Intracellular pH and intracellular free calcium responses to protein kinase C activators and inhibitors in *Xenopus* eggs

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Summary

Cell activation during fertilization of the egg of *Xenopus laevis* is accompanied by various metabolic changes, including a permanent increase in intracellular pH (pHi) and a transient increase in intracellular free calcium activity ([Ca$^{2+}$]). Recently, it has been proposed that protein kinase C (PKC) is an integral component of the *Xenopus* fertilization pathway (Bement and Capco, *J. Cell Biol.* 108, 885–892, 1989). Indeed, activators of PKC trigger cortical granule exocytosis and cortical contraction, two events of egg activation, without, however, releasing the cell cycle arrest (blocked in second metaphase of meiosis). In the egg of *Xenopus*, exocytosis as well as cell cycle reinitiation are supposed to be triggered by the intracellular Ca$^{2+}$ transient. We report here that PKC activators do not induce the intracellular Ca$^{2+}$ transient, or the activation-associated increase in pHi. These results suggest that the ionic responses to egg activation in *Xenopus* do not appear to depend on the activation of PKC. In addition, in eggs already pretreated with phorbol esters, those artificial activators that act by releasing Ca$^{2+}$ intracellularly, triggered a diminished increase in pHi. Finally, sphingosine and staurosporine, two potent inhibitors of PKC, were found to trigger egg activation, suggesting that a decrease in PKC activity might be an essential event in the release of the metaphase block, in agreement with recent findings on the release of the prophase block in *Xenopus* oocytes (Varnold and Smith, *Development* 109, 597–604, 1990).

Key words: *Xenopus*, protein kinase C, egg activation, intracellular pH, intracellular free calcium.

Introduction

Recently, it has been proposed that protein kinase C (PKC) is an integral component of the *Xenopus* fertilization pathway (Bement and Capco, 1989). Indeed, three activators of PKC, phorbol 12-myristate 13-acetate (PMA), phorbol 12,13-didecanoate (PD) and 1-oleoyl-2-acetyl-sn-glycerol (OAG), were shown to trigger cortical granule exocytosis and cortical contraction in *Xenopus* eggs (Bement and Capco, 1989). Activation or fertilization of eggs of the amphibian *Xenopus laevis* also involves the participation of inositol-1,4,5-trisphosphate [Ins(1,4,5)P$_3$] (Busa et al. 1985) which, like diacylglycerol (DAG), the presumed activator of PKC, is an intermediate produced by the activity of phospholipase C in the phosphoinositide pathway (reviewed by Berridge, 1984).

In other systems, such as cultured mammalian cells and sea urchin eggs, PKC activation following cell activation (stimulation with growth factors or fertilization) results in an increase in intracellular pH (pHi) via phosphorylation of a Na$^+$/H$^+$ exchanger in the plasma membrane (Burns and Rozengurt, 1983; Swann and Whitaker, 1985). In *Xenopus* eggs, fertilization is also accompanied by an increase in pHi (Webb and Nuccitelli, 1981). However, unlike that in cultured mammalian cells and sea urchin eggs, the activation-associated increase in pHi in *Xenopus* eggs is not due to a Na$^+$/H$^+$ exchange system (Webb and Nuccitelli, 1982). This, together with the fact that PKC is involved during *Xenopus* egg activation (Bement and Capco, 1989), prompted us to measure pHi in *Xenopus* eggs in response to activation of PKC by phorbol esters and synthetic diacylglycerols. We report that activators of PKC do not produce the activation-associated increase in pHi in *Xenopus* eggs. In addition, activation by Ca$^{2+}$-dependent activators (pricking or A23187) of eggs pretreated with phorbol esters produced a much smaller increase in pHi than in control non-pretreated eggs.

The exocytosis of cortical granules located in the peripheral cytoplasm of *Xenopus* eggs has long been thought to be Ca$^{2+}$-mediated, because it can be triggered by pricking the egg cortex only in the presence of Ca$^{2+}$ (Wolf, 1974), or by application of the calcium ionophore A23187 (Steinhardt et al. 1974) or by Ca$^{2+}$ ionophoresis in a specific manner (Cross, 1981). Bement and Capco (1989) have recently reported that PKC activators triggered a complete reaction of cortical granule exocytosis in *Xenopus* eggs. Intracellular free
calcium levels ([Ca$^{2+}$]) had not been measured under such circumstances. However, Bement and Capco (1990) recently reported the failure of PKC activators to trigger the intracellular Ca$^{2+}$ transient rise normally triggered upon egg activation (Busa and Nuccitelli, 1985). In agreement with Bement and Capco's findings, we report that treatment of unactivated eggs of Xenopus with PKC activators does not produce any change in [Ca$^{2+}$].

To assess further the role of PKC in Xenopus egg activation, we used inhibitors of PKC, and report that 1 μM staurosporine or 20 μM sphingosine did not block A23187- or prick-induced activation. At the higher dose of 100 μM, a concentration previously reported to inhibit cortical contraction and cortical granule exocytosis in response to A23187 or PMA (Bement and Capco, 1990), sphingosine was surprisingly found to activate a large proportion of the egg populations. Similarly, staurosporine was found to activate the eggs when used at a minimal concentration of 20 μM. These results are incompatible with the idea that the triggering of egg activation in Xenopus is controlled by the activation — or an increase in the activity — of PKC. Our results are compared to those of Bement and Capco (1990), and the role of PKC in Xenopus egg activation is discussed.

Materials and methods

Eggs were obtained from Xenopus laevis, reared in the laboratory, stimulated with 800 to 1000 i.u. human chorionic gonadotropin (Organon). Eggs were dejellied with 2% cysteine in F1 solution, modified from Hollinger and Corton (1980), which contained (mM): NaCl, 31.2; KCl, 1.8; CaCl$_2$, 1.0; MgCl$_2$, 0.1; NaHCO$_3$, 2.0; NaOH, 1.9, buffered with 10 mM Heps, pH7.4. Morphological criteria for successful egg activation, detectable under a stereomicroscope, were: (1) the elevation of the vitelline envelope, a consequence of cortical granule exocytosis; (2) the cortical contraction, a transient uniform contraction of the pigmented area (the animal hemisphere) of the egg towards the animal pole; (3) the disappearance of the maturation spot, a consequence of meiosis resumption due to the migration deeper into the cytoplasm of the egg nucleus (evolving from the metaphase II stage of meiosis to the pronucleus stage). Electrical and ionic criteria for successful egg activation, detectable in eggs impaled with microelectrodes (see below), were: (1) the activation potential, a rapid Cl$^-$-dependent plasma membrane depolarization; (2) a transient increase in [Ca$^{2+}$]; (3) a permanent increase in pH. Respect to the activation potential, which is the earliest event that can be detected at egg activation, 2 to 5 s after pricking the egg cortex or stimulation with the calcium ionophore A23187 (two classical artificial activators of Xenopus eggs), the subsequent events of egg activation could be observed starting at 1-2 min (elevation of the vitelline envelope), 1-5 min (intracellular Ca$^{2+}$ transient), 3-4 min (cortical contraction), 7-10 min (increase in pH), 20-25 min (disappearance of the maturation spot).

Each egg, bathed in F1 solution, was impaled with a H$^+$-sensitive microelectrode, measuring intracellular pH (pHi) (fabricated as described in Charbonneau et al., 1983) or a calcium-sensitive microelectrode, measuring intracellular free calcium (pCa, the negative logarithm of free Ca$^{2+}$ activity) (fabricated as described in Busa, 1986) and with a potential microelectrode measuring the membrane potential. Membrane potential value was subtracted from the pH or calcium microelectrode output at the pen recorder (Linseis) input (Charbonneau and Grandin, 1983). A complete description of the methods used during electrophysiological recording has been published elsewhere (Grandin and Charbonneau, 1989). Both neutral carriers used for selective intracellular microelectrodes were from Fluka: hydrogen ion ionophore 1 (ref. 95291) and calcium ionophore ETH 1001 (ref. 24048). Ca$^{2+}$-sensitive microelectrode and pH microelectrode calibration traces are shown in Fig. 1.

The two phorbol esters, PMA (phorbol 12-myristate 13-acetate) and PD (phorbol 12,13-didecanoate) and the inactive PMA analogue PDD (4-alpha-phorbol 12,13-didecanoate) were prepared as stock solutions of 1 mM in dimethylsulphoxide (DMSO). The synthetic diacylglycerol SAG (1-stearoyl-2-arachidonyl-sn-glycerol), another synthetic diacylglycerol, was prepared as a stock solution of 5 mg ml$^{-1}$ in ethanol. OAG (1-oleoyl-2-acetyl-sn-glycerol), another synthetic diacylglycerol, was prepared as a stock solution of 100 mM in DMSO. The protein kinase C inhibitors, staurosporine and sphingosine, were respectively prepared as stock solutions of 1 and 100 mM in DMSO. All chemicals were purchased from Sigma.

Results

Activation of protein kinase C is not accompanied by any variation in [Ca$^{2+}$].

We examined the effects of an activator of PKC, the phorbol ester phorbol 12-myristate 13-acetate (PMA), on [Ca$^{2+}$], and membrane potential of Xenopus laevis eggs. Fig. 2A shows that PMA, although producing two normal events of egg activation, cortical granule exocytosis and cortical contraction (Bement and Capco, 1989), did not induce any change in [Ca$^{2+}$]. An absence of an effect of PMA on [Ca$^{2+}$] in unactivated eggs of Xenopus was observed in all twenty-four experiments. In PKC-stimulated eggs, the extent of cortical granule exocytosis was the same as in normally activated eggs, although exocytosis proceeded at a slower rate (Bement and Capco, 1989). In the case of a normal activation triggered by agents elevating the [Ca$^{2+}$], (entry of external Ca$^{2+}$ caused by pricking the egg cortex or intracellular release of Ca$^{2+}$ from intracellular stores induced by the calcium ionophore A23187), we measured a transient increase in [Ca$^{2+}$] (Fig. 2B), in agreement with previous findings (Busa and Nuccitelli, 1985).

In addition, PMA did not trigger the activation potential, a Cl$^-$-dependent plasma membrane depolarization, the earliest event that can be detected upon egg activation (Cross and Elinson, 1980). However, PMA was found to have dramatic effects on the membrane potential of unactivated eggs: an initial slow depolarization (peak value around $-10$ to $-2$ mV) generated within 10 min, followed by a very large hyperpolarization of $-30$ to $-70$ mV, 30-45 min after treatment (Figs 2A, 3A). In those cases in which the membrane potential attained values as large as $-50$ to $-70$ mV, spontaneous and rapid depolarizations were frequently recorded. These 'spikes' had a peak potential close to
Activation of protein kinase C does not produce the increase in pHi associated with egg activation

In many types of cultured mammalian cells, as well as in sea urchin eggs, a phosphorylating activity of PKC seems to be responsible for the activation of a Na\(^+\)/H\(^+\) exchange system (Burns and Rozengurt, 1983; Swann and Whitaker, 1985), which produces the cell activation-associated increase in pHi (Roos and Boron, 1981; Busa and Nuccitelli, 1984). Although Xenopus eggs do not seem to possess a Na\(^+\)/H\(^+\) antiporter (Webb and Nuccitelli, 1982), it was interesting to know whether PKC might be implicated in their pHi regulation system. Measurements with intracellular pH microelectrodes demonstrated that the PKC activator PMA did not induce the normal activation-associated increase in pHi (data not shown). Indeed, intracellular pH did not change for at least 90 min following PMA treatment (3 \(\mu\)M), as measured in twelve experiments, although in five other experiments PMA produced a slow decrease in pHi (0.10–0.15 pH unit) (Fig. 3A). Phorbol 12,13-didecanoate (PD), 3 \(\mu\)M, had no effect on pHi levels in all five experiments performed.

Fig. 1. Calibration traces of Ca\(^{2+}\) (A) and pH (B) microelectrodes. We chose to show the calibration traces corresponding to the microelectrodes used in the experiments described in Fig. 2A and 3A. (A) Ca\(^{2+}\)-selective microelectrodes were calibrated in pCa 6.0 and pCa 7.0 solutions, as described in Busa and Nuccitelli (1985), which contained, respectively, 10.0 mM EGTA, 5.0 mM CaCl\(_2\), 10.0 mM Pipes, 45.0 mM KOH, 15.0 mM KCl, pH 6.77 (at 23°C), and 10.0 mM EGTA, 5.0 mM CaCl\(_2\), 10.0 mM Mops, 35.5 mM KOH, 29.3 mM KCl, pH 7.27 (at 23°C). At the beginning of the experiment, this microelectrode had a slope of 33 mV between pCa 6.0 and pCa 7.0. Just after calibration, the solution was replaced by the physiological Fl solution, which is responsible for the large upward deflection in the trace (‘Fl’). This is because the Fl solution contains much more free Ca\(^{2+}\) than the pCa 6.0 solution. Eggs were then immersed in Fl solution, in the recording chamber, and impaled, each with a Ca\(^{2+}\)-selective microelectrode and a potential microelectrode (arrow ‘in’). The beginning and end of the recording of [Ca\(^{2+}\)] in this egg (72 min recording not shown) correspond to the egg recording shown in Fig. 2A. At the end of this experiment, the voltage followers were turned off, the microelectrodes removed from the eggs (arrow ‘Out’), and the eggs and Fl solution removed and replaced by pCa 7.0 calibration solution. pH microelectrodes were calibrated, as described in Charbonneau et al. (1985), in buffers of pH 7.78, 7.27 and 6.77 containing, respectively, 10.0 mM Hepes, 10.0 mM Mops or 10.0 mM Pipes, plus 10.0 mM EGTA, 5.0 mM CaCl\(_2\) and 75.0 mM KCl. However, we noticed that, in some cases, pH microelectrodes displayed signs of instability or difficulty in rapidly attaining a stable level when further immersed in Fl solutions. Therefore, we chose to calibrate our pH microelectrodes directly in Fl solutions at pH 6.50, 7.50 and 8.50 (or about these values: the exact values were measured with a Knick 654 pH meter immediately beforehand) buffered, respectively, with 10.0 mM Pipes, 10.0 mM Hepes and 10.0 mM 3-[dimethyl(hydroxymethyl)-methylamino]-2-hydroxypropane sulfonic acid (Ampso). Just after calibration, the eggs were immersed in Fl solution at pH 8.50. Achievement of egg impalement was indicated by the large deflection of the pHi trace as the microelectrode entered the egg cytoplasm (arrow ‘in’). Three minutes after impalement, the Fl solution at pH 8.50 around the eggs was replaced by Fl solution at pH 7.50 (arrowhead), which is marked on the trace by the slight acidification of the egg cytoplasm. The beginning and the end of recording of pHi level in this egg (114 min recording not shown) correspond to the trace shown in Fig. 3A. At the end of the experiment, the voltage followers were turned off, the microelectrodes pulled out of the eggs (arrow ‘Out’), and the Fl solution in the recording chamber was replaced by a fresh solution that had been adjusted at pH 7.50 immediately beforehand.

Pretreatments with phorbol esters diminish the pHi and [Ca\(^{2+}\)] responses to egg activation

As previously reported, phorbol esters do not lead to
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pus eggs, and are therefore partial activators (Bement and Capco, 1989). In particular, PKC activators are not capable of triggering the [Ca\(^{2+}\)]\(_i\) and pH\(_i\) increases, or the activation potential, as seen above. Could these ionic events be triggered by mobilization of intracellular Ca\(^{2+}\) stores following partial activation with PKC activators? Eggs pretreated with 3 \(\mu\)M PMA or 3 \(\mu\)M PD displayed an increase in pH\(_i\) when further challenged with Ca\(^{2+}\) -dependent activating signals (Fig. 3A). However, the activation-associated increase in pH\(_i\) in eggs pretreated with 3 \(\mu\)M PMA for at least 30 min and stimulated with 0.5 or 1 \(\mu\)M A23187 was much smaller, 0.09±0.04 pH unit (s.d., \(n=14\)), than in control eggs (Table 1). The kinetics of the A23187-induced increase in pH\(_i\) in eggs pretreated with phorbol esters suggested that these changes were physiological. In order to rule out the possibility of an artefactual change in pH\(_i\) in phorbol ester-pretreated eggs, due to the Ca\(^{2+}\)/H\(^{+}\) exchange properties of A23187, Xenopus eggs were impaled with pH microelectrodes, activated by prick-

ing, and, once pH\(_i\) had attained a stable elevated value (around 40 min after egg activation), treated with 2 \(\mu\)M A23187. Under such conditions, A23187 produced, around 10 min after addition, only a slight change in pH\(_i\), a 0.02 to 0.04 pH unit cytoplasmic acidification, as measured in seven eggs.

In addition, an intracellular Ca\(^{2+}\) transient was recorded in only three out of fourteen experiments in which eggs were pretreated with 3 \(\mu\)M PMA for at least 30 min and stimulated with 0.5 or 1 \(\mu\)M A23187. In contrast, an intracellular Ca\(^{2+}\) transient was recorded in all nine control experiments (no pretreatment). In contrast to the pH\(_i\) and intracellular Ca\(^{2+}\) responses to egg activation, which were modified after pretreatment with phorbol esters, as seen above, the activation potential remained similar to that in controls. Indeed, the peak value of the activation potential after stimulation with 0.5 or 1 \(\mu\)M A23187 was found to be around +5 mV in control non-pretreated and PDD-

treated eggs, as well as in PMA- and PD-treated eggs (Table 1). Since the only known mode of action of artificial activators of Xenopus eggs, such as A23187, is to release Ca\(^{2+}\) intracellularly, it seemed reasonable to assume that A23187 did produce an intracellular Ca\(^{2+}\) transient in PMA-pretreated eggs. However, in such eggs, that Ca\(^{2+}\) transient would not have been detected by the Ca\(^{2+}\) microelectrodes, possibly due to the fact that it was smaller than that in non-pretreated eggs (see Discussion). In order to evaluate such a possibility, PMA-pretreated eggs (3 \(\mu\)M, 20–30 min) were microinjected with 100 mM BAPTA, a potent chelator of Ca\(^{2+}\) ions, and further challenged with 1 \(\mu\)M A23187. Under these conditions, the eggs did not activate, that is did not display the activation potential, as observed in all eight experiments performed (Fig. 4). Although the eggs reasonably supported the microinjection of BAPTA, the subsequent addition of A23187 rapidly produced their complete lysis. The period of time between A23187 addition and egg lysis, sufficient to assess the absence of an activation potential in PMA-

pretreated eggs microinjected with BAPTA, was not long enough to record pH\(_i\) until the moment corre-

sponding to the physiological changes (7–10 min after

Fig. 2. (A) Effect of the phorbol ester PMA (phorbol 12-

myristate 13-acetate) on the membrane potential (E\(_m\)) and intracellular free calcium activity (pCa) of an unactivated Xenopus egg. The egg, bathed in F1 solution, was impaled with a potential microelectrode, measuring membrane potential (top trace) and a calcium-selective microelectrode, measuring the activity of intracellular free calcium (bottom trace). Addition of 3 \(\mu\)M PMA (arrow 'PMA') induced, within a few minutes, a small depolarization, followed by a dramatic hyperpolarization from -12 to -73 mV with, at intervals, appearance of dramatic transient depolarizations. However, PMA did not induce any changes in the intracellular free calcium level, which remained at pCa 6.42 (0.38 \(\mu\)M). The egg was rinsed with F1 solution (arrow 'F1') 30 min after PMA addition to avoid possible lysis. All measurements performed in the study on the effects of phorbol esters indicate, in unactivated eggs, a basal [Ca\(^{2+}\)] of 0.37±0.11 \(\mu\)M (s.d., \(n=37\)) and a membrane potential, measured in eggs impaled with two microelectrodes (a potential microelectrode and a calcium or pH microelectrode), of -11.0±2.2 mV (s.d., \(n=93\)). (B) Representative changes in membrane potential (top trace) and intracellular free calcium (bottom trace) following activation in an egg of Xenopus. The egg, impaled with a potential microelectrode and a calcium-selective microelectrode, was bathed in F1 solution containing 3 \(\mu\)M of the inactive PMA analogue, PDD (4-alpha-phorbol 12,13-didecanoate). The egg was activated by pricking (arrow 'Pricking'), 45 min after PDD addition. A transient increase in intracellular free calcium concentration was detected 5 min after egg activation, indicated by the occurrence of the activation potential (arrow 'AP\(_P\)')
Fig. 3. (A) Changes in intracellular pH in an egg of *Xenopus* following treatment with phorbol 12-myristate 13-acetate (PMA) and activation with the calcium ionophore A23187. The egg, bathed in F1 solution, was impaled 20 min before the beginning of the trace shown here with a potential microelectrode, measuring the membrane potential (Em; top trace), and a pH-selective microelectrode, measuring the intracellular pH (pHi; bottom trace). Addition of 3 μM PMA (arrow 'PMA') induced a slow cytoplasmic acidification, from pH 7.47 to pH 7.38, and a large hyperpolarization of the membrane. The egg was activated with 1 μM A23187 (arrow 'A23187'), 35 min after PMA addition, which generated a large activation potential (arrow 'AP'), from the potential level attained after PMA treatment (-40 mV) to the equilibrium potential for chloride ions (+2 mV). Activation also triggered a transient acidification followed by a small alkalization, from pH 7.38 to pH 7.53 (see mean values in Table 1). The egg was rinsed with F1 solution (arrow 'F1') a few minutes after addition of A23187. Rinsing is probably responsible for the re-depolarization following the rapid hyperpolarization which occurred soon after the plateau value of the AP had been attained. Indeed, that large hyperpolarization, abnormal for an activation potential, was probably due to the presence of PMA in the recording chamber. Removal of PMA upon rinsing brought the membrane potential back to a less negative value, typical of a normal AP. In the whole study on the effects of phorbol esters, pHi in unactivated eggs, before treatment, was 7.48±0.07 (S.D., n=35).

(B) Normal changes in intracellular pH following activation of a *Xenopus* egg. The egg, impaled with a potential microelectrode and a pH-selective microelectrode, was incubated for 30 min in F1 solution containing 3 nM of the inactive PMA analogue, PDD (4-alpha-phorbol 12,13-didecanoate). Activation, induced by 1 μM A23187 (arrow 'A23187'), triggered a typical activation potential from -12 to +3 mV (arrow 'AP'; top trace) and, following a small transient acidification, a large alkalization of the egg cytoplasm, from pH 7.45 to pH 7.77 (see mean values in Table 1).

Absence of effects of synthetic diacylglycerols on pHi and [Ca2+]i in *Xenopus* eggs

To extend our findings concerning the relationships between PKC activation and [Ca2+]i, and pHi changes, we also used two synthetic diacylglycerols: 1-oleoyl-2-acetyl-sn-glycerol (OAG) and 1-stearoyl-2-arachidonoyl-sn-glycerol (SAG). Both OAG and SAG have previously been shown to activate PKC (Bell, 1986). In addition, OAG stimulates cortical granule exocytosis in *Xenopus* eggs (Bement and Capco, 1989). Neither OAG, at concentrations up to 100 μM, nor SAG, at concentrations as high as 77 μM (50 μg ml⁻¹), produced any change in pHi (six experiments with OAG, five with SAG) or [Ca2+]i (eight experiments with OAG, thirteen with SAG) in unactivated eggs of *Xenopus*. In addition, eggs pretreated with 100 μM OAG or 77 μM SAG, for at least 45 min, remained perfectly activatable by A23187, and generated an intracellular Ca2+ transient (five experiments with OAG, eight with SAG) and an increase in pHi (eight experiments with OAG, four with SAG) identical to those in non-pretreated eggs. Thus, synthetic diacylglycerols seem to be less potent than PKC activators in affecting the pHi and intracellular Ca2+ responses to Ca2+-mobilizing agents.

Effects of PKC inhibitors on *Xenopus* egg activation

We used staurosporine and sphingosine which, although not entirely specific, are potent inhibitors of PKC (Davis *et al.* 1989; Huang, 1989). Treatment of unactivated eggs for at least 2 h with 1 μM staurosporine or 20 μM sphingosine had no effect on egg activation.
Indeed, in response to either pricking or A23187 (0.5 \( \mu \)M), events characteristic of egg activation, such as the activation potential, cortical contraction, elevation of the vitelline envelope, pH increase and meiosis resumption were triggered in eggs pretreated with either of the PKC inhibitors used.

Bement and Capco (1990) recently reported an inhibition of cortical contraction and cortical granule exocytosis, in response to A23187 or PMA, in eggs pretreated with 100 \( \mu \)M sphingosine. Our results are somewhat different. Indeed, we noted that 100 \( \mu \)M sphingosine alone activated the majority of the eggs (75-90 %) in a mixture of eggs from two or three females. In some females (taken individually), 100 \( \mu \)M sphingosine was even found to activate 100 % of the eggs. The activating effect of 100 \( \mu \)M sphingosine was observed in all four experiments performed, using eggs from nine females (at least thirty eggs for each experiment). Activation by 100 \( \mu \)M sphingosine was evident morphologically, under a stereomicroscope: elevation of the vitelline envelope, cortical contraction and disappearance of the maturation spot. Morphologically, the first signs of sphingosine-induced egg activation were evident as early as 5 min after treatment. The majority of the egg population was activated around 15 min after treatment: elevation of the vitelline envelope and disappearance of the maturation spot. Morphologically, sphingosine-induced egg activation was evident in about 50 % of the egg population around 15 min after treatment: elevation of the vitelline envelope and disappearance of the maturation spot. The maximal percentage of egg activation (80-100 %) induced by 20 \( \mu \)M staurosporine was achieved around

\[ \begin{array}{|c|c|c|}
\hline
\text{Treatment} & \text{Activation potential peak value}^a (\text{mV}) & \text{pHi increase}^b (\text{pH unit}) \\
\hline
\text{PMA}^b & +4.3±2.3 & 0.09±0.04 \\
(n=27) & & \\
\text{PD}^b & +5.2±1.3 & 0.22±0.09 \\
(n=5) & & \\
\text{PDD}^b & +4.0±1.7 & 0.31±0.03 \\
(n=14) & & \\
\text{non-pretreated eggs} & +4.9±1.7 & 0.31±0.04 \\
(n=25) & & \\
\hline
\end{array} \]

\(^a\) Results are expressed as mean values±Standard Deviations (number of experiments).

\(^b\) The two phorbol esters, PMA (phorbol 12-myristate 13-acetate) and PD (phorbol 12,13-didecanoate), and the inactive PMA analogue PDD (4 alpha-phorbol 12,13-didecanoate) were used at a final concentration of 3 \( \mu \)M. Treatment of unactivated dejellied eggs of \textit{Xenopus} was for 30 to 45 min. Then, the eggs were stimulated with the calcium ionophore A23187 (0.5 or 1 \( \mu \)M). Equilibrium potential attained at the plateau of the egg activation-induced activation potential (Cl– dependent plasma membrane depolarization).

\(^c\) Amplitude of the egg activation-induced pH increase measured at the plateau level (30 to 40 min after egg activation).

\textit{Xenopus} eggs was shared with other inhibitors of PKC, we studied the effects of staurosporine at concentrations higher than 1 \( \mu \)M, an ineffective concentration, as seen above. Interestingly, staurosporine also triggered activation of \textit{Xenopus} eggs when its concentration in the surrounding medium was at least 20 \( \mu \)M. Morphologically, staurosporine-induced egg activation became evident in about 50 % of the egg population around 15 min after treatment: elevation of the vitelline envelope and disappearance of the maturation spot. The maximal percentage of egg activation (80-100 %) induced by 20 \( \mu \)M staurosporine was achieved around

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\hline
\end{array} \]
The present study demonstrates that activation of protein kinase C (PKC) by phorbol esters does not produce the physiological changes in [Ca$^{2+}$], and pH$i$ associated with activation of *Xenopus* eggs. This implies that cortical granule exocytosis, which can be triggered by phorbol esters in *Xenopus* eggs (Bement and Capco, 1989), can occur without noticeable variation in [Ca$^{2+}$]. Moreover, the absence of [Ca$^{2+}$], and pH$i$ variations during phorbol-ester-induced activation correlates well with the observations that phorbol esters cannot trigger meiosis resumption in *Xenopus* eggs (Bement and Capco, 1989) and that protein kinase C appears to act downstream of the egg-activation-associated [Ca$^{2+}$], rise to trigger cortical granule exocytosis (Bement and Capco, 1990). In contrast, our results with sphingosine and staurosporine, two inhibitors of PKC, suggest that the release of the metaphase block might be correlated with a decrease in PKC activity.

**Absence of an effect of PKC activators on pH$i$ in *Xenopus* eggs can be correlated with an absence of Na$^+/H^+$ exchange system**

On first analysis, the absence of an effect of phorbol esters on the intracellular pH of unactivated eggs of *Xenopus* can be explained by the fact that these eggs lack a Na$^+/H^+$ exchange system (Webb and Nuccitelli, 1982; Grandin and Charbonneau, 1989b). Indeed, in other systems, phorbol esters or synthetic diacylglycerols activate a Na$^+/H^+$ exchanger with a resultant pH$i$ rise, for example in sea urchin eggs (Swann and Whitaker, 1985; Lau et al. 1986; Shen and Burgart, 1986) and cultured mammalian cells (Burns and Rozengurt, 1983; Besterman and Cuatrecasas, 1984; Moo lenar et al. 1984). Since phorbol esters and synthetic diacylglycerols presumably activate PKC specifically (Nishizuka, 1984, 1986, 1988), it appears that the Na$^+/H^+$ exchanger is directly turned on by this kinase. The egg of *Xenopus* appears to represent a particular system in which PKC, although involved in the activation pathway as recently demonstrated (Bement and Capco, 1989), does not seem to be responsible for the activation-associated pH$i$ rise, as shown in the present study.

**Phorbol-ester-induced cortical granule exocytosis occurs in the absence of [Ca$^{2+}$], variations**

Our results demonstrate that the phorbol ester PMA, previously shown to trigger a complete exocytosis of the cortical granules in *Xenopus* eggs (Bement and Capco, 1989), does not induce any variation of [Ca$^{2+}$], thus confirming a recent report (Bement and Capco, 1990). This has already been described in human neutrophils, in which PMA triggers exocytosis of secondary granules even when [Ca$^{2+}$], is lowered 10–20 times below the normal resting level (Di Virgilio et al. 1984). This was later confirmed in several systems (see references in Knight et al. 1989) and interpreted as resulting from a phorbol-ester-induced increase in the sensitivity of the granules to [Ca$^{2+}$], (Knight et al. 1989). In other words, the Ca$^{2+}$-independent exocytosis observed in several systems appears to be due to an increased sensitivity of the secretory process to intracellular Ca$^{2+}$ rather than to a Ca$^{2+}$-independent pathway (Knight et al. 1989). The observations by Bement and Capco (1989) that

**Discussion**

The present study demonstrates that activation of protein kinase C (PKC) by phorbol esters does not produce the physiological changes in [Ca$^{2+}$], and pH$i$.
subthreshold concentrations of PMA and A23187 act synergistically to trigger exocytosis in *Xenopus* eggs, together with the present finding and that by Bement and Capco (1990) that PMA triggers exocytosis without [Ca$^{2+}$], variation, suggest that cortical granule exocytosis in *Xenopus* eggs represents one of these so-called types of Ca$^{2+}$-independent exocytosis, which is, in fact, a Ca$^{2+}$-dependent exocytosis in which activation of PKC increases the sensitivity of the secretory granules to [Ca$^{2+}$].

A role for protein kinase C in *Xenopus* egg activation

So far, the only evidence for a precise role of PKC in *Xenopus* egg activation is that provided by the recent work of Bement and Capco (1989), who have demonstrated that the two phorbol esters PMA and PD and the synthetic diacylglycerol OAG trigger cortical granule exocytosis and cortical contraction. Using the same activators of PKC, at the same concentrations (3 µM for PMA and PD, 100 µM for OAG), we have demonstrated that PKC did not appear to be involved in the physiological [Ca$^{2+}$], and pH$\upi$ changes accompanying activation of *Xenopus* eggs. In addition, we have demonstrated that two potent inhibitors of PKC, staurosporine and sphingosine, at 1 and 20 µM, respectively, did not interfere with any event of egg activation. Yet, phorbol esters, principally PMA, seem to have dramatic effects on the membrane potential of *Xenopus* eggs, but not of the kind that are triggered during normal egg activation. These changes in membrane potential might be related to the previously described modulation, in various cell types, of ion conductance by PKC, via phosphorylation of ion channel proteins, ion exchange proteins or pumps (reviewed by Nishizuka, 1986). However, in *Xenopus* eggs, it cannot be excluded that PMA might act on some ion channel conductance independently of its effect as an activator of PKC, as has been recently proposed concerning the depression of Ca$^{2+}$ current in chick sensory neurones by the phorbol esters O-tetradecanoylphorbol-13-acetate and phorbol-12,13-diacetate (Hockberger et al. 1989).

Although activation of PKC and the associated cortical granule exocytosis in *Xenopus* eggs do not appear to be coupled to pH$\upi$ or [Ca$^{2+}$], changes, as seen above, it is interesting to note that pretreatment with one of the two phorbol esters used, PMA, but not with the synthetic diacylglycerols OAG and SAG, prevented the intracellular Ca$^{2+}$ transient and strongly reduced the increase in pH$\upi$ following stimulation with A23187 (see Table 1). The kinetics of the reduced increase in pH$\upi$ in PMA-pretreated eggs stimulated with A23187, identical to those in normally activated eggs, as well as our experiments on the effects of A23187 on pH$\upi$ of already activated eggs, demonstrate that the increase in pH$\upi$ in PMA-pretreated eggs is not artefactual. During normal egg activation, the intracellular Ca$^{2+}$ transient precedes the increase in pH$\upi$ by 5 to 10 min. Presumably, the increase in pH$\upi$ is a consequence of the intracellular Ca$^{2+}$ transient, because the intracellular Ca$^{2+}$ transient is believed to represent some sort of triggering signal during egg activation. How can we explain the occurrence, in PMA-pretreated eggs stimulated with A23187, of an increase in pH$\upi$, even with a reduced amplitude, in the absence of an intracellular Ca$^{2+}$ transient? It might well be possible that, in such eggs, [Ca$^{2+}$], did change following stimulation with A23187, but in an altered way, perhaps because the change did not proceed as a wave as is normally the case (Busa and Nuccitelli, 1985). We believe that [Ca$^{2+}$], changes in *Xenopus* eggs are more reliably detected with the Ca$^{2+}$ microelectrodes when they correspond to a massive propagating front, whereas smaller [Ca$^{2+}$], changes occurring simultaneously in various distinct regions of the cytoplasm might be missed by the Ca$^{2+}$ microelectrode tip. We propose that, in PMA-pretreated eggs, A23187 would produce more discrete [Ca$^{2+}$], changes than those taking place during normal egg activation. These smaller [Ca$^{2+}$], variations, which, in addition, might not be propagating as a wave, might in turn result in a reduced increase in pH$\upi$. The assumption that A23187 did produce [Ca$^{2+}$], variations in PMA-pretreated eggs is confirmed by the observation that microinjection of BAPTA, a Ca$^{2+}$ chelator, into such eggs could prevent the occurrence of the activation potential in response to A23187. This demonstrates that some intracellular Ca$^{2+}$ must be released into the egg cytoplasm, although it cannot be detected with the Ca$^{2+}$ microelectrode for reasons explained above, in order to trigger the activation potential.

In contrast to the results of Bement and Capco (1990), who recently reported a blockade of A23187 or PMA action by 100 µM sphingosine, we observed a direct activation of a very large proportion of eggs by 100 µM sphingosine. Sphingosine competes with the natural diacylglycerol on the regulatory domain of PKC (reviewed by Huang, 1989). Sphingosine, although not entirely specific, is known to be a potent inhibitor of PKC (Davis et al. 1989; Huang, 1989). The situation found here, in which sphingosine (100 µM) activates *Xenopus* eggs, is very similar to that recently described in *Xenopus* oocytes in which sphingosine (170 µM in the incubation experiments) induced oocyte maturation (Varnold and Smith, 1990). The activating effect of an inhibitor of PKC on *Xenopus* eggs is confirmed, in the present study, by the utilization of staurosporine (20 µM), another potent inhibitor of PKC. A straightforward explanation of the present finding, that inhibition of PKC triggers egg activation in *Xenopus*, is that a decrease in PKC activity is required for egg activation, as appears to be the case for oocyte maturation (Varnold and Smith, 1990). Both events, oocyte maturation and egg activation, correspond to the release of a meiotic block, in prophase and metaphase, respectively. However, it should be kept in mind that at these very high concentrations, 20 and 100 µM, respectively, staurosporine and sphingosine might have effects unrelated to PKC. It is therefore premature to conclude that a decrease in PKC activity is associated with — or required for — *Xenopus* egg activation. In addition, our observation that sphingosine and staurosporine, two
inhibitors of PKC, activate the eggs of *Xenopus* might explain our other observation that the pH$_i$ and [Ca$^{2+}$]$_i$ responses to Ca$^{2+}$-dependent activating signals are diminished when eggs are pretreated with activators of PKC (Fig. 3; Table 1). Indeed, if a decrease in PKC activity is required during egg activation, then it is logical that activation of PKC prior to application of activating stimuli might interfere with the ionic responses to egg activation. We cannot explain why our results with 100 µM sphenosine are totally opposite to those of Bement and Capco (1990), who reported an inhibition of A23187-induced egg activation by the same drug at the same concentration. Recent experiments on the effects of various agonists and antagonists of PKC on *Xenopus* oocyte maturation have also led to quite variable, and sometimes opposing, results (see references in Varnold and Smith, 1990). In the case of *Xenopus* egg activation, inhibition of PKC seems to be involved in the resumption of the cell cycle (release of the metaphase block), as shown here, while, on the other hand, activation of PKC appears to be required for cortical granule exocytosis. This is not a unique situation, since in hamster eggs the response to GTP[S], which generates diacylglycerol and activates PKC, is enhanced by the PKC inhibitor sphenosine, suggesting the existence of a feedback inhibition of that response by PKC (Swann et al. 1989). Future experiments on the role of PKC in *Xenopus* egg activation should be directed at examining the kinetics of changes in the activities of the natural diacylglycerol DAG and of PKC, associated with egg activation.

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