Micromolar free calcium exposes ouabain-binding sites in digitonin-permeabilized *Xenopus laevis* oocytes

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As demonstrated previously, digitonin-permeabilized *Xenopus* oocytes have a large internal pool of sodium pumps which are inaccessible to cytosolic ouabain [Schmalzing, Kröner & Passow (1989) *Biochem. J.* **260**, 395–399]. Access to internal ouabain-binding sites required permeabilization of inner membranes with SDS. In the present study, micromolar free Ca^{2+} was found to stimulate ouabain binding in the digitonin-permeabilized cells ($K_{0.5}$ $0.5 \mu\text{M}$ - Ca^{2+} , h 1.9, average of seven experiments) without disrupting intracellular membranes. Sustained incubation at $9 \mu\text{M}$ - Ca^{2+} was as effective as SDS in inducing access to the ouabain-binding sites of the internal sodium pumps. Omission of either Mg^{2+} or ATP completely abolished the Ca^{2+} effect. Half-maximal stimulation by Ca^{2+} required approx. 0.4 mM - MgATP . Of a variety of nucleotides tested, none was as effective as ATP {rank order $\text{ATP} > \text{ADP} > \text{ATP[S]}$ (adenosine 5'-[γ -thio]triphosphate) $> \text{CTP} > \text{UTP} > \text{ITP} = \text{XTP} > \text{GTP}$ }. P_i , AMP, cyclic AMP, cyclic GMP, GTP[S] (guanosine 5'-[γ -thio]triphosphate) and a stable ATP analogue p[NH]ppA (adenosine 5'-[β -imido]triphosphate), were ineffective. The metalloendoproteinase inhibitor carbobenzoxy-Gly-Phe-amide reduced the Ca^{2+} effect by some 50 %. Inhibitors of chymotrypsin and the Ca^{2+} proteinase calpain had no effect. Ca^{2+} ionophores (A23187 and ionomycin) and the polycations neomycin and polymixin B blocked the Ca^{2+} response entirely. Neomycin also abolished a Ca^{2+} -independent stimulation of ouabain binding by the wasp venom mastoparan. The requirements for increasing the accessibility of ouabain-binding sites are remarkably similar to those for exocytosis in secretory cells, suggesting that oocytes and eggs possess a Ca^{2+} -regulated pathway for the plasma membrane insertion of sodium pumps.

INTRODUCTION

Integral cell surface proteins are incorporated into the plasma membrane by either constitutive or regulated exocytosis [1]. In the constitutive pathway, Golgi-derived vesicles fuse continuously with the plasma membrane. By contrast, in the regulated pathway, a portion of the cell surface proteins is stored in intracellular granule membranes until fusion with the plasma membrane is initiated by some external or internal stimulus. Exocytotic insertion of specific proteins into the plasma membrane allows the cell to respond rapidly to hormones and other alterations in the external or internal milieu [2–5]. Subsequent return of the cell to the unstimulated state is due to the endocytotic retrieval of the transporters from the plasma membrane to the intracellular compartment. Fusion of cytoplasmic vesicles containing transporters with the plasma membrane and their endocytotic removal has been demonstrated to be involved in the regulation of water permeability, glucose uptake, Na^+ permeability and H^+ transport (see refs. [2–5] for reviews).

We have recently demonstrated that half of the sodium pumps of prophase-arrested oocytes of *Xenopus laevis* are located intracellularly [6]. This intracellular pool of sodium pumps increases during meiotic maturation, when the capacity of the plasma membrane to transport Na^+ and K^+ and to bind ouabain is completely lost [7]. The inhibition of active Na^+/K^+ exchange is not due to some covalent modification of surface sodium pumps *in situ*, but to a selective removal of the pump molecules from the plasma membrane [8]. When maturation is completed with the arrest of mature oocytes (eggs) in the second meiotic metaphase, all sodium pumps reside in the cell interior.

In the present study, we examined the possibility that oocytes and eggs of *Xenopus* possess a regulated pathway for the

recruitment and plasma membrane insertion of intracellular sodium pumps. In order to manipulate the internal milieu, the plasma membrane of oocytes and eggs was rendered leaky with digitonin, which, in a variety of secretory cells, has been found not to disrupt their secretory function [9,10]. Since exocytosis generally requires Ca^{2+} , we have challenged digitonin-permeabilized oocytes and eggs with Ca^{2+} buffers. The results provide indirect support for the hypothesis that Ca^{2+} stimulates the exocytotic insertion of intracellular sodium pumps into the plasma membrane.

Some of these results have been presented previously in abstract form [11].

EXPERIMENTAL

Reagents

[^3H]Ouabain (21 and 31 Ci/mmol), [^3H]inulin (2.3 Ci/mmol) and [^{14}C]sucrose (10 mCi/mmol) were purchased from Amersham-Buchler (Braunschweig, Germany). Collagenase (0.6–0.8 units/mg), SDS, GTP[S] (guanosine 5'-[γ -thio]triphosphate), and digitonin were from Serva (Heidelberg, Germany). Proteinase inhibitors, mastoparan, Na_3VO_4 , BAPTA and *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminidase were obtained through Sigma (München, Germany). Ionomycin, EGTA and the D-gluconate salts of potassium, sodium, calcium and magnesium were from Fluka (Neu-Ulm, Germany). A23187, nucleotides and nucleotide analogues (except GTP[S]), in the form of their sodium or lithium salts, were purchased from Boehringer (Mannheim, Germany). All other chemicals were purchased from Merck (Darmstadt, Germany), and were analytical or Suprapur grade.

Abbreviations used: BAPTA, 1,2-bis-(2-aminophenoxy)ethane-*NNN'*-tetra-acetic acid; K_a , apparent dissociation constant; B_{max} , ouabain binding capacity; p[NH]ppA, adenosine 5'-[β -imido]triphosphate; ATP[S] , GTP[S] , adenosine and guanosine 5'-[γ -thio]triphosphates; CBZ, carbobenzoxy; PIP₂, phosphatidylinositol 4,5-bisphosphate; PIP, phosphatidyl 4-monophosphate; IC_{50} , concn. causing half-maximal inhibition.

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Oocyte isolation and maturation *in vitro*

Ovaries were removed surgically from anaesthetized *Xenopus laevis* (Xenopus Ltd., Redhill, Surrey, U.K.) and digested with collagenase as described [7]. To reinitiate meiosis *in vitro*, defolliculated oocytes of oogenesis stages 5 and 6 [12] were selected and exposed to 10 μM -progesterone for 20 min at 22 °C. Following incubation for 12 h at 17 °C in oocyte Ringer solution (ORI: 90 mM-NaCl/3 mM-KCl/1 mM- CaCl_2 /10 mM-Hepes, pH 7.4) supplemented with 1 mM- MgCl_2 and 50 μg of gentamycin/ml, maturation was scored by the presence of a white spot at the animal pole which indicates the release of the first polar body. Some of the cells were dissected after fixation in 5% trichloroacetic acid to verify dissolution of the nucleus. Selected oocytes of the same batch that had not received progesterone were also cultured at 17 °C and served as controls. Viability was assessed routinely by measuring membrane potentials of some cells of a batch with 1 M-KCl-filled micro-electrodes (resistance 8–10 M Ω). Results shown in the present study were obtained from batches of cells having membrane potentials of at least –50 mV before maturation.

Cell permeabilization

If not indicated otherwise, groups of up to 45 cells were rendered leaky in 10 ml of a permeabilization medium (110 mM-sodium gluconate, 2 mM-magnesium gluconate, 10 mM-Tris, pH 7.0, 1 mM-ATP, 10 mM-EGTA and 10 μM -digitonin). After 30 min at 5 °C under slow horizontal shaking, the cells were transferred to fresh permeabilization medium without digitonin and incubated for an additional 10 min at 5 °C. The permeabilization medium differed from the one described previously [6] in that gluconate was substituted for chloride and that ATP was included. These changes did not alter the time-course or extent of the release of the cytosolic markers K^+ and lactate dehydrogenase nor those of the Ca^{2+} -independent ouabain binding to the leaky cells. Less than 0.5% of the lysosomal marker β -hexosaminidase was released during the permeabilization procedure (results not shown). A cytosol-like medium of exactly the same composition, except that Na^+ was replaced by K^+ , was used in some of the experiments.

Ca^{2+} stimulation and ouabain-binding assay in permeabilized cells

In all experiments, the cells were first rendered leaky with digitonin in EGTA and then stimulated separately with Ca^{2+} . Chloride-free Ca^{2+} buffers were prepared with calcium gluconate. If not indicated otherwise, the permeabilized cells were incubated in a sodium gluconate/magnesium medium (110 mM-sodium gluconate, 2 mM-magnesium gluconate, 10 mM-Tris, pH 7.0, adjusted with Hepes) supplemented with 1 mM-ATP, 5 mM- Ca -EGTA and 50 nM- ^3H ouabain. According to K_d values of 7–10 nM determined by Scatchard analysis at 30 °C (Figs. 2b and 3b), ouabain binding data correspond to 83–86% of B_{max} (maximum binding capacity). In experiments designed to study the effects of K^+ , ATP, Mg^{2+} and putative inhibitors, the Ca^{2+} stimulation step was separated from ouabain binding to avoid direct influences of additions on ouabain binding. Permeabilized cells were challenged with Ca^{2+} as above, except that ^3H ouabain was omitted from the reaction mixture. At the desired time, Ca^{2+} was removed at 5 °C by two 30 min washes in sodium gluconate/magnesium containing 10 mM-EGTA. For determination of ouabain binding, the cells were reincubated for 2 h at 30 °C in the Ca -free sodium gluconate/magnesium medium supplemented with 1 mM-ATP, 10 mM-EGTA and 40–50 nM- ^3H ouabain. Radioactivity bound to individual cells was determined as described [6]. Non-specific binding determined in parallel with 1 mM unlabelled ouabain was subtracted from all data.

Ca^{2+} determination

Ca^{2+} standards were prepared as described [13], except that NaCl (Suprapur, Merck) was used instead of KCl as the principal electrolyte. Medium $[\text{Ca}^{2+}]$ was checked on occasion with a Ca^{2+} -sensitive minielectrode (World Precision Instruments, New Haven, CT, U.S.A.). The Ca /EGTA ratios and measured Ca^{2+} concentrations were: 0.2, 0.18 μM ; 0.4, 0.31 μM ; 0.6, 0.71 μM ; 0.8, 2.0 μM ; 0.9, 4.7 μM ; 0.95, 8.9 μM ; 0.98, 22.4 μM . These values were higher by a factor of 1.6 ± 0.3 (S.D.) than those calculated by a computer program (EQCAL; Biosoft, Cambridge, U.K.) using the stability constants given by Sillén & Martell [14]. Ca^{2+} concentrations of media with BAPTA or without Ca^{2+} chelator were also determined with the Ca^{2+} electrode.

β -Hexosaminidase release from permeabilized cells

Portions of the incubation medium were assayed for β -hexosaminidase as described by Barrett [15] using *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminidine as a substrate. All results were normalized to the total enzyme activity of supernatants of non-incubated cells homogenized in 10 mM-Tris/HCl (pH 7.4) with 0.1% Triton X-100 and centrifuged for 5 min at 10000 g.

Data analysis and presentation of results

If not indicated otherwise, data of specific ouabain binding are given as the means $\pm$ S.D. of ten separate determinations in individual oocytes. The results are representative of at least three similar experiments. Correlation coefficients (*r*) were derived from linear regression analysis. Fits to the Hill equation were carried out by non-linear least-squares regression using a commercially available computer program (Enzfitter; Biosoft, Cambridge, U.K.).

RESULTS

Stimulation by Ca^{2+} of ouabain binding to digitonin-permeabilized cells

As demonstrated previously [6], ouabain binding to oocytes permeabilized by 10 μM -digitonin attained a plateau after 2–4 h of incubation at 25 °C in the Ca^{2+} -free EGTA medium (Fig. 1a). In the absence of Ca^{2+} and SDS, the ouabain-binding capacity of permeabilized oocytes was approximately equal to that of the surface of intact cells. Access to the ouabain-binding sites of pumps located in the cell interior required the additional permeabilization of intracellular membranes with the non-selective detergent SDS.

Fig. 1(a) shows that intracellular sodium pumps can also be rendered accessible to ouabain in the absence of SDS by introducing Ca^{2+} into the cell. At 9 μM - Ca^{2+} in digitonin-permeabilized oocytes, ouabain binding increased continuously such that after 10 h at 25 °C, 330 ± 19 c.p.m. of ouabain was bound per oocyte (Fig. 1a). This value is close to the 352 ± 23 c.p.m. bound per SDS-treated oocyte of the same batch, indicating that Ca^{2+} induced access to nearly all sodium pumps in a cell. Comparison of the amount of Ca^{2+} -independent ouabain binding in the absence and presence of SDS suggests that, in this particular cell batch, only about one-third of the sodium pumps were located in the plasma membrane.

A Ca^{2+} -induced increase in ouabain binding was also observed in meiotically mature oocytes (i.e. eggs, Fig. 1b). In the absence of Ca^{2+} or SDS, these cells bind less ouabain than oocytes (see Figs. 1a, 1b, 2 and 3), since their plasma membranes are devoid of sodium pumps. The surface sodium pumps are endocytosed during meiotic maturation and reside in an intracellular compartment [8] where they are inaccessible to ouabain even after permeabilization of the plasma membrane (see Fig. 12). Because

Fig. 1. Time and temperature dependence of ouabain binding to digitonin-permeabilized cells at 1 nM- and 9 μ M- Ca^{2+}

(a), (b) Time dependence. Digitonin-permeabilized oocytes (a) and eggs matured *in vitro* (b) were incubated at 25 °C in sodium gluconate/magnesium supplemented with 1 mM-ATP and 50 nM-[³H]ouabain. Ca^{2+} in the medium was buffered to 1 nM with 10 mM-EGTA (○) or to 9 μ M with 5 mM-Ca/EGTA (●). (c) Temperature dependence. Digitonin-permeabilized eggs were incubated as above, except that [³H]ouabain was omitted during Ca^{2+} stimulation and incubation temperature was varied as indicated. After 4 h the cells were washed in EGTA and reincubated for 2 h at 25 °C in Ca-free sodium gluconate/magnesium supplemented with 10 mM-EGTA and 40 nM-[³H]ouabain.

of the low basal level of ouabain binding, the Ca^{2+} effect was more pronounced in the eggs than in the oocytes (Fig. 1b). Within 4 h at 9 μ M- Ca^{2+} and 25 °C, ouabain binding increased by up to 3-fold. Comparison with ouabain binding in eggs treated in parallel with 0.02% SDS indicated that almost all intracellular sodium pumps became labelled with ouabain when incubation was continued for at least 10 h. All further data except those depicted in Fig. 2 were obtained with eggs.

The Ca^{2+} effect on ouabain binding increased steeply with increasing incubation temperature, as illustrated in Fig. 1(c). At 30 °C and 9 μ M- Ca^{2+} , maximal ouabain binding was obtained within 6–8 h (results not shown). For technical reasons, in all experiments described below the cells were challenged with Ca^{2+} for 4 h at 30 °C.

Quantitative analysis of Ca^{2+} stimulation of ouabain binding

The dependence of ouabain binding to permeabilized oocytes on the Ca^{2+} concentration is shown in Fig. 2(a). In the presence of 1 mM-ATP and 2 mM-magnesium gluconate, the increase in ouabain binding occurred over a narrow range of Ca^{2+} concentrations, indicating positive co-operativity. Hill analysis yielded a $K_{0.5}$ of $0.4 \pm 0.1 \mu\text{M}$ - Ca^{2+} and a Hill coefficient (h) of 1.8 ± 0.2 (mean $\pm$ s.d. of three experiments). The typical Scatchard plots given in Fig. 2(b) demonstrate that the Ca^{2+} effect is not due to a change in the affinity for ouabain (the K_d value actually increased from 7 to 10 nM), but to a doubling of the ouabain-binding capacity (B_{max}). The B_{max} values derived from x-axis intercepts of the straight lines ($r > 0.984$) averaged 8.6 and 17.1 fmol per cell in the absence and presence respectively of Ca^{2+} . A subset of oocytes was exposed to 0.02% SDS to determine the total number of ouabain-binding sites per cell (Fig. 2b). In the presence of SDS, Ca^{2+} had little effect on B_{max} (28.6 versus 29.6 fmol/cell, $r > 0.993$).

A typical Ca^{2+} activation curve obtained with permeabilized eggs is shown in Fig. 3(a). As in immature oocytes, Ca^{2+} increased ouabain binding with positive co-operativity. A Ca^{2+} concentration of $0.6 \pm 0.1 \mu\text{M}$ was half-maximally stimulatory (h 2.2 ± 0.2 , average of three experiments). The Scatchard graphs in Fig. 3(b) indicate that Ca^{2+} induced a 4–5-fold increase in B_{max} from 3.4 fmol ($r = 0.966$) to 15.6 fmol per cell ($r = 0.996$), but had little influence on the K_d values. B_{max} values determined in the presence of SDS at 1 nM- or 9 μ M- Ca^{2+} amounted to 29.0 ($r = 0.983$) and 31.6 ($r = 0.999$) fmol/cell respectively. These data

can be compared directly with those of Fig. 2(b), since oocytes and eggs were derived from the same oocyte batch.

Effect of the major cation or anion on the Ca^{2+} response

Since K^+ is the principal intracellular cation, an incubation medium which contains K^+ as the major cation would mimic the intracellular milieu more closely. The reason for using a Na^+ -based medium in most of the present experiments was that K^+ would greatly reduce the apparent affinity of the sodium pump for ouabain. To examine whether the Ca^{2+} effect also occurs in the presence of K^+ , the cells were first stimulated with Ca^{2+} and MgATP in the absence of [³H]ouabain in a medium containing K^+ in place of Na^+ . Controls were incubated in parallel in sodium

Fig. 2. Ca^{2+} dependence of ouabain binding to digitonin-permeabilized oocytes

Digitonin-permeabilized oocytes were exposed to Ca^{2+} buffered with 5 mM-EGTA (●) or 5 mM-BAPTA (▲) in sodium gluconate/magnesium containing 1 mM-ATP. The indicated Ca^{2+} concentrations were determined using a Ca^{2+} -sensitive electrode. (a) Typical Ca^{2+} activation curve after 4 h and 30 °C at 40 nM-[³H]ouabain. The line drawn through the data points is a non-linear least-squares fit to the Hill equation ($K_{0.5}$ $0.4 \pm 0.1 \mu\text{M}$, Hill coefficient 2.0 ± 0.5 , s.d. of the least-squares fit). (b) Typical Scatchard plots for ouabain binding at 1 nM- (○, □) and 9 μ M- Ca^{2+} (●, ■) after 4 h at 30 °C in the absence (○, ●) or presence (□, ■) of 0.02% SDS. [³H]Ouabain ranged between 1 and 50 nM. Bound/free has units of $\text{fmol} \cdot \text{cell}^{-1} \cdot \text{nM}^{-1}$.

Fig. 3. Ca^{2+} dependence of ouabain binding to digitonin-permeabilized eggs

Digitonin-permeabilized eggs were incubated as described in the legend to Fig. 2. (a) Ca^{2+} -activation curve after 4 h at 30 °C and 40 nM-[3H]ouabain. The line drawn through the data points is a non-linear least-squares fit to the Hill equation ($K_{0.5}$ $0.7 \pm 0.1 \mu M$, h 2.3 ± 0.5 , s.d. of the least-squares fit). (b) Scatchard plots for ouabain binding at 1 nM- ($\circ$, $\square$) and 9 μM - Ca^{2+} ($\bullet$, $\blacksquare$) after 4 h at 30 °C in the absence ($\circ$, $\bullet$) or presence ($\square$, $\blacksquare$) of 0.02% SDS. [3H]ouabain ranged between 1 and 50 nM. Similar Scatchard plots of ouabain binding to immature oocytes of the same batch from which the eggs were derived are shown in Fig. 2(b). Bound/free is given in $fmol \cdot cell^{-1} \cdot nM^{-1}$.

gluconate/magnesium. After 4 h, Ca^{2+} and K^+ were removed by washing the cells with EGTA to determine [3H]ouabain binding in the absence of Ca^{2+} and K^+ . As demonstrated in Fig. 4, Ca^{2+} was as effective in the K^+ medium as in the Na^+ medium in increasing ouabain binding. A detailed investigation of the Ca^{2+} dependence showed that essentially identical activation curves were obtained irrespective of whether ouabain was present simultaneously with Ca^{2+} in a Na^+ medium (Fig. 3a) or was added subsequent to stimulation in a K^+ medium ($K_{0.5}$ $0.5 \pm 0.1 \mu M$ - Ca^{2+} ; h 1.7 ± 0.3 , average of two experiments).

Exocytosis in electroporated chromaffin cells is dependent on the nature of the major anion. In particular, Cl^- has been shown to interfere with exocytosis [16]. In permeabilized eggs, however, Cl^- did not diminish the Ca^{2+} -mediated increase in ouabain binding relative to that observed in the presence of the impermeant anion gluconate (Fig. 4). By contrast, substitution of glutamate or isethionate for Cl^- or gluconate was consistently found to augment the Ca^{2+} effect (Fig. 4). Thiocyanate impaired the Ca^{2+} -mediated rise in ouabain binding at 10 mM and above (results not shown).

Release of β -hexosaminidase

In order to examine whether the Ca^{2+} -dependent increase in ouabain binding was associated with the liberation of intravesicular protein, the extracellular medium was assayed for the lysosomal enzyme β -hexosaminidase. During 4 h of incubation at 30 °C, the release of total β -hexosaminidase amounted to $0.4 \pm 0.2\%$ at 1 nM- Ca^{2+} and $0.5 \pm 0.2\%$ at 9 μM - Ca^{2+} (means of twelve experiments). Higher Ca^{2+} concentrations also failed to release significant amounts of the enzyme. Since the release of β -hexosaminidase apparently did not parallel the increase in ouabain binding, the sodium pumps made accessible by Ca^{2+} cannot reside in the lysosomes.

ATP and Mg^{2+} requirement of Ca^{2+} -stimulated ouabain binding

Ouabain binding in digitonin-treated *Xenopus* oocytes and eggs is stimulated at a Ca^{2+} concentration range similar to that known to induce exocytosis in a variety of permeabilized secretory

Fig. 4. Influence of monovalent cations and anions on Ca^{2+} -stimulated ouabain binding

Eggs were permeabilized with digitonin in sodium gluconate/magnesium as usual. Subsequent incubations were performed in media containing 10 mM-Tris (pH 7.0, adjusted with HEPES), 1 mM-ATP, 2 mM-magnesium gluconate, 110 mM of the electrolyte indicated in the Figure ($\square$, Na^+ ; $\blacksquare$, K^+), and 1 nM- or 9 μM - Ca^{2+} . After 4 h at 30 °C, the cells were washed twice for 30 min at 5 °C in Ca -free sodium gluconate/magnesium containing 10 mM-EGTA. Ouabain binding was determined by incubating the cells for an additional 2 h at 30 °C in Ca -free sodium gluconate/magnesium supplemented with 10 mM-EGTA, 1 mM-ATP, and 40 nM-[3H]ouabain. Data are means $\pm$ s.d. of three independent experiments. Ca^{2+} -induced ouabain binding was calculated by subtracting basal binding from total binding in cells exposed to 1 nM- Ca^{2+} or 9 μM - Ca^{2+} respectively. The results were normalized to Ca^{2+} -induced ouabain binding to cells in the sodium gluconate medium, which amounted to 150, 243 and 276 c.p.m. in the three experiments.

Fig. 5. Orthovanadate and phosphate do not support the Ca^{2+} effect on ouabain binding

Oocytes were permeabilized with digitonin as usual, except that ATP was omitted from the permeabilization medium. The permeabilized cells were incubated for 4 h at 30 °C, 40 nM-[3H]ouabain and 1 nM- ($\square$) or 9 μM - Ca^{2+} ($\blacksquare$) in sodium gluconate/magnesium supplemented with sodium orthovanadate, sodium phosphate (P_i) or ATP as indicated.

Fig. 6. MgATP dependence of the Ca²⁺ effect on ouabain binding

(a) Mg²⁺-dependence. Eggs were rendered leaky as usual, but Mg²⁺ was omitted from the washing step following digitonin permeabilization. The permeabilized cells were prestimulated at 1 mM- (○) or 9 μM-Ca²⁺ (●) in sodium gluconate supplemented with 1 mM-ATP and magnesium gluconate at the concentrations indicated. After 4 h at 30 °C, the cells were washed twice for 30 min at 5 °C in Ca²⁺-free sodium gluconate with 10 mM-EGTA. For the determination of ouabain binding, the cells were incubated for an additional 2 h at 30 °C in Ca²⁺-free sodium gluconate/magnesium containing 10 mM-EGTA, 1 mM-ATP and 40 nM-[³H]ouabain. (b) ATP-dependence. Eggs permeabilized with digitonin in the absence of ATP were prestimulated as above, except that the ATP concentrations were varied as indicated at a constant concentration of 2 mM-magnesium gluconate. [³H]Ouabain binding was determined as described above after removal of Ca²⁺ by washing the cells in EGTA.

Fig. 7. Nucleotide specificity of Ca²⁺-stimulated ouabain binding

Digitonin-permeabilized eggs were stimulated at 9 μM-Ca²⁺ in the sodium gluconate/magnesium medium with 1 mM of the nucleotides shown (■). Ca²⁺-free controls were incubated in parallel in the presence of 10 mM-EGTA (□). After 4 h at 30 °C, the cells were washed and assayed for [³H]ouabain binding as described in the legend to Fig. 6. The asterisk indicates that 1 mM-diadenosine pentaphosphate was also present.

Nucleotide specificity

A number of nucleotides were examined for their ability to support the Ca²⁺ effect on ouabain binding. To avoid direct effects of nucleotides on ouabain binding, the digitonin-permeabilized eggs were prestimulated with 1 mM of a particular nucleotide in the presence of 9 μM-Ca²⁺ and 2 mM-magnesium gluconate. After removal of Ca²⁺ and nucleotides, ouabain binding was assessed in the Ca²⁺-free EGTA medium containing 1 mM-ATP. The results are illustrated in Fig. 7. The most pronounced increase in ouabain binding was induced by ATP, followed by ADP > ATP[S] > CTP > UTP > ITP = XTP > GTP. p[NH]ppA (adenosine 5'-[γ-imido]triphosphate, a non-phosphorylating analogue of ATP), cyclic AMP and AMP were without effect. The effect of ADP could be reduced by blocking the conversion of ADP into ATP with diadenosine pentaphosphate (Fig. 7), a myokinase inhibitor [22]. None of the nucleotides affected the level of Ca²⁺-independent ouabain binding. Exactly the same rank order of potency was found when permeabilized oocytes were exposed to Ca²⁺ and the various nucleotides (results not shown).

Studies in permeabilized secretory cells showed that GTP and non-hydrolysable GTP analogues increased the Ca²⁺ sensitivity of secretion [23–25]. However, in permeabilized *Xenopus* oocytes and eggs, ouabain binding stimulated with 0.4 μM-Ca²⁺ and 1 mM-MgATP was not affected significantly by 1 mM-GTP or 10 μM-GTP[S] (results not shown).

Ca²⁺-stimulated entrapment of incubation medium

Stimulation by 9 μM-Ca²⁺ caused non-specific ouabain binding to increase from a control level of 17 ± 5 to 43 ± 16 c.p.m./cell (means of nine experiments). Since exocytosis is usually followed by endocytosis that removes membrane from the cell surface, the increase in non-specific binding might result from entrapment of medium in an endocytotic compartment. To address this question, the Ca²⁺-dependent cellular accumulation of fluid phase markers was measured. On the basis of the uptake of [¹⁴C]sucrose and [³H]inulin (5.2 kDa) determined in two independent experiments, 9 μM-Ca²⁺ induced the entrapment of 16 ± 4 nl and 9 ± 3 nl respectively of incubation medium in digitonin-permeabilized cells incubated for 4 h and 30 °C in the presence of

cells [9,16–19]. Exocytosis from permeabilized cells is further characterized by its rather specific requirement for Mg²⁺ and ATP ([9,16,18,19], for additional references, see [20]). The question of whether MgATP is also necessary for the Ca²⁺ effect on ouabain binding was examined by a series of experiments taking into account the fact that ouabain binding needs the phosphorylated conformation of the sodium pump supported by ATP or P_i. In the first set of experiments, the cells were stimulated with Ca²⁺ in an ATP-free medium. Ouabain binding was facilitated with P_i or orthovanadate [21]. As is apparent from Fig. 5, basal ouabain binding in the absence of Ca²⁺ was lower with P_i or orthovanadate than with ATP. Nevertheless, the results depicted in Fig. 5 indicate very clearly that Ca²⁺ completely failed to stimulate ouabain binding in the presence of 2 mM-magnesium gluconate when ATP was absent.

In the second set of experiments, the dependence on the ATP concentration of Ca²⁺-stimulated ouabain binding was studied. The cells were first challenged in the absence of [³H]ouabain with 9 μM-Ca²⁺ and various concentrations of ATP at a constant concentration of 2 mM-magnesium gluconate. The reaction was stopped by washing in EGTA. [³H]Ouabain binding was assayed in a medium containing ATP and EGTA but no Ca²⁺. As shown in Fig. 6(a), stimulation of ouabain binding by Ca²⁺ required the presence of ATP in the medium. About 0.4 mM-ATP produced a half-maximal effect. Essentially the same results were obtained when the cells were prestimulated with Ca²⁺ under otherwise identical conditions at 25 °C instead of 30 °C (results not shown).

In the third series of experiments, the concentration of Mg²⁺ was varied at a constant concentration of added ATP of 1 mM. As is apparent from Fig. 6(b), ATP was unable to support the stimulating effect of Ca²⁺ in the absence of Mg²⁺. About 0.4 mM-Mg²⁺ was necessary to give rise to half-maximal stimulation of ouabain binding by Ca²⁺.

Fig. 8. Inhibition by neomycin and polymyxin B of Ca^{2+} -evoked ouabain binding

(a) Digitonin-permeabilized eggs were challenged at 30 °C with 9 μM - Ca^{2+} and 1 mM-ATP in sodium gluconate/magnesium containing neomycin in concentrations as indicated. Controls were incubated in the presence of 10 mM-EGTA (1 nM- Ca^{2+}). After 4 h, the cells were washed and assayed for [^3H]ouabain binding as described in the legend to Fig. 6. (b) Cells were challenged with Ca^{2+} and assayed for [^3H]ouabain binding as above, except that polymyxin B was present during Ca^{2+} stimulation. The polymyxin B concentrations are based on a M_r value of 1000. The data points are means from a representative experiment, giving s.d. values of ten separate determinations in individual oocytes as vertical bars. The line drawn through the data points at 9 μM - Ca^{2+} (●) is a non-linear least-squares fit to the Hill equation (IC_{50} 173 $\pm$ 9 μM ; h 1.79 $\pm$ 0.15; s.d. of the least-squares fit).

1 mM-MgATP. Uptake of [^{14}C]sucrose was reduced to 3 $\pm$ 1 nl/cell when ATP was omitted from the medium. The Ca^{2+} -dependent increase in non-specific [^3H]ouabain binding is consistent with the entrapment of 17 nl of medium/cell, corresponding to about 3% of the water space of an oocyte.

Effect of some putative inhibitors of exocytosis

Calmodulin. To further explore the mechanism of the stimulation by Ca^{2+} of ouabain binding, we studied the effects of a variety of putative inhibitors of exocytosis. Calmodulin has been implicated in exocytosis in chromaffin cells [26] and sea urchin eggs [27]. However, neither calmodulin (1000 units/ml) nor the non-specific calmodulin antagonists trifluoperazine (20 μM) and calmidazolium (compound R24571, 10 μM) affected Ca^{2+} -sensitive ouabain binding. Calcineurin (5 units/ml), a calmodulin-binding protein, was also without any effect.

Neomycin. The aminoglycoside antibiotic neomycin has been reported to inhibit exocytosis from sea urchin egg cortices [28], electropermeabilized chromaffin granules [29] and transiently permeabilized mast cells [24]. As illustrated in Fig. 8(a), neomycin blocked the stimulatory effects of 9 μM - Ca^{2+} on ouabain binding, with 1 mM-neomycin being approximately half-maximally effective. Increasing the free Ca^{2+} concentration up to 100 μM did not reduce the inhibition (results not shown) arguing against a direct competition between Ca^{2+} and neomycin. Neomycin had little effect on basal ouabain binding to digitonin-permeabilized oocytes at 1 nM- Ca^{2+} (controls in Fig. 8a) and no effect on binding of ouabain to intact oocytes (results not shown). Gentamycin, another aminoglycoside antibiotic, and the polyamines spermine and spermidine were about one order of magnitude less effective than neomycin, whereas streptomycin (10 mM) was ineffective.

Polymyxin B. The inhibition of exocytosis by the cationic antibiotic neomycin has been attributed to a blockade of the

Fig. 9. Stimulation of ouabain binding and β -hexosaminidase release by mastoparan

Digitonin-permeabilized eggs were challenged with 100 μM -mastoparan at 1 nM- Ca^{2+} (□), 9 μM - Ca^{2+} (■) or 1 nM- Ca^{2+} plus 0.02% SDS (▨). When indicated 10 mM-neomycin was additionally present. After 4 h at 30 °C, the medium was assayed for β -hexosaminidase and the cells for [^3H]ouabain binding as described in the legend to Fig. 6.

hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) and subsequent activation of protein kinase C. The amphipathic peptide antibiotic polymyxin B, another inhibitor of protein kinase C [30], was also found to abolish the Ca^{2+} effect on ouabain binding (Fig. 8b). Half-maximal inhibition occurred at approx. 0.13 mM (h = 1.7). In contrast, H-7 [1-(5-isoquinolinesulphonyl)-2-methylpiperazine, 100 μM], a reported specific inhibitor of protein kinase C [31], had no effect.

Effect of mastoparan, a putative stimulator of secretion

Mastoparan, an amphiphilic tetradecapeptide from wasp venom, has been reported to stimulate secretion from a variety of cell types [32] and to affect phosphoinositide hydrolysis [33,34]. As shown in Fig. 9(a), mastoparan induced a pronounced increase in ouabain binding in digitonin-permeabilized eggs which was Ca^{2+} -independent. The stimulating effect of mastoparan was accompanied by a marked release of the lysosomal marker β -hexosaminidase (Fig. 9b), suggesting that mastoparan causes direct lysis of intracellular organelles in a similar way to the non-specific detergent SDS. It should be noted, however, that the effect of mastoparan on ouabain binding could be completely blocked by neomycin (Fig. 9a) without abolishing the release of β -hexosaminidase (Fig. 9b).

Proteinase inhibitors

In view of the suggested role of metalloendoproteases in exocytosis from mast cells and chromaffin cells [35] and in the acrosome reaction in sea urchin sperm [36], the effect of the metalloendoprotease inhibitor CBZ-Gly-Phe-amide on Ca^{2+} -stimulated ouabain binding was studied (Fig. 10). A maximally inhibitory concentration of 4 mM-CBZ-Gly-Phe-amide reduced the Ca^{2+} effect by about 50% [IC_{50} (concn. causing half-maximal inhibition) approx. 1 mM]. In contrast, the Ca^{2+} response was relatively unaffected by the control dipeptides CBZ-Gly-Phe (Fig. 10) and 2 mM-Gly-Phe-amide (results not shown) which do not inhibit metalloendoproteases. The metalloendoprotease inhibitors 1,10-phenanthroline (0.5 mM) and phosphoramidon (20 μM), the calpain inhibitors antipain and leupeptin (1 mM each [37]) and the chymotrypsin inhibitors phenylmethanesulphonyl fluoride (0.1 mM), *N*-acetyl-L-Tyr ethyl ester (1 mM, [38]) and aprotinin (up to 1 unit/ml) were without any effect on Ca^{2+} -stimulated ouabain binding (results not shown).

Fig. 10. Effect of CBZ-dipeptides on Ca²⁺-stimulated ouabain binding

Digitonin-permeabilized eggs were prestimulated at 9 μ M-Ca²⁺ and 30 °C in sodium gluconate/magnesium containing 1 mM-ATP and CBZ-Gly-Phe-amide (○, ●) or CBZ-Gly-Phe (□, ■) at the concentrations indicated. Controls were incubated in the presence of 10 mM-EGTA (1 nM-Ca²⁺, ○, □). The CBZ-dipeptides were added from stock solutions in dimethyl sulphoxide (final dimethyl sulphoxide concentration 0.2%). After 4 h the cells were washed and assayed for [³H]ouabain binding as described in the legend to Fig. 6.

Fig. 11. Inhibition by Ca²⁺ ionophores of Ca²⁺-evoked ouabain binding

Digitonin-permeabilized eggs were incubated for 4 h at 30 °C and 40 nM-[³H]ouabain in sodium gluconate/magnesium containing 1 mM-ATP and A23187 (a) or ionomycin (b) at the concentrations indicated. The ionophores were added from stock solutions in dimethyl sulphoxide (final solvent concentration 0.2%). Controls were incubated in the presence of 10 mM-EGTA (1 nM-Ca²⁺, ○). The data points are means of a representative experiment, giving s.d. values of ten separate determinations in individual oocytes as vertical bars. The lines drawn through the data points at 9 μ M-Ca²⁺ (●) are non-linear least-squares fits to the Hill equation. A23187: IC₅₀ 73 $\pm$ 12 nM; *h* 0.57 $\pm$ 0.05. Ionomycin: IC₅₀ 9 $\pm$ 1 nM; *h* 0.71 $\pm$ 0.06 (s.d. of the least-squares fit).

Inhibition of the Ca²⁺ response by Ca²⁺ ionophores

Vesicular transport to the cell surface can be blocked by dissipating transmembrane pH gradients of vesicles with chloroquine, a lysosomotropic agent, or with the carboxylic ionophore monensin, which catalyses Na⁺-H⁺ and K⁺-H⁺ exchange. At concentrations of up to 100 μ M, chloroquine failed to block the

Ca²⁺ effect on ouabain binding, whereas 30 μ M-monensin reduced Ca²⁺-dependent ouabain binding by some 10%. Of a number of other ionophores tested, carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), valinomycin and nigericin (10 μ M each) were without any effect. However, the Ca²⁺ response could be completely inhibited by low concentrations of the Ca²⁺ ionophores A23187 (IC₅₀ 73 nM) and ionomycin (IC₅₀ 9 nM), as illustrated in Fig. 11; Hill analysis yielded Hill coefficients of 0.6 and 0.7 respectively.

DISCUSSION

Evidence that Ca²⁺-stimulated ouabain binding in permeabilized cells results from exocytosis

Secretory cells permeabilized in various ways have been shown to keep their secretory function and to discharge their granule contents such as neurotransmitters, hormones and enzymes when challenged with micromolar Ca²⁺ [9,10,16,18–20,25,39]. In addition to Ca²⁺, Mg²⁺ and ATP are required for compound secretion from most permeabilized cell systems [9,16,18–20]. The requirements found here for increasing the number of accessible ouabain binding sites in permeabilized oocytes and eggs are remarkably similar to those for secretory exocytosis, i.e. half-maximal stimulation by 0.5 μ M-Ca²⁺ (Figs. 2b and 3b) and absolute dependence on MgATP (Figs. 5 and 6). Taking into account the fact that *Xenopus* oocytes and eggs have a large number of internal ouabain-binding sites that are not directly accessible from the cytosol [6], it is intriguing to assume that the Ca²⁺- and MgATP-stimulated ouabain binding reflects exocytotic fusion of vesicles containing sodium pumps with the plasma membrane. The possible orientation of sodium pumps in internal and surface membranes is shown schematically in Fig. 12. Inaccessibility from the cytosol indicates that the ouabain-binding sites of intracellular sodium pumps are enclosed in an intracellular compartment that is insensitive to permeabilization by 10 μ M-digitonin. After Ca²⁺-stimulated fusion with the cell surface, the interior of the intracellular membrane faces out, such that the sodium pumps assume their plasma membrane orientation, in which the binding sites for ouabain and ATP are on the external and internal sides respectively. An increase in cytosolic Ca²⁺ has been previously shown to cause the exocytotic insertion of H⁺-ATPases into the plasma membranes of intact turtle bladder cells [40,41]. To our knowledge, the present study is the first to suggest that exocytotic insertion of integral membrane proteins can also be induced in permeabilized cells.

Further support for the view that membrane fusion is involved in the stimulation of ouabain binding by Ca²⁺ comes from inhibition studies. Neomycin, in concentrations known to inhibit exocytosis in various permeabilized cells including sea urchin eggs [24,28,29], completely blocked the Ca²⁺ effect on ouabain binding (Fig. 8a). In addition, CBZ-Gly-Phe-amide, a substrate dipeptide which has been reported to inhibit secretion in intact mast cells and chromaffin cells by blocking metalloendoproteinase [35], reduced Ca²⁺-stimulated ouabain binding in digitonin-permeabilized eggs (Fig. 10). The role of metalloendoproteinases in exocytosis is unclear, since inhibition of secretion by metalloendoprotease inhibitors in chromaffin cells has been found to be linked with a depression in the rise in cytosolic Ca²⁺ [42]. Moreover, in digitonin-permeabilized cells metalloendoprotease inhibitors blocked secretion invoked by millimolar Ca²⁺, but not by the more relevant micromolar Ca²⁺ concentrations [35]. Irrespective of their mode of action, however, it is well established that metalloendoprotease inhibitors block a wide variety of membrane fusion events, including myoblast fusion [43], the acrosome reaction in sea urchin sperm [36] and

Fig. 12. Model for the orientation of sodium pumps in internal and surface membranes

In intact cells, ouabain binds to an ectodomain of the membrane-spanning α -subunit of a sodium pump located in the plasma membrane. The ouabain-binding domains of sodium pumps which reside in intracellular membranes are assumed to face the interior of vesicles since the binding domain is inaccessible from the cytosol. Fusion of vesicles with cell surface causes the sodium pumps to assume their plasma membrane orientation. ●, Ouabain-binding site; □, ATP cleavage site.

exocytotic transport of new synthesized protein [44]. Therefore the partial inhibition of Ca^{2+} -stimulated ouabain binding by CBZ-Gly-Phe-amide adds indirect evidence that membrane fusion is involved in the Ca^{2+} effect.

Possible involvement of phosphatidylinositol in membrane fusion

Polyamines and other polycations such as neomycin are known to interact strongly, by virtue of their positively charged groups, with acidic phospholipids, especially phosphatidylinositol 4-phosphate (PIP) and PIP_2 [45–47]. The inhibition of exocytosis by neomycin [24,28] has been attributed to a blockade of the hydrolysis of PIP_2 catalysed by phospholipase C.

More recently, however, McLaughlin & Whitaker [48] showed that neomycin, by binding to phosphatidylinositol, reduces the negative surface potential of membranes, which in turn increases the Ca^{2+} requirement for exocytosis. A similar non-specific mechanism of action of neomycin in the present study might be deduced from the finding that other polycations such as polymyxin B (Fig. 8b), spermine and spermidine (results not shown) were also inhibitors of Ca^{2+} -stimulated ouabain binding. Increasing the free Ca^{2+} concentration, however, failed to reduce the inhibitory effect of neomycin in permeabilized *Xenopus* eggs (results not shown). An alternative explanation for the neomycin effect might be that phosphatidylinositol is involved in membrane fusion or surface assembly of plasma membrane proteins, as has been inferred from inositol starvation of yeast [49]. Such a view gains support from the observation of Ca^{2+} -independent stimulation of ouabain binding by mastoparan, which has been reported to interfere directly with polyphosphoinositides [33,34]. Mastoparan binds to acidic phospholipids, presumably because it contains three positively-charged lysine residues at neutral pH [33]. The release of β -hexosaminidase from digitonin-permeabilized eggs (Fig. 8b) would suggest that mastoparan

causes lysis of intracellular organelles (cf. [50]) in a similar fashion to the detergent SDS used in this and previous studies to gain access to intracellular ouabain-binding sites. However, the mastoparan effect on ouabain binding could be completely blocked with neomycin (Fig. 9a), suggesting that neomycin and mastoparan compete for a common binding site. The high concentrations of neomycin required for inhibition of the Ca^{2+} response as compared with $1\ \mu\text{M}$ -neomycin required to bind PIP_2 in phospholipid vesicles has been explained by the fact that, in biological membranes, most PIP_2 is protein-bound [46].

The Ca^{2+} -stimulated ouabain binding was also inhibited by polymyxin B, a reported inhibitor of protein kinase C [30]. Protein kinase C has been implicated in exocytosis from electrically permeabilized cells [29]. However, since protein kinase C is known to leak out rapidly from digitonin-permeabilized cells [51], this enzyme is unlikely to play a role in the Ca^{2+} effect observed in the present study. In agreement with this view, phorbol 12-myristate 13-acetate failed to affect either the magnitude or the Ca^{2+} -sensitivity of Ca^{2+} -stimulated ouabain binding when introduced directly into digitonin-permeabilized oocytes and eggs (results not shown).

Exocytosis versus granule lysis in permeabilized cells

By permeabilizing inner membranes with SDS, we have previously demonstrated that a close correlation exists between labelling of intracellular ouabain-binding sites and the release of the lysosomal marker β -hexosaminidase [6]. The Ca^{2+} -induced access to intracellular sodium pumps was not accompanied by a marked release of β -hexosaminidase, indicating that intracellular sodium pumps are not located in lysosomes (or cortical granules, see below), but in a distinct compartment with a sensitivity towards detergents similar to that of other intracellular membranes. Since intracellular membranes may behave differently towards Ca^{2+} , it cannot be excluded that Ca^{2+} exerts its effect simply by increasing the permeability of this particular compartment for ouabain. Although the MgATP requirement of the Ca^{2+} effect and its inhibition by calcium ionophores or neomycin and polymyxin B argue against this possibility, definite proof of exocytosis will require immunocytochemical demonstration that sodium pumps are incorporated into the plasma membrane.

Of the two electrophoretically distinct β -hexosaminidase activities of *Xenopus* eggs, one appears to be associated with cortical granules and is released from the egg at fertilization [52]. Since exocytosis of the cortical granule contents is triggered by a rise in cytosolic Ca^{2+} , it is surprising that permeabilized eggs did not secrete β -hexosaminidase when exposed to Ca^{2+} . Isolated cortices of unfertilized sea urchin eggs have for instance been shown to discharge their attached granules [53] completely when exposed to $1\text{--}3\ \mu\text{M}$ free Ca^{2+} in the presence of MgATP [17]. A possible explanation might be that the digitonin-permeabilized *Xenopus* eggs have lost a cytoplasmic factor (cf. [54]) required for exocytosis of cortical granule contents in these cells. The sodium pumps may populate a subset of vesicles with requirements for membrane fusion distinct from those containing secretory proteins.

Modulation of sodium pumps by Ca^{2+}

Most data reported so far show that intracellular Ca^{2+} inhibits sodium pump activity [55]. Up-regulation of the amount of sodium pumps in the plasma membrane in response to certain stimuli has been demonstrated to result from increases in the synthesis of the α and β subunits regulated at the level of transcription, but not from a redistribution of preformed pump molecules [56]. Intracellular Na^+ appears to play an important role in the regulation of the expression of sodium pumps at the

cell surface of excitable cells (see [57] for review). Raising intracellular Ca²⁺ by exposing myotubes to ionophore A23187 failed to change cell surface levels of sodium pumps [57]. By contrast, in non-permeabilized *Xenopus* eggs parthenogenetic activation with A23187 induced a reappearance of sodium pumps at the cell surface [8].

Possible role of Ca²⁺-stimulated insertion of sodium pumps

Activation of eggs both by calcium ionophore and by fertilization results from a rise in cytosolic Ca²⁺ [58], followed by the massive fusion of cortical granules with the plasma membrane. Since Ca²⁺ and cell fusion are involved, it is reasonable to assume that the reappearance of surface sodium pumps on activation [8] and the Ca²⁺-induced access to intracellular sodium pumps in permeabilized cells observed in the present study have a common mechanism, i.e. insertion of pump molecules into the plasma membrane. The increase in the number of ouabain-binding sites is slow in comparison with compound exocytosis. This might be attributed to the presence of a low number of organelles containing sodium pumps immediately beneath the cell surface, such that the transport of more intracellularly located organelles to the plasma membrane is rate limiting. In addition, compound exocytosis such as catecholamine release from digitonin-treated chromaffin cells is limited to 10–15% of the cellular content [9,10], whereas under present experimental conditions exocytosis appears to proceed until all sodium pumps are inserted into the plasma membrane.

Assuming that an egg resembles a smooth ball with a diameter of 1.1–1.2 mm, the 2–3 fmol of ouabain bound per activated egg [8] corresponds to about 400 sodium pump molecules per μm^2 . Although this represents a rather high density of pumps, it should be noted that activated eggs have five times less sodium pumps in their plasma membranes than the oocytes from which the eggs were derived, whereas the total number of sodium pumps is the same in both cell types. In activated eggs, approx. 90% of the total number of sodium pumps remain in the cell interior, as compared with 50% in oocytes. Insertion of no more than one-tenth of the internal sodium pumps might be attributed to the fact that the rise in cytosolic Ca²⁺ at fertilization is only transient. Treatment with A23187 results also in a transient increase in cytosolic Ca²⁺ only. In addition, the inhibition of the Ca²⁺ response in permeabilized cells by Ca²⁺ ionophores (Fig. 11) raises the possibility that A23187 is inhibitory in such experiments with intact cells. From the present findings in permeabilized oocytes and eggs (Figs. 2a and 2b), it may be concluded that all intracellular sodium pumps are available for insertion into the plasma membrane, provided that the rise in Ca²⁺ persists for several hours. Ca²⁺ transients have also been reported to accompany the mitotic cell divisions that follow fertilization [59]. If we assume that the main function of the Ca²⁺-regulated pathway is the delivery of sodium pumps to the growing surface of the early *Xenopus* embryo, it might be speculated that each Ca²⁺ transient is associated with the recruitment of a certain fraction of the intracellular sodium pumps, depending on the duration and magnitude of the Ca²⁺ rise. Such a regulatory mechanism would closely adapt plasma membrane assembly of sodium pumps to the growth of cell surface.

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