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Activation of Protein Kinase C by Phorbol Ester Induces Downregulation of the Na^+/K^+-ATPase in Oocytes of *Xenopus laevis*

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Summary. Full-grown prophase-arrested oocytes of *Xenopus laevis* were treated with 50 nM phorbol 12-myristate 13-acetate (PMA), an activator of protein kinase C, or with 50 nM 4α-phorbol 12,13-didecanoate (4αPDD) that does not activate protein kinase C. The effects on membrane currents and capacitance, inulin uptake and ouabain binding, and on membrane morphology were analyzed.

(*i*) During application of PMA, current generated by the Na^+/K^+ pump decreases; in addition, Cl^- and K^+ channels become inhibited. This general decrease in membrane conductance reaches steady state after about 60 min. 4αPDD was ineffective.

(*ii*) Ouabain binding experiments demonstrate that PMA ($K_{1/2}$ = 7 nM), but not 4αPPD, induces a reduction of the number of pump molecules in the surface membrane. Permeabilization of oocytes by digitonin plus 0.02% SDS renders all binding sites present prior to PMA treatment again accessible for ouabain. The K_D value for ouabain binding is not influenced. 4αPDD was ineffective.

(*iii*) Exposure of oocytes to PMA reduces membrane capacitance and stimulates uptake of inulin suggesting an increase in endocytosis. Electron micrographs show that PMA reduces the number and length of microvilli, leading finally to a smooth membrane surface with a reduced surface area.

From these results we conclude that stimulation of protein kinase C leads to downregulation of the sodium pump. A major portion of this inhibition is brought about by reduction in area of surface membrane with a concomitant internalization of pump molecules. In addition to this mode of downregulation, a direct effect of stimulation of protein kinase C on the pump molecule cannot be excluded.

Key Words *Xenopus* oocyte · phorbol ester · protein kinase C · sodium pump · endocytosis · ouabain binding · cell permeabilization · electron microscopy

Introduction

One of the most prominent transport proteins in nearly all animal cells is the Na^+/K^+-ATPase. Under physiological conditions, this enzyme mediates the transport of 3 Na^+ ions out of the cell and 2 K^+ ions into the cell per ATP molecule that is split. By this active transport, transmembrane gradients for Na^+ and K^+ are maintained that are essential for a large variety of cellular functions (*see*, e.g., De Weer, 1985). The oocytes of the clawed toad *Xenopus laevis* are particularly suited for studying this transport. The large size of the cells allows detection of electrogenic currents and performance of binding studies on a single cell. The resulting net current generated by the pump can easily be analyzed in voltage-clamp experiments (Lafaire & Schwarz, 1985, 1986). The specific inhibitor of the Na^+/K^+-ATPase, ouabain, has a low rate of dissociation; therefore, radioactively labeled ouabain can be used for counting pump molecules, which is possible even for single cells (Richter, Jung & Passow, 1984).

The rate of transport by the Na^+/K^+-ATPase depends on a variety of parameters; in the oocytes, dependencies on extracellular K^+ (Lafaire & Schwarz, 1986; Marx, Ruppersberg & Rüdel, 1987; Rakowski, Vasilets & Schwarz, 1990), intracellular Na^+ and membrane potential (Lafaire & Schwarz, 1986) have been studied. Second messengers released during intracellular signaling for the phosphorylation of membrane proteins by protein kinase C (Nishizuka, 1984, 1986) play a key role in regulation of membrane permeabilities (*see* Kaczmarek, 1987). For the Na^+/K^+-ATPase in somatic cells, it has been suggested that activation of protein kinase C by phorbol esters can modulate the rate of Na^+/K^+ pumping (Lynch et al., 1986; Hootman, Brown & Williams, 1987; Bertorello & Aperia, 1989). Phorbol esters have been demonstrated to activate protein kinase C also in *Xenopus* oocytes (Laurent et al., 1988), to modulate membrane currents (Dascal et al., 1985; Lupu-Meiri, Shapira & Oron, 1989), and to induce resumption of oogenesis (Bement & Capco, 1989).

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In this contribution, we demonstrate that the Na^+/K^+ pump is downregulated by application of nanomolar concentrations of phorbol ester. Two distinct processes are involved in the inhibition: (*i*) endocytotic removal of pump molecules from the plasma membrane, and (*ii*) reduced activity of those pump molecules that remain in the plasma membrane. Part of the results has been reported previously (Vasilets et al., 1989*a*; Vasilets, Schmalzing & Schwarz, 1989*b*).

Materials and Methods

Oocytes

X. laevis were anesthetized with 2 g/liter 3-aminobenzoic acid-ethylester methanesulfonate (MS222, Sandoz, Basel, Switzerland), and parts of the ovary were removed. Full-grown, prophase-arrested oocytes of stages V and VI (Dumont, 1972) were selected after removal of the enveloping tissue by treatment of the parts of ovary with collagenase (0.6–0.8 U/ml in oocyte Ringer solution, ORI, *see below*) and subsequent washing in Ca^{2+}-free ORI. The time of enzyme treatment varied between 7 and 12 hr depending on seasonal variations in the connective tissue and on the batch of collagenase used. If not stated otherwise, experiments were performed at room temperature (about 21°C).

Electrophysiological Measurements

Voltage-clamp experiments were performed by conventional two-microelectrode techniques (*see* Lafaire & Schwarz, 1986). For determination of current-voltage dependencies, rectangular voltage pulses of varying amplitude and 500-msec duration were applied every 4 sec, and steady-state current was averaged during the last 100 msec. Between the pulses, the holding potential was set to the respective resting potential (zero-current potential).

The current-voltage curves were recorded every 10 min during superfusion of the oocyte with control solution or solution containing phorbol ester. For determination of membrane capacitance we used a method of charge estimation as described by Adrian and Almers (1974) that does not depend on a particular model for membrane capacitor. Five rectangular voltage pulses of 20 mV were applied from the resting potential, and the resulting transient current was sampled at 10 or 20 kHz. The signal, averaged from the five pulses, was integrated and represents the charges necessary to load the membrane capacitor. The error made by neglecting the finite establishment of the rectangular potential step and the low leakage conductance was less than 1%.

Measurements of Ouabain Binding

Oocytes were treated with phorbol ester or the carrier (ethanol, final concentration 0.1%) in K^+-free ORI for defined times and then were transferred to fresh medium containing 2 μM ^{3}H-ouabain (0.37 TBq/mmol, Amersham-Buchler, Braunschweig, FRG) and 0.1 MBq/ml ^{14}C-sucrose (370 MBq/mmol, Amersham-Buchler) as an extracellular marker (total volume 70 μl). After 20 min at 21°C, unbound label was removed by washing. Individual oocytes were dissolved separately in 5% SDS and counted in Quickszint 2000 (Zinsser, Frankfurt, FRG). ^{14}C-sucrose was included in the reaction mixture to identify cells that became leaky during treatment by PMA. Cells with intact plasma membrane accumulated less than 10 cpm sucrose under present conditions. Ouabain binding data of cells that exhibited uptake of more than 20 cpm ^{14}C-sucrose were rejected.

In some experiments, oocytes were pretreated with PMA as above, washed in EGTA, and permeabilized with 10 μM digitonin as described by Schmalzing, Kröner & Passow, (1989). ^{3}H-ouabain binding was quantified by incubating the cells for 4 hr at 25°C with 1–40 nM ^{3}H-ouabain in the NaCl-Tris medium containing (in mM): 110 NaCl, 2 $MgCl_2$, 1 EGTA, 1 ATP, 10 Tris/HCl (pH 7.0) with or without 0.02% SDS. Nonspecific binding determined in the presence of 1 mM unlabeled ouabain was subtracted from all data. Less than 0.5% of the lysosomal marker β-hexosaminidase (Barrett, 1972) was released from both controls and PMA-treated oocytes during the permeabilization procedure (*results not shown*).

Measurements of Inulin Uptake

For detecting endocytosis, the fluid phase marker ^{3}H-inulin (5.2 kDa, 85 MBq/mmol, Amersham-Buchler) was applied to the cells at a final concentration of 0.4 MBq/ml (final volume 0.2 ml) just after addition of phorbol ester. At defined times, cells were washed in ice-cold ORI and assayed for radioactivity as described above.

Electron Microscopy

In order to visualize endocytosis by electron microscopy, oocytes were incubated as for the measurements of inulin uptake except that the medium was supplemented with 10 mg/ml horseradish peroxidase (250 U/mg, Serva 31943). After 20 and 40 min at 21°C, the cells were briefly washed in ice-cold ORI and fixed in 2.5% glutaraldehyde in 0.1 M Na-cacodylate (pH 7.2) for 30 min at 4°C and for 2 hr at 21°C. The peroxidase reaction was performed according to Graham and Karnovsky (1966). After washing in Na-cacodylate buffer, the cells were refixed for 16 hr at 21°C in 2.5% glutaraldehyde in cacodylate buffer and postfixed for 1 hr at 4°C in 1% OsO_4. In some cases, the oocytes were additionally treated with 0.5% tannic acid (Simionescu & Simionescu, 1976). After bloc-staining with 2% uranyl acetate, the oocytes were dehydrated in ethanol and embedded in Spurr's epoxy resin (Spurr, 1969) with propylene oxide as intermedium or in LR White medium (Craig & Miller, 1984). Thin sections were cut with the Ultracut (Reichert, Austria), double stained with uranyl acetate and lead citrate, and viewed in a Philips 300 electron microscope.

Solutions

The composition of the ORI solution was (in mM): 110 NaCl, 3 KCl, 2 $CaCl_2$, and 5 N-2-hydroxyl-ethyl-piperazine-N′-ethanesulfonic acid (HEPES, pH 7.6). Stock solutions of phorbol 12-myristate 13-acetate (PMA), 4α-phorbol 12,13-didecanoate (4αPDD), and strophantidine were prepared in ethanol which was present at a final concentration of 0.1% in most of the experiments. Ethanol contents of up to 1% were without detectable effects on membrane currents. In solutions with reduced Cl^-, all NaCl was replaced by sodium methylsulfate ($NaMeSO_4$).

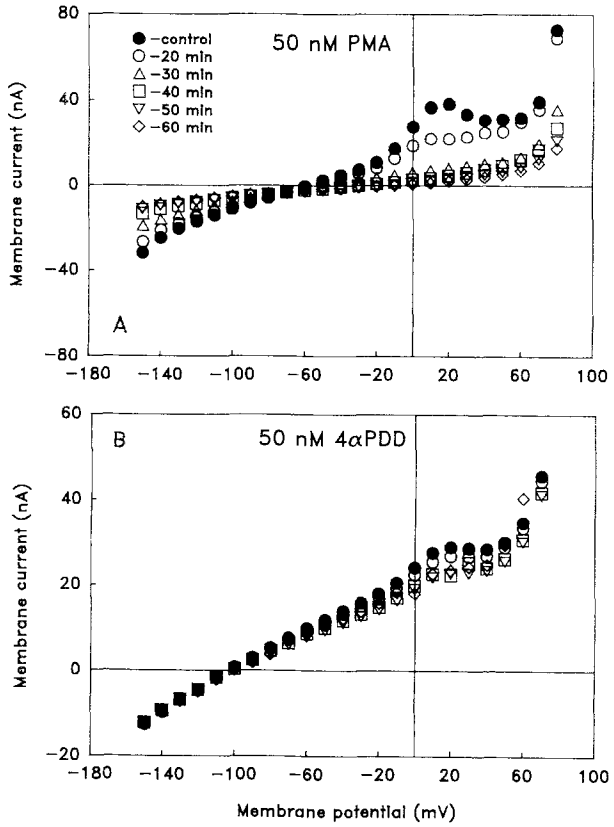

Fig. 1. Alterations in the current-voltage dependencies during application of phorbol esters. Oocytes were continuously superfused. After registration of an *I-V* curve in ORI without phorbol ester (filled circles), 50 nM PMA (*A*) or 4αPDD (*B*) was applied, and additional *I-V* curves were recorded. The indicated times refer to the time of exposure to phorbol esters

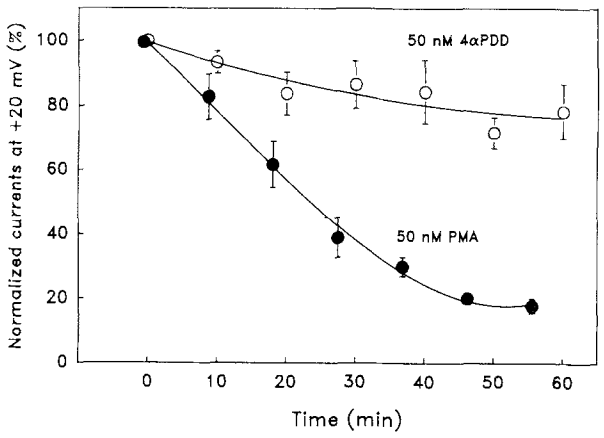

Fig. 2. Time course of decrease of membrane current at +20 mV during application of 50 nM PMA (filled circles) or 4αPDD (open circles). Data represent average values of the currents (± SEM) from 5–8 experiments normalized to the respective current at +20 mV in control solution

Results

As in other cells, ouabain acts as a selective inhibitor of the Na^+/K^+-ATPase also in the oocytes of *X. laevis*. Submicromolar concentrations of ouabain produce complete inhibition of pump activity and saturation of all ouabain binding sites. To assess Na^+/K^+ pump activity, that component of membrane current was measured that can be inhibited by 10 μM ouabain, dihydroouabain (DHO) or strophantidine. For simplicity, this current component will be called pump current or ouabain-sensitive current. Estimates of the number of pump molecules were obtained by studying ouabain binding using ^{3}H-labeled ouabain at a concentration of about 2 μM.

Effects of Phorbol Esters on Electrical Parameters

Conductance

Preliminary experiments showed that, 1 hr after application, 50 nM PMA produced maximum effects on the membrane currents. The electrophysiological experiments described below were, therefore, performed at 50 nM of the phorbol esters.

Typical steady-state current-voltage curves determined in voltage-clamp experiments are shown in Fig. 1. The total membrane conductance consists of at least three major specific components; passive conductances for Cl^- (*see*, e.g., Robinson, 1979; Miledi, 1982; Barish, 1983; Peres & Bernardini, 1983; Parker & Miledi, 1988*a*) and K^+ (*see*, e.g., Peres et al.,1985; Parker & Miledi, 1988*b*; Schweigert, Lafaire & Schwarz, 1988), and the electrogenic contribution of the 3 Na^+/2 K^+ pump (Lafaire & Schwarz, 1986). Figure 1*A* demonstrates that the phorbol ester PMA, known to stimulate activity of protein kinase C (Castagna et al., 1982; Nishizuka, 1984), induces a gradual decrease of total membrane current at all membrane potentials. The resting potential usually shows complex time-dependent changes with hyperpolarizing and depolarizing phases (*see*, e.g., Fig. 7*A*). If, instead of PMA, 50 nM of the phorbol ester analogue 4αPDD (ineffective in stimulating protein kinase C; Castagna et al., 1982) is applied, changes in membrane currents and resting potential remain insignificant (Fig. 1*B*).

To demonstrate the time course of current inhibition, average values of the relative changes of membrane current at +20 mV are plotted in Fig. 2. The dramatic decrease of membrane current induced by PMA reaches steady state within 1 hr. The slow decline in the presence of 4αPDD reflects normal run down, also seen in control solutions without additions (*not demonstrated*). Occasionally an initial increase of membrane current could be detected immediately after addition of PMA (*see* Fig. 3). This

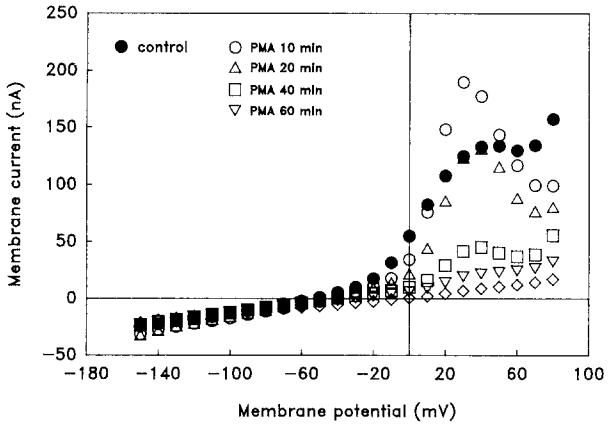

Fig. 3. Transient stimulation followed by inhibition of membrane current by application of 50 nM PMA. Current-voltage dependence before (filled circles) application and different times after application of PMA (open symbols)

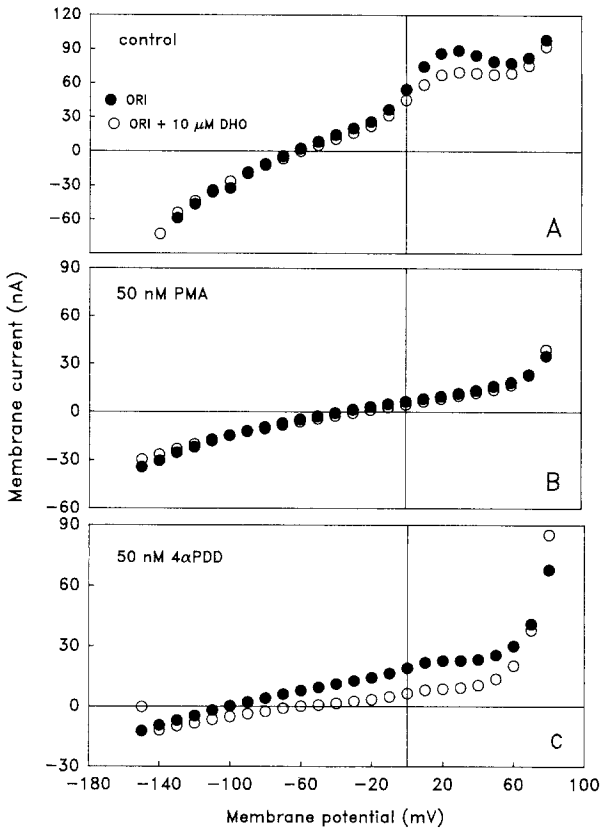

Fig. 4. Effect of 10 μM DHO on current-voltage dependencies before (*A*) and after application of 50 nM PMA (*B*) or 50 nM 4αPDD (*C*)

was particularly observed for outwardly directed membrane current. However, stimulation was transient and always followed by the reduction of total membrane current as described above.

Figure 4*A* shows the inhibition by DHO of the current component generated by the Na^+/K^+ pump in the absence of phorbol esters. After 60 min of exposure to PMA, DHO has nearly no effect on the membrane current persisting in the PMA-treated oocytes (Fig. 4*B*). Obviously, PMA induces a pronounced downregulation of pump current. In contrast, 4αPDD does not reduce the ouabain-sensitive current; 60 min after addition of 4αPDD, a large fraction of total membrane current can still be inhibited by DHO (Fig. 4*C*). Figure 5 summarizes the effects of a series of experiments on the ouabain-sensitive current determined at +20 mV. While 4αPDD has no significant effect on the pump current, PMA induces a reduction by a factor of about 8.3, a small fraction of about 12% is still detectable indicating that after 70 min of PMA application a pronounced, but not complete, inhibition is obtained.

To examine which other conductances may be modulated during the action of PMA, current-voltage relations were determined in experiments where the pump was inhibited by 10 μM DHO. Again, the residual membrane current decreases over the whole potential range (Fig. 6*A*). If, in addition to downregulation of the pump, the Cl^- conductance is reduced by replacement of most Cl^- by $MeSO_4^-$, currents are primarily carried by K^+ ions. PMA still leads to a strong reduction of this remaining current (Fig. 6*B*). If, instead of reducing the Cl^- conductance, the K^+ conductance is reduced by adding 5 mM $BaCl_2$ to the bath solution (*see* Schweigert et al., 1988), the remaining current again becomes reduced

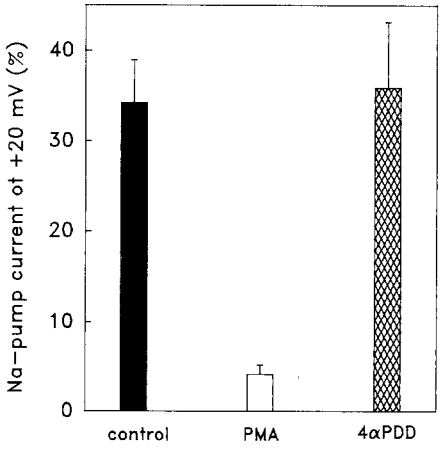

Fig. 5. Effect of 50 nM PMA or 4αPDD on the DHO-sensitive current component measured at +20 mV. Histogram represents average values of 9 (for control), 10 (for PMA), and 5 (for 4αPDD) experiments (± SEM). Data were normalized to the respective total membrane current at +20 mV

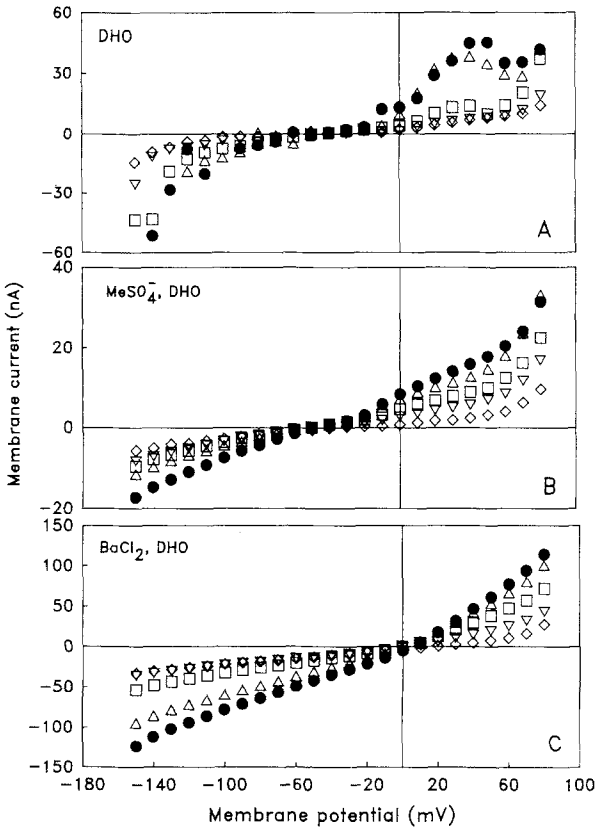

Fig. 6. Alterations of the current-voltage dependencies during application of 50 nM PMA, in an oocyte: (*A*) with the sodium pump inhibited by 10 μM DHO, (*B*) with inhibited pump and reduced Cl^- conductance (by replacement of the NaCl by $NaMeSO_4$ in the bath solution), and (*C*) with inhibited pump and blocked K^+ conductance (by addition of 5 mM $BaCl_2$)

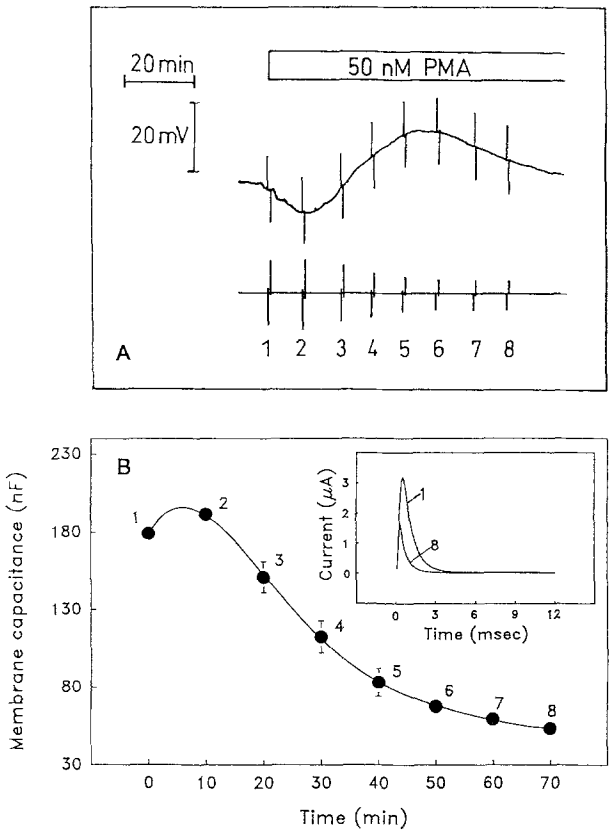

Fig. 7. Effects of PMA on membrane capacitance. The numbers indicate the times where membrane capacitance was determined by application of a series of 20-mV depolarizing and hyperpolarizing voltage-clamp pulses. (*A*) Time course of membrane potential (upper curve) and clamp current (lower trace) recorded on a pen recorder before and during application of 50 nM PMA. The membrane potential at the beginning of the experiment was about −60 mV; upward deflections represent depolarizations. The current signals during the clamp pulses are in arbitrary units. (*B*) Time course of decrease of membrane capacitance during application of 50 nM PMA. Capacitance was determined by integration of the transient currents. The inset shows the currents elicited by depolarizing pulses before (*1*) and 70 min after (*8*) application of PMA. Data represent average values (± SEM) of five experiments. The line represents a polynomial fit to the data

in the presence of PMA (Fig. 6*C*). These data suggest that, in addition to the pump currents, K^+ and Cl^- currents are blocked by a phorbol ester that activates protein kinase C. During the transient stimulation of membrane current usually hyperpolarizations were observed, but in a few cases immediate depolarizations were detected. This phenomenon was not further analyzed but suggests that Cl^- as well as K^+ conductances can be stimulated by PMA temporarily.

Capacitance

Activation of protein kinase C has been reported to be involved in stimulation of meiotic maturation (Laurent et al., 1988). On the other hand, maturation involves endocytotic uptake of membrane proteins. This has been shown to be responsible for the down-regulation of pump activity during the maturation process of *Xenopus* oocytes (Schmalzing et al., 1990). Endocytotic activity that involves a reduction in membrane surface should be detectable by measuring the membrane capacitance. The electrical capacitance of oocytes was estimated from transient currents elicited by nearly rectangular potential steps (*see* inset in Fig. 7*B*). Figure 7*A* shows a typical registration of membrane potential and clamp current on a pen recorder during an experiment. The current trace already indicates that during application of PMA the transient current gradually decreases. Figure 7*B* summarizes the results of a series of such experiments where the membrane capacitance was calculated from integration of transient

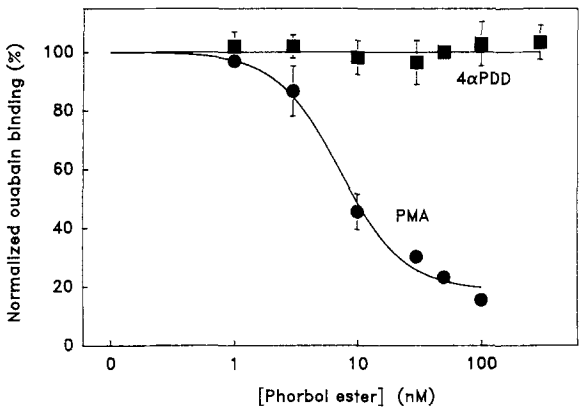

Fig. 8. Concentration dependence of the effect of phorbol esters on ouabain binding. Intact oocytes were challenged at 21°C with PMA (circles) or 4αPDD (squares) in concentrations as indicated. After 50 min, the cells were transferred to fresh medium and assayed for ^{3}H-ouabain binding as described under Materials and Methods. The line drawn through the circles is a nonlinear least-squares fit to the Hill equation ($K_{1/2}$ = 7 nM, Hill coefficient 1.7). Each data point is the mean ± SEM of 9–11 separate determinations in individual cells of a representative experiment. 100% corresponds to about 17 fmol of ouabain bound per cell

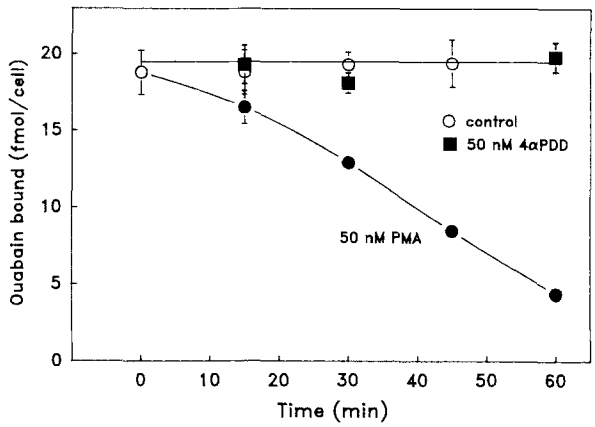

Fig. 9. Time course of the effect of phorbol esters on ouabain binding; data for untreated control oocytes are represented by open circles. Intact oocytes were challenged at 21°C with 50 nM PMA (filled circles) or 50 nM 4αPDD (filled squares) for the indicated times and then assayed for ^{3}H-ouabain binding. Each data point is the mean ± SEM of 9–11 separate determinations in individual cells of a representative experiment. Ouabain bound is approximately 90% of B_{max}

membrane currents (*see* inset Fig. 7*B* and Materials and Methods). Under control conditions, the average capacitance is 179 ± 5 nF. During the first 10 min of exposure to PMA the capacitance slightly increases but then decreases by a factor of 3.4 to 53 ± 1 nF. The time course is similar to that of the reduction of membrane current, and steady state is reached after about 50 min (*compare* Fig. 2). This suggests that the decrease in membrane current could at least partially be due to a reduction in membrane surface area and removal of transport proteins induced by the activation of protein kinase C. Application of 4αPDD does not affect membrane capacitance. In one batch of oocytes, we found for untreated oocytes 230 ± 6 nF (n = 10) and for oocytes treated for at least 1 hr with 50 nM 4αPDD 229 ± 5 nF (n = 10).

Effect of Phorbol Esters on Ouabain Binding

The number of Na^+/K^+ pump molecules in the plasma membrane can be assessed with ^{3}H-ouabain that binds specifically in a one-to-one stoichiometry to an ectodomain of the catalytic α-subunit of the pump. Exposure of intact oocytes to ^{3}H-ouabain for no more than 20 min leads to half-maximum binding at 0.1–0.3 μM (*results not shown*). Accordingly, the ouabain binding data derived at 2 μM ^{3}H-ouabain and depicted in Figs. 8 and 9 correspond to approximately 90% of the ouabain binding capacity (B_{max}). As shown in Fig. 8, PMA induces a concentration-dependent decrease of ouabain binding, whereas 4αPDD has no effect. As in electrophysiological measurements, 50 nM PMA gives nearly the maximal response. At higher PMA concentrations, an increasing number of cells becomes leaky for compounds of low molecular weight such as ^{3}H-ouabain and ^{14}C-sucrose.

The time course of the effect of 50 nM PMA on ouabain binding is depicted in Fig. 9. Within 60 min of incubation, the number of ouabain binding sites on the outer cell surface is reduced by a factor of 3 to 4. Incubation for additional 20 min gave no further reduction, suggesting that steady state is reached after 60 min.

To examine the question whether surface pumps are inactivated by PMA *in situ* or translocated from the plasma membrane to an internal compartment, we quantitated ouabain binding in PMA-treated oocytes permeabilized with detergents. B_{max} of oocytes which have been permeabilized selectively with digitonin is approximately equal to that of intact cells (Schmalzing et al., 1989). Additional permeabilization of inner membranes with the nonselective detergent SDS discloses intracellular Na^+/K^+ pumps in oocytes. The time course of PMA pretreatment on ouabain binding in digitonin-permeabilized cells is illustrated in the inset of Fig. 10. Exposure of oocytes to 50 nM PMA before permeabilization greatly reduces ouabain binding to the permeabilized cells. The time course parallels the reduction of ouabain

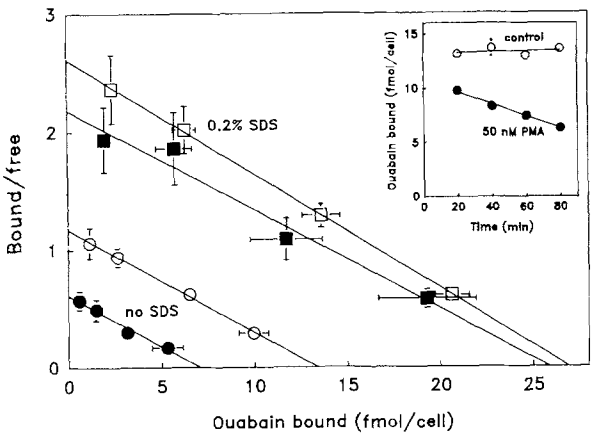

Fig. 10. Ouabain binding to permeabilized oocytes pretreated with PMA. Intact oocytes were treated with 0.1% ethanol (open symbols) or 50 nM PMA (filled symbols) in K^+-free ORI. After 50 min at 21°C, the cells were permeabilized with digitonin and incubated for 4 hr at 25°C with 1–40 nM ^{3}H-ouabain in NaCl-Tris in the absence (circles) and presence (squares) of 0.02% SDS. *Inset:* Time dependence of the effect of PMA pretreatment on ouabain binding to digitonin-permeabilized cells. Intact oocytes were treated with 0.1% ethanol (open circles) or 50 nM PMA (filled circles). At the indicated times, cells were permeabilized with digitonin and labeled for 3 hr at 25°C with 50 nM ^{3}H-ouabain in NaCl-Tris. Each data point is the mean ± SD of 8–10 separate determinations in individual cells of a representative experiment

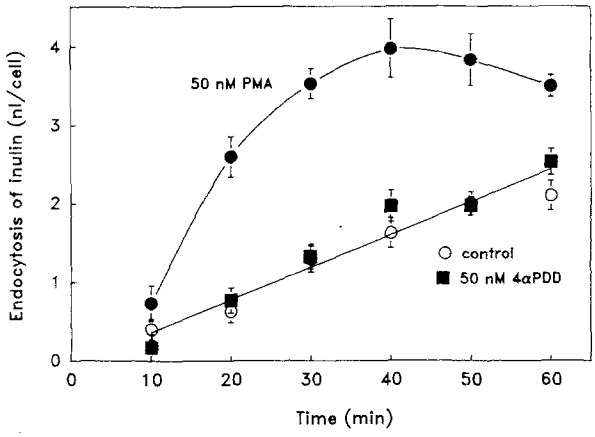

Fig. 11. Effect of phorbol esters on inulin uptake in oocytes. Intact oocytes were incubated with ^{3}H-inulin and 50 nM PMA (filled circles) or 50 nM 4αPDD (filled squares) for the indicated times. Controls (open circles) were run in parallel. Each data point is the mean ± SEM of 9–11 separate determinations in individual cells of a representative experiment

binding in nonpermeabilized cells (*cf.* Fig. 9). According to the Scatchard graphs presented in Fig. 10, PMA treatment before permeabilization decreases B_{max} from 13 to 7 fmol/cell, but does not influence the apparent affinity of the pump for ouabain (K_D = 11 ± 1 nM).

Permeabilization of inner membranes with SDS increases B_{max} from 13 to 27 fmol/cell in control oocytes and from 7 to 26 fmol/cell in PMA-pretreated oocytes. Since the B_{max} values determined in the presence of SDS are not significantly different in both cell types, PMA-treated oocytes apparently possess the same total number of functional Na^+/K^+ pumps as before exposure to PMA. This suggests that the PMA-induced loss of ouabain binding sites on intact oocytes results from a redistribution of surface pumps to the SDS-sensitive compartment such that now a larger fraction of the total pump molecules resides in the cell interior.

Effect of Phorbol Esters on Endocytotic Uptake of Inulin

The parallel reduction of total conductance, pump current, membrane capacitance, and surface pump molecules suggests that the PMA effect is brought about by a reduction in surface membrane. Since the surface of prophase-arrested full-grown oocytes is highly folded (*see* Dumont, 1972), a reduction in membrane area could result from endocytotic removal of microvilli. To examine whether PMA stimulates endocytosis, the uptake of the fluid phase marker ^{3}H-inulin was monitored. Results are presented in Fig. 11. Untreated control oocytes show constant endocytotic activity of 2–3 nl/hr (note that the internal water space is about 500 nl). Addition of 50 nM PMA, but not of 4αPDD, results in a strong temporary increase in endocytosis to up to more than 10 nl/hr. After 40 min, endocytotic activity ceases in PMA-treated oocytes, but continues at a constant rate in the presence of 4αPDD.

Demonstration of Endocytosis by Electron Microscopy

Endocytotic removal of the microvillous structure can directly be demonstrated by electron microscopy. Figures 12 and 13 show cross sections through the cortical regions of control and PMA-treated oocytes. The surface of control oocytes is enlarged on both the animal (Fig. 12*A*) and vegetal (Fig. 12*B*) hemispheres by numerous and partially branched microvilli extending into the perivitelline space (*PV*). Sometimes single endocytotic vesicles can be seen to originate between the microvilli (*see* arrows in Fig. 12*A* and *B*).

The micrographs depicted in Fig. 12*C* and *D* are samples which were incubated with peroxidase and treated with tannic acid to stain the membrane coat for better visualization of the plasma membrane.

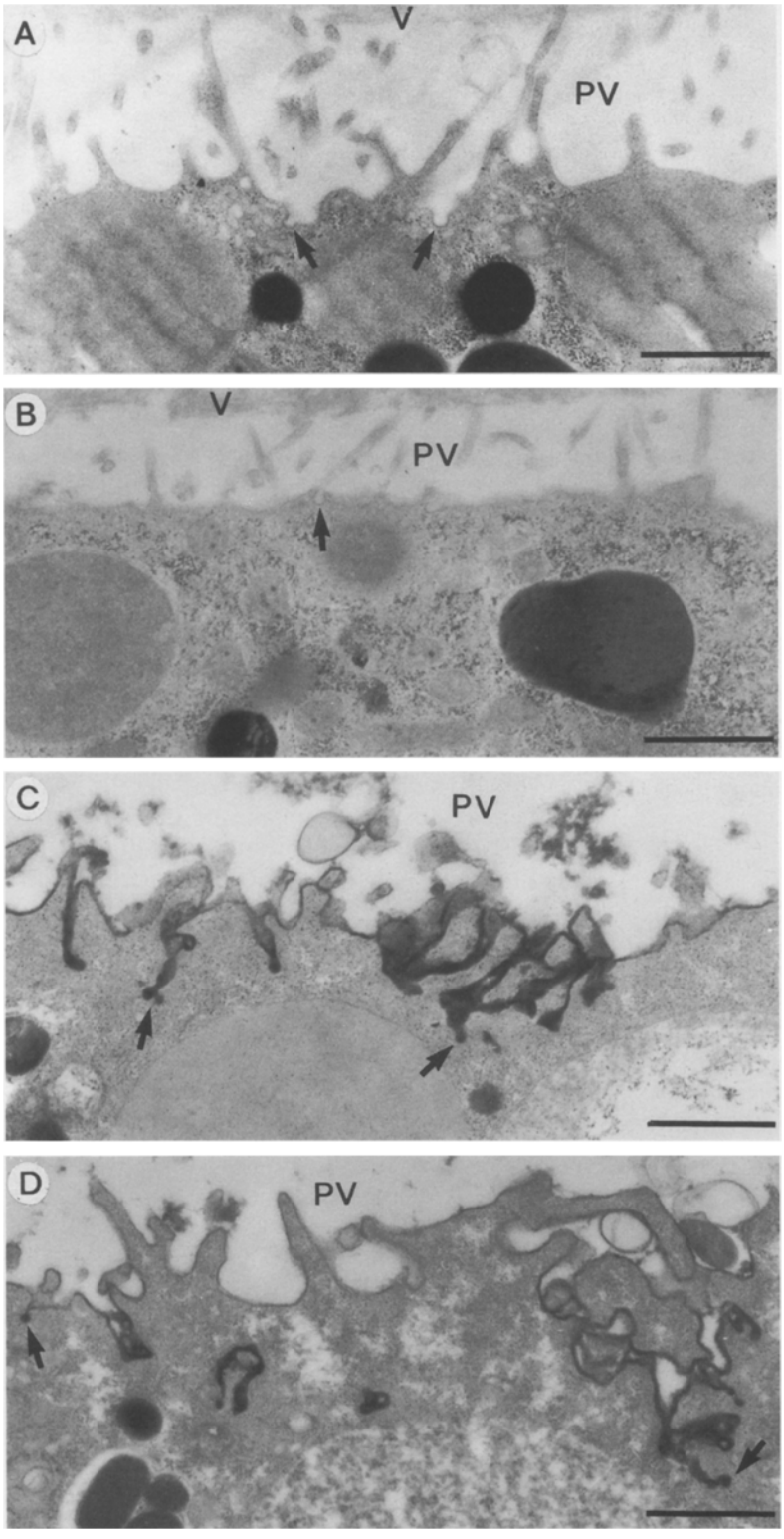

Fig. 12. Electron micrographs of cortical regions of PMA-treated oocytes. Micrographs are from a control oocyte embedded in LR White medium (*A* and *B*) and from oocytes incubated for 20 min with 50 nM PMA in the presence of horseradish peroxidase (*C* and *D*). Peroxidase-labeled oocytes were mordanted with tannic acid and embedded in Spurr's resin. *A* and *C*, animal poles; *B* and *D*, vegetal poles; *PV*, perivitelline space; *V*, vitelline layer; arrows, endocytotic processes; bar = 1 μm

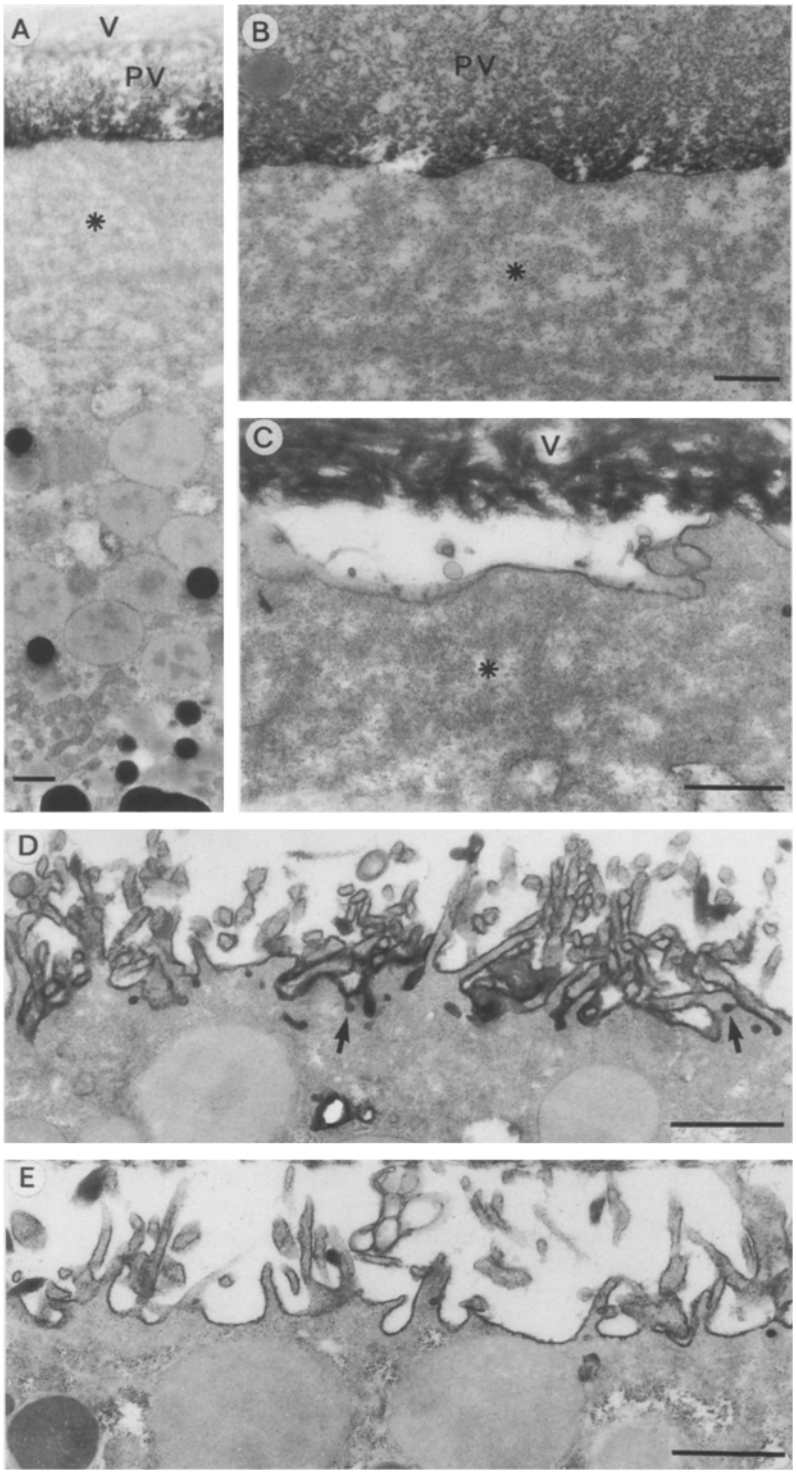

Fig. 13. Electron micrographs of cortical regions of PMA-treated oocytes. Micrographs are from oocytes incubated for 40 min with 50 nM PMA (*A–C*) or 50 nM 4αPDD (*D* and *E*) in the presence of horseradish peroxidase. Peroxidase-labeled oocytes were mordanted with tannic acid and embedded in Spurr's resin. *A*, *B*, and *D*, animal poles; *C* and *E*, vegetal poles; *PV*, perivitelline space; *V*, vitelline layer; arrows, endocytotic processes; asterisks, granular cytoplasm; bar = 1 μm

Already after 20 min of treatment with 50 nM PMA, the morphology of the oocyte surface has drastically changed. Microvilli are reduced in length and number, and appear to be more irregularly orientated. Numerous endocytotic vesicles originate from deep membrane invaginations (arrows) on both the animal (Fig. 12*C*) and the vegetal (Fig. 12*D*) poles.

Prolonged exposure to PMA results in an almost complete smoothening of the plasma membrane (Fig. 13*A–C*). After 40 min of PMA treatment, oo-

cytes have lost their microvilli. The cytoplasmic organelles are located deeper in the cell interior and a layer of granular material (asterisk) is now present just underneath the plasma membrane (Fig. 13*A*). In addition, a flocculent material appears in the perivitelline space (*PV*, Fig. 13*B*).

Electron micrographs of oocytes treated for 40 min with 4αPDD as a control are shown in Fig. 13*D* and *E*. The oocyte surface appears unaltered and resembles that of untreated cells (Fig. 12*A* and *B*). Organelles are located immediately underneath the plasma membrane and some basic endocytosis of plasma membrane material can be observed (arrows). The same observation of a smoothed membrane surface by treatment with PMA but not with 4αPDD has been found in all cross sections from oocytes of four animals.

Discussion

PMA in concentrations known to stimulate protein kinase C in a variety of cells (Castagna et al., 1982; Nishizuka, 1986) reduces in full-grown *Xenopus* oocytes current generated by the Na^+/K^+ pump (Figs. 4 and 5) and conductances sensitive to Cl^- or K^+ (Fig. 6), membrane capacitance (Fig. 7), and ouabain binding capacity (Figs. 8 and 9). These changes follow a similar time course, and a steady state is reached after about 40 to 60 min. Modulations in membrane currents are accompanied by an endocytotic reduction of the surface of the plasma membrane, as demonstrated by decrease of capacitance (Fig. 7), increase of inulin uptake (Fig. 11), and electron microscopy (Figs. 12 and 13). As discussed below, these findings suggest that the alterations in the membrane characteristics are primarily due to an endocytotic reduction in membrane surface. Particularly, pump current and ouabain binding capacity are reduced by the internalization of the pump molecules. Most likely, the changes are mediated by stimulation of protein kinase C, since PMA, but not 4αPDD, can induce the alterations.

Activation of Protein Kinase C by Phorbol Ester Stimulates Endocytosis

Stimulation of endocytosis by PMA has been demonstrated in macrophages (Swanson, Yirinec & Silverstein, 1985). On the other hand, in a variety of other cell types, activation of protein kinase C by phorbol ester has been demonstrated to stimulate exocytosis (*see* Knight, Sugden & Baker, 1988). Apparently, activation of protein kinase C may have dual effects, depending on the cellular system involved.

There are several lines of evidence that activation of protein kinase C stimulates endocytosis in *Xenopus* oocytes. Most direct evidence comes from the enhanced uptake of the fluid phase marker ^{3}H-inulin (Fig. 11). The ceasing of inulin uptake after 40 to 60 min is consistent with the electron micrographs showing that within the time the membrane lost its microvilli and a nearly smooth surface is assumed (*see* Figs. 12 and 13). The reduction in capacitance from about 180 nF to about 50 nF (Fig. 7*B*) is also in line with these observations. A typical biological cell membrane has a specific capacity of about 1 $\mu F/cm^2$ (*see*, e.g., Adrian & Almers, 1974). This value is indeed obtained after PMA treatment when the oocyte resembles a smooth ball assuming an average diameter of 1.2 mm. The decrease in capacitance by PMA treatment suggests that the original full-grown oocytes have a membrane surface that is more than three times larger due to microvilli.

Reduction of Pump Current Results from Endocytosis

Due to PMA-stimulated endocytosis, surface membrane together with transport proteins are transferred to the cell interior. This is apparent particularly for the Na^+/K^+-ATPase (Fig. 10) but can probably also be anticipated for the other transport systems that are downregulated during activation of protein kinase C. For instance, unspecific removal of transport proteins from the surface membrane may be involved in the reduction of Cl^- or K^+ current; Fig. 6 shows that, after pump inhibition, membrane currents become reduced by PMA in solution that in addition inhibits either Cl^- or K^+ currents.

Comparison of Figs. 7*B* and 9 indicates that capacitance and number of binding sites decrease with a similar time course. This is more directly illustrated in Fig. 14, showing that number of binding sites and capacitance determined after various times of PMA treatment are linearly correlated. Comparison of these data with the pump current, on the other hand, shows that stimulation of protein kinase C may have an additional effect on the ATPase activity. While the membrane surface is reduced by a factor of about 3, the ouabain-sensitive current becomes reduced by a factor of 8. A possible explanation might be that the activity of sodium pumps remaining in the plasma membrane is reduced, for instance by phosphorylation at a modifier site (Ling & Cantley, 1984), changes of intracellular free calcium (Yingst, 1988), or reductions in intracellular Na^+ or ATP. Inhibition of K^+ currents by phorbol ester in *Xenopus* oocytes has been reported previously (Dascal et al., 1985), and one might speculate

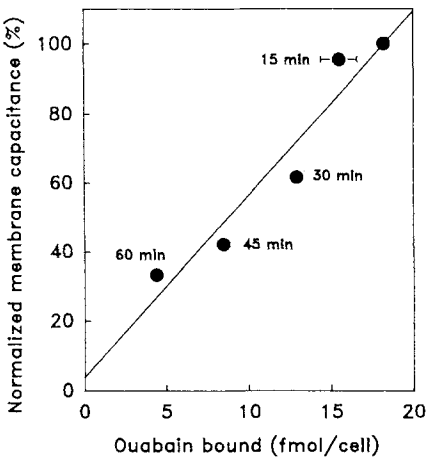

Fig. 14. Correlation between number of ouabain binding sites and membrane capacitance. Data are taken from Figs. 9 and 7. The numbers in the figure give the time of exposure to PMA. Correlation coefficient $r = 0.961$

that this is also, at least partially, due to endocytosis rather than direct phosphorylation of the K^+ channel.

Correlations to Meiotic Maturation

Treatment of *Xenopus* oocytes with PMA has been reported to induce meiotic maturation in the absence of progesterone (Stith & Maller, 1987; Laurent et al., 1988). In fact, in several aspects the PMA-induced changes in the properties of the oocyte plasma membrane observed here resemble those occurring during progesterone-induced maturation. Similarities include a decrease in K^+ permeability with subsequent membrane depolarization (Wallace & Steinhardt, 1977; Kado, Marcher & Ozon, 1981), decrease of chloride permeability, and reduction of the number of active sodium pumps in the plasma membrane (Richter et al., 1984). These alterations might in part also be due to a nonspecific reduction of surface membrane including integral membrane proteins as judged from disappearance of microvilli and decrease in membrane capacitance from 170 to 60 nF (*see* Kado et al., 1981) similar to that observed here (Fig. 7*B*). By quantifying ouabain binding in detergent-permeabilized oocytes as in the present study (*cf.* Fig. 10), we have previously shown that inactivation of sodium pumps during progesterone-induced maturation results also from an internalization of pump molecules (Schmalzing et al., 1990).

Despite such general similarities, however, there are differences in the downregulation of the sodium pump. During progesterone-induced maturation, the capacity of the oocyte plasma membranes to bind ouabain and to transport Na^+ and K^+ is completely abolished (Richter et al.,1984), whereas PMA induces only an incomplete downregulation of surface sodium pumps (*see* Figs. 8–10). This suggests that during meiotic maturation a specific sorting event precedes endocytosis such that all pump molecules can be internalized. This effect of progesterone is not mimicked by PMA. Moreover, sections through paraffin-embedded oocytes show that PMA treatment does not induce dissolution of the nucleus (*results not shown*), arguing against the notion that PMA-treated oocytes are meiotically mature (Stith & Maller, 1987).

In this paper we have demonstrated that application of the phorbol ester PMA leads to downregulation of the Na^+/K^+-ATPase in oocytes of *X. laevis*. A major portion of this downregulation is brought about by endocytotic removal of pump molecules from the plasma membrane. An additional direct effect of stimulation of protein kinase C on the pump molecule cannot be excluded. To further investigate this possibility, inhibitors and other activators of the protein kinase have to be tested.

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