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Biochemistry

Activated α subunit of G_o protein induces oocyte maturation

(*Xenopus laevis*/protein kinase C/c-mos translation)

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ABSTRACT **The capability of various activated guanine nucleotide binding regulatory protein (G protein) α subunits to induce meiotic maturation was studied. Activated G_o protein α subunit (α_o^*) but not the three inhibitory G protein α subunits triggered meiotic maturation in *Xenopus* oocytes. The effect was concentration dependent with a half-maximal effect in the 100–200 pM range. Injection of α_o^* stimulated protein kinase C activity. Coinjection of the peptide containing residues 19–36 of protein kinase C [PKC-(19–36)], a specific protein kinase C inhibitor, blocked the α_o^*- but not progesterone-induced maturation. Cycloheximide and the injection of antisense oligonucleotides specific to the c-*mos* transcript blocked α_o-induced maturation. Immunoprecipitation with a mos protein-specific monoclonal antibody showed that α_o-injected oocytes had phosphorylated mos protein. When PKC-(19–36) was coinjected with α_o^*, phosphorylated mos protein was not observed. These observations indicate that α_o^*, through protein kinase C and the translation of c-mos, can trigger meiotic division of *Xenopus* oocytes. Our results raise the possibility that persistently activated G proteins through cellular protooncogenes may regulate cell-cycle resumption.**

Many receptors that regulate growth have their cellular effects through guanine nucleotide binding regulatory proteins (G proteins) (1). G proteins harboring activating mutations have been found in tumors and it has been suggested that the activated G protein α subunit may contribute to neoplastic transformation (2, 3). Yet a direct demonstration between activation of a known G protein and regulation of cell division is missing. We had found that the bovine brain heterotrimeric G protein G_o can function as a signal transducer in the receptor-regulated inositol phospholipid pathway of *Xenopus* oocytes (4). Many growth factors and oncogenes that activate entry into the G_1 phase of the cell cycle alter inositol phospholipid metabolism (5). Hence it appeared feasible that the activated G_o protein could trigger cell division.

The *Xenopus* oocyte is a useful model to study cell-cycle regulation (6). The capability of various molecules to release oocytes from prematuration phase arrest and allow their progression through meiosis I serves as a measure of their participation in processes regulating entry into the cell cycle. This process, termed "oocyte maturation," is characterized by germinal vesicle breakdown (GVBD) and the appearance of condensed chromosomes (6, 7). As a measure of the capability of various G proteins to trigger cell division, we tested whether the activated α subunit of G_o protein (α_o^*) and α subunits of the three inhibitory G proteins could cause oocyte maturation.

MATERIALS AND METHODS

Materials. Female *Xenopus laevis* were purchased from Nasco (Fort Atkinson, WI). A peptide containing residues 19–36 of protein kinase C [PKC-(19–36)] was from Bachem and the protein kinase C assay kit was from Amersham. Oligonucleotides were synthesized at the Brookdale Oligonucleotide Synthesis Facility of the Mount Sinai School of Medicine.

Injection of Oocytes and Measurement of GVBD. Stage VI oocytes were surgically removed from female *Xenopus laevis* under tricaine anesthesia and defolliculated by collagenase treatment (8). G proteins were purified and characterized, and guanine 5′-[γ-thio]triphosphate (GTP[γ-S])-activated α subunits were prepared (4). G protein α subunits were injected by use of a Drummond microinjector superficially in the equatorial region between the animal and vegital poles of the oocyte. Activated α-subunit storage vehicle was 25 mM Hepes·NaOH/20 mM 2-mercaptoethanol/100 mM NaCl/2 mM $MgCl_2$/2–10 mM potassium phosphate, pH 7.5. This storage solution was used for vehicle injections. $\beta\gamma$ or α_o^* plus $\beta\gamma$ subunits were also injected in a final volume of 50 nl. The $\beta\gamma$ dimers were diluted 1:10 in 100 mM KCl to yield a final concentration of 120 nM and 25 nl of this $\beta\gamma$-containing solution was mixed with α_o^* or vehicle alone for injection. When PKC-(19–36) was used, a stock solution of 1 mM in distilled water was stored at −20°C. When oligonucleotides were used, they were purified by ethanol precipitation and resuspended in 100 mM KCl to achieve a concentration of 4 mg/ml. When mixtures of oligonucleotides were used, the final concentration of the individual oligonucleotides was 2 mg/ml, and 50 nl of this solution was injected per oocyte.

GVBD was measured by the appearance of the white spot surrounded by a brown ring on the animal pole using 15–30 oocytes for each assay condition. After addition of progesterone or injection of G-protein subunits, oocytes were not preselected to discard fast-maturing oocytes. In each group, there were one or two oocytes that displayed GVBD within 2–4 hr. These were also included in the final results. To verify GVBD, oocytes were fixed in 10% (wt/vol) trichloroacetic acid, sliced, and visually inspected under a low-power (×10) dissecting microscope.

Protein Kinase C Assay. Vehicle or α_o^* (50 nl) was injected into oocytes (final concentration, 3 nM). Fifteen minutes after injection, groups of 20 oocytes were homogenized in 50 mM Tris·HCl/5 mM EDTA/10 mM EGTA/0.3% 2-mercaptoethanol/1 mM phenylmethylsulfonyl fluoride (freshly prepared)/10 mM benzamidine/leupeptin (5 mg/ml), pH 7.5. Each oocyte was homogenized in 17 vol (17 ml) of homogenization medium, and 25 ml was assayed for protein kinase activity by measurement of ^{32}P transfer from [γ-^{32}P]ATP to [Thr^{25}]PKC-(19–36), the internal pseudosubstrate sequence of protein kinase C (RFARKGALRQKNVHEVKN) (ref. 9), except that Ala-25 has been changed to threonine. The assay was performed essentially as described in the manual provided with the Amersham protein kinase C enzyme assay system (code RPN77).

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Abbreviations: G protein, guanine nucleotide binding regulatory protein; α_o, G_o protein α subunit; α_o^*, activated α_o; GVBD, germinal vesicle breakdown.

Immunoprecipitation of ^{32}P-Labeled mos Protein. Oocytes were incubated for 4 hr in ND96 frog Ringers medium with 25 mCi of ^{32}P (1 Ci = 37 GBq). Typically 100 oocytes were incubated in 5 ml of radioactive medium. After 4 hr, groups of 25 oocytes were injected with vehicle, α_o^*, or α_o^* plus PKC-(19–36). Final concentration of α_o^* was 3 nM and of PKC-(19–36) was 10 mM. When used, final concentrations of progesterone was 10 mM. After 6 hr, the oocytes were homogenized in RIPA buffer (100 ml per oocyte) as defined by Watanabe *et al.* (10). pp39^{mos} protein was immunoprecipitated using the S5 monoclonal antibody raised against *Xenopus* mos protein expressed in *Escherichia coli*. (11).

Replication of Results. All experiments were repeated at least twice. Some experiments, including α_o^* induction of GVBD and blockade of PKC-(19–36), was repeated six to eight times. Qualitatively similar results were obtained. Typical data are shown.

RESULTS

Persistently Activated α_o^* Induces Oocyte Maturation. The heterotrimeric G protein G_o was purified from bovine brain and activated with Mg^{2+} and GTP[γ-S]. α_o^* was purified (4) and injected into oocytes. GVBD manifests itself as a white spot in the dark animal pole and was observed under a dissecting microscope. α_o^* induced GVBD (Fig. 1*A*), as did progesterone. Background response in vehicle-injected eggs may be due to 2-mercaptoethanol, which causes GVBD (6). Since G protein α subunits are labile without sulfhydryl group reducing agents, the vehicle was not altered. Activated inhibitory G protein type 2 α subunits (Fig. 1*A*) did not induce substantial GVBD. Activated inhibitory G protein types 1 and 3 α subunits did not appear to induce GVBD. Heterotrimeric G_o protein also did not induce GVBD (data not shown). Though in the experiment in Fig. 1*A* α_o^*-induced maturation was slower than that induced by progesterone, temporal differences may not be biologically significant since the time course of progesterone induction is variable (compare Figs. 1*A* and 3*C*). The α_o^* effect was concentration dependent. Half-maximal effects were observed at ≈150 pM assuming α_o^* equilibrates fully within the cell (Fig. 1*B*).

Prolonged exposure to muscarinic receptor agonists did not induce maturation (data not shown). This could be due to desensitization of the receptor signal. In contrast, when G_o is activated with GTP[γ-S], the α_o subunit should be persistently activated. Hence, we tested the effect of coinjected G protein $\beta\gamma$ subunits that preferentially associate with the GDP-bound form of α_o and should not affect α_o^*. Coinjection of $\beta\gamma$ subunits did not affect α_o^*-induced GVBD (Fig. 1*C*). The concentration of $\beta\gamma$ subunits used produces a substantial reduction in the muscarinic-receptor-evoked Cl^- current (12). This observation indicates that the GTP[γ-S]-bound α_o stays persistently activated in the oocyte.

To ascertain that α_o^*-injected oocytes had completed the first meiotic cycle, we looked for condensed and clustered chromosomes (7). Oocytes that had undergone GVBD were fixed in 10% formaldehyde and stained with Hoechst 33258 dye, and chromosomes were visualized by epi-illumination. Injection of α_o^* resulted in chromosome condensation similar to that seen in progesterone-treated oocytes (compare Fig. 2 *A* and *B*), indicative of the completion of at least meiosis I (7).

Protein Kinase C in the α_o^*-Induced Maturation. Since α_o^* is thought to stimulate phospholipase C, it should activate protein kinase C through diacylglycerol. We measured protein kinase C activity in homogenates from vehicle- and α_o^*-injected cells using a specific peptide substrate [Thr^{25}]-PKC-(19–36). We found at least a 2.5-fold stimulation of protein kinase C in α_o^*-injected cells (Fig. 3*A*) by two criteria. (*i*) Protein kinase C specific activity in homogenates of α_o^*-injected oocytes was 2- to 2.5-fold higher than that of vehicle-injected oocytes. (*ii*) The protein kinase C activity in homogenates of α_o^*-injected oocytes was elevated and further stimulation by phorbol esters was only 40%, in contrast to vehicle-injected homogenates where phorbol esters stimulated protein kinase C 3.5-fold (Fig. 3*A*).

To determine if increased protein kinase C activity was required for the α_o^* effect, we coinjected α_o^* with a specific peptide inhibitor, PKC-(19–36), which is the pseudosubstrate region in the regulatory domain of protein kinase C (9).

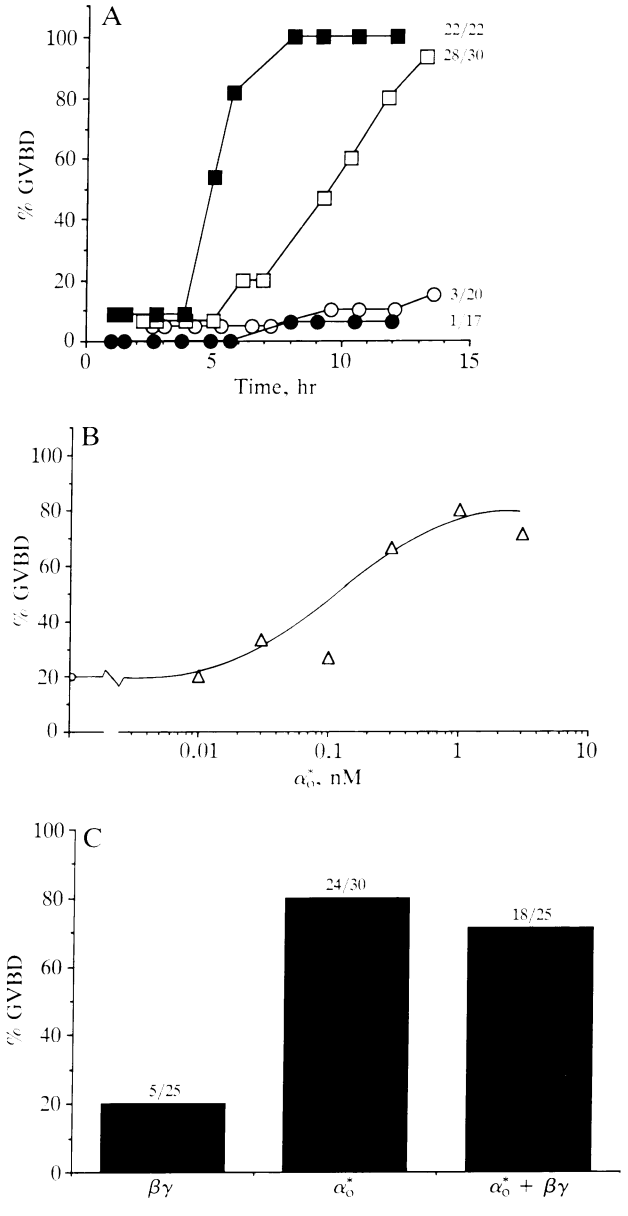

FIG. 1. Intracellular injection of persistently activated α_o^* induces *Xenopus* oocyte maturation characterized by GVBD. (*A*) Time course of GVBD induced by injection of α_o^* (□) or bath application of 10 mM progesterone (■). Controls included injection of vehicle (○) or the inhibitory G protein type 2 α subunit (●). A 50-nl sample of control vehicle or α subunits was injected. Concentration of the α_o^* solution was 63 nM and the inhibitory G protein type 2 α subunit was 50 nM. (*B*) Effect of the injection of various concentrations of α_o^* on the induction of GVBD. The α_o^* protein was diluted into the vehicle and then injected at a constant volume (50 nl) to yield the desired final concentration in the oocytes. Final concentration was calculated assuming a uniform distribution and an oocyte volume of 1 ml. (*C*) Lack of effect of co-injection of $\beta\gamma$ subunits on GVBD induced by α_o^*. Final concentrations of $\beta\gamma$ was 20 nM and of α_o^* was 3 nM. Fractions in *A* and *C* are the number of oocytes that display GVBD/total number of oocytes in the group.

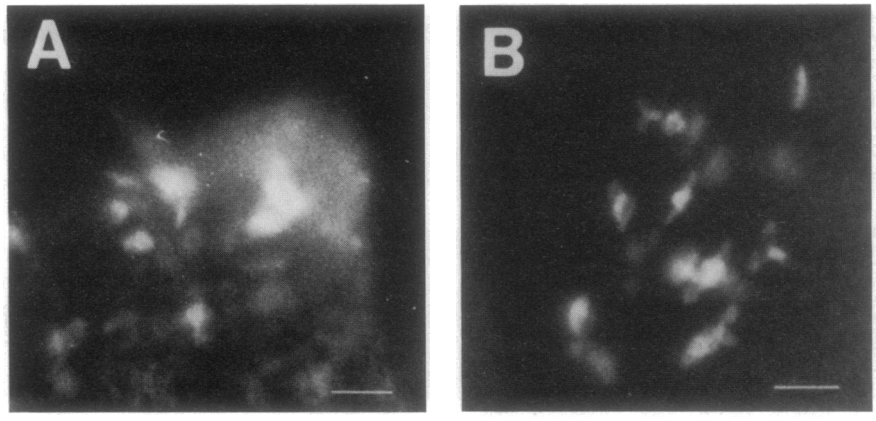

FIG. 2. Condensed chromosomes in progesterone-treated (*A*) or α_o^*-injected (*B*) oocytes. *Xenopus* oocytes were treated with 10 mM progesterone in the medium or injected with α_o^* (final concentration, 3 nM) and allowed to mature up to the appearance of the white spot in the animal pole. At this stage, the oocytes were fixed in 10% formaldehyde, stained with Hoechst 33258 dye, and visualized by epi-illumination by the procedure of Gerhart *et al.* (7). The images were photographed onto Kodak Pan X black and white film. (Bar = 10 m.)

Coinjection of the PKC-(19–36) blocked α_o^*-induced maturation (Fig. 3*B*), but by itself did not induce maturation in excess of that of vehicle injection. PKC-(19–36) did not affect progesterone-induced GVBD (Fig. 3*C*), indicating that protein kinase C activation was not essential for the progesterone-induced pathway. PKC-(19–36) inhibition of α_o^*-induced maturation was concentration dependent with an approximate half-maximal effect in the 10–20 nM range (Fig. 3*D*).

c-mos- and α_o^*-Induced Maturation. The progesterone effect is mediated at least in part through translation of $pp39^{mos}$, a serine/threonine kinase protein product of the protooncogene c-mos^{xe} (10, 11). Since protein kinase C is also a

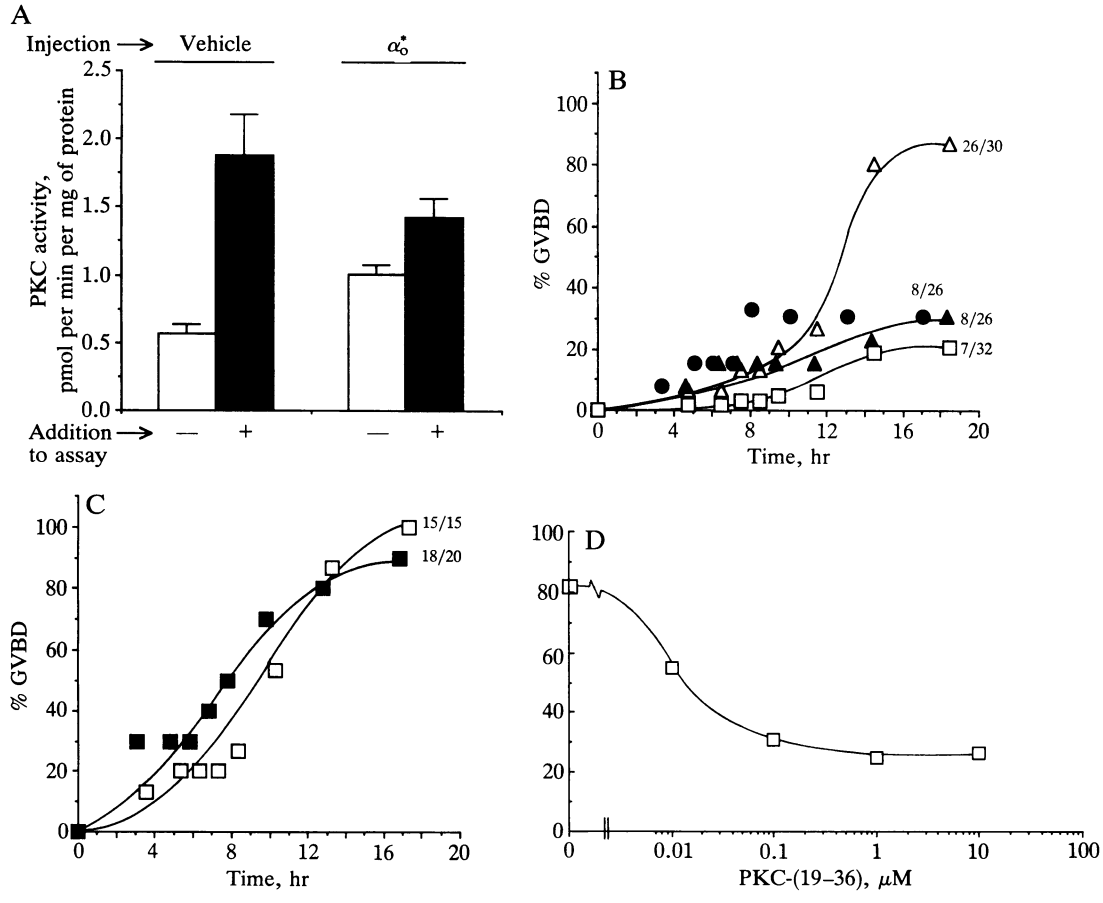

FIG. 3. Role of protein kinase C in the α_o^*-induced maturation. (*A*) Comparison of protein kinase C (PKC) activity in the absence (−) and presence (+) of phorbol 12-myristate 13-acetate in homogenates prepared from vehicle- and α_o^*-injected oocytes. (*B*) Time of α_o^*-induced GVBD and its blockade by coinjection of the protein kinase C inhibitor peptide PKC-(19–36). Maturation was triggered by the injection of α_o^* with (▲) and without (△) PKC-(19–36). Control injections included vehicle (□) or PKC-(19–36) in the vehicle (●). Final concentration of α_o^* was 3 nM and of PKC-(19–36) was 10 mM. (*C*) Lack of effect of PKC-(19–36) on progesterone-induced GVBD. Maturation was triggered by the bath application of 10 mM progesterone in control (□) and 10 mM PKC-(19–36)-injected (■) oocytes. (*D*) Effect of injection of the PKC inhibitor peptide PKC-(19–36) on α_o^*-induced GVBD. Fractions to the right in *B* and *C* are the number of oocytes that display GVBD/total number of oocytes in the group.

serine/threonine kinase and required for the α_o^* effect but not the progesterone effect, it was not *a priori* obvious whether the α_o^* effect would require protein synthesis. Hence we tested whether cycloheximide would block the α_o^*-induced maturation. Treatment of oocytes with cycloheximide for 4 hr prior to the injection of α_o^*, blocked the α_o^* effect (Fig. 4*A*). Translation of the c-mos transcript is necessary to trigger progesterone-induced maturation (10, 11). We therefore determined whether the α_o^* effect also required translation of c-mos mRNA. Two antisense oligonucleotides to the c-mos transcript (11) were tested for their capability to block the α_o^*-induced maturation. It was found that both antisense oligonucleotides, when injected individually or together 4 hr prior to treatment, inhibited α_o^*- and progesterone-induced maturation (Fig. 4*B*). Corresponding sense oligonucleotides did not have significant effects. Hence it appears that translation of the c-mos transcript is necessary for the α_o^* effect.

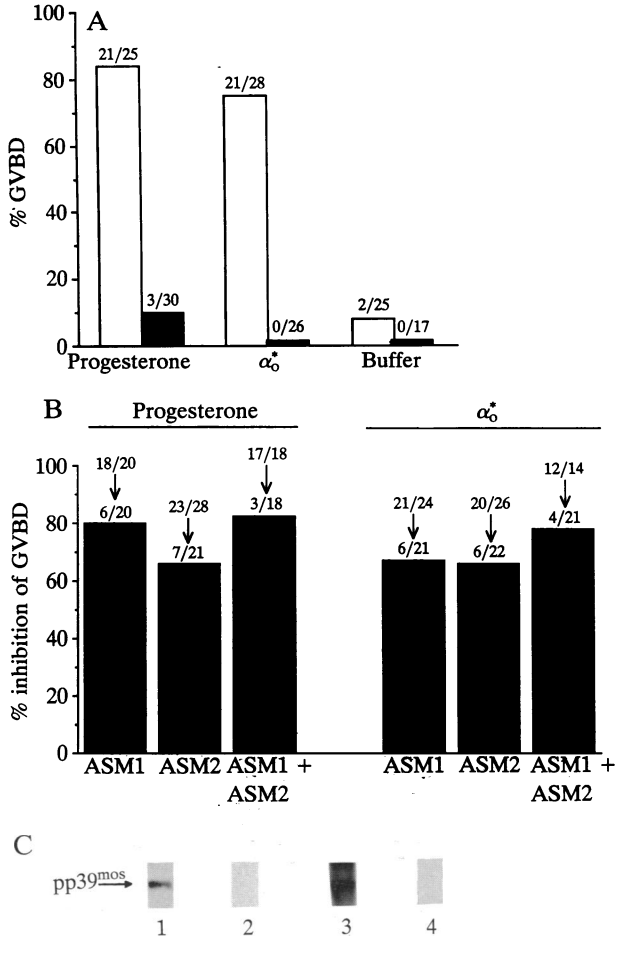

FIG. 4. Role of pp39^{mos} synthesis in the α_o^*-induced maturation. (*A*) Effect of a 4-hr cycloheximide (10 mg/ml) pretreatment (solid bars) on progesterone- and α_o^*-induced GVBD. Open bars are the control without cycloheximide treatment. (*B*) Effect of injection of antisense oligonucleotides to the mos mRNA on progesterone- and α_o^*-induced GVBD. ASM1 is an oligonucleotide complementary to nucleotides +178 to +198 in the coding region of c-*mos*. ASM2 is complementary to nucleotides −21 to +6 and spans the ATG initiation codon (11). The numbers on top of the graph represent the number of oocytes that displayed GVBD/total number in the group comparing corresponding sense (upper set) and antisense (lower set) oligonucleotide injections. (*C*) Immunoprecipitation analysis of pp39^{mos} in progesterone-treated and vehicle-, α_o^*-, and α_o^*- plus PKC-(19–36)-injected oocytes (lanes 1–4, respectively). The oocytes were labeled with ^{32}P and phosphorylated mos was immunoprecipitated with the mos-specific monoclonal antibody S5 (11) and analyzed by SDS/PAGE and autoradiography. Since immunoprecipitation from the α_o^*-injected oocytes have had a much higher background, contact-positives with various exposure times were made of the autoradiograms. The progesterone-, vehicle-, and α_o^*- plus PKC-(19–36)-injected samples had 20-sec exposures, whereas the α_o^*-injected sample had 90-sec exposure to obtain contact positives with Kodak EDF film.

We next analyzed the role of protein kinase C in the α_o^* effect on mos protein. Watanabe *et al.* (10) have shown that the c-mos protein production is not present in measurable amounts in immature oocytes and maturation results in translation and phosphorylation of c-mos. At 4–6 hr after the start of the maturation signal, the amount of mos protein identified by ^{35}S and ^{32}P labeling was similar. Hence we utilized ^{32}P labeling of oocytes to determine the effect of protein kinase C activity on the identification of mos by immunoprecipitation with a mos-specific monoclonal antibody S5 (11). No labeled protein was detected in buffer-injected oocytes but labeled protein could be detected in progesterone-treated oocytes. Injection of α_o^* resulted in the appearance of the 39-kDa band. When PKC-(19–36) was injected, no 39-kDa immunoprecipitatable band was observed. The experiments in Fig. 4 indicate that mos synthesis is required for the α_o^* effect. Since mos is synthesized and phosphorylated in the absence of protein kinase C activation in progesterone-matured oocytes, it appears that the presence of ^{32}P-labeled mos in α_o^*-injected oocytes and its absence in α_o^*- plus PKC-(19–36)-injected oocytes indicate that phosphorylation and possibly synthesis of mos by α_o^* is regulated through protein kinase C.

DISCUSSION

Our studies show that α_o^* can trigger oocyte maturation through activation of protein kinase C. Previous studies from our laboratories have demonstrated that α_o^* induces a Cl^- current using Ca^{2+} from inositol trisphosphate-sensitive stores (4). Thus these data indicate that α_o^* initiates its action in oocytes by stimulation of phosphatidylinositol bisphosphate hydrolysis. The subsequent stimulation of protein kinase C is essential for α_o^*-induced maturation, since the specific inhibitor PKC-(19–36) blocks the α_o^*-induced process. The activation of protein kinase C is not a general requirement of oocyte maturation since PKC-(19–36) does not affect progesterone-induced maturation. Thus there may be multiple pathways by which external stimuli can trigger oocyte maturation.

Agonist occupancy of native muscarinic receptors that function through G_o protein does not trigger maturation, but muscarinic agents at physiologically relevant concentrations promote progesterone-induced maturation (13). In contrast, α_o^* subunit induces maturation in the absence of other stimuli, possibly due to prolonged activation of the phosphatidylinositol bisphosphate hydrolysis system and protein kinase C. At a conceptual level the use of α_o^* in our experiments is not dissimilar to the overexpression of serotonin-1C receptors in NIH 3T3 cells and their prolonged stimulation by agonists to induce oncogenic transformation (14). In both cases persistent stimulation of the inositol phospholipid pathway appears to be essential in obtaining the observed growth effects. Thus, not only the appearance of the signal but also its duration may be crucial in determining the cellular growth response.

Our findings indicating that α_o^* through protein kinase C may induce the translation of the c-mos mRNA suggest that α_o^* can be a putative oncoprotein. c-*mos* and v-*mos*, the Moloney sarcoma virus oncogene, are similarly oncogenic. The *Xenopus* oocyte c-*mos*, when overexpressed, transforms fibroblasts (15). In oocytes, c-*mos* cannot cause unregulated proliferation due to built-in constraints, including its destruction by calpain during fertilization (10). However, in other tissues, such as brain, where both α_o and c-mos exist (11), it

is possible that α_o^* through c-*mos* could deliver a proliferative signal.

We have used α_o^* since phospholipase C regulation in oocytes is pertussis-toxin-sensitive and responds to α_o^*. In many other cell types, G-protein regulation of phosphatidylinositol bisphosphate hydrolysis is pertussis-toxin-insensitive (16), suggesting that other G proteins are involved. These G proteins, too, when persistently activated by mutations in the GTP hydrolysis domain, such as those found in the stimulatory and inhibitory G proteins (2, 3), could be putative oncoproteins. These possibilities need to be further explored.

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