ROLE OF CALCIUM IONS IN THE CONTROL OF EMBRYOGENESIS OF *XENOPUS*

Changes in the Subcellular Distribution of Calcium in Early Cleavage Embryos after Treatment with the Ionophore A23187

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ABSTRACT

Treatment of stage 5 *Xenopus* embryos with the ionophore A23187 for only 10 min, in the absence of extracellular Mg^{2+} and Ca^{2+}, causes cortical contractions and a high incidence of abnormal embryos during subsequent development. Cation analysis shows that divalent ions are not lost from the embryos, but that Ca^{2+} is redistributed within the subcellular fractions. Ca^{2+} is probably released from yolk platelets and/or pigment granules by the action of A23187, $[Ca^{2+}]$ rises in the cytosol, and the mitochondria attempt to take up this free Ca^{2+}. The mitochondria concomitantly undergo characteristic ultrastructural transformations, changing towards energized-twisted and energized-zigzag conformations. A23187 allows these changes to be demonstrated *in situ*. Extracellular divalent cations (10^{-4} M) interfere with this intracellular action of A23187. Intracellular accumulation of Na^{+} (by treatment with ouabain) or Li^{+} also causes abnormal development, probably by promoting a release of Ca^{2+} from the mitochondria. It is suggested (*a*) that all these treatments cause a rise in $[Ca^{2+}]_i$ which interferes with normal, integrated cell division, so causing, in turn, abnormal embryogenesis, (*b*) that levels of $[Ca^{2+}]_i$ are of importance in regulating cleavage, (*c*) that the mitochondria could well have a function in regulating $[Ca^{2+}]_i$ during embryogenesis in *Xenopus*, and (*d*) that vegetalizing agents may well act by promoting a rise in $[Ca^{2+}]_i$ in specific cells in the amphibian embryo.

KEY WORDS calcium · embryogenesis · ionophore · *Xenopus* · cell division · lithium

There is considerable evidence that a variety of internal signals function as regulators of cell division. Cyclic AMP, cyclic GMP, and Ca^{2+} have all been implicated in the control of growth; the evidence has been reviewed by Berridge (7) who has advanced the unifying hypothesis that the primary signal responsible for switching on cell division is an increase in the intracellular level of calcium ($[Ca^{2+}]_i$). In this hypothesis, the ability of cAMP to influence division in a variety of cells is accounted for on the basis of its positive or negative feedback effects on $[Ca^{2+}]_i$. Amphibian embryos represent a convenient system for the

study of the intricate control of cell division during early development and have been used in the present investigation.

Exposure of embryos to Li^+ produces characteristic abnormalities in which the normal balance of cell populations and the control of morphogenesis are disturbed (44, 45). It seems clear that intracellular accumulation of Li^+, which is not readily pumped out of the cells, causes an interference in the proper regulation of cell division (34). Intracellular accumulation of Li^+ has a number of microphysiological effects in other cells, including a dramatic increase in the spontaneous rate of release of transmitter at the presynaptic terminals of the frog neuromuscular junction (10). We believe that this acceleration of spontaneous release is related to a rise in $[Ca^{2+}]_i$ (46, 47) which is, in turn, a consequence of the rise in $[Li^+]_i$ (48). We have, therefore, explored the possibility that changes in $[Ca^{2+}]_i$ can affect normal morphogenesis in *Xenopus* embryos. In many of the experiments, we have made use of the divalent cation ionophore A23187 which transports divalent cations, but not alkali metal cations, across biological membranes (46, 49), and our results suggest that changing levels of $[Ca^{2+}]_i$ are important in the control of normal morphogenesis and that $[Ca^{2+}]_i$ could be regulated, at least in part, by the mitochondria.

Early studies by Mazia (29) suggested that $[Ca^{2+}]_i$ was responsible for triggering cell division after fertilization, and more recently it has been argued (4, 5, 6, 31) that inducing compounds in amphibian development, both natural and unnatural, have as a common property an alteration in membrane properties, resulting in the internal release and redistribution of inorganic ions to new binding sites. Baker and Warner (3) have monitored changes in $[Ca^{2+}]_i$ in *Xenopus* embryos with the calcium-sensitive protein aequorin and have suggested that the average concentration of free intracellular $[Ca^{2+}]_i$ may increase at the time of cleavage.

Our results also demonstrate that an experimental modification of $[Ca^{2+}]_i$ by the use of A23187, rapidly causes cortical contractions. A number of papers have shown previously that Ca^{2+} is probably also important in the activation of a contractile filament system. The studies on this system have utilized such techniques as the application of A23187 in frog eggs (41) and squid embryos (1), injections of cytochalasin B (28), and applications of highly charged polycations (20) to *Xenopus* embryos, treatment of *Ambystoma* neurulae with papaverine (31), and an electron microscope study of the microfilament system in the surface layers of early embryos of *Triturus* and *Xenopus* (36). This Ca^{2+}-activated system has been shown to be important in cleavage and wound healing, and its activation is frequently accompanied by pigment movements in amphibian embryos (41).

MATERIALS AND METHODS

Obtaining Synchronously Developing, Jelly-Free Embryos

Adult *Xenopus laevis* were maintained in the laboratory at 20°C with a 12-h light, 12-h dark regime and fed on chopped liver. Eggs were obtained from either normal or artificial fertilization. In the latter technique, 15–20 h after injection of 750 IU of chorionic gonadotrophin (Chorulon; Intervet, Cambridge, U. K.), *Xenopus laevis* females were gently squeezed to obtain several hundred unfertilized eggs. These were artificially inseminated using methods adapted from Wolf and Hedrick (54) and were allowed to develop to early cleavage stages. Embryos were staged according to Nieuwkoop and Faber (33), and in most experiments were at stage 5 (16 cells) when first placed in the test solution, although some tests with stage 6 (32 cells) embryos were also carried out. The embryos were then dejellied by treatment with 2% L-cysteine hydrochloride plus 0.01% papain in 10% Steinberg solution (50), adjusted to pH 7.8 with NaOH (after Dawid [13]). Dejellied eggs were washed in four changes of 10% Steinberg solution and maintained in 10% Steinberg solution in small petri dishes at 20°C (±0.5°C).

Solutions

The standard 10% Steinberg solution used contained 6×10^{-3} M NaCl, 6.7×10^{-5} M KCl, 8×10^{-4} M $MgSO_4$, 3×10^{-5} M $Ca(NO_3)_2$, 7×10^{-3} M Tris buffered to pH 7.1 with HCl. In early experiments the Ca^{2+} was adjusted and maintained constant by a Ca^{2+}-ethylene glycol-bis(β-aminoethyl ether)*N,N,N',N'*-tetraacetate (EGTA) buffer (38); 0.5 mM EGTA was added before addition of HCl, and the appropriate volume of AnalaR standard volumetric $CaCl_2$ was added in place of the $Ca(NO_3)_2$. Later experiments with unbuffered Ca^{2+} were prepared in distilled water which had been passed through an ion-exchange column and stored in plastic bottles to minimize Ca^{2+}-exchange. All plasticware and glassware were acid-washed. A23187 was dissolved in ethanol so that the stock solution contained 1 mg A23187 ml^{-1} ethanol. The final concentration of A23187 used in most experiments was 2.5 μg ml^{-1} saline, and the concentration of ethanol was then 2.5 μl ml^{-1}. Control solutions contained the same concentration of ethanol, although this concentration had no

significant effect on either abnormalities or survival of the embryos. In the experiments concerned with the effect of exposure to monovalent cations on development, LiCl, NaCl, or sucrose was added to the 10% Steinberg solution, as appropriate.

Exposure to Test Solutions

The embryos were washed twice in 10% Steinberg solution containing the appropriate concentration of divalent cations lacking ionophore and then transferred to the test solution (8 ml) at 20°C. After exposure, embryos were washed once in 10% Steinberg solution containing the original concentration of divalent cations but lacking the test agent, followed by three washes in standard 10% Steinberg solution. All experiments were carried out with appropriate controls in solutions with the same ionic composition but lacking ionophore. Embryos were allowed to develop at 20°C (±0.5°C) and were examined for survival and abnormalities after 24 and 48 h.

Pigment Movements

Pigment movements during exposure to A23187 were observed under a stereomicroscope at a low level of illumination. Since the effects of A23187 were dependent on temperature, photography under bright lights proved to be impossible. After exposure to A23187, embryos were placed in Smith's fixative for 10 min, washed, and then photographed with a Wild photomicroscope. Wild Heerbrugg Instruments, Farmingdale, N. Y.

Electron Microscopy

Embryos were transferred to fixative at selected intervals after initial exposure to A23187. They were fixed whole after removal of the vitelline membrane and were divided into animal and vegetal halves during fixation. Fixation was for 3–5 h in 2.5% glutaraldehyde, 2% paraformaldehyde, 2.5×10^{-3} M $CaCl_2$, 0.1 M sodium cacodylate buffer at pH 7.2 (modified from Karnovsky [24]), followed by washes in several changes of 0.1 M cacodylate buffer containing 2.5×10^{-3} M $CaCl_2$ for ½–1 h. Material was postfixed for 2–3 h in 1% osmium tetroxide in 0.1 M cacodylate buffer and then washed several times in fresh 0.1 M cacodylate buffer over 2 h. After dehydration the material was embedded in Spurrs firm composition epoxy resin mixture (Emscope Labs., London). Sections were cut at 60–90 nm, stained in uranyl acetate (20 min), poststained with lead citrate (5 min) and examined in an AEI Corinth 272 electron microscope.

Cell Fractionation

The cell fractionation procedure used was essentially that of Dawid (14). All operations were carried out at 0°–4°C. After exposure to A23187, embryos were rinsed three times in 0.25 M sucrose, 0.03 M Tris-HCl (pH 7.4) before being homogenized in the same buffered sucrose solution with a teflon-glass homogenizer (125 embryos/2 ml). The homogenate was centrifuged at 480 *g* for 15 min and the pellet, consisting mainly of yolk platelets and most of the pigment granules, was resuspended in buffered sucrose, recentrifuged at 480 *g* for 15 min, and the combined supernates were centrifuged at 12,000 *g* for 20 min; the resulting pellet was clearly divided into a lower layer of black pigment granules and an upper yellow-brown layer of mitochondria. The mitochondria were carefully suspended in buffered sucrose without disturbing the pigment granules and centrifuged at 12,000 *g* for 20 min. Both pellets were then resuspended in 1 ml of ion-free water, and 200-μl aliquots were assayed for protein according to Lowry et al. (27). The remainder of the cell fractions was used for the determination of total Ca^{2+} and Mg^{2+} content. The 12,000 *g* supernate was analyzed in the same way as the other cell fractions.

Cation Analysis

Cell fractions were digested with 2 vol of a 1:1 (vol/vol) mixture of fuming nitric acid:perchloric acid overnight at room temperature. The digests were diluted with ion-free water and were analyzed for Ca^{2+} and Mg^{2+} with a Varian Techron 1100 flame spectrophotometer (Varian Associates, Instrument Div., Palo Alto, Calif).

Chemicals

All inorganic salts and sucrose were AnalaR grade. Ouabain and Tris were obtained from Sigma Chemical Co., St. Louis, Mo. L-cysteine and papain from BDH Chemicals Ltd., Poole, U.K. and Chorulon from Intervet Labs., Cambridge U.K. A23187 was a gift from Eli Lilly Research Ltd., Windlesham, Sy, U. K.

RESULTS

Application of A23187 (at the standard concentration of 2.5 μg ml^{-1}) in standard 10% Steinberg solution had only limited effects on *Xenopus* embryos. However, when the concentrations of Ca^{2+} and Mg^{2+} were appropriately adjusted this divalent cation ionophore was able to produce two different types of effects, even after short periods of exposure, that proved to be both characteristic and dramatic: (*a*) a movement of the pigment in the embryos, frequently accompanied by "contraction" of the cells of the animal pole, and (*b*) production of abnormalities that became apparent during subsequent development of the embryos, together with a much lower survival than in control groups.

Pigment Movements

Treatment of stage 5 embryos with A23187 in

a Ca^{2+}- and Mg^{2+}-free saline at 20°C produced a sequence of pigment movements that are illustrated in Fig. 1, *A–L*, which shows embryos fixed at different intervals during exposure. The following stages in the sequence were recognized: (*a*) Normal embryos (Fig. 1 *A*). (*b*) Pigment moves to the cell boundaries where it becomes localized and concentrated. These concentrations of pigment are adjacent to each other in neighbouring cells (Fig. 1 *E* and *F*). (*c*) Pigment appears to move to the apical region of the embryo (Fig. 1 G and *H*). This effect is apparently achieved by the movement of the pigment in each cell (including those in the vegetal region) towards the apical region. (*d*) Cells at the animal pole "contract" and ultimately a depression appears in this region. The pigment then appears to be more centrally located in each cell (Fig. 1 *I*). (*e*) A partial reversal of these effects. The cells in the animal region relax and the pigment returns to a more normal distribution (Fig. 1 *J–L*).

A similar sequence of changes are found when

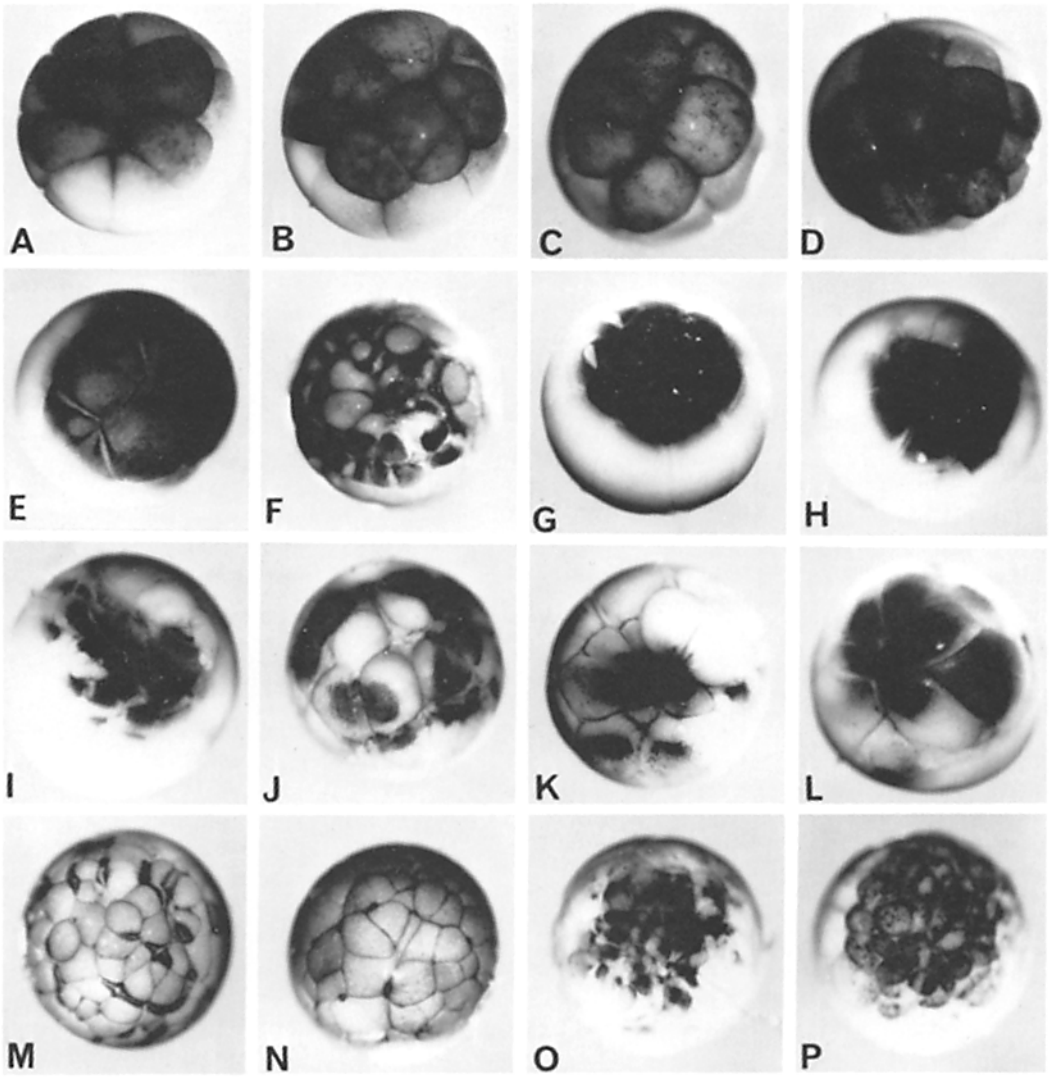


FIGURE 1 Sequence of changes in pigment distribution in *Xenopus* embryos after A23187 treatment in Mg^{2+}- and Ca^{2+}-free saline at 20°C. *A–L*: stage 5 embryos, *M–P*: stage 6 embryos. Exposures: *A*, 0 min. *B*, 1 min. *C*, 2 min. *D*, 3.5 min. *E*, 4.5 min. *F*, 5.5 min. *G*, 5.5 min. *H*, 7 min. *I*, 10 min. *J*, 10 min. *K*, 15 min. *L*, 20 min. *M*, 5.5 min, *N*, 5.5 min, *O*, 10 min, *P*, 25 min.

stage 6 embryos are exposed to A23187 in the absence of Ca^{2+} or Mg^{2+} in the saline (Fig. 1 *M–P*), although stage *b*, in which the pigment concentrates on adjacent sides of the boundaries of the cells, is much more clearly seen (Fig. 1 *M* and *N*).

Production of Abnormalities

Treatment of stage 5 embryos with A23187 in a saline containing appropriate concentrations of divalent cations produced embryos with abnormalities that were clearly evident on inspection 24 or 48 h later. Table I summarizes the different classes of abnormality that were recognised, in approximate order of severity. Classes 2–3 were regarded as slight abnormalities, involving small deformations and oedema, classes 4–5 as medium abnormalities, and classes 6–7, with severe dorsal flexure and open neural folds and even lack of form, as gross abnormalities.

Experiments Involving Ca^{2+} Buffered Salines

In early experiments, the $[Ca^{2+}]_o$ was accurately controlled by a Ca^{2+}-EGTA buffer and, after appropriate washing (see Materials and Methods), the embryos were exposed to A23187. Under these conditions, almost all of the embryos either died or showed gross abnormalities, even after only 10-min exposure to the ionophore. A range of Ca^{2+} concentrations was employed at either 0 or 8×10^{-4} M $[Mg^{2+}]_o$. Salines lacking both Mg^{2+} and Ca^{2+} were the most deleterious, with little or no survival of the embryos 24 h after a 10-min exposure. Survival was improved by the addition of either 10^{-4} M Ca^{2+} or 8×10^{-4} M Mg^{2+}. Control experiments showed that exposure of the embryos to EGTA in the absence of A23187 was not deleterious. In subsequent experiments, this chelating agent was omitted and precautions were taken to minimize Ca^{2+}-contamination as far as possible (see Materials and Methods).

TABLE I

Description of Abnormalities Produced in Xenopus 48 h after A23187 Treatment

Class no.	Description
1	Normal embryos
2	Ventral oedema only
3	Various slight deformations: slight flexures and curvatures, fin defects, ventral swelling ± oedema
4	Stunted ± oedema, ± ventral wasting, some combination with factors from 3
5	Medium to severe dorsal flexure ± some features found in 4
6	Severe dorsal flexure, open neural folds, bifurcated tail, loss of dorsal fin
7	Lack of form
8	Death

All solutions containing Ca^{2+} buffered with EGTA also promoted pigment movements when exposed to A23187. Omission of Mg^{2+} caused pigment movements to stage *b* in 2 min, to stage *c* in 3.5 min, and to stage *d* in 5.5 min. Solutions containing 8×10^{-4} M Mg^{2+}, irrespective of Ca^{2+} supplementation, caused pigment movements in the embryos to stage *b*, but no further changes were recorded.

Effect of Divalent Cation Concentrations in Salines not Containing EGTA

The effect of variation of $[Mg^{2+}]_o$ and $[Ca^{2+}]_o$ on the development of the embryos is shown in Tables II and III. Exposure for 10 min to A23187 in the absence of both $[Mg^{2+}]_o$ and $[Ca^{2+}]_o$ causes a 50% mortality of the embryos, and the majority of the survivors show abnormalities during subsequent development. Exposure to ionophore for longer periods (30 or 60 min) produces no rise in mortality but 100% of the embryos show abnormalities (Table II, Exp. 1C). Increasing $[Ca^{2+}]_o$ in these salines lacking EGTA, when $[Mg^{2+}]_o$ = zero, provides no protection at 10^{-7} to 10^{-6} M, although survival improves at 10^{-5} M $[Ca^{2+}]_o$ (Exp. 1F) and the percentage of abnormalities also falls markedly at 10^{-4} M. Excellent survival and a low percentage of abnormalities were found in control experiments, in which A23187 is omitted (Exps. 1A and 1B), and also in a saline with high extracellular divalent cations (Exp. 1H), in the presence of the ionophore.

Table III shows that, even in the absence of $[Ca^{2+}]_o$, exposure to A23187 in salines containing 8×10^{-5} M to 2×10^{-3} M $[Mg^{2+}]_o$ causes very little mortality, and survival is excellent even after 60-min exposure (Exps. 2B–2E, Table III). Increasing the $[Mg^{2+}]_o$ (when $[Ca^{2+}]_o$ = zero) also progressively reduces the percentage of embryos showing abnormalities 24 and 48 h after the ionophore treatment, until 2×10^{-3} M $[Mg^{2+}]_o$

TABLE II

Effect of Variation of $[Ca^{2+}]_0$ on Percentage Survival and Production of Abnormalities in Xenopus Embryos when $[Mg^{2+}]_0$ = Zero, 24 h after A23187 Treatment

Exp. no.	Ionic concentrations	Survival: Exposure time (min) 10	30	60	Abnormalities: Exposure time (min) 10	30	60
	M	%	%	%	%	%	%
1A	Control, O Mg, O Ca	96 (80)	96 (90)	99 (78)	24 (80)	27 (90)	10 (78)
1B	Control, O Mg, 10^{-7} Ca	91 (64)	–	–	33 (64)	–	–
1C	OMg, OCa	55 (150)	63 (64)	63 (49)	85 (150)	94 (64)	100 (49)
1D	OMg, 10^{-7} Ca	25 (120)	55 (20)	37 (35)	94 (120)	100 (20)	100 (35)
1E	OMg, 10^{-6} Ca	65 (20)	50 (20)	65 (20)	75 (20)	100 (20)	100 (20)
1F	OMg, 10^{-5} Ca	80 (35)	63 (49)	78 (18)	54 (35)	92 (50)	100 (20)
1G	OMg, 10^{-4} Ca	93 (40)	100 (20)	80 (20)	45 (40)	30 (20)	65 (20)
1H	8×10^{-4} Mg, 10^{-4} Ca	100 (35)	–	–	17 (35)	–	–

EGTA not included in medium. Abnormalities equal those in class numbers 2-8, Table I. Figures in parentheses equal number of embryos treated. Note good survival and low incidence of abnormalities when $[Mg^{2+}]_0 = 8 \times 10^{-4}$ M, $[Ca^{2+}]_0 = 10^{-4}$ M (exp. 1H). A23187 (2.5 μg ml^{-1}) omitted in controls which were treated with ethanol (2.5 μl ml^{-1}) only.

TABLE III

Effect of Variation of $[Mg^{2+}]_0$ on Percentage Survival when $[Ca^{2+}]_0$ = Zero, after A23187 Treatment

Exp. no.	Ionic concentrations	Survival: Exposure time (min) 10			30			60		
	M	%	%		%	%		%	%	
2A	Control, O Mg, O Ca	96	93	(80)	96	86	(90)	99	95	(78)
2B	O Mg, O Ca	55	44	(150)	63	38	(64)	62	12	(50)
2C	8×10^{-5} Mg, O Ca	93	83	(30)	100	87	(30)	100	67	(30)
2D	8×10^{-4} Mg, O Ca	97	86	(65)	93	91	(45)	100	90	(30)
2E	2.0×10^{-3} Mg, O Ca	100	100	(30)	97	90	(30)	97	93	(30)

Details as Table II. For each exposure time, the first column gives survival 24 h and the second survival 48 h after ionophore treatment.

allows almost all embryos to develop normally, even after a 60-min exposure to A23187 (Fig. 2). When $[Mg^{2+}]_0 = 8 \times 10^{-5}$ M, the length of time that the embryos are treated with A23187 has an effect on the proportion that develop abnormally, whereas the length of time of exposure proved less important at higher concentrations. Increasing $[Mg^{2+}]_0$ not only reduces the proportion of embryos that develop abnormally after ionophore treatment but also substantially reduces the severity of the abnormalities found 48 h later (Table IV). Thus, after exposure to A23187 in the absence of both extracellular Ca^{2+} and Mg^{2+}, a high proportion of the embryos have abnormalities characterized by a severe dorsal flexure and bifurcated tail, combined with a short neural plate and open neural folds. The incidence of these abnormalities is progessively reduced by increasing $[Mg^{2+}]_0$. Although a detailed study of the morphological abnormalities produced after ionophore treatment has not been made, it is likely that they are the result of disturbances in either gastrulation and/or neurulation.

In addition to their effects on development, extracellular Ca^{2+} and Mg^{2+} are also of importance in determining the degree of pigment movement observed during exposure to A23187 when these ions are unbuffered (i.e., EGTA omitted from the saline). Omission of both Mg^{2+} and Ca^{2+} caused the rapid sequence of pigment movements through to stage *e* (i.e., partial reversal, after 15 min). Addition of either 8×10^{-4} M Mg^{2+} or 10^{-5} M Ca^{2+} markedly suppressed the magnitude of the effects, the pigment movements being classified as stage *b* or *c* even after 15 min.

These experiments with A23187 were repeated

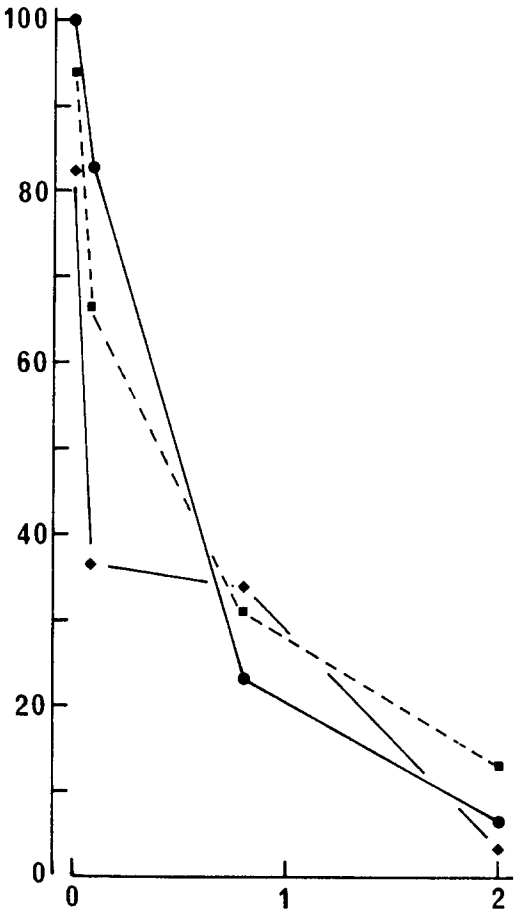


FIGURE 2 Effect of varying the concentration of Mg^{2+} in the saline when $[Ca^{2+}]_o$ = zero. Ordinate: percentage of abnormalities (classes 2–8, Table I) recorded 48 h after exposure to A23187 at 20°C. Abscissa: $[Mg^{2+}]_o$, mM. Duration of exposure: ●—● = 60 min; ■—■ = 30 min; ◆ — ◆ = 10 min.

with embryos from which the vitelline membrane had been manually removed. The same pattern of pigment movements was observed, provided Mg^{2+} and Ca^{2+} were omitted from the saline (which was full-strength Steinberg solution).

The effects of different concentrations of the ionophore at 20°C were tested; exposure was for 10 min in Ca^{2+}- and Mg^{2+}-free saline. Clear effects on survival and the production of abnormalities were found at 2.5 and 1.25 μg A23187 ml^{-1}, whereas lower concentrations had no marked action.

Movement of Divalent Cations during A23187 Treatment

Since the same pattern of pigment movements was found in Ca^{2+}- and Mg^{2+}-free saline, irrespective of whether the embryos were enclosed in the vitelline membrane, it is possible that the ionophore acts at the plasma membrane, causing the efflux of these ions from the cells. However, cation analysis of the bathing saline during, and at the conclusion of, exposure to A23187 revealed no detectable rise in Ca^{2+} or Mg^{2+} concentration. We conclude that, if any divalent cations are lost by the embryos, the amounts must be very small.

Stage 5 embryos were exposed to A23187 for 5 min in Ca^{2+}- and Mg^{2+}-free saline, by which time pigment movements had reached stage *d*. They were then rinsed and homogenized. An analysis was then made of the total Ca^{2+} and Mg^{2+} content of the embryos and of the different subcellular fractions. Table V shows that there is no significant change in the total content of either divalent cation after A23187 treatment. However, the ionophore promotes substantial changes in the distribution of intracellular Ca^{2+}, whereas the distribution of Mg^{2+} is largely unaffected. Table V shows that Ca^{2+} is lost from the pellet that is formed of the yolk platelets and most of the pigment granules. On the other hand, both the mitochondrial pellet and the final supernate show marked rises in Ca^{2+} concentration after exposure of the embryos to ionophore.

Action of High Concentrations of Monovalent Cations

The effect of exposing the embryos for 3 h to high concentrations of alkali metal ions is shown in Table VI. Exp. 4B confirms previous reports that exposure to 100 mM LiCl causes an increase in mortality, and all the embryos subsequently develop abnormalities 24 h later that are typically vegetalized. It seems clear from experiments previously reported that the abnormal development must be associated with Li^+ moving into the cells of the embryo and accumulating intracellularly, since this ion is not readily transported out of the cells. Parallel experiments (Exp. 4C) might indicate that this effect is specific to the lithium ion, since development proceeds normally in 100 mM NaCl. However, when the embryos are placed in 100 mM NaCl plus 10^{-4} M ouabain, all survive but the incidence of abnormalities 24 h later is now 100% (Exp. 4D). Ouabain is an inhibitor of the Na^+-K^+-ATPase associated with the active extrusion of Na^+ and, in its presence, Na^+ presumably accumulates intracellularly. Exp. 4D suggests, therefore, that an intracellular accumulation

TABLE IV

Classification of Abnormalities Produced 48 h after A23187 Treatment in Salines Containing Different Concentrations of Mg^{2+} ($[Ca^{2+}]_0$ = Zero)

Class no.	O Mg			8×10^{-5} M Mg			8×10^{-4} M Mg			2×10^{-3} M Mg			Control		
	Exposure time (min)														
	10	30	60	10	30	60	10	30	60	10	30	60	10	30	60
1	11	6	0	63	32	17	66	69	77	97	87	93	76	80	85
2	2	0	2	0	7	17	0	4	0	0	0	0	9	0	1
3	11	9	0	13	36	10	5	13	13	3	0	0	7	2	5
4	4	2	0	7	10	0	8	0	0	0	3	0	2	3	3
5	1	2	2	0	0	3	0	0	0	0	0	0	0	0	1
6	15	19	6	0	3	20	6	4	0	0	0	0	0	0	0
7	1	0	2	0	0	0	0	0	0	0	0	0	0	0	0
8	55	63	88	17	13	33	15	9	10	0	10	7	7	14	5
n	114	74	50	30	30	30	65	45	30	30	30	30	45	90	78

Class nos. of abnormalities defined in Table I. Controls equal 0 $[Mg^{2+}]_0$, 0 $[Ca^{2+}]_0$, minus A23187. Figures equal percentage in each class. A23187 = 2.5 μg ml^{-1}; 20°C.

TABLE V

Ca^{2+} and Mg^{2+} Content of Embryos and of Different Cell Fractions from Stage 5 Embryos that had been Exposed to A23187 for 5 min

Exp. no.	Total Ca^{2+} content	μg Ca^{2+}/mg protein			Total Mg^{2+} content	μg Mg^{2+}/mg protein		
		480 *g* pellet	12,000 *g* pellet	12,000 *g* supernate		480 *g* pellet	12,000 *g* pellet	12,000 *g* supernate
	μg				μg			
				Control				
3A	15.40	0.610	0.602	0.315	34.04	1.881	0.133	0.573
3B	12.97	0.586	0.607	0.309	31.17	1.842	0.197	0.668
3C	16.43	0.692	0.489	0.346	32.32	1.888	0.131	0.512
Mean	14.93	0.629	0.566	0.323	32.51	1.870	0.154	0.584
S.D.	1.78	0.056	0.067	0.020	1.44	0.025	0.038	0.079
				A23187				
3A	17.02	0.442	0.877	0.738	28.57	1.768	0.145	0.660
3B	11.96	0.292	0.965	0.638	32.18	1.997	0.191	0.680
3C	13.60	0.380	0.985	0.448	28.39	2.043	0.109	0.412
Mean	14.19	0.371	0.942	0.608	29.71	1.936	0.148	0.584
S.D.	2.58	0.075	0.058	0.147	2.14	0.147	0.041	0.149
p	n.s.	<0.01	<0.01	<0.05	n.s.	n.s.	n.s.	n.s.

Total Ca^{2+} and Mg^{2+} given for samples of 125 embryos

of either Li^+ or Na^+ can produce abnormal morphogenesis. Ouabain (10^{-4} M) in normal 10% Steinberg solution does not cause a high incidence of abnormalities (Exp. 4E); under these conditions $[Na^+]_0$ is only 6×10^{-3} M, the inward flux of Na^+ would be low and one would expect only small accumulations of Na^+ intracellularly, even in the presence of ouabain. It is unlikely that the effects of these alkali metal ions is an osmotic one since embryogenesis is normal in 200 mM sucrose (Exp. 4F). It is noteworthy that, at even higher concentrations, Li^+ (200 mM) or Na^+ (200 mM, no ouabain) produce very similar results. Thus, after exposure to either ion at this concentration, cell division is clearly retarded and the cells tend to become separated. After return to normal 10% Steinberg solution, the cells flatten, re-establish contact, and division continues. However, normal morphogenesis is apparently not possible and all embryos are dead 24 h later (Exps. 4G and 4H). These changes are again not seen in 400 mM sucrose and embryogenesis remains normal in the majority of embryos after 3 h exposure (Exp. 4I).

Electron Microscopy

The results of the ion analyses reported above

TABLE VI

Effect of Alkali Metal Ions on Morphogenesis in Xenopus Stage 5 Embryos

Exp. no.	Saline	Survival	Abnormalities	n
		%	%	
4A	Control	94	17	387
4B	0.1 M LiCl	80	100	1712
4C	0.1 M NaCl	98	2	41
4D	0.1 M NaCl + 10^{-4} M ouabain	100	100	20
4E	10^{-4} M ouabain	100	20	20
4F	0.2 M sucrose	92	10	39
4G	0.2 M LiCl	0	100	100
4H	0.2 M NaCl	0	100	40
4I	0.4 M sucrose	98	33	60

Incubation for 3 h in 10% Steinberg solution plus additions as indicated at 20°C. Percentage survival and abnormalities recorded 24 h after treatment. Abnormalities equal stages 2–8, Table I.

suggest that, after A23187 treatment, Ca^{2+} leaves the yolk platelets and/or pigment granules and is taken up by the mitochondria from the cytosol. Examination of the embryos fixed at intervals during exposure to A23187 in a Mg^{2+}- and Ca^{2+}-free saline reveals that the mitochondria very quickly show clear conformational changes (Table VII).

Ultrastructural changes have been recognized in mitochondria, both isolated and *in situ*. A variety of structural states have been recognized and various forms of nomenclature have been proposed (22, 25, 35). In the present study, the configurational states of the mitochondria of stage 5 *Xenopus* embryos have been ascribed to one of the following seven categories (Figs. 3–11). (A) Cristal membranes approximately parallel along their length. Intracristal spaces small. Matrix space large with low electron density (Fig. 3). (B) Intracristal space still generally small, but wider in places, giving characteristic triangular shapes. Matrix remains large but there is an increase in electron density (Figs. 4 and 5). (C) Cristal membranes remain broadly parallel along their length, but the intracristal space is large. Matrix space reduced and more electron dense (Figs. 5 and 6). (D) Intracristal space large, similar to C. Matrix electron dense. An apparent "twisted" conformation of the cristal space and matrix is evident, although the matrix is still continuous. Membranes not parallel (Fig. 7). (E) Numerous interconnections between intracristal spaces and the continuity of the matrix is lost, the latter often appearing as lacunae enclosed by cristal membranes. Matrix therefore appears "rounded up" and is electron dense. Membranes of cristae not parallel (Figs. 8 and 9). (F) Intermediate condition between E and G. Cristae begin to show angular conformations characteristic of G. Matrix electron dense (Fig. 10). (G) Characteristic zigzag appearance. Cristal membranes widely spaced; matrix electron dense (Fig. 11).

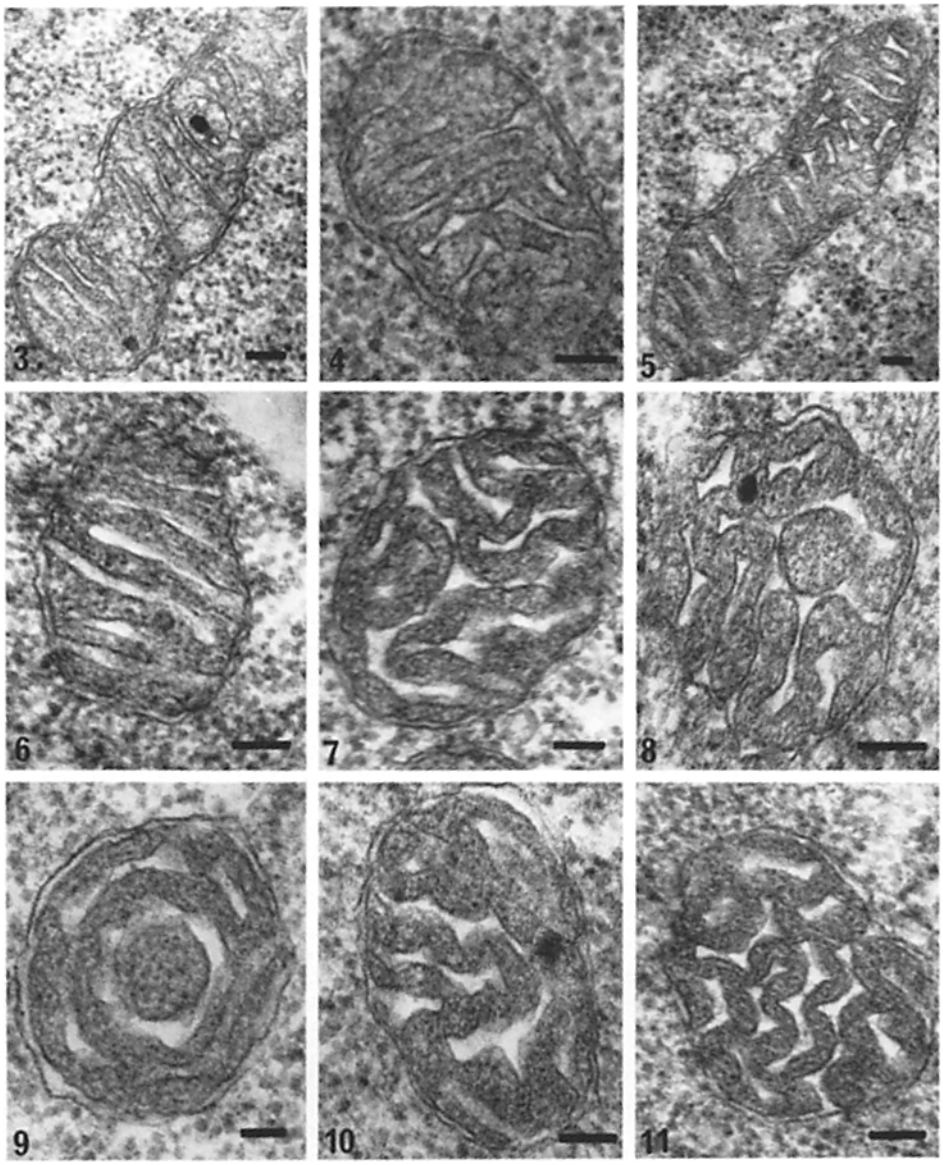
We believe that stages A–G form an approximate sequence and regard A, C, E, and G as clearly-defined stages, whereas B, D, and F are probably intermediate conditions. The effect of A23187 treatment on the configuration of the mitochondria is shown in Table VII; Mg^{2+} and Ca^{2+} were omitted from the bathing saline in these experiments. The embryos were fixed at various time intervals after the beginning of exposure, and electron micrographs of corresponding areas near the cell surface of each were prepared. All mitochondria in the field were classified as to their configurational state. Before application of the ionophore (see also control experiments, Table VIII and Fig. 12) the majority of mitochondria are in state C, although inspection reveals a range from states A to D, with a small percentage in state E. Exposure to A23187 causes a progressive change; after 3.5 min, the majority of mitochondria (>80%) are in configuration D or E and at 4.5–5.5 min there is a progressive shift to configuration E and a small percentage appear in configuration F (Table VII, Fig. 13). After 10-min exposure, there are signs of a partial reversal of these changes and 30 min after the initial application of ionophore the majority of the mitochondria (40%) have returned to configuration D.

TABLE VII

Configurational Changes in Mitochondria after A23187 Exposure

Mitochondrial configuration	Time of exposure (min)								
	0	1	2	3.5	4.5	5.5	10	25	30
A	9	8	3	–	–	–	–	4	–
B	27	15	15	1	–	–	2	12	4
C	53	46	32	17	7	7	14	28	32
D	10	25	33	39	25	12	34	12	40
E	1	5	16	43	67	76	48	44	23
F	–	–	–	–	1	5	1	–	–
G	–	–	–	–	–	–	–	–	–
n	109	61	99	77	81	157	85	57	47

Values equal percentage of mitochondria in each configuration. $[Mg^{2+}]_o$, $[Ca^{2+}]_o$ = zero.



FIGURES 3–11 Configurational states of the mitochondria of *Xenopus laevis* embryos *in situ*, as described in the text. Fig. 3: State A, × 55,000. Fig. 4: State B, × 80,400. Fig. 5: State B, × 42,000. Fig. 6: State C, × 75,000. Fig. 7: State D, × 69,000. Fig. 8: State E, × 90,000. Fig. 9: State E, × 57,000. Fig. 10: State F, × 75,000. Fig. 11: State G, × 75,000. Bar, 0.1 μm.

Such configurational changes were not found when the embryos were exposed to ionophore in saline containing 8×10^{-4} M Mg^{2+} ($[Ca^{2+}]_o$ = zero). Table VIII shows that no state E mitochondria were found, and after 10-min exposure the majority (96%) were in configurational states A–C (Fig. 14). The typical sequence of configurational changes is still found when $[Ca^{2+}]_o$ is raised to 10^{-5} M in the absence of extracellular Mg^{2+} (Table VIII). At 10^{-4} M, however, extracellular Ca^{2+}, like Mg^{2+}, has a protective effect and the ultrastructural changes in mitochondrial configuration are not seen.

DISCUSSION

The pigment movements seen in the present study

TABLE VIII

Effect of Extracellular Mg^{2+} and Ca^{2+} in Modifying Changes in Mitochondrial Configuration after A23187 Treatment

Mitochondrial configuration	Control 10 min	Control 30 min	8×10^{-4} M Mg O Ca 10 min	8×10^{-4} M Mg O Ca 30 min	10^{-5} M Ca O Mg 10 min	10^{-5} M Ca O Mg 30 min
A	22	15	21	17	14	–
B	22	14	35	29	10	8
C	25	32	40	41	10	25
D	21	22	5	14	8	12
E	10	16	–	–	59	54
F	–	–	–	–	–	–
G	–	1	–	–	–	2
n	158	104	86	66	51	65

Values equal percentage of mitochondria in each configuration. Control: $[Mg^{2+}]_o$, $[Ca^{2+}]_o$ = zero.

are identical to those produced in *Rana pipiens* (41) and can be attributed to the action of a subcortical contractile system. The cortical cytoplasm associated with these events is occupied by arrays of microfilaments (Osborn and Duncan, manuscript in preparation) and is similar to that found beneath cleavage furrows (28), near closing wounds (20), or at the sites of polycation administration (20). Exposure of *Xenopus* embryos to A23187 for brief periods has little effect either on cortical contractions or on subsequent embryogenesis, provided extracellular concentrations of Mg^{2+} and Ca^{2+} are maintained at reasonably high levels. When $[Mg^{2+}]_o = 8 \times 10^{-4}$ M and $[Ca^{2+}]_o = 10^{-4}$ M (Exp. 1H), mortality is low and embryogenesis proceeds normally. Treatment with A23187 under these conditions is therefore not deleterious. Cortical contractions and a high incidence of abnormalities and mortality follow when $[Mg^{2+}]_o$ and $[Ca^{2+}]_o$ are maintained at low levels. These effects seem to be exacerbated when EGTA is included in the extracellular medium, even when included in a Ca^{2+}-EGTA buffer system. We believe that cortical contractions and the modification of the subsequent embryogenesis are a direct consequence of changes in $[Ca^{2+}]_i$.

There are a number of ways in which A23187 could be acting: (*a*) The ionophore acts at the plasma membrane, allowing divalent cations to move down their concentration gradients. It is possible that the vitelline membrane fluid contains a high concentration of $[Ca^{2+}]$ (43), and A23187 permits entry of this ion. However, the same changes in pigment movement and in abnormal development are seen in embryos from which the vitelline membrane has been removed. Since the major effects of ionophore are seen in the absence of extracellular Mg^{2+} and Ca^{2+}, the ionophore could promote only an efflux of divalent ions. However, the ion analysis studies reveal no such rise in $[Ca^{2+}]_o$ or $[Mg^{2+}]_o$ during treatment, nor is the total Mg^{2+}- and Ca^{2+}-content of the embryos affected by A23187, and we conclude that A23187 does not have a major effect at the plasma membrane. (*b*) A23187 causes the release of Ca^{2+} from intracellular sites, so causing a rise in $[Ca^{2+}]_i$.

The evidence obtained in the present study and in experiments conducted with A23187 in other cellular systems strongly suggests that the latter hypothesis is correct: (*a*) There is a considerable body of evidence that cortical contractility and cleavage in developing embryos are all dependent on the local level of free $[Ca^{2+}]_i$ (1, 3, 31, 41). The cortical contractions recorded in this study are therefore explicable in terms of a rise in $[Ca^{2+}]_i$ as a consequence of the A23187 treatment. (*b*) Ion analysis of the subcellular fractions (Table V) confirms that $[Ca^{2+}]$ rises in the cytosol, probably after release from the yolk platelets and/or pigment granules, and that the mitochondria attempt to correct this by taking up Ca^{2+}. It has been suggested (32) that the bulk of the cell Ca^{2+} is associated with the pigment granules and yolk platelets in the early cleavage embryos of *Rana pipiens* and that amphibian liver melanocytes have functional similarities with mitochondria (53). The pigment granules in the stage 5 embryos are sited in the periphery of the cells, close to the layer of subcortical microfilaments. (*c*) A variety of different systems of nomenclature have been proposed for the configurations that have been observed in mitochondria from different sources. We believe that the structural states that we have designated A–G can probably be related to the classifications proposed by other workers (21, 23, 25, 35), i.e., state A equivalent to the nonenergized (orthodox) form, state C equivalent to the energized form, state E equivalent to the energized-twisted form, and state G equivalent to the energized-zigzag form. Table VII shows that A23187 treatment of *Xenopus* embryos is rapidly followed by configurational changes in the mitochondria, from the nonenergized/energized condition to energized-twisted and towards energized-zigzag. These changes are prevented when the ionophore exper-

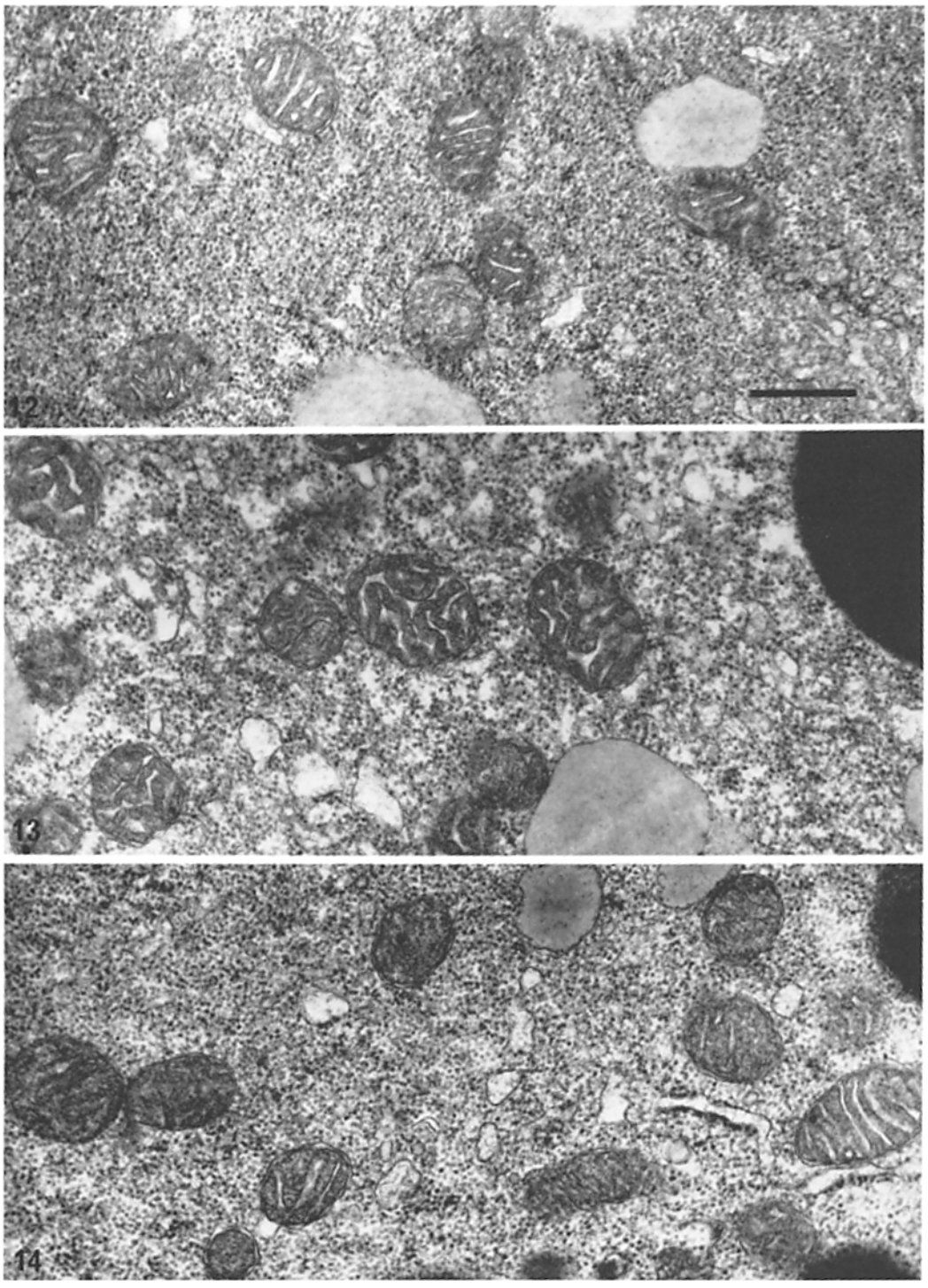


FIGURE 12 Control embryo before exposure to A23187 at 20°C. × 31,200. Bar, 0.5 μm, which is also the scale for Figs. 13 and 14.

FIGURE 13 Embryo in Mg^{2+}- and Ca^{2+}-free saline, 4.5 min after exposure to A23187 at 20°C. × 31,200.

FIGURE 14 Embryo in saline containing 8×10^{-4} M Mg^{2+}, 0 Ca^{2+}, 10 min after exposure to A23187 at 20°C. × 31,200.

iments are repeated with the extracellular concentration of Mg^{2+} or Ca^{2+} raised to $\sim 10^{-4}$ M. These extracellular divalent cations therefore not only protect the embryos during A23187 treatment from the pigment movements and abnormal development, but also prevent the characteristic configurational changes of the mitochondria. We suggest that the ultrastructural changes in the mitochondria are associated with Ca^{2+}-uptake as the organelles attempt to regulate $[Ca^{2+}]_i$. (*d*) We have shown (49) that the effect of A23187 on the resting potential of frog skeletal muscle is dependent on external divalent cations. Only when $[Ca^{2+}]_o$ and $[Mg^{2+}]_o$ are very low does the ionophore cause a fall in resting potential. (*e*) At low concentrations, A23187 acts primarily on intracellular Ca^{2+}-storage sites in bovine spermatozoa, rather than at the plasma membrane (2).

We conclude from these experiments, therefore, firstly that Ca^{2+} and Mg^{2+} in the extracellular medium are able to interfere with the action of A23187 at low concentration on *Xenopus* embryos. This view is supported by studies with A23187, in which the ultraviolet and fluorescent spectral properties of the ionophore are shown to be dramatically altered by highly selective complexing with unsolvated Ca^{2+} or Mg^{2+} (37). Furthermore, studies on the intracellular uptake of A23187 into pancreatic acinar cells, using measurements of the fluorescence excitation spectrum of the ionophore, showed that uptake was rapid in the absence of extracellular Ca^{2+} and Mg^{2+} ($t_{\frac{1}{2}}$ = 1 min) and very much slower in the presence of these ions ($t_{\frac{1}{2}}$ = 20 min). The cell-associated ionophore was found to be largely intracellular, rather than being located at the plasma membrane (9). Omission of Ca^{2+} and Mg^{2+} from the extracellular medium is effective, therefore, in promoting the effect of A23187 on *Xenopus* embryogenesis because these ions can interfere with the ionophore action, and not because A23187 acts at the plasma membrane, allowing the efflux of Mg^{2+} and Ca^{2+} when the electrochemical gradient for these ions is outwards.

Secondly, we conclude that A23187 operates primarily at intracellular sites, causing the release of stored Ca^{2+} and a consequent rise in $[Ca^{2+}]_i$ which in turn causes the observed pigment movements. This conclusion is supported by the redistribution of Ca^{2+} in the subcellular fractions after A23187 treatment (Table V).

Thirdly, the evidence suggests that the mitochondria are involved in the control of $[Ca^{2+}]_i$. They undergo ultrastructural changes after A23187 treatment, and it is noteworthy that the protection from subsequent abnormal embryogenesis afforded by divalent cations in the external medium during ionophore exposure is reflected in the absence of mitochondrial changes under these conditions. It is of interest that the partial reversal of pigment movements after some 10-min exposure to A23187 is also accompanied by the partial reversal of the mitochondrial configurational changes after this time.

The experiments concerning the abnormalities produced by Li^+ or ouabain also support the thesis that rises in $[Ca^{2+}]_i$ cause abnormal development. A variety of agents have been described with a teratogenic action on amphibian and echinoderm embryos, and have been classified as having animalizing or vegetalizing effects. Li^+ has been known for many years to cause vegetalization in amphibian embryos, as we have confirmed (Table VI). Li^+ is known to alter markedly the frequency of spontaneous release at presynaptic terminals (10), a process that is believed to be governed by $[Ca^{2+}]_i$ (16, 46, 47). There is now accumulating evidence that increasing $[Na^+]_i$ causes a rise in $[Ca^{2+}]_i$ in a variety of cells; the evidence suggests that $[Li^+]_i$ has the same effect, the distinction between the two ions being that Na^+ is normally readily removed from the cells whereas Li^+ is not (15, 48). Thus, a rise in $[Na^+]_i$ or $[Li^+]_i$ causes a rise in $[Ca^{2+}]_i$ in synaptosomes, probably either increasing Ca^{2+}-uptake or decreasing Ca^{2+}-efflux (8) whereas elevation of $[Na^+]_i$ in isolated islets of Langerhans apparently causes the release of Ca^{2+} from intracellular pools (26). Both Na^+ and Li^+ have been shown to promote a dramatic release of Ca^{2+} from isolated mitochondria from a variety of tissues (11, 12).

If Na^+ influx is increased in *Xenopus* embryos by raising $[Na^+]_o$ for 3 h (Table VI), there are few deleterious effects and embryogenesis is normal. The cells are presumably able to maintain $[Na^+]_i$ constant by increasing active Na^+ efflux. However, if this active efflux of Na^+ is inhibited by ouabain (Exp. 4D), the incidence of abnormalities is 100%. Such results suggest that an intracellular accumulation of either Li^+ or Na^+ is sufficient to interfere with integrated embryogenesis. There are clear parallels, therefore, between the effects of these alkali metal ions on morphogenesis and their reported action on the modification of $[Ca^{2+}]_i$ in a variety of cells (15, 48). The cells are unable to remove Li^+ readily, in contrast with the

active transport of Na^+ where the effects of an intracellular accumulation are only demonstrable when the cation pump is inhibited. We therefore suggest that some of the teratogenic effects of Li^+ are probably a consequence of an intracellular accumulation of an alkali metal ion which produces, in turn, a modification of $[Ca^{2+}]_i$ and, indeed, inspection of many of the agents known to cause either animalization or vegetalization (45) suggests that they (e.g., DNP, CN^-, azide, heavy metals) may operate via a modification of $[Ca^{2+}]_i$ in specific areas of the embryo. Whether they are primarily animalizing or vegetalizing would depend on which cells in the embryo are most rapidly affected and in which $[Ca^{2+}]_i$ first changes above a critical level.

Experiments with stage 5 embryos of *Xenopus* (34) suggest that a major effect of Li^+ treatment is to slow the rate of cell division, and these findings confirm those of Flickinger et al. (17) on the effect of Li^+ on the length of the cell cycle. We therefore suggest that the regulation of $[Ca^{2+}]_i$ is of particular importance in the control of cell division. Abnormal morphogenesis follows if the accurate sequence of cell division during cleavage is modified.

If the integrated control of embryogenesis in *Xenopus* is dependent on the cells being able to alter markedly the levels of $[Ca^{2+}]_i$, some internal site for Ca^{2+}-storage and release will be required in a self-sufficient system which is isolated in a freshwater environment. Barth and Barth (4, 5, 6) have suggested the importance of the involvement of ions in amphibian development, particularly Ca^{2+} during neurulation, and have advanced a general theory concerning the regulation of induction during normal gastrulation by Na^+ and Ca^{2+} (and possibly Mg^{2+}) movements; they suggest that the blastocoel and the yolk platelets are major sites of ion sequestration. However, no true blastocoel is present in stage 5 embryos, and our experiments suggest that it is Ca^{2+} that is of particular importance in regulating cell division. (Table V shows that the distribution of Mg^{2+} is unaffected by ionophore treatment.) The mitochondria undergo rapid and marked configurational changes during A23187 treatment, and the observation that both Li^+ and Na^+ cause the release of Ca^{2+} from isolated heart mitochondria (11) supports the idea that these organelles represent one intracellular site for the control of $[Ca^{2+}]_i$ in *Xenopus* embryos.

From these studies, two points emerge: (*a*) Normal, integrated cell division during the embryogenesis of *Xenopus* can probably be impaired if $[Ca^{2+}]_i$ is experimentally raised. It seems that it is important that the cells are able to alter $[Ca^{2+}]_i$ accurately, rapidly and reversibly, since an exposure of only 10 min to A23187 in the absence of extracellular Mg^{2+} and Ca^{2+} is markedly deleterious. (*b*) It is not yet clear whether $[Ca^{2+}]_i$ acts primarily in the control and triggering of cell division, or whether it is merely required for the activation of such events as cell cleavage. Changes in $[Ca^{2+}]_i$ may be linked with changes in intracellular pH (52). Nevertheless, many experiments confirm the importance of Ca^{2+} during embryogenesis; some are summarized in the Introduction, and recently it has been shown that $[Ca^{2+}]_i$ is low in the unfertilized eggs of *Oryzias latipes*, but that the Ca^{2+}-level rises dramatically and rapidly when activated by sperm. $[Ca^{2+}]_i$ then slowly returns to the resting level (19, 39). Thus, Ca^{2+} is released from an intracellular site at fertilization in the sea urchin egg (51), and mammalian oocytes are activated by an intracellular injection of Ca^{2+} (18). Finally, a Ca^{2+}-sensitive cytoplasmic factor has been extracted from mature oocytes of frog, but not from immature oocytes or fertilized embryos, which is capable of inducing cleavage arrest at metaphase when microinjected into one blastomere of a cleaving embryo (30).

The level of $[Ca^{2+}]_i$ produces a graded control of junctional permeability in *Chironomus* salivary gland cells and therefore constitutes a powerful mechanism for regulating intercellular communication by providing a means of selective transmission of intercellular molecular signals (40). These authors point out that selective uncoupling by molecular signals could provide an economical means of producing cellular differentiation during embryogenesis. The cells in *Xenopus* embryos up to stage 7 are electrically coupled, but junctional permeability is restricted since fluorescein molecules are unable to pass between cells, unlike many other electrically-coupled cells (42). If the Ca^{2+}-control proves to be a truly selective restriction of permeability (rather than being a result of a nonselective reduction in the number of channels), it is suggested that it will be interesting to see whether $[Ca^{2+}]_i$ is elevated at the stage in development when free intercellular communication is established (40). It is clear that an experimentally-induced rise in $[Ca^{2+}]_i$ in stage 5 *Xenopus* embryos by A23187 treatment, as described in the present study, would interfere in the deli-

cate control of intercellular communication and hence markedly affect embryogenesis.

We therefore advance the following tentative hypotheses concerning events during early embryogenesis in *Xenopus*: (*a*) The mitochondria are able to store Ca^{2+}. (*b*) A23187 causes the release of Ca^{2+} from intracellular sites, such as the pigment granules and yolk platelets. (*c*) The uptake (and possibly the cyclic flux) of Ca^{2+} at the mitochondrial membrane is accompanied by ultrastructural transformations. The use of A23187 allows these to be demonstrated *in situ*. (*d*) An intracellular accumulation of Li^+ or Na^+ also causes a rise in $[Ca^{2+}]_i$, probably by promoting Ca^{2+}-efflux from the mitochondria. (*e*) $[Ca^{2+}]_i$ has an important role in the regulation of cell division and interference with this process during cleavage apparently causes modifications of subsequent embryogenesis. (*f*) The mitochondria could well have a function in regulating $[Ca^{2+}]_i$ during embryogenesis in *Xenopus*.

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