Progesterone inhibits protein kinase A (PKA) in *Xenopus* oocytes: demonstration of endogenous PKA activities using an expressed substrate

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Summary

3′-5′ cyclic adenosine monophosphate (cAMP)-dependent protein kinase, PKA, is thought to be a key enzyme that controls prophase arrest in vertebrate oocytes. It has long been established that overexpression of the catalytic subunit of PKA inhibits hormone-induced frog oocyte maturation whereas overexpression of the regulatory subunits induces hormone-independent oocyte maturation. However, the activities of endogenous oocyte PKA, or its regulation by the maturation-inducing hormone progesterone, have never been directly demonstrated in frog oocytes. We have developed a novel expressed substrate for PKA in live oocytes by constructing a fusion protein containing an N-terminal myristylation sequence (derived from the Src tyrosine kinase) followed by an antigenic epitope tag and a substrate motif (the C-terminal cytoplasmic domain of β_2 adrenergic receptor). Following mRNA injection, the phosphorylation status of the substrate was determined by two-dimensional electrophoresis followed by epitope immunoblotting, or alternatively by SDS-PAGE followed by immunoblotting using antibodies specifically recognizing the PKA-phosphorylated form of the substrate. In prophase oocytes, the expressed protein, myr-HA-β_2AR-C, was fully phosphorylated on a single PKA site (Ser^{346} of human β_2 adrenergic receptor). Within one hour of the addition of progesterone, the PKA site became mostly dephosphorylated. No re-phosphorylation of the PKA site, and therefore no reactivation of PKA, was observed throughout the entire maturation process. To demonstrate the generality of this PKA substrate, we analyzed its phosphorylation status in COS-7 cells following transfection. We show that dibutyryl cAMP rapidly stimulates phosphorylation of the PKA site. These results represent the first biochemical demonstration of regulation of endogenous *Xenopus* oocyte PKA by progesterone. Furthermore, myr-HA-β_2AR-C should be widely adaptable as an in vivo PKA activity indicator.

Key words: cAMP-dependent protein kinase, Two-dimensional gel electrophoresis, In vivo substrate, Progesterone, Meiosis, COS-7 cells, C terminus of β_2 adrenergic receptor

Introduction

In *Xenopus laevis*, full-grown oocytes are physiologically arrested at the diplotene stage of meiotic prophase (commonly known as G2 arrest). Progesterone is thought to be the natural ovarian hormone responsible for the re-initiation of meiosis or oocyte maturation. Injection of the regulatory subunit of PKA (PKAr), or a peptide inhibitor (PKI), causes hormone-independent oocyte maturation whereas injection of catalytically active PKA (PKAc) prevented progesterone-induced oocyte maturation (Maller and Krebs, 1977). These results clearly suggest that endogenous PKA plays a critical role in maintaining G2 arrest. Consistent with this notion, many previous studies show that progesterone causes a modest reduction (20%) of intracellular cAMP (Smith, 1989). In contrast, the mechanism by which PKA helps to maintain G2 arrest is not well understood. Over the years, many investigators have searched for physiological substrates for PKA in frog oocytes. Earlier studies have suggested that PKAc can phosphorylate and inhibit the germ-cell-specific protein kinase Mos (Yang et al., 1996). However, as Mos protein is absent in G2-arrested oocytes (Sagata et al., 1988), phosphorylation of Mos cannot be the primary mechanism by which PKA maintains G2 arrest. The best characterized PKA substrate in frog oocytes appears to be the dual specificity phosphatase Cdc25C (Schmitt and Nebreda, 2002; Duckworth et al., 2002). PKA catalyzed phosphorylation of Cdc25C inhibits the ability of the latter to dephosphorylate and activate Cdc2 (the catalytic subunit of maturation promoting factor or MPF). On the other hand, Cdc25C cannot be the only physiological substrate of PKA in frog oocytes, as PKAc clearly inhibits Mos translation, which precedes Cdc25C dephosphorylation and activation (Matten et al., 1994; Lazar et al., 2002). Furthermore, PKA apparently also has an inhibitory role in G2 oocytes that is independent of its kinase activity, as a catalytically inactive PKAc is able to inhibit progesterone-induced oocyte maturation as potently as wild-type PAKc (Schmitt and Nebreda, 2002).

Despite the importance of PKA in G2 arrest and its proposed

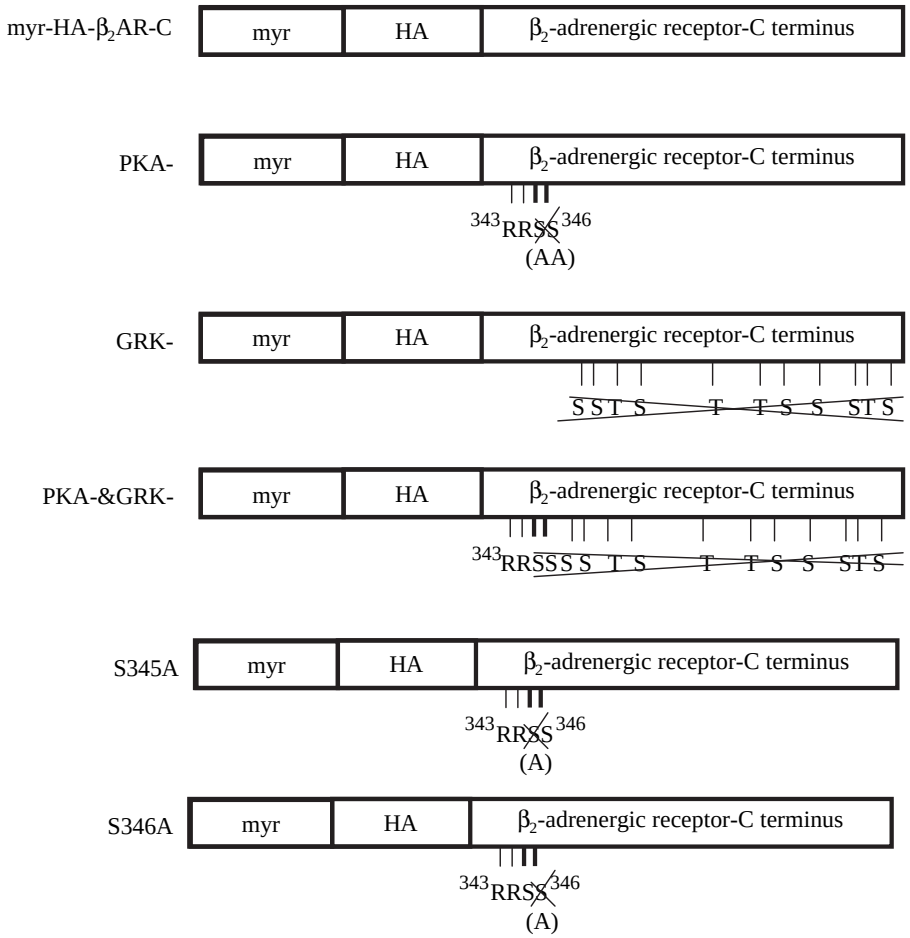

Fig. 1. Schematic representation of the plasmid constructs used in this study. To generate a potential expressed substrate for PKA we added two features to the C-terminus of β_2AR. The first was to add a myristylation sequence to target the truncated protein to the membranes, where β_2AR normally resides. We also included a hemagglutinin (HA) antigenic epitope to facilitate detection by immunoblotting. In addition to myr-HA-β_2AR-C, we also constructed myr-HA-β_2AR-C/PKA$^-$ (substituting both Ser^{345} and Ser^{346} with Ala), myr-HA-β_2AR-C/GRK$^-$ [substituting all 11 GRK sites with either Ala or Gly, as described previously (Hausdorff et al., 1989)] and myr-HA-β_2AR-C/PKA$^-$&GRK$^-$ (containing neither PKA nor GRK sites). myr, myristylation; HA, hemagglutinin tag.

regulation by progesterone during the initiation of frog oocyte maturation, activities of endogenous PKA have never been directly demonstrated. Inactive (cAMP-free) PKA exists as a holoenzyme that consists of two regulatory subunits (PKAr) and two catalytic subunits (PKAc). Upon binding of cAMP to the regulatory subunits, the two catalytic subunits are released from the PKAr dimer and become active (Francis and Corbin, 1994). Three subtypes of PKAc (PKAcα, PKAcβ, PKAcγ) and four subtypes of PKAr (PKArIα, PKArIβ, PKArIIα, PKArIIβ) have been identified in mammalian cells (Skalhegg and Tasken, 1997). As the cAMP-binding subunit, PKAr determines the activation constant of PKA for cAMP, the activation constants of PKA I (containing PKArI) and PKA II (containing PKArII) for cAMP are 0.1 μM and 0.5 μM respectively (Dostmann and Taylor, 1991). PKA II appears to be the major PKA in frog oocytes (Masaracchia et al., 1979; Schmitt and Nebreda, 2002).

As PKA is regulated by intracellular cAMP, measuring PKA activities after cell lysis may not accurately determine the kinase activities in live cells. Therefore, we sought to develop a novel approach to determine PKA activities in live cells by expressing a PKA substrate whose phosphorylation status can be analyzed following cell lysis. We constructed a tripartite fusion protein containing an N-terminal myristylation sequence derived from the c-Src protein (Aronheim et al., 1997), followed by an antigenic epitope tag (hemagglutinin or HA) and a PKA substrate motif (the C-terminal cytoplasmic domain of β_2-adrenergic receptor (β_2-AR) (Kemp et al., 1977; Zamah et al., 2002). We used this construct to demonstrate, for the first time, PKA activities in G2 oocytes and its inhibition by progesterone.

Materials and Methods

Chemicals

Polyacrylamide gel electrophoresis materials (for both first dimension mini-tube gels and second dimension slab gels) were purchased from BioRad. All cell culture materials were purchased from Invitrogen. H89 was purchased from Calbiochem. Antibodies against phospho-MAP kinase were from Upstate Biotechnology. Other chemicals were from Sigma unless otherwise indicated. PKAc was from Promega and stored in aliquots at –80°C (350 mM potassium phosphate buffer, pH 6.8, 0.1 mM DTT).

Oocyte isolation and mRNA injection

Stage VI Oocytes were manually isolated from sexually mature female *Xenopus laevis* (NASCO) 3-7 days after priming with pregnant mare serum gonadotropin (50 IU per frog). Oocytes were injected in OR2 medium without Ca^{2+} (83 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, 5 mM HEPES, pH 7.8). Each oocyte received 14 nl of mRNA (1 μg/μl). Following mRNA injection, oocytes were incubated overnight at 18°C in OR2 medium containing gentamycin (100 μg/ml) and Ca^{2+} (1 mM) to allow protein expression. mRNA was generated through in vitro transcription using linearized plasmid DNA as template and the MessageMachine kit with Sp6 polymerase (Ambion, Texas).

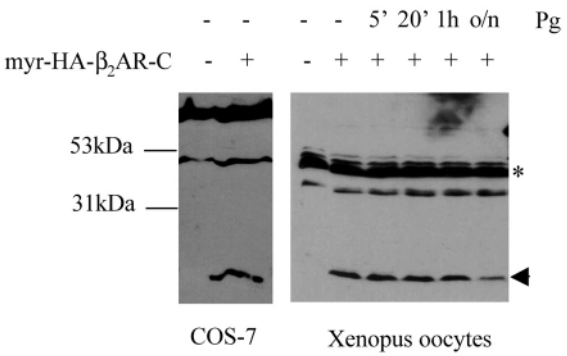

Fig. 2. Expression of myr-HA-β_2AR-C in *Xenopus* oocytes and in COS-7 cells. COS-7 cells mock transfected (–) or transfected with myr-HA-β_2AR-C (+) were lysed and analyzed by immunoblotting with antibodies against HA (left panel). Frog oocytes were injected with water (–) or mRNA for myr-HA-β_2AR-C (+). Oocytes injected with myr-HA-β_2AR-C mRNA were incubated in OR2 (–) or with progesterone for the indicated periods. Oocytes were lysed and analyzed by immunoblotting with antibodies against HA (right panel). Oocytes treated overnight (o/n) with progesterone (Pg) exhibited 100% germinal vesicle breakdown (GVBD). The arrow indicates the position of myr-HA-β_2AR-C and the asterisk indicates a nonspecific protein, that apparently exists in both frog oocytes and in COS-7 cells.

Expression plasmids

Full-length cDNA constructs containing the wild-type human β_2-adrenergic receptor (β_2AR) and its various phosphorylation site mutants (Fig. 1) were provided by Dr Robert J. Lefkowitz. The nucleotide sequence encoding the C-terminus of β_2AR (amino acids 328-413) was PCR-amplified using the following primers: Forward: 5′-TAT GGA TCC ATG GGG TAC CCA TAC GAT GTT CCA GAT TAC GCT CGG AGC CCA GAT TTC AGG AT-3′; Reverse: 5′-TAT GAA TTC ACA GCA GTG AGT CAT TTG TAC TA-3′. The amplified segment was digested with *Bam*HI and *Eco*RI, and then ligated into pCS2$^+$ vector (Turner and Weintraub, 1994), which had been similarly digested. The resultant plasmid encoded HA tagged β_2-adrenergic receptor C terminus (HA-β_2AR-C). This plasmid was further linearized with *Nco*I. Double stranded oligos encoding the Src myristylation sequence (Forward: 5′-C ATG GGG AGT AGC AAG AGA AAG CCT AAG GAC CCC AGC CAG CGC CGG CC-3′; Reverse: 5′-CAT GGG CCG GCG CTG GCT GGG GTC CTT AGG CTT GCT CTT GCT ACT CCC-3′) containing cohesive *Nco*I ends was then inserted into the linearized HA-β_2AR-C, resulting in myr-HA-β_2AR-C. The myr-HA-β_2AR-C mutants lacking PKA-phosphorylation site or GRK-phosphorylation sites, or both PKA and GRK phosphorylation sites, were produced in the same way as wild-type myr-HA-β_2AR-C, with the exception that the corresponding full-length β_2AR mutants (Hausdorff et al., 1989) were used as PCR template. To generate the two single Ser to Ala (S345A and S346A) mutants of myr-HA-β_2AR-C, we used the two-step PCR mutagenesis protocol (Vallette et al., 1989), using myr-HA-β_2AR-C as a template. PCR products containing SP6 sequence at the 5′ end and T3 sequence at the 3′ end, including the SV40 poly(A) tail, were used directly for in vitro transcription. Both PCR products were also analyzed by DNA sequencing to confirm the respective single mutation (S345A and S346A).

Preparation of oocyte extracts and two-dimensional (2D) immunoblotting

Oocytes were lysed in 2 μl ice-cold extraction buffer (20 mM HEPES pH 7.2, 50 mM glycerophosphate, 10 mM EDTA, 2.5 mM $MgCl_2$, 0.25 M sucrose, 0.1 M NaCl, 1% Triton X-100, 200 μM PMSF, 10 μg/ml leupeptin, 1 μM sodium orthovanadate, 10 μM H89 and 1 μM Okadaic acid) by forcing through pipette tips. The extracts were centrifuged for 15 minutes at 13,500 rpm (in a refrigerated Eppendorf centrifuge). The clarified supernatant was mixed with an equal volume of first dimension sample buffer (8 M urea, 2% triton X-100, 5% β-mercaptoethanol, 1.6% Bio-lyte 5/7 ampholyte, 0.4% Bio-lyte 3/10 ampholyte). 20 μl of each sample were loaded onto mini-tube gels for first dimension electrophoresis. Electrophoresis was carried out at 750 V for 3.5 hours. Subsequently, the tube gels were extruded and loaded onto the SDS polyacrylamide slab gels. We typically loaded two tube gels side-by-side to reduce the number of slab gels. In addition, an extra sample in SDS sample buffer was loaded directly onto the second dimension slab gel as a marker (see Fig. 3H for example). Transfer of proteins onto nitrocellulose membranes and the subsequent immunoblotting with HA antibodies and ECL detection were all standard procedures as described in previously (Sheng et al., 2001; Ma et al., 2003).

Protein phosphatase assays

Groups of four oocytes were lysed in 20 μl phosphatase assay buffer (20 mM Tris pH 7.5, 4 mM EDTA, 15 mM β-mercaptoethanol, 200 μM PMSF, 10 μg/ml leupeptin, 25 μg/ml benzamadine and 1% Triton X100). Extracts were clarified by centrifugation (13,500 rpm for 5 minutes). The supernatants were further diluted 50 times with the same buffer. Phosphatase reaction was started by mixing 8.5 μl assay buffer and 4 μl of the diluted lysates with 12.5 μl synthetic phosphopeptide (Ser/Thr phosphatase Assay kit, Upstate Biotechnology) in wells of a microtiter plate. The reaction was carried out at room temperature for 30 minutes and stopped by addition of 100 μl Malachite Green phosphate detection solution (Ser/Thr phosphatase Assay kit, Upstate Biotechnology). Absorbance was measured at 650 nm and readings were converted to amounts of free phosphate released from reaction by using a standard curve. The relative ratios of free phosphate released were normalized by defining a group without progesterone stimulation as 100%.

COS-7 cell culture

COS-7 cells were grown and maintained in DMEM supplemented with 10% fetal bovine serum (FBS), antibiotics (penicillin and streptomycin) and Fungizone at 37°C in a humidified 5% CO_2 atmosphere. Cells in 6 cm dishes were transfected using 8 μg wild-type myr-HA-β_2AR-C or myr-HA-β_2AR-C/PKA$^-$ DNA and 20 μl lipofectamine™ 2000 reagent according to the manufacturer's protocol. After transfection, the cells were incubated for 24 hours in DMEM containing 10% FBS. Then the transfected cells were starved in serum-free DMEM for another 24 hours. Subsequently, dibutyl-cAMP (db-cAMP, final concentration 1.5 mM) was added to the medium and incubation continued for 40 minutes at 37°C. Finally, the cells were directly lysed in first dimension sample buffer (4 M urea, 1% Triton X-100, 2.5% β-mercaptoethanol, 0.8% 5/7 ampholyte and 0.2% 3/10 ampholyte, 200 μM PMSF, 10 μg/ml leupeptin).

Results

The C-terminal intracellular domain of the β_2AR contains one of the two well-characterized PKA phosphorylation sites (in the context of RRSS^{346}; the other is in the third intracellular loop) and all 11 potential phosphorylation sites for G protein-coupled receptor kinases or GRKs (Kemp et al., 1977; Hausdorff et al., 1989; Fredericks et al., 1996; Zamah et al., 2002). Phosphorylation of β_2AR by PKA and GRKs represents an important regulatory mechanism that controls receptor desensitization (Hausdorff et al., 1989; Hausdorff et al., 1990).

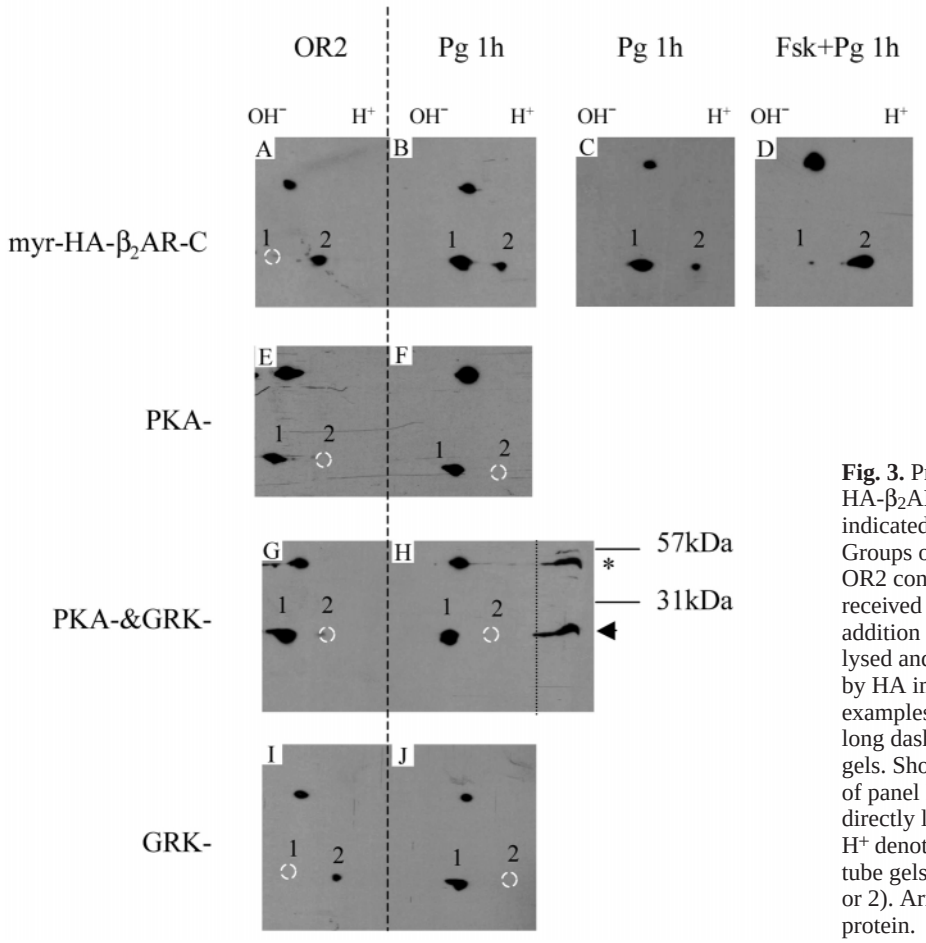

Fig. 3. Progesterone-induced dephosphorylation of myr-HA-β_2AR-C. (A-J) *Xenopus* oocytes injected with the indicated mRNAs were incubated in OR2 for 24 hours. Groups of at least 20 oocytes were incubated in OR2 or OR2 containing 1 μM progesterone (Pg). Group D received 50 μM forskolin (Fsk) 20 minutes prior to the addition of progesterone. One hour later, oocytes were lysed and analyzed by 2D gel electrophoresis followed by HA immunoblotting. Shown are representative examples of three to ten independent experiments. The long dashed line separates two first dimension mini-tube gels. Shown in the PKA⁻&GRK⁻ panel (on the right side of panel H) is one example of an extra SDS sample directly loaded on the second-dimension gel. OH^- and H^+ denote the anion and cation ends, respectively, of the tube gels. Circles represent positions of missing spots (1 or 2). Arrow, myr-HA-β_2AR-C; asterisk, nonspecific protein.

To generate a potential expressed substrate for PKA, we added two features to the C-terminus of β_2AR. The first was to add a myristylation sequence to target the truncated protein to the membrane, where β_2AR normally resides. We also included a hemagglutinin (HA) antigenic epitope to facilitate detection by immunoblotting. In addition to myr-HA-β_2AR-C, we also constructed myr-HA-β_2AR-C/PKA⁻ (substituting both Ser^{345} and Ser^{346} with Ala), myr-HA-β_2AR-C/GRK⁻ (substituting all 11 GRK sites with either Ala or Gly) and myr-HA-β_2AR-C/PKA⁻&GRK⁻ (containing neither PKA nor GRK sites) (Fig. 1).

mRNAs for myr-HA-β_2AR-C and the three mutant forms were individually injected into G2 oocytes. Following overnight incubation, extracts were prepared and analyzed by

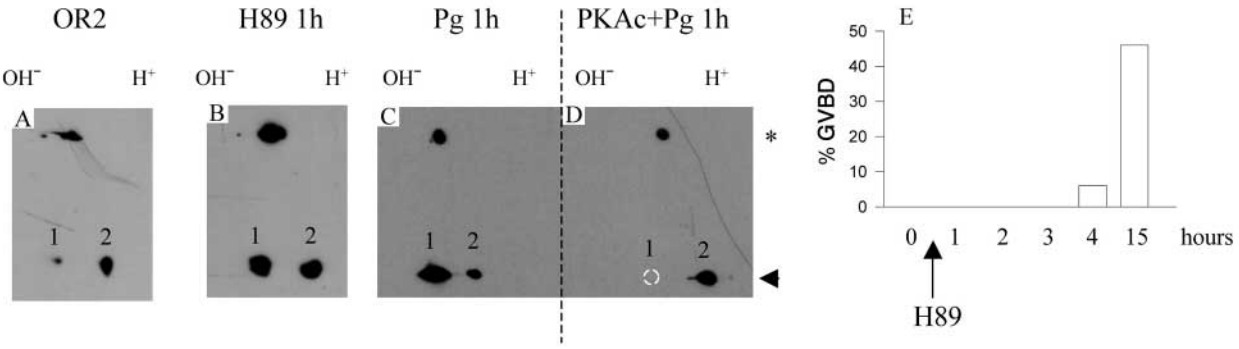

Fig. 4. Protein kinase A phosphorylated myr-HA-β_2AR-C in *Xenopus* oocytes. (A-D) Oocytes injected with mRNA for myr-HA-β_2AR-C were incubated in OR2 for 24 hours. One group received a further injection of 10 nl PKAc per oocyte. One hour after the PKAc injection, oocytes were incubated in OR2 or OR2 containing H89 (100 μM) or progesterone (Pg), as indicated. One hour later, 20 oocytes were retrieved from each group for lysis and 2D electrophoresis and immunoblotting with HA antibodies. Circle in (D) represents the position of missing spot 1. Arrow, myr-HA-β_2AR-C; asterisk, nonspecific protein. (E) The remaining oocytes in each group were scored for GVBD at the indicated time following the addition of H89 or progesterone. Only the H89 data are shown here (representative of three independent experiments).

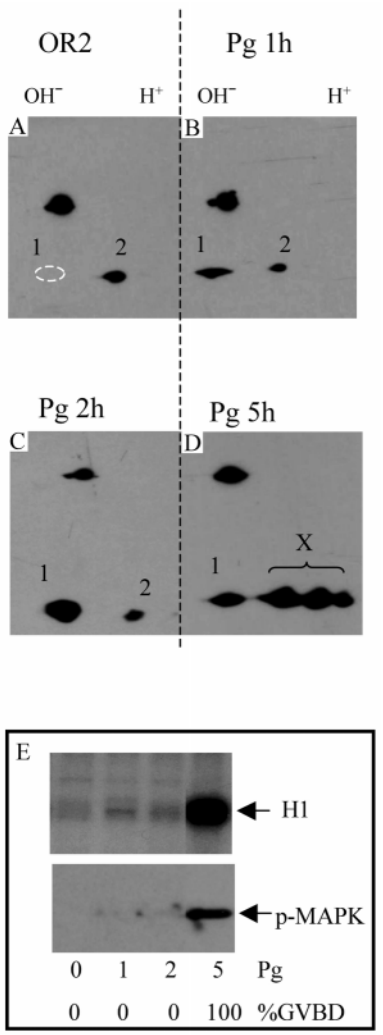

Fig. 5. A time course of myr-HA-β_2AR-C dephosphorylation caused by progesterone. (A-D) *Xenopus* oocytes injected with mRNA for myr-HA-β_2AR-C were incubated for 24 hours. Twenty oocytes were set aside as control (OR2) and the rest were incubated with progesterone (Pg). At each of the indicated times, 20 oocytes were removed and all were then subjected to 2D electrophoresis and HA immunoblotting. (D) Multiple phosphorylated forms of myr-HA-β_2AR-C were seen in oocytes that had undergone GVBD (represented by X). (E) Progesterone-treated oocytes were scored for GVBD. The same lysates were also analyzed for histone H1 kinase (MPF) activities (H1) and for MAP kinase phosphorylation (p-MAPK). Representative examples from three independent experiments are shown.

SDS-PAGE followed by immunoblotting using antibodies against HA. These experiments showed that myr-HA-β_2AR-C (Fig. 2, right panel) and its phosphorylation mutants (not shown, but see Fig. 9B) were properly expressed. When the corresponding cDNAs were transfected into COS-7 cells, the same protein was produced (Fig. 2, left panel). These experiments also indicated that myr-HA-β_2AR-C exhibited identical migration patterns whether the oocytes were treated with progesterone or not, suggesting that changes in the phosphorylation status of myr-HA-β_2AR-C, if they occurred, did not result in any shift on one-dimensional gels (Fig. 2, right panel). We therefore carried out two-dimensional gel electrophoresis followed by HA immunoblotting. As shown in Fig. 3, myr-HA-β_2AR-C (panel A) and myr-HA-β_2AR-C/GRK$^-$ (panel I) migrated as a single and identical spot (designated as 2; identity confirmed by 2D analyses of mixed sample, data not shown). Myr-HA-β_2AR-C/PKA$^-$ (Fig. 3E) and myr-HA-β_2AR-C/PKA$^-$&GRK$^-$ (Fig. 3G) also appeared as a single and identical spot (designated as 1; identity similarly confirmed), but distinct from spot 2 in the first dimension. Clearly, spot 1 and spot 2 differed only in their isoelectric points, with spot 2 being more acidic as a result of higher levels of phosphorylation. The correlation of spot 2 (phosphorylated form) with the presence of the PKA phosphorylation site suggested that myr-HA-β_2AR-C (or myr-HA-β_2AR-C/GRK$^-$) was fully phosphorylated by PKA in G2 oocytes. An unknown endogenous protein (indicated by the asterisk in Fig. 3) that was recognized by HA antibodies and that did not undergo progesterone-induced migration pattern change (compared to first dimensional protein markers with various pHi values, not shown) served as a convenient reference point in our analyses.

Following a 1 hour incubation with progesterone, the more phosphorylated form (spot 2) diminished for both myr-HA-β_2AR-C (Fig. 3B,C) and myr-HA-β_2AR-C/GRK$^-$ (Fig. 3J). Correspondingly, spot 1 (dephosphorylated form) was the predominant form for both. In contrast, the migration pattern of myr-HA-β_2AR-C/PKA$^-$ or myr-HA-β_2AR-C/PKA$^-$&GRK$^-$ did not change and remained as a single spot (spot 1). In the presence of forskolin, progesterone was unable to cause dephosphorylation of myr-HA-β_2AR-C and therefore it remained predominantly as spot 2 (Fig. 3D). Forskolin completely blocked progesterone-induced oocyte maturation (not shown). These data clearly demonstrated that progesterone caused dephosphorylation of the PKA site in intact oocytes (Fig. 3B,C,J).

To confirm that spot 2 represented the PKA-phosphorylated form of myr-HA-β_2AR-C, we employed a highly specific PKA inhibitor, H89 (Chijiwa et al., 1990). Indeed, treating oocytes with H89 for 1 hour resulted in a significant decrease in concentration of spot 2 and a corresponding increase of spot 1 (Fig. 4B). In contrast, prior injection of PKAc completely blocked progesterone-induced dephosphorylation of myr-HA-β_2AR-C (Fig. 4, compare D to C). Not surprisingly, oocytes incubated with H89 eventually underwent hormone-independent germinal vesicle breakdown (GVBD; Fig. 4E). However, we typically observed that less than 50% of the treated oocytes underwent GVBD, in contrast to earlier reports of higher percentages of GVBD when oocytes were injected with other PKA inhibitors, PKAr or PKI (Maller and Krebs, 1977). Injection of PKAc completely blocked progesterone-induced GVBD (not shown), as reported previously (Maller and Krebs, 1977; Schmitt and Nebreda, 2002; Duckworth et al., 2002).

Based on the results presented in Figs 3 and 4, we were confident that spot 2 was the PKA-phosphorylated form of myr-HA-β_2AR-C. We therefore used myr-HA-β_2AR-C to determine endogenous PKA activity during progesterone-induced oocyte maturation. In G2 oocytes, myr-HA-β_2AR-C was always fully phosphorylated on the PKA site (Fig. 5A), indicating that PKA is activated in G2-arrested oocytes. When these oocytes were examined 1 hour (Fig. 5B) or 2 hours (Fig.

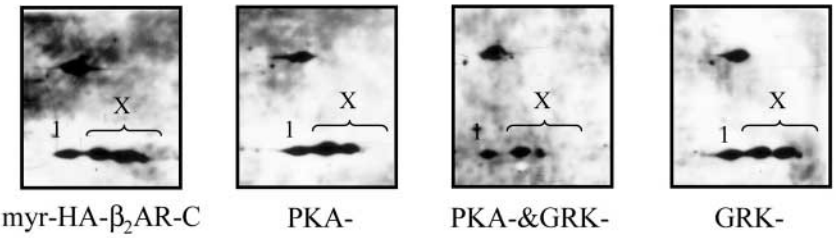

Fig. 6. myr-HA-β_2AR-C is phosphorylated by an unidentified kinase(s) in GVBD oocytes. *Xenopus* oocytes injected with the indicated mRNAs were incubated for 24 hours before the addition of progesterone. Following progesterone-induced GVBD, oocytes were lysed and analyzed by 2D electrophoresis and immunoblotting with hemagglutinin (HA). Representative examples from two independent experiments are shown. X, multiple phosphorylated forms of myr-HA-β_2AR-C.

5C) following the addition of progesterone, myr-HA-β_2AR-C appeared predominantly as spot 1, suggesting that progesterone-induced PKA inhibition was persistent and not transient. In some batches of oocytes, we examined the phosphorylation status of myr-HA-β_2AR-C 30 minutes following the addition of progesterone and found very similar shift from spot 2 to spot 1 (not shown, but see Fig. 9). The time course of progesterone-induced dephosphorylation of PKA site, and hence inhibition of endogenous PKA, was clearly ahead of the activation of maturation-specific protein kinases (e.g. MAP kinase and MPF) and GVBD (Fig. 5E, also see Fig. 9). In oocytes that had undergone GVBD (5 hours after the addition of progesterone in this particular experiment), myr-HA-β_2AR-C exhibited multiple phosphorylated forms (collectively designated as X) (Fig. 5D). Similar analyses of the various mutant forms of myr-HA-β_2AR-C in oocytes that had undergone GVBD indicated that all four constructs exhibited more or less the same pattern of multiple phosphorylated forms (X) (Fig. 6). These results argued strongly that a kinase(s) other than PKA or GRK was responsible for myr-HA-β_2AR-C phosphorylation in oocytes that had undergone GVBD.

As G2 oocytes contained myr-HA-β_2AR-C that was fully phosphorylated at its PKA site, the decrease in PKA phosphorylation following progesterone stimulation could also be the result of activation of protein phosphatases such as PP1 and/or PP2A, two enzymes thought to be mainly responsible for dephosphorylating PKA sites (Cormier et al., 1990; Wadzinski et al., 1993; Zhang and Lee, 1997). We carried out in vitro phosphatase assays to determine whether progesterone caused any significant changes in oocyte phosphatase activities. As shown in Fig. 7, G2 oocytes contained significant levels of phosphatase activity capable of dephosphorylating a synthetic phosphopeptide (K-R-pT-I-R-R) (Harder et al., 1994). The phosphatase activities in G2 oocytes were eliminated by okadaic acid (100 nM), a highly specific inhibitor of PP2A (IC_{50}=1 nM) and, to a lesser extent, PP1 (IC_{50}=100-500 nM) (Bialojan and Takai, 1988). However, no significant changes were observed in the levels of phosphatase activities throughout progesterone-induced GVBD (Fig. 7).

To determine whether myr-HA-β_2AR-C could be adopted as an indicator of in vivo PKA activities in mammalian cells, we transfected COS-7 cells with myr-HA-β_2AR-C cDNA. Preliminary experiments indicated that unsynchronized COS-7 cells (transfected with myr-HA-β_2AR-C) that had been cultured in 10% FBS contained a single spot that co-migrated with spot 2 in frog oocytes (not shown). However, after culturing these cells for 24 hours in the absence of serum, myr-HA-β_2AR-C appeared as two spots of more or less equal intensities, corresponding to spot 1 and spot 2, respectively, in frog oocytes (Fig. 8A, left panel). A brief incubation of these cells with db-cAMP resulted in disappearance of spot 1 such that myr-HA-β_2AR-C migrated as a single spot (spot 2) (Fig. 8A, right panel). In contrast, myr-HA-β_2AR-C/PKA$^-$ migrated as a single spot 1 with or without db-cAMP (Fig. 8B).

To overcome the limitation of 2D electrophoresis to distinguish phosphorylation events caused by PKA compared to other protein kinases (e.g. Fig. 6), we sought to develop antibodies against PKA-phosphorylated myr-HA-β_2AR-C. We first wished to determine whether myr-HA-β_2AR-C was phosphorylated at Ser^{345}, Ser^{346} or both, although previous studies simply assumed β_2AR was doubly phosphorylated at both (Hausdorff et al., 1989). We generated two more myr-HA-β_2AR-C constructs, each bearing a single Ser to Ala mutation. As shown in Fig. 9A, in G2 oocytes myr-HA-β_2AR-C(S345A) appeared exclusively as spot 2 whereas myr-HA-β_2AR-C(S346A) only as spot 1. These results indicated that myr-HA-β_2AR-C was phosphorylated only at Ser^{346}. Our results represented the first direct biochemical evidence that the PKA phosphorylation site at the C-terminus of β_2AR was RRSp-S^{346}.

Antibodies against artificially phosphorylated PKA sites (RRp-S^{345}p-S^{346}) within the C-terminus of β_2AR are now

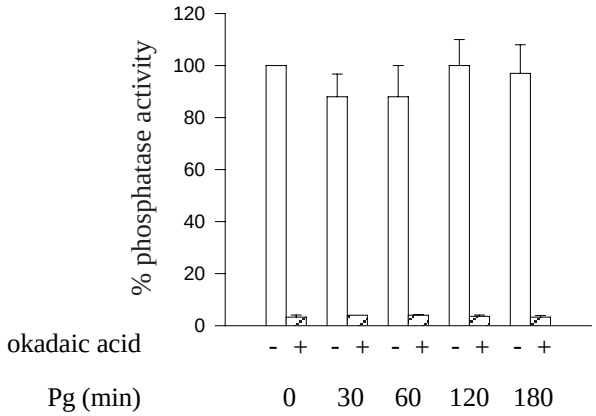

Fig. 7. Progesterone does not alter *Xenopus* oocyte serine/threonine phosphatase activities. Phosphatase activities were determined in the absence or presence of 100 nM okadaic acid. Means±s.e. of three (with okadaic acid) and five (without okadaic acid) independent experiments are shown. At least 50% of the oocytes at 180 minutes had undergone GVBD. The basal phosphatase activities in G2 oocytes were 76±6.5 nmol phosphate released per oocyte in 30 minutes.

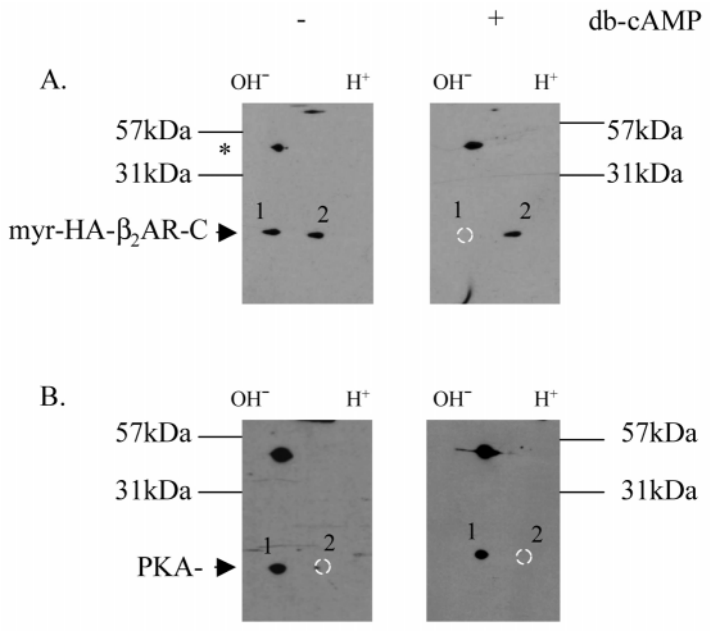

Fig. 8. db-cAMP stimulates PKA phosphorylation of myr-HA-β_2AR-C in COS-7 cells. COS-7 cells were transfected with myr-HA-β_2AR-C (A) or myr-HA-β_2AR-C/PKA$^-$ (B). The transfected cells were starved for 24 hours in serum-free DMEM and then either left untreated (left panels) or treated with 1.5 mM db-cAMP for 40 minutes (right panels). Cell lysates were prepared and analyzed by 2D electrophoresis and HA immunoblotting. Representative examples from three independent experiments are shown. Circles indicate missing spots. Asterisk indicates position of a nonspecific protein.

available (Santa Cruz Biotechnology). To test whether these antibodies recognize PKA-phosphorylated myr-HA-β_2AR-C in G2 oocytes, we injected mRNA for myr-HA-β_2AR-C (Fig. 9B, lane 5) and the various phosphorylation site mutants (lanes 1 to 4). Clearly, despite the fact that the antibodies were raised against doubly (artificially) phosphorylated (RRp-S^{345}p-S^{346}) peptide, they recognized myr-HA-β_2AR-C singly phosphorylated at Ser^{346} (lanes 1, 4 and 5). The weaker recognition of the S345A mutant (comparing lane 4 to lane 1) was probably caused by amino acid mismatch (Ala instead of Ser at position 345). As expected, progesterone treatment caused a complete loss of antibody recognition (lane 6), as did H89 treatment (lanes 9 and 10). In contrast, injection of PKAc restored antibody recognition, even in the presence of progesterone (lane 7). Expression and stability of the various forms of the substrate were confirmed by anti-HA immunoblotting.

With these phosphorylation-specific antibodies, we re-investigated the time course of myr-HA-β_2AR-C dephosphorylation during progesterone-induced oocyte maturation. Within 30 minutes of the addition of progesterone, myr-HA-β_2AR-C was significantly dephosphorylated (Fig. 9C, comparing lane 2 to lane 1). As our 2D analyses suggested, myr-HA-β_2AR-C was never re-phosphorylated at its PKA site during progesterone-induced oocyte maturation (lanes 3 to 5), indicating that PKA was never reactivated. Progesterone-induced dephosphorylation of myr-HA-β_2AR-C, and therefore inactivation of PKA, occurred long before the almost simultaneous accumulation/activation of Mos (Fig. 9C, xMos blot), MAP kinase activation (phosphorylation-specific shift of xMAP kinase) and MPF activation (phosphorylation-specific shift of cyclin B2). Interestingly, the protein synthesis inhibitor cycloheximide, which blocked oocyte maturation and activation of any of the protein kinases, did not affect progesterone-induced dephosphorylation of myr-HA-β_2AR-C, and hence inhibition of PKA (Fig. 9C, lanes 6-10).

Discussion

We have developed a novel approach to determine the activities of PKA in live cells. We chose the C-terminus of β_2AR as our candidate substrate motif for the following reasons. First, phosphorylation of the C-terminal RRSS^{346} of β_2AR by PKA has been demonstrated both in vitro and in vivo (Kemp et al., 1977; Doronin et al., 2000; Zamah et al., 2002). Second, PKA-catalyzed phosphorylation of β_2AR serves an important physiological function in promoting receptor desensitization (Hausdorff et al., 1989; Hausdorff et al., 1990). Third, the C-terminus of β_2AR, when expressed in isolation, retains its ability to be an efficient and specific substrate for PKA in vitro (Doronin et al., 2000). The decision to target β_2AR-C to the membrane was based on the belief that a membrane-bound β_2AR-C will more closely mimic its physiological precursor, β_2AR.

Despite the importance of cAMP and PKA in intracellular signal transduction, direct measurement of cAMP and PKA in live cells had not been possible until very recently. The best known are the several fluorescence resonance energy transfer (FRET)-based assays to analyze intracellular cAMP concentrations (Zaccolo et al., 2000) and PKA activities (Nagai et al., 2000; Zhang et al., 2001). These important advances clearly pave the way for analyzing PKA activities in live cells/organisms. However, the intrinsic background FRET (in the absence of PKA activation) and the overlapping fluorescence spectra of the donor and acceptor green fluorescence proteins (GFPs) result in rather modest FRET changes following pharmacological activation of PKA (with forskolin or db-cAMP) in live cells (Nagai et al., 2000). On the other hand, neither withdrawing db-cAMP from the culture medium nor the inclusion of PKA inhibitor (H-89) reverses the FRET change induced by db-cAMP (Zhang et al., 2001), raising the possibility that the substrate motif within AKAR (A-kinase activity reporter) may be phosphorylated by other protein kinases in addition to PKA in live cells. Recently, another group has reported the development of a cell-based

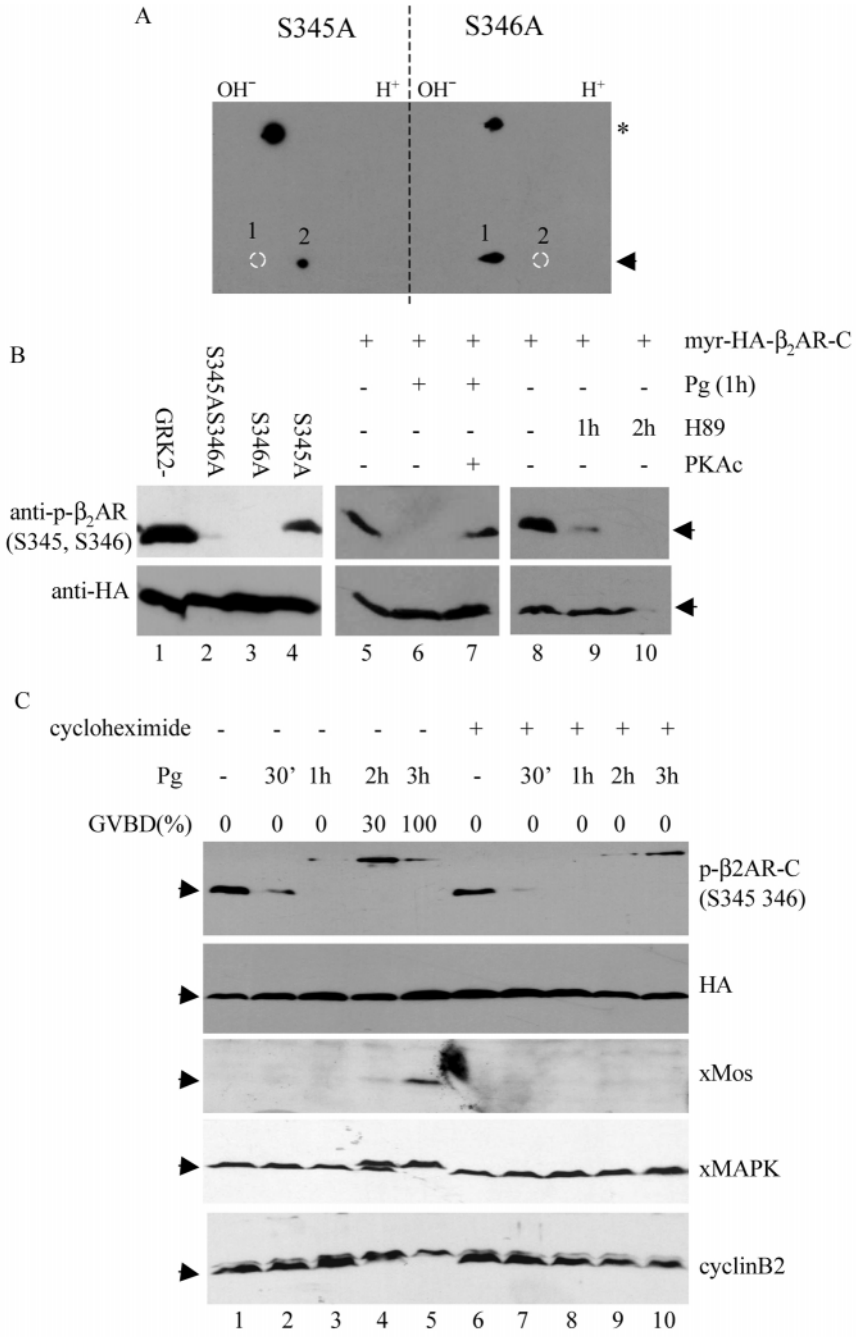

Fig. 9. Inhibition of PKA in the absence of de novo protein synthesis. (A) Oocytes were injected with mRNAs for myr-HA-β_2AR-C (S345A) or myr-HA-β_2AR-C(S346A). Following 24 hours incubation, PKA phosphorylation of the two mutants was analyzed by 2D electrophoresis followed by anti-HA immunoblotting. Arrow, myr-HA-β_2AR-C; asterisk, nonspecific protein. (B) Oocytes were injected with myr-HA-β_2AR-C/GRK2- (lane 1), myr-HA-β_2AR-C/PKA$^-$ (lane 2), myr-HA-β_2AR-C/S346A (lane 3), myr-HA-β_2AR-C/S345A (lane 4) or myr-HA-β_2AR-C (lanes 5-10). After 24 hours of incubation, oocytes in lanes 1-4 were directly lysed and analyzed by SDS-PAGE followed by immunoblotting with anti-phospho-β_2AR-C (S345 346) (upper panel) or anti-HA (lower panel). Oocytes in lane 6 were treated with progesterone for 1 hour. Oocytes in lane 7 were injected with PKAc (0.8 units) 1 hour prior to the progesterone treatment. Oocytes in lanes 9 and 10 were treated with 100 μM H89 for 1 or 2 hours respectively. Representative examples from three to five independent experiments are shown. (C) Oocytes were injected with mRNA for wild-type myr-HA-β_2AR-C. After 24 hours of incubation, half of the oocytes were pre-incubated for 2 hours in OR2 containing 50 μg/ml cycloheximide. Both groups were then incubated with progesterone. At the indicated times following the addition of progesterone, oocytes were scored for GVBD and ten oocytes were randomly withdrawn for lysis. The extracts were analyzed by SDS-PAGE and immunoblotted with the indicated antibodies. Shown are representative examples of five independent experiments.

assay to monitor intracellular PKA activity by using capillary electrophoresis and green fluorescence protein (GFP)-tagged substrate containing the PKA phosphorylation site consensus RRRp-SIN (Zarrine-Afsar and Krylov, 2003). However, it appears that in intact cells the peptide only exists in phosphorylated form, with no more than 1% of the substrate existing in unphosphorylated form under any conditions (judged from the data presented in the paper). Again, it seems possible that such a minimal consensus sequence, in the context of a carrier protein (GFP), may be subjected to more promiscuous phosphorylation by other protein kinases.

In contrast, myr-HA-β_2AR-C exhibited almost 100% convertibility between spot 1 and spot 2 when expressed in frog oocytes. In COS-7 cells, the convertibility was about 50%. Presumably, serum deprivation did not completely inhibit PKA activities in COS-7 cells. Furthermore, the RRSS^{346} motif appeared to be strictly a PKA phosphorylation site, as well as the only PKA phosphorylation site within myr-HA-β_2AR-C. Mutant proteins carrying RRAA^{346} in its position (PKA$^-$ and PKA$^-$&GRK$^-$) were not phosphorylated in G2 oocytes (Fig. 3E,G and Fig. 9), nor were they phosphorylated in COS-7 cells stimulated with db-cAMP (Fig. 8B). In GVBD oocytes, on the other hand, myr-HA-β_2AR-C appeared to be phosphorylated by one or more protein kinases that become activated at this time. However, the fact that all four constructs exhibited the same pattern of phosphorylation argued strongly that neither

the PKA site (RRSS^{346}) nor any of the GRK sites were phosphorylated in GVBD oocytes. Nonetheless, the presence of these multiple phosphorylation forms precluded the use of 2D analyses of myr-HA-β_2AR-C to determine PKA activities in GVBD oocytes. This was confirmed using antibodies against the β_2AR peptides carrying doubly phosphorylated PKA site (RRp-Sp-S^{346}) (Fig. 9C).

Earlier studies have clearly implicated cAMP and PKA as major players in maintaining G2 arrest. Forskolin (Schorderet-Slatkine and Baulieu, 1982) and cholera toxin (Maller et al., 1979), two highly specific cAMP-elevating agents, are both potent inhibitors of progesterone-induced oocyte maturation. Furthermore, whereas injection of PKAc inhibits progesterone-induced oocyte maturation, injection of PKAr or another peptide inhibitor of PKA (PKI) induces hormone-independent oocyte maturation (Maller and Krebs, 1977). On the other hand, considerable controversies still exist regarding the endogenous cAMP levels during progesterone-induced oocyte maturation. Overall, there appears to be a consensus that progesterone causes a rapid (within minutes) but modest (~20%) reduction of cAMP (Smith, 1989). However, some have also reported that this reduction is transient, with levels of cAMP recovered quickly (within 10-20 minutes) (Maller et al., 1979), whereas others have reported more persistently reduced levels of cAMP throughout the maturation process (Bravo et al., 1978). The basal cAMP concentration in G2 oocytes is estimated as 1 pmol/oocyte (Maller et al., 1979) (V. Montplaisir and X. J. Liu, unpublished data) or 1 μM (assuming 1 μl as the average volume of a sphere of 1.2-1.3 mm diameter). This is close to the apparent activation constant of type II protein kinase A (or PKA II, determined by the regulatory subunit RII) for cAMP (0.54 μM) (Dostmann and Taylor, 1991). Significantly, PKA II is the predominant form of PKA in frog oocytes (Masaracchia et al., 1979; Schmitt and Nebreda, 2002). Therefore, a modest reduction of oocyte cAMP by progesterone would have functionally significant effects on PKA activities in intact oocytes.

For the first time, we have demonstrated that endogenous PKA is activated in G2 oocytes by showing that a PKA substrate expressed in these oocytes is fully phosphorylated on the PKA site. Furthermore, we have demonstrated that progesterone caused an almost complete loss of PKA phosphorylation of the substrate within 30-60 minutes, long before MAP kinase activation or MPF activation that usually occurred simultaneously several hours after the addition of progesterone. The rapid loss of PKA phosphorylation site was probably the result of dephosphorylation by the significant levels of okadaic acid-sensitive phosphatase activities that were present in G2 oocytes and remained unchanged throughout the maturation process (Fig. 7). In G2 oocytes, PKA was dominant and therefore myr-HA-β_2AR-C was fully phosphorylated on its PKA site, despite these phosphatase activities. Progesterone caused a rapid reduction of oocyte cAMP and the corresponding inhibition of PKA. In the absence of PKA activities, myr-HA-β_2AR-C was rapidly dephosphorylated. However, not all PKA substrates were dephosphorylated equally in the absence of PKA activities. For example, dephosphorylation of the inhibitory PKA site on Cdc25C occurs much later and coincides with activation of MAP kinase and MPF (Duckworth et al., 2002). Presumably, the PKA phosphorylation site of Cdc25C is protected from phosphatase activities until this threshold point in the maturation process: the 'switch' point described by Ferrell and Machleder (Ferrell and Machleder, 1998).

Perhaps the most important contribution of our study is the demonstration that progesterone caused a rapid and permanent inhibition of PKA during oocyte maturation (Figs 5, 6 and 9). This was in contrast to the prevailing view that progesterone causes a 'transient' reduction of cAMP. The notion of a transient reduction of cAMP (or inhibition of PKA) was clearly not compatible with several recent studies demonstrating that PKA phosphorylates Cdc25C and inhibits its activities towards Cdc2 (Schmitt and Nebreda, 2002; Duckworth et al., 2002). As Cdc25C is an immediate activator of Cdc2 and its activation coincides with activation of MAP kinase and MPF (Izumi and Maller, 1993; Duckworth et al., 2002), PKA must be inhibited at this time. Our data clearly support such a notion.

Finally, the demonstration of PKA inhibition in the absence of de novo protein synthesis (Fig. 9C) established that progesterone-induced PKA inhibition was truly an early event dissociable from all other biochemical (activation of numerous protein kinases) and cytological (germinal vesicle breakdown) events that are dependent on de novo protein synthesis. The inhibition of PKA and the maintenance of PKA inhibition did not require any feedback mechanism involving Mos-MAP kinase pathway or MPF activity. Together with earlier work that demonstrates inhibition of PKA alone is sufficient to induce oocyte maturation (Maller and Krebs, 1977), our data clearly establish that PKA plays a dominant role in maintaining oocyte G2 arrest and that rapid and permanent suppression of PKA activity is the most prominent biochemical event in hormonal induction of re-initiation of meiosis (oocyte maturation).

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