphase or telophase at 80-min post-fertilization, whereas, A23187-activated cells were in prophase or in a "prometaphase-like" stage. Mitotic spindle material appeared later than usual, such that when the nuclear membranes broke down between 80- and 100 min, the chromosomes were deposited directly into "cytoplasm" or into small areas of clear spindle-like material. Fibrous monasters were occasionally seen at 100 and 125 min, either associated with highly-condensed metaphase chromosomes or with newly-reformed nuclei. By 145 and 180 min, most cells were in telophase or interphase with one nucleus, but occasional binucleate cells were seen.

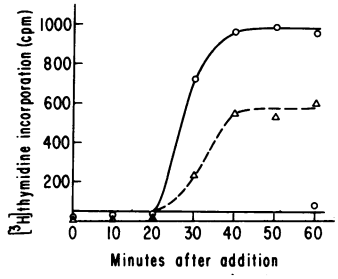

FIG. 4. Changes in DNA synthesis following ionophore A23187 activation or fertilization of 0.1% suspension of *L. pictus* eggs. O——O, fertilized; Δ- - -Δ, 5 μM A23187.

Comparisons of ionophore activation in different ionic media

To test whether A23187 required the presence of any particular ion in the external medium in order to activate, we exposed *L. pictus* eggs to ionophore in different ionic media. Our comparisons include membrane elevation, respiration. and protein synthesis.

Membrane Elevation. Membrane elevation was observed in all media tested, 0 Ca^{++} SW, 0 Mg^{++} SW, 0 Na^{+} SW, 0 K^{+} SW, and 0.55 M KCl plus 2 mM EDTA. This was also true for *S. purpuratus* eggs. These results indicate that no single ion or combination of ions is required for ionophore activation of membrane elevation.

The test of membrane elevation in 0.55 M KCl plus 2 mM EDTA was the only indicator of activation we could use in the total absence of divalent cations. Eggs lyse in absence of divalents if stirred, and stirring is required for other metabolic measurements (see below). Membrane potentials can be measured without stirring, but not in the absence of calcium. We could not test activation in the total absence of salts since eggs lyse and quickly die in isotonic non-electrolyte media.

Respiration. A23187 activated the burst of O_2 uptake in the absence of calcium or magnesium (Fig. 5). The O_2 uptake in 0 Ca^{++} or 0 Mg^{++} media was slightly less than the fertilized sea-water control. This slight reduction might be an effect of the ionic media directly on respiration rather than on the

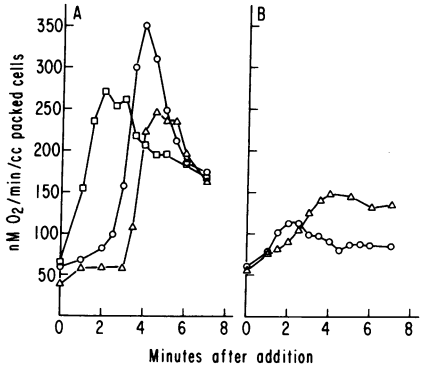

FIG. 5. Changes in respiration following ionophore A23187 activation (7.5 μM) or fertilization of *L. pictus* eggs in singly substituted ionic media. Fertilization in 0 Ca^{++} SW or 0 Mg^{++} SW were not possible, so comparisons with fertilization could not be made. (*A*) O——O, artificial SW; □——□, 0 Mg^{++} SW; Δ——Δ, 0 Ca^{++} SW. (*B*) O——O, fertilized in 0 Na^{+} SW; Δ——Δ A23187 in 0 Na^{+} SW.

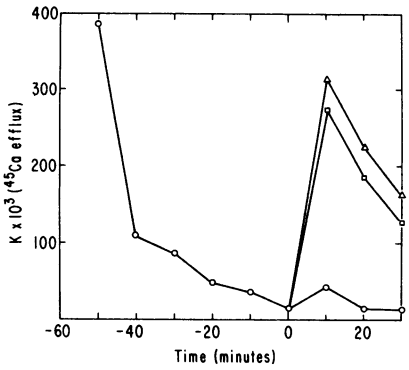

FIG. 6. ^{45}Ca efflux following ionophore A23187 activation or fertilization of 5% suspension of *L. pictus* eggs. O——O, unfertilized; □——□, fertilized; Δ——Δ, 5 μM A23187. The K values of ^{45}Ca efflux refer to the fraction $\times 10^3$ of the radioactive calcium released during the preceding 10-min period. In order to calculate the fraction released, we need the amount of isotope remaining in the eggs for each period. This is determined by taking the ^{45}Ca remaining at the end of the experiments. Efflux is then added back to the midpoint of each period to give total ^{45}Ca for each point. The minus time values refer to the efflux before treatment. At time zero the eggs were divided into three batches and treated with A23187, sperm, or a DMF–ethanol blank. (The numbers on the ordinate have been multiplied by 10^3.)

activation of respiration but we could not test this point since fertilization is not possible in either 0 Ca^{++} or 0 Mg^{++} artificial seawaters. In 0 Na^+ SW, the ionophore activation actually stimulated more O_2 uptake than sperm activation, but respiration was considerably reduced in that media for both types of activation. The possibility that the respiratory burst reflects energy used to pump out Na^+ is currently being investigated.

Protein Synthesis. A23187-activated protein synthesis in the absence of calcium, magnesium, or sodium is shown in Table 1. Ionophore activations in 0 Na^+ SW show a reduced rate. However, this reduction of rate is also seen with normally fertilized eggs after they are put in 0 Na^+ media (legend, Table 1).

Divalent cations

Ca^{++} Efflux. Since the data above is consistent with an ionophore-stimulated release of intracellular stores of calcium, we tested for the release of preloaded ^{45}Ca by A23187 and by normal fertilization. A 6.8% suspension of *L. pictus* eggs was preloaded with ^{45}Ca (18 μCi/ml) for 1 hr at 4°. The preloaded eggs were washed four times with sea water and then their efflux was followed until they reached low equilibrium levels. The eggs were then divided into three batches and treated. The DMF/ethanol blank remained at low levels of ^{45}Ca efflux, returning to equilibrium values within the next 30 min. Both the A23187-activated eggs and fertilized eggs showed a 20-fold increase in calcium efflux which remained high over the 30-min period sampled after activation (Fig. 6).

"Free" and "Bound" Calcium and Magnesium in the Unfertilized Egg. Since A23187 can bind to and transport Mg^{++} as well as Ca^{++}, we examined the proportions of these ions which were already available as "free" cation. *S. purpuratus* eggs were washed twice in 0.55 KCl and then disrupted in an ice-cold Dounce homogenizer in a variety of media (Table 2). The extracts were immediately centrifuged at 37,000 × *g* for 30 min and separated into "soluble" and "insoluble" fractions. The supernatant fractions were analyzed directly by atomic absorption, and the insoluble fractions were extracted overnight in concentrated HNO_3, neutralized, and then analyzed. As seen (Table 2), 70–80% of the magnesium is found in the soluble fraction in all the homogenation media. This contrasts markedly with calcium, which ranges from 9% soluble in 0.55 M KCl, pH 6, to 47% soluble in 0.60 M sucrose. These results indicate that whereas magnesium is generally available, the amount of calcium present in the soluble fraction depends on the conditions of homogenation. This suggests that calcium is usually in the bound state but in a form that can be transfered to the soluble phase.

TABLE 1. *Protein synthesis in singly-substituted ionic media*

Activator and type of seawater (SW)	Percent incorporation at 30 min	60 min
Sperm, artificial SW	18.8	18.7
A23187, ASW	20.2	16.4
A23187, 0 Ca^{++} SW	32.0	15.2
A23187, 0 Mg^{++} SW	24.4	23.3
A23187, 0 Na^+ SW	11.9	10.8
Unfertilized	1.5	

Eggs were activated with sperm or ionophore (5 μM) and pulsed for 5 min with [^{3}H]valine 30 and 60 min later. The depression of incorporation in 0 Na^+ SW is most likely a general effect on cell synthesis and not an effect on activation. In experiments using choline-substituted 0 Na^+ SW, protein synthesis of embryos was reduced 59%.

DISCUSSION

Every parameter of normal activation with sperm that we examined is also observed when eggs are exposed to divalent transporting ionophore. Membrane elevation, membrane conductance changes, the respiratory burst, and increases in protein and DNA synthesis are all initiated in the usual fashion by micromolar concentrations of A23187. However, differences develop as time goes on. Protein synthesis falls behind in the ionophore-activated eggs after 40 min, and mitosis is delayed and was not successfully completed. Monasters eventually formed in most cells and subsequent

TABLE 2. *"Free" Calcium and Magnesium in S. purpuratus eggs*

Homogenation media	% Free Mg^{++} *	% Free Ca^{++} †
0.55 M KCl, pH 6	47	9
0.55 M KCl, pH 7	62	22
0.25 M KCl, 0.40 M sucrose, pH 7	61	43
0.60 M sucrose, pH 7	56	47
0.55 M KCl, 0.005 M EDTA, pH 7	51	19

Values determined by atomic absorption; pH 7 extracts buffered with 0.05 M imidazole.

* Total Mg^{++} is 17.9 mM.

† Total Ca^{++} is 7.1 mM.

events were similar to those seen for other parthenogenetic agents when cleavage is unsuccessful (10).

We were unable to obtain normal embryonic development, even when we washed *L. pictus* eggs in fresh sea water 5 min after A23187 activation. However, we have made no concentrated efforts to promote normal development with secondary treatments, such as hypertonic seawater.

These physiological, biochemical, and cytological observations point to a fairly normal activation by ionophore with lesions emerging as development proceeds. Since it is difficult to obtain parthenogenetic development with *L. pictus* eggs, it is not clear whether these lesions result from the ionophore *per se* or are characteristic of parthenogenetic activation of these eggs.

A23187 can bind to and transport Ca^{++} and Mg^{++}, but not K^+, ions through lipid phases (5). Like X-537A (11), A23187 has been shown to act across cell membranes and show dependence (12) or independence (13) of the ionic composition of external solutions. A23187 activation of sea urchin eggs is independent of ions in the external solution and suggests divalent ion release from subcellular stores. Since Ca^{++} ion activity was previously shown to increase with fertilization (1), we conclude that most probably intracellular calcium release is the normal activating agent in fertilization and in activation by A23187.

Our results showing a 20-fold increase in ^{45}Ca effiux with ionophore activation or normal fertilization support the calcium hypothesis of activation. A23187 could theoretically activate by Mg^{++} movements. However, our data on free and bound Ca^{++} and Mg^{++} in homogenates of unfertilized eggs would discount that possibility. Most of the Mg^{++} is already available, whereas the low levels of free Ca^{++} in the unfertilized egg make calcium the likely candidate for release by divalent ionophore.

We thank D. Mazia, T. Schroeder, P. Reed, C. Mehard, B. Volcani, and R. Hamill for helpful discussion and E. Baker for technical assistance. This work was supported in part by grants from the National Science Foundation and the Population Council.

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