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Original Article

Activation of an 85 kDa ribosomal S6 kinase during serotonin-induced oocyte maturation

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ABSTRACT **Oocytes from the Japanese clam *Ruditapes philippinarum* are naturally blocked at the prophase-I stage of meiosis. Following physiological activation by the neurohormone serotonin (5HT), oocytes undergo germinal vesicle breakdown (GVBD) and reach a second cell cycle arrest in metaphase-I. To identify the kinases activated during meiosis reinitiation, we used a phosphorylation assay following sodium dodecyl sulphate-polyacrylamide gel electrophoresis and *in situ* renaturation. A soluble 85-kDa serine/threonine kinase (PK85) was highly and consistently activated (up to 17-fold) within 5 minutes following addition of the hormone. This activation occurred 5 to 10 minutes before GVBD and only when 5HT concentration was sufficient to induce meiosis reinitiation. The calcium ionophore A23187 and NH_4Cl, two compounds known to induce GVBD by increasing intracellular calcium concentration, also activate PK85. In crude oocyte extracts, the presence of ß-glycerophosphate, NaF, okadaic acid, calyculin A or microcystin, prevented inactivation of PK85, suggesting that it is activated by phosphorylation. Partial purification of PK85 followed by Western blotting showed that this kinase is related to the ribosomal S6 kinase $pp90^{rsk}$. PK85 phosphorylates the peptides LRRASLG (kemptide) and PLARTLSVAGLPGGK (syntide-2), and to a lesser extent the synthetic polyamino acids poly(R_3:S_1) while myelin basic protein (MBP), histone III-S, casein, the peptides PEKRPSQRSKYL ($(pGlu^4)$-MBP 4-14), GTFRASIRRLAARRR (NIMA kinase substrate), the protein kinase C (PKC) substrate LRTLRR and the synthetic polyaminoacids poly(R_1:P_1:T_1) were poor substrates. 5HT-induced GVBD and PK85 activation are both inhibited by the phorbol ester 12-myristate 13-acetate (PMA) and this inhibition can be reversed by 5 µM of the bisindolylmaleimide GF109203X, a potent PKC inhibitor. PMA inhibitory action appears to take place between 5HT binding to its receptor and the intracellular calcium surge since it has no effect on GVBD induced by calcium ionophore A23187 and thapsigargin. Taken together, these results suggest that serotonin-induced activation of PK85 occurs after the intracellular calcium surge in a PKC-independent pathway.**

KEY WORDS: *renaturation, RSK, signal transduction, cell cycle*

Introduction

Protein phosphorylation plays a major role in the regulation of cell cycle progression during meiosis and mitosis (for review, see Pelech *et al.*, 1990). The use of oocytes from marine invertebrates has become a system of choice for studies on protein kinase cascades activated during meiotic and mitotic cell cycle progression, since they can be easily obtained in large amounts and because they respond quite synchronously to external stimuli. In oocytes from various bivalve species which are blocked at the first meiotic prophase stage, the neurohormone serotonin (5HT) is the natural signal that induces the G2/M transition in the cell cycle (Hirai *et al.*, 1988). This event is accompanied by an easily observable phenomenon, the dissolution of their huge nucleus, also termed germinal vesicle breakdown (GVBD), and depends upon activation of the universal M phase-promoting factor (MPF) (for reviews see Eckberg, 1988; Guerrier *et al.*, 1990;

Abbreviations used in your paper: ASW, artificial sea water; CAPS, 3-[cyclohexyl-amino]-1-propanesulfonate; DAG, diacylglycerol; ERK, extracellular signal regulated kinase; 5HT, 5-hydroxytryptamine; IP_3, inositol 1,4,5-trisphosphate; MAP, microtubule-associated protein; MBP, myelin basic protein; MOPS, 3-[N-morpholino]propane sulfonate; PKI, protein kinase inhibitor; PMA, phorbol 12-myristate 13-acetate; PMA^{met}, phorbol 12-myristate 13-acetate 4-O-methyl ether; PMSF, phenylmethylsulfonyl fluoride; poly[R_1:P_1:T_1], polymer of arginine:proline:threonine (1:1:1); poly[R_3,S_1], copolymer of arginine:serine (3:1); poly[E_4,Y_1], copolymer of glutamate:tyrosine (4:1); pS, phosphoserine; pT, phosphothreonine; PTK, protein tyrosine kinase; PVDF, polyvinylidenedifluoride; pY, phosphotyrosine; RSK, ribosomal protein S6 kinase; SBTI, soybean trypsin inhibitor; TCA, trichloroacetic acid.

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Pelech *et al.*, 1990; Honigberg *et al.*, 1993). In oocytes of the Japanese clam *Ruditapes philippinarum*, 5HT binding to a specific membrane receptor induces GVBD within 12-18 min and formation of the metaphase-I spindle. A second arrest in the cell cycle then occurs which can be released by binding and penetration of the spermatozoa, leading to extrusion of the two polar bodies, formation of the male and female pronuclei, and cell division.

The intracellular mediators that link stimulation of the 5HT-receptors to dissolution of the nuclear envelope are presently unknown. Recent work suggests that an intracellular calcium surge constitutes an essential step preceding GVBD in both *Ruditapes philippinarum* and the surf clam *Spisula solidissima* (for review see Abdelmajid *et al.*, 1993). Indeed drugs such as calcium ionophores, thapsigargin, ammonia and procaine, which trigger an elevation of intracellular calcium, also induce prophase-I arrested oocytes to resume meiosis (Dubé, 1992; Guerrier *et al.*, 1993). In *Spisula* oocytes, protein kinase C (PKC) is suspected to play a role in the process of meiosis reinitiation since the phorbol ester PMA can induce GVBD (Dubé *et al.*, 1987; Eckberg *et al.*, 1987). On the other hand, the intracellular cascade induced by 5HT differs in *Ruditapes* as PMA inhibits 5HT-induced maturation in a dose-dependent fashion (Gobet *et al.*, 1994).

In this study, we used an *in situ* renaturation method after protein electrophoresis (Kameshita and Fujisawa, 1989; Durocher *et al.*, 1992) to characterize the modulation of protein kinase activities that follows addition of 5HT to *Ruditapes philippinarum* oocytes. Our results show that among multiple renatured kinases, a major soluble serine/threonine kinase of 85 kDa (PK85) is rapidly activated. The same activation may be obtained following treatment with ionophore A23187 or NH_4Cl, suggesting that it follows the intracellular calcium surge. Partial purification followed by Western blotting and substrate specificity assays showed that this kinase is related to the ribosomal S6 kinase $pp90^{rsk}$. Finally, it appears that the intracellular pathway leading to its activation is independent of PKC.

Results

Early activation of an 85 kDa kinase by 5HT

The pattern of renatured protein kinases modulated *in vivo* by 5HT is shown in Figure 1A. Among multiple renatured kinases, a strong activation (11.6±4.2-fold, n= 5) of an 85 kDa (PK85) one consistently occurred within 5 min following the addition of the hormone, and remained over basal activity for at least 60 min. Figure 1B shows the relationship between the kinetics of PK85 activation and GVBD. While GVBD occurred in about 12-18 min at room temperature, maximal PK85 activation was observed within 5 min after 5HT addition. A decrease in PK85 activity was then observed and varied among different batches of oocytes, ranging between 20% and 90% of the peak level. Slight activation of two other kinases of 67 kDa and 60 kDa, together with an increase in the apparent molecular weight of PK160 after 20 min also occurred. Activation of a 33 kDa kinase after 20 min of treatment and inhibition of two kinases of 63 kDa and 43 kDa was also observed, but somewhat less consistently.

Serotonin concentration dependence for GVBD and PK85 activation

Forty minutes after the addition of increasing 5HT concentrations, the percentage of oocytes which underwent GVBD was estimated (Fig. 2, upper panel); the activity of PK85 was monitored in parallel using the renaturation assay following SDS-PAGE (lower panel). Comparison of panel A with panel B shows that the minimum 5HT concentration required to induce oocyte maturation (10^{-7} M) is the same as that required for full PK85 activation. This concentration is in the physiological range already described for 5HT-competent oocytes (Guerrier *et al.*, 1993).

PK85 is activated by mimetics

Previous studies have shown that an increase in intracellular free calcium is an essential step for meiosis reinitiation in clam oocytes (Dubé, 1992; Guerrier *et al.*, 1993). Indeed, the calcium

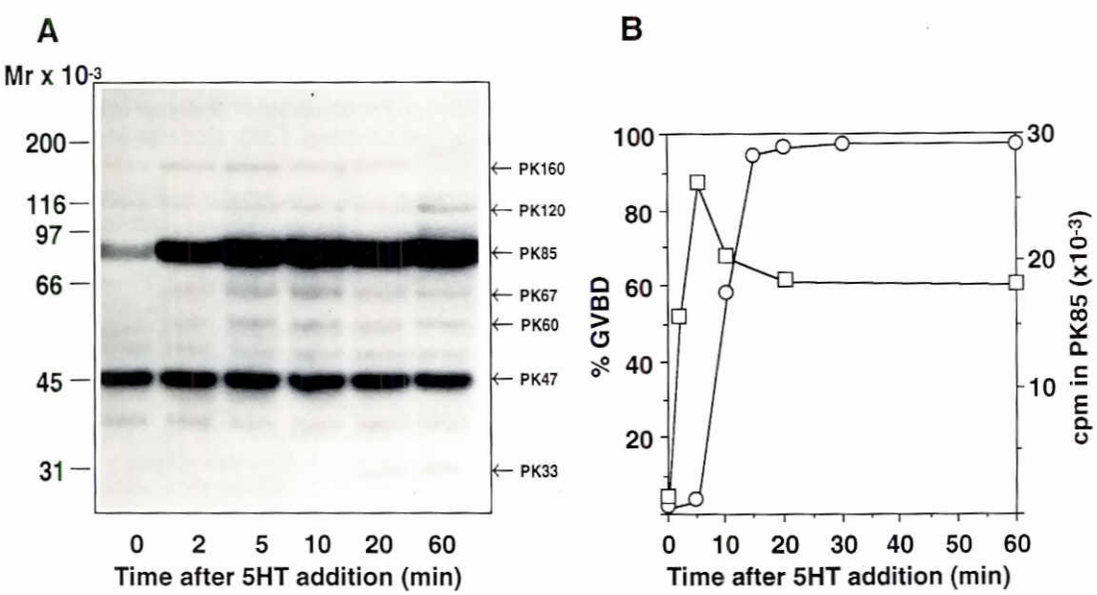

Fig. 1. Time course of 5HT-induced GVBD and kinases modulation in *Ruditapes* oocytes. *Oocytes were exposed to 1 μM 5HT and rapidly harvested by centrifugation followed by solubilization in SDS-sample buffer for in situ renaturation and phosphorylation following electrophoresis as described in Materials and Methods.* **(A)** *^{32}P-labeling was revealed by autoradiography (4 h exposure at -80°C).* **(B)** *^{32}P incorporation in PK85 was determined by liquid scintillation (open squares) and compared to oocyte activation as determined by GVBD estimation (open circles).*

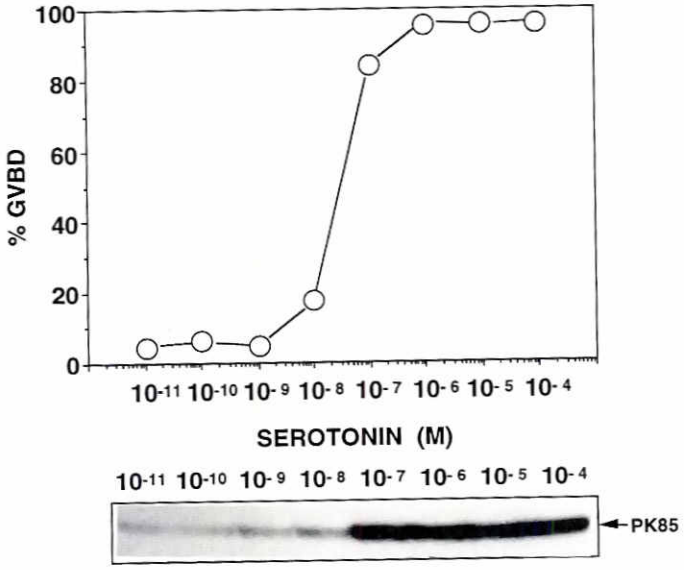

Fig. 2. Serotonin concentration dependence for GVBD and activation of PK85. *Oocytes were treated with the indicated concentrations of 5HT and aliquots were kept for GVBD determination after 40 min* **(upper panel).** *The remaining oocytes were harvested 20 min after 5HT addition and processed for in situ renaturation and phosphorylation* **(lower panel)**.

ionophore A23187 and ammonia, two compounds known to induce an intracellular calcium surge in *Ruditapes* oocytes, also induce GVBD (Guerrier *et al.*, 1993). Their effects on PK85 activation were thus monitored. As shown in Figure 3, both A23187 (lane 3) and NH_4Cl (lane 4) induced GVBD (upper panel) and PK85 activation (lower panel).

PK85 activity is stabilized by phosphatase inhibitors

Since the activated state of PK85 resists heat-denaturation in the presence of SDS and β-mercaptoethanol, a covalent modification of the enzyme should be responsible for its activation. In view of the important loss of kinase activity that occurs when crude homogenates are prepared in the absence of phosphatase inhibitors (Fig. 4: compare lane 2 with lane 1), this modification is likely to be a phosphorylation. Indeed, PK85 activity can be effectively stabilized by the presence of 50 mM β-glycerophosphate (lane 4), 50 mM sodium fluoride (lane 5), 200 nM okadaic acid (lane 6), 200 nM calyculin A (lane 7) and 200 nM microcystin (lane 8). Quantification of ^{32}P-incorporation into PK85 is graphically represented in the upper panel. Molybdate (50 mM) and vanadate (500 μM) were ineffective as inhibitors (not shown).

PK85 has a strict Ser/Thr-kinase activity

Phosphoamino acid analysis of the major renatured kinases observed in Figure 1 (PK160, PK120, PK85, PK67, PK60, PK47 and PK33) revealed that the γ phosphate transferred from (γ-^{32}P)ATP occurred exclusively on serine and threonine residues (not shown). To ascertain that PK85 has a strict Ser/Thr-kinase activity and to detect a putative protein tyrosine kinase (PTK) activity, we added the tyrosine-containing synthetic copolymer poly(E_4:Y_1) directly to the gel prior to polymerization (Durocher *et al.*, 1992). Following electrophoresis and *in situ* renaturation and phosphorylation, two main phosphotransferases of 85 and 47 kDa were detected in the presence of this polymer (Fig. 5), but PK85 activity appeared lower than that seen without the synthetic substrate. Phosphoamino acid analysis demonstrates that while PK47 is able to phosphorylate tyrosine residues present in the added copolymer, PK85 has a strict Ser/Thr-kinase activity. However, PK47 activity did not appear to increase following 5HT stimulation since its phosphotyrosine content remained constant for up to 20 min (not shown).

PK85 is related to the ribosomal S6 kinase II

Subcellular fractionation of 5HT-treated oocytes into particulate (P) and soluble (S) fractions showed that PK85 is a soluble kinase (Fig. 6B). Further characterization of PK85 was undertaken by partial purification on a cation exchange chromatography column (Fig. 6A). Renaturation of aliquots from collected fractions was performed in order to detect PK85 activity. Figure 6B shows that PK85 is mainly found in the NaCl eluate (SE) from the Fast-S Sepharose cation exchange column. Preliminary studies using various antibodies directed toward potentially activated kinases during serotonin-induced oocyte activation (MEKK, B-Raf1, PKCζ, $pp90^{rsk}$, $p70/p85^{s6k}$ and NIMA) showed that only anti-$pp90^{rsk}$ clearly recognized a protein of 85 kDa in oocyte lysates. Immunodetection of the same fractions as in panel B shows that an 85 kDa immunoreactive band co-elutes with PK85 (Fig. 6C). This eluate was then precipitated with ammonium sulfate, applied on a Mono-Q anion exchange col-

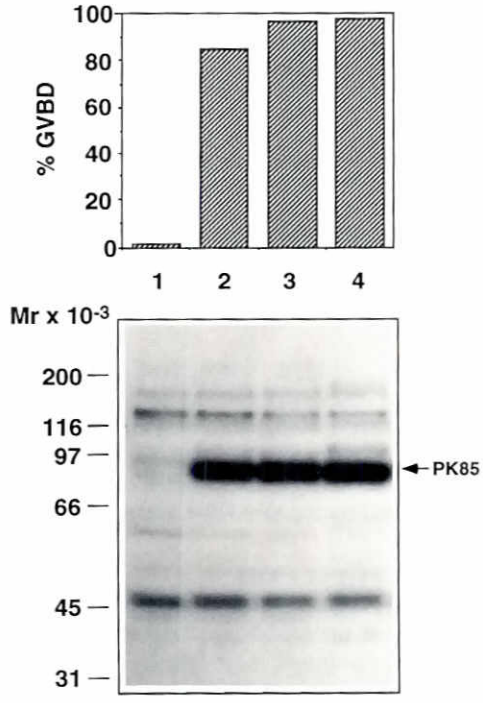

Fig. 3. PK85 activation by mimetics. *Oocytes were stimulated with the following drugs for 20 min: (1) control; (2) 1 μM 5HT; (3) 10 μM A23187; (4) 20 mM NH_4Cl (in ASW buffered with 5 mM Tris pH 8.0). GVBD was estimated on an aliquot fraction* **(upper panel)** *and the remaining oocytes were processed for in situ renaturation and phosphorylation* **(lower panel)**.

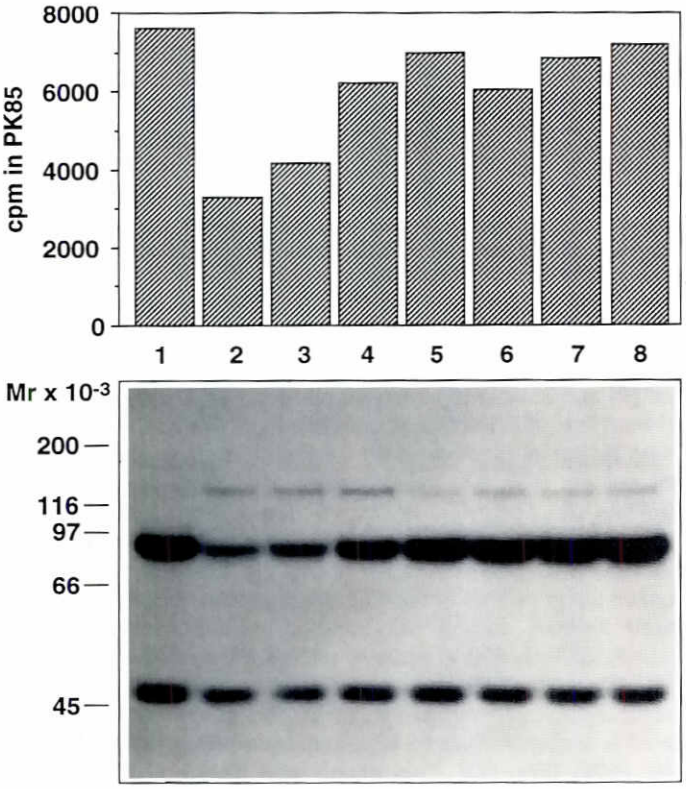

Fig. 4. Stabilization of PK85 activity in the presence of phosphatase inhibitors. *Oocytes were activated for 20 min with 1 μM 5HT and solubilized in a non-denaturing buffer containing 20 mM MOPS pH 7.2, 0.2% Thesit®, 50 μg/ml SBTI, 50 μg/ml leupeptin and 20 μg/ml pepstatin. Lysates were incubated in the absence (lane 2) or presence of 10 mM ß-glycerophosphate (lane 3), 50 mM ß-glycerophosphate (lane 4), 50 mM NaF (lane 5), 200 nM okadaic acid (lane 6), 200 nM calyculin A (lane 7) or 200 nM microcystin (lane 8). After 30 min incubation at room temperature, the reactions were stopped by addition of 2X SDS-sample buffer and boiling. For the control (lane 1), 2X SDS-sample buffer was immediately added to the lysate. The samples were then processed for in situ renaturation and phosphorylation* **(lower panel)**. *Radioactivity incorporated into PK85 was quantified by liquid scintillation counting* **(upper panel)**.

umn and eluted using a linear NaCl gradient (Fig. 6D). Renaturation and immunodetection of the collected fractions (Fig. 6E and 6F, respectively) clearly show that PK85, eluting in fractions 19-21, is indeed related to the ribosomal S6 kinase pp90^{rsk}, since an 85 kDa protein present in the same fractions is strongly recognized by the anti-RSK antiserum.

Substrate specificity of PK85

Crude cytosolic extracts from resting or 5HT-treated oocytes were first assayed toward exogenous substrates. Figure 7A shows that 5HT-treatment significantly increases phosphotransferase activities toward MBP, kemptide and histone III-S. Kemptide kinase activation was further evidenced when the assay was performed in the presence of 500 nM PKI, a potent protein kinase A inhibitor. Fractions 19-21 from the Mono-Q column were then pooled and assayed to determine the substrate specificity of PK85 (Fig. 7B). Among substrates tested and as already described for pp90^{rsk} (Erikson and Maller, 1988; Erikson *et al.*, 1991), kemptide was the best phosphoacceptor for PK85. This figure also shows that the purified fraction is essentially devoid of MBP, histone and PKI-sensitive kinase activities.

5HT-induced GVBD and PK85 activation are blocked by phorbol ester

The phorbol ester PMA has been shown to induce GVBD in oocytes from various species. However, in the Japanese clam *Ruditapes philippinarum*, PMA alone is unable to promote meiosis reinitiation, but rather blocks the effect of 5HT in a dose-dependent fashion (Gobet *et al.*, 1994). Figure 8A shows that the inhibitory effect of PMA on GVBD can be partially reversed by increasing 5HT concentration while the biologically inactive phorbol ester PMA^{met} is without effect. Moreover, while 5 μM of the potent PKC inhibitor GF109203X (Toullec *et al.*, 1991) has no effect on GVBD, it completely prevents PMA-mediated inhibition, showing that this phorbol ester acts through the activation of a PKC. We previously showed that PMA must be added during the 5HT-dependent period to exert its negative effect, which results in the inhibition of the intracellular calcium surge (Gobet *et al.*, 1994). To further support the hypothesis that the inhibition occurs before the intracellular calcium surge, we showed that PMA has no effect on GVBD induced by the calcium ionophore A23187 and thapsigargin (Fig. 8B), an inhibitor of microsomal Ca^{2+} ATPases (Thastrup *et al.*, 1989).

The effect of PMA on 5HT-induced activation of PK85 was also studied. Figure 9 shows that while PMA alone cannot induce GVBD (lane 3, upper panel) nor modulate the activity of renatured kinases (lower panel, compare with lane 1), it strongly inhibits 5HT-induced PK85 activation (lane 4). While the bisindolylmaleimide GF109203X has no effect on 5HT-induced PK85 activation (compare lane 5 with lane 2), it reverses the inhibitory action of PMA on this kinase (lane 6).

Discussion

Using a method for renaturing protein within the gel matrix following SDS-PAGE, we have evidenced a soluble Ser/Thr-kinase of 85 kDa (PK85) whose activity increased up to 17-fold within 5 min following serotonin addition to *Ruditapes philippinarum* oocytes. Since PK85 is also activated by calcium ionophore A23187 and ammonia, two compounds which increase intracellular free calcium in *Ruditapes philippinarum* (Guerrier *et al.*, 1993), this suggests that PK85 activation occurs downstream of the calcium surge. The observed stability of PK85 activity in the presence of Ser/Thr-phosphatase inhibitors suggests that phosphorylation by an upstream kinase is responsible for its activation. Partial purification of PK85 by ion exchange chromatographies followed by Western blotting using a polyclonal antiserum (Rachie *et al.*, 1993) directed against residues 707-724 of the mouse *rsk1* gene (Alcorta *et al.*, 1989) showed that it is immunologically related to the ribosomal S6 kinase pp90^{rsk}. This was further supported by substrate specificity where kemptide is the best phosphoacceptor assayed. Thus, PK85 represents a marine invertebrate homolog of the growing family of ribosomal S6 kinases (for review see Pelech and Shangera, 1992; Blenis, 1993).

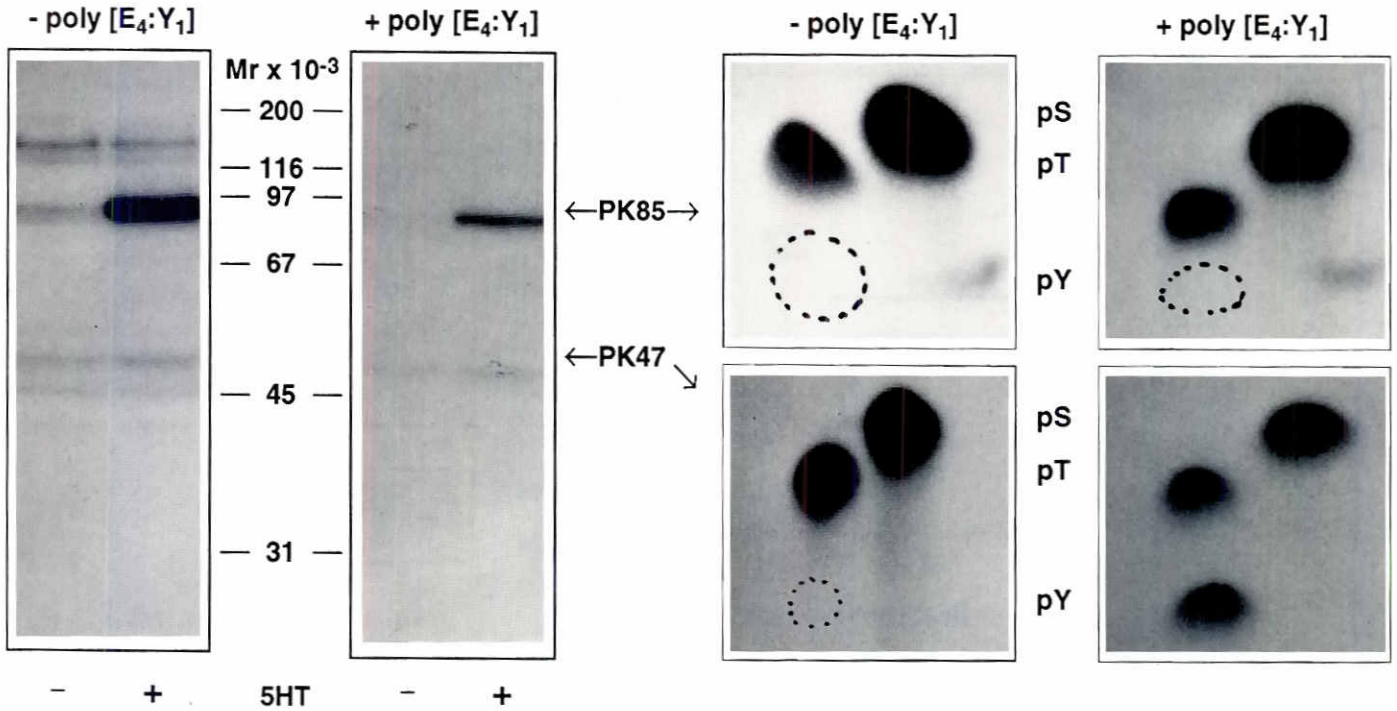

Fig. 5. PK85 has a strict Ser/Thr kinase activity. *Oocytes were stimulated (+) or not (–) with 1 μM 5HT for 10 min and processed for renaturation and in situ phosphorylation (left panels) in a control gel [-poly(E_1:Y_4)] or a gel containing 100 μg/ml poly(E_1:Y_4) as a substrate for putative renatured PTKs as described in Materials and Methods. PK85 and PK47 from 5HT-treated oocytes were excised from the gels for phosphoamino acids analysis by two-dimensional thin layer electrophoresis (right panels). pS, phosphoserine; pT, phosphothreonine; pY, phosphotyrosine.*

Signal transduction cascade linking 5HT receptors to PK85 activation and GVBD

Recent data suggest that 5HT receptors of *Ruditapes philippinarum* must be coupled to G proteins to be functional (Gobet *et al.*, 1994). Indeed, mastoparan and mas-7, two G-protein activators (Higashijima *et al.*, 1990), facilitate oocyte maturation when delivered simultaneously with subthreshold levels of 5HT. Iontophoresis of GTP and GTPγS triggered GVBD in 14 out of 19 oocytes tested. Also, a significant increase of inositol 1,4,5-trisphosphate (IP_3) has been clearly demonstrated which peaks 3 min after addition of 5HT. Thus, 5HT signal transduction is mediated via phospholipase C which produces IP_3 and diacylglycerol (DAG), the former inducing the observed intracellular calcium surge (Guerrier *et al.*, 1993) while the latter would activate a phorbol ester/DAG-sensitive PKC. Indeed, PMA has been shown to induce GVBD in oocytes from the surf clam *Spisula solidissima* (Dubé *et al.*, 1987; Eckberg *et al.*, 1987), the annelid *Chaetopterus* (Eckberg and Palazzo, 1992) and the frog *Xenopus laevis* (Stith and Maller, 1987; Chung *et al.*, 1992). It has also been reported to activate MAP kinase and $pp90^{rsk}$ *in vivo* (Meier *et al.*, 1991; Gause *et al.*, 1993) and *in vitro* when added to a mixture containing recombinant PKC, Raf and MEK (Marquardt *et al.*, 1994). In *Ruditapes philippinarum* however, PKC is unlikely to be required for PK85 activation and GVBD: first, those phenomena were never induced under the influence of PMA; second, both 5HT-induced PK85 activation and GVBD are abolished *in vivo* by this phorbol ester, and third, the PKC-specific inhibitor GF109203X neither blocks 5HT-induced PK85 activation nor GVBD (when present at 5 μM) but rather releases oocytes from PMA-induced inhibition. The PMA inhibitory action on *Ruditapes* oocytes is similar to that found in starfish oocytes where it has been shown to block 1-methyladenine-induced GVBD (Kishimoto *et al.*, 1985).

We also showed that PMA-induced inhibition of GVBD represses the 5HT-generated intracellular calcium surge in *Ruditapes philippinarum* (Gobet *et al.*, 1994), a situation which has also been described in hamster eggs (Miyazaki *et al.*, 1990). The observation that PMA must be added within 3 min after 5HT addition to effectively block GVBD, an interval which corresponds to the hormone-dependent period for 5HT and which is immediately followed by the intracellular calcium surge (Gobet *et al.*, 1994), could explain why PMA has no effect on meiosis reinitiations induced by calcium ionophore A23187 or thapsigargin. Thus, PKC may inhibit transduction of the 5HT signal through phosphorylation of the receptor itself, its associated G-proteins or phospholipase C. Taken together, these results also suggest that PK85 activation occurs following the elevation of intracellular calcium. As already described for RSK (Erikson, 1991), PK85 activity appears to be positively regulated by its phosphorylation. Since MAP kinase may participate in the phosphorylation and activation of RSK and has been shown to be activated in less than 3 min following 5HT-induced meiosis reinitiation (Abdelmajid *et al.*, 1994), it may be the kinase responsible for PK85 phosphorylation and activation. Interestingly, a calcium-dependent but PKC-independent activation of MAP kinase has been demonstrated in fibroblasts and A431 cells using thapsigargin (Friedman *et al.*, 1989; Chao *et al.*, 1992).

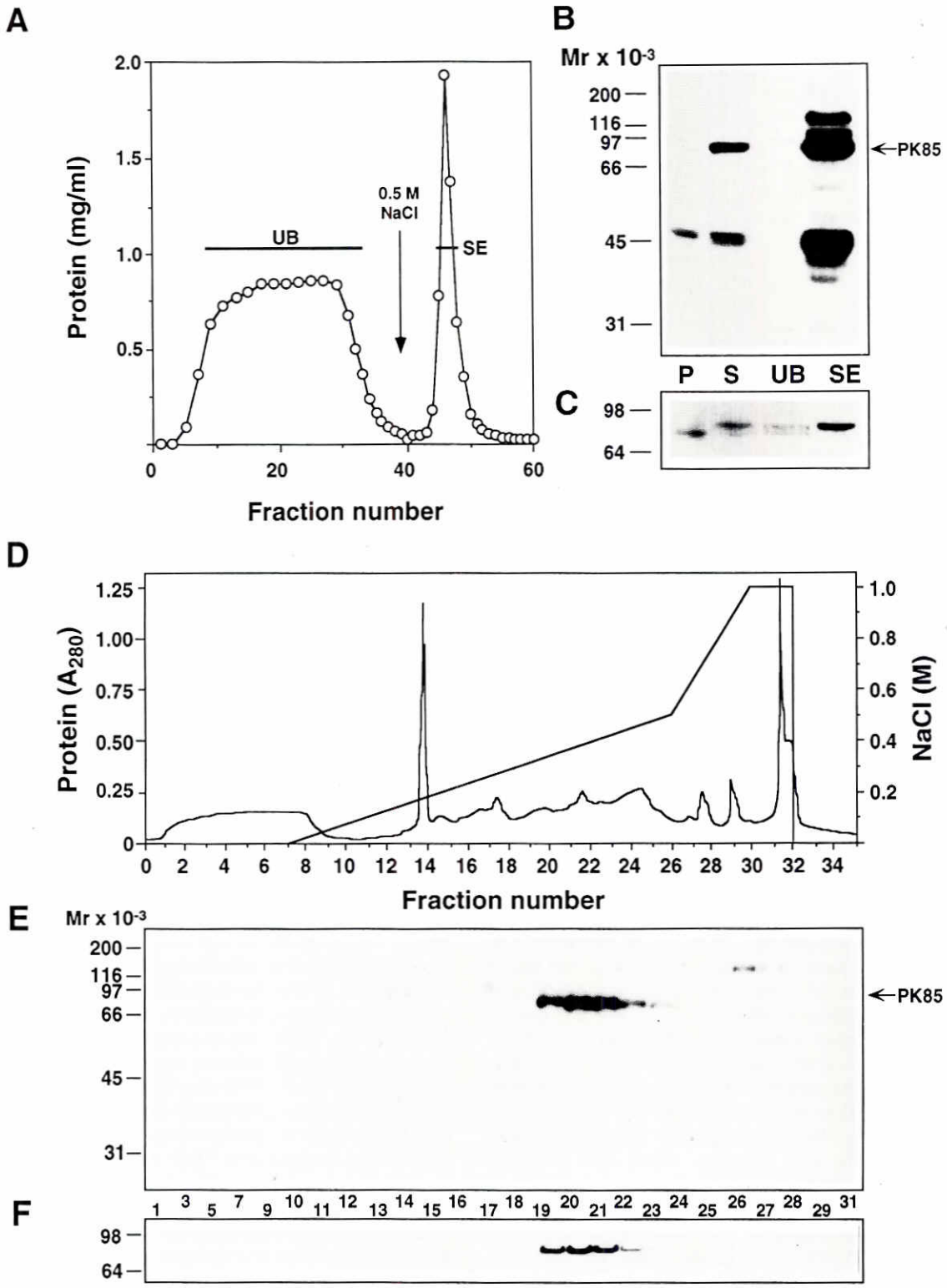

Fig. 6. Subcellular distribution, partial purification and immunodetection of PK85 with anti-pp90^{rsk} antiserum. *Metaphase-I oocytes (20 min after 5HT addition) were fractionated in soluble (S) and particulate (P) fractions and the soluble fraction used for partial purification of PK85 on a Fast-S Sepharose column* **(panel A)**. *Aliquots (25 µg of protein) from pooled (delimited by solid bars) unbound (UB) and salt-eluted (SE) fractions from panel A, as well as particulate and soluble fractions were submitted to electrophoresis for in situ renaturation and phosphorylation* **(panel B)** *or Western blotting using anti-pp90^{rsk} antiserum* **(panel C)**. *Further purification of pooled SE fractions was performed on a Mono-Q column as described in Materials and Methods (***D,** *protein elution profile;* **E,** *in situ renaturation and phosphorylation;* **F,** *immunodetection with anti-pp90^{rsk} antiserum).*

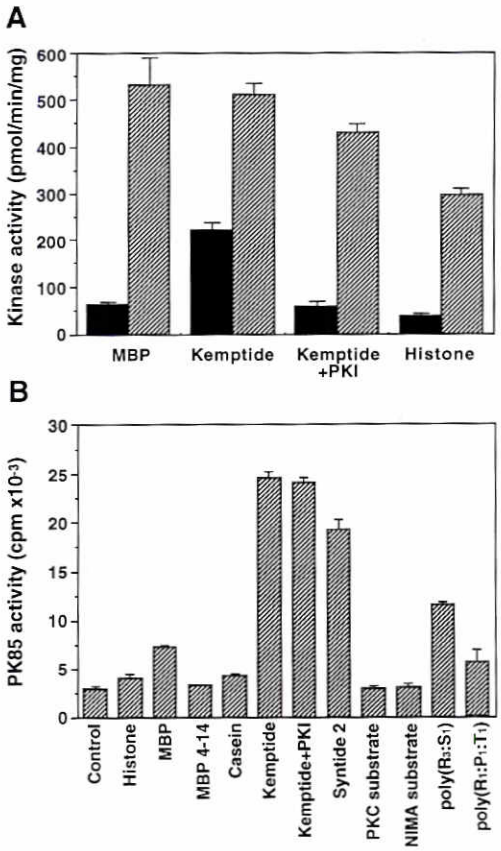

Fig. 7. Kinase activities of crude cytosolic extracts and substrate specificity of partially purified PK85. (A) *Crude cytosolic extracts from resting (closed box) or 5HT-activated (hatched box) oocytes were used for kinase assay toward MBP, kemptide (alone or in the presence of 500 nM PKI) and histone III-S.* **(B)** *Fractions 19-21 from Mono-Q column (Fig. 6) were pooled and used to study substrate specificity of PK85 as described in Materials and Methods. For both panels, mean±SEM of triplicate determinations are shown.*

Potential intracellular substrates of RSK

In progesterone-treated *Xenopus* oocytes, maximal activation of a 92 kDa ribosomal S6 kinase occurs just prior to GVBD (Cicirelli *et al.*, 1988; Martin-Perez *et al.*, 1988; Erikson and Maller, 1989) while in 1-methyladenine treated starfish oocytes maximal S6 kinase activation occurs shortly after GVBD (Pelech *et al.*, 1987). This contrasts with the situation observed in *Ruditapes philippinarum* where maximal activity of PK85 clearly precedes GVBD. While one of the best known *in vitro* substrates of RSK is the ribosomal protein S6, this protein instead appears to be the physiological substrate of $p70^{s6k}$ (Blenis, 1993). Accordingly, in progesterone-treated *Xenopus* oocytes, a 10-fold activation of $p70^{s6k}$ occurs within 1 h, 3 h earlier than that of $pp90^{rsk}$ (Lane *et al.*, 1992), and would be responsible for the early S6 phosphorylation observed during meiotic maturation in this species (Cicirelli *et al.*, 1990). Among physiological substrates for RSK, nuclear lamins have been described as potent candidates (Ward and Kirschner, 1990). Lamin phosphorylation is indeed causally related to the transient disassembly of the nuclear lamina during mitosis (for reviews see Gerace and Burke, 1988; Nigg, 1992; Moir and Goldman, 1993). However, in the clam *Spisula solidissima*, the $p34^{cdc2}$/cyclin B complex alone appears sufficient for the release of the 67 kDa lamin (L67) from isolated oocyte nuclei (Dessev *et al.*, 1991). A 49-kDa nucleoplasmin-like protein which is extensively phosphorylated 5 min after activation of *Spisula* oocytes has been identified (Herlands and Maul, 1994). Whether this protein could be a substrate for PK85 remains to be verified.

In conclusion, 5HT-induced meiosis reinitiation in *Ruditapes philippinarum* is associated with an early and PKC-independent activation of an 85 kDa S6 kinase. Its role in the phosphorylation cascade linking G-protein coupled membrane receptors to the dissolution of the germinal vesicle remains to be elucidated.

Materials and Methods

Materials

(γ-^{32}P)ATP (5000 Ci/mmol) was purchased from Amersham. SDS was purchased from Serva and N,N-bisacrylamide from BDH. Molecular weight markers and Tween-20 were obtained from Bio-Rad and leupeptin from Boehringer Mannheim. Cellulose thin-layer plates and propanol-2 were purchased from Merck. PVDF membranes, QMA Sep-Pak®-Light and C_{18} Sep-Pak® cartridges were from Waters (Millipore Corp.). Calyculin A, GF109203X and microcystin were from BioMol and okadaic acid from Gibco BRL. All other chemicals were from Sigma. A stock solution of imidazole/iminodiacetic acid (500 mM/280 mM) was made and purified over activated charcoal.

Handling of oocytes

Japanese clams were obtained at the IFREMER Laboratory of La Tremblade or from various commercial sources in the Golfe du Morbihan and maintained in running sea water tanks. Oocytes were recovered from ovaries by dissection, filtered through 4 layers of cheesecloth and washed at least 5 times in natural or artificial sea water (ASW). Oocytes were then diluted in ASW to a final concentration of 0.5-2% (v/v) and kept at 16-18°C until used. Within this temperature range, no spontaneous maturation was observed even after 8 h of incubation. Oocytes from at least 5 females were always mixed together for experiments. Germinal vesicle breakdown was scored by random counting of 100-200 oocytes.

Gel electrophoresis and renaturation of protein kinases

Except where mentioned, oocytes were directly solubilized in 2X SDS-sample buffer (4% SDS, 10% β-mercaptoethanol, 50 mM Tris-HCl pH 6.8, 20% glycerol, 0.02% bromophenol blue) and incubated for 5 min in a boiling water bath. The mixtures were then submitted to electrophoresis (20-50 μg of protein per well) using a Bio-Rad mini slab gel apparatus (acrylamide:bisacrylamide, 30:0.8; stacking: 4%, separation: 8 to 10%) at 30 mA/gel. To renature protein kinases, gels were then treated as previously described (Durocher *et al.*, 1992) with some modifications (volumes applies for two mini-gels of 1.5 mm thickness): SDS was first removed from gels by two successive washes (1st, 100 ml, 45 min or overnight; 2nd, 100 ml, 45 min) in 50 mM imidazole, 28 mM iminodiacetic acid, pH 7.0 containing 20% isopropanol, followed by one wash (200 ml, 45 min) in Buffer A (50 mM imidazole, 28 mM iminodiacetic acid, 10 mM ß-mercaptoethanol, pH 7.0), all at 22°C. To further unfold proteins within the gel matrix, gels were incubated in the presence of 20 ml of 8 M guanidine-HCl in Buffer A containing 50 mM ß-mercaptoethanol (90 min at 22°C). Proteins were then allowed to renature at 22°C using five incubations of 45 min each with gentle shaking in 100 ml of renaturation buffer (25 mM imidazole, 14 mM iminodiacetic acid, 0.04% Tween 20, 10% sucrose, 50 mM NaF, 50 mM KCl and 10 mM ß-mercaptoethanol); an incubation overnight in 200 ml may substitute for two washes of 45 min. Gels were subsequently equilibrated (45 min at 22°C) in 200 ml of Buffer B (10

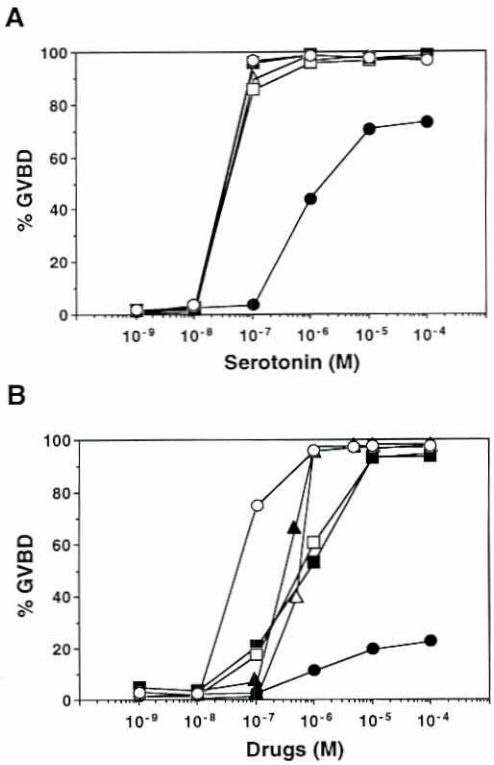

Fig. 8. Effect of PKC modulators on GVBD. (A) *Oocytes were preincubated for 10 min in the presence of the indicated drugs before addition of 5HT. Open circles, control; closed circles, 1 μM PMA; open triangles, 1 μM PMA^{met}; open squares, 5 μM GF109203X; closed squares, 1 μM PMA + 5 μM GF109203X. GVBD was determined 40 min after addition of 5HT.* **(B)** *Control oocytes (open symbols) or oocytes preincubated for 10 min in the presence of 1 μM PMA (closed symbols) were activated with various concentrations of the indicated drugs and GVBD was determined 40 min later (circles, 5HT; squares, ionophore A23187; triangles, thapsigargin). Different batches of oocytes were used for A and B.*

mM HEPES-NaOH pH 7.4, 10 mM ß-mercaptoethanol, 5 mM $MnCl_2$, 50 mM NaF, and 100 μM Na_3VO_4) and phosphorylation was carried out (90 min at 22°C) in 20 ml of Buffer B containing 10-50 μCi (γ-^{32}P)ATP (1000-5000 Ci/mmol). This incubation time is in the linear portion of the ^{32}P-incorporation curve as assessed for three kinases (PK160, PK85, and PK47; not shown). Gels were then successively washed 5 times with 100 ml of 5% (w/v) TCA containing 1% (w/v) sodium phosphate and 1% (w/v) sodium pyrophosphate (45 min each), stained with Coomassie R250, dried and exposed to X-ray film in order to visualise the pattern of renatured kinases.

Phosphoamino acid analysis

Bands containing ^{32}P-labeled polypeptides of interest were excised from the gel, minced and partially hydrolyzed in 6 M HCl at 110°C for 90 min. Released phosphoamino acids were partially purified on C18 and QMA cartridges (Durocher and Chevalier, 1994) prior to their separation by two-dimensional electrophoresis on cellulose thin-layer plates (Cooper *et al.*, 1983).

Oocyte fractionation

Sedimented oocytes were homogenized on ice with a potter in 5-10 volumes of fractionation buffer (Buffer F: 10 mM MOPS-NaOH pH 7.3, 50 mM ß-glycerophosphate, 50 mM NaF, 500 nM microcystin, 100 μM Na_3VO_4, 0.3 M sucrose, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF, 10 μg/ml SBTI, 10 μg/ml leupeptin and 2 μg/ml pepstatin). The homogenate was then centrifuged at 100,000g for 60 min at 4°C and the resulting soluble and particulate fractions were stored in aliquots at -80°C. Protein concentration was determined using a Coomassie Blue dye binding assay (Sedmak and Grossberg, 1977).

Partial purification of PK85 and immunodetection

Soluble fractions were applied onto a Fast-S Sepharose cation exchange column (1.0x16 cm) equilibrated in Buffer F at a flow rate of 1 ml/min. After washing the column with 50 ml of Buffer F, proteins were eluted with 15 ml of Buffer F containing 500 mM NaCl. Following addition of mercaptoethanol to a final concentration of 0.01% the eluate was precipitated with ammonium sulfate (70% saturation). The precipitate was then desalted by chromatography on a Sephadex-G25 column equilibrated in Buffer Q (50 mM ß-glycerophosphate, 50 mM NaF, 1 mM EDTA) containing 1 mM PMSF, 10 μg/ml SBTI, 10 μg/ml leupeptin and 2 μg/ml pepstatin. The samples were applied at 1 ml/min onto a Mono-Q HR 5/2 anion exchange column equilibrated in Buffer Q and eluted with 17 ml of a 0-500 mM NaCl linear gradient in the same buffer. 0.75 ml fractions were collected and 20 μl aliquots were used for the renaturation assay or Western blotting. For immunodetection, proteins were transferred to PVDF membranes at 250 mA for 30 min in 10 mM CAPS pH 11 containing 20% methanol (4°C). After blocking with 5% BSA in Tris buffered saline (TBS), membranes were incubated for 2 h with rabbit anti-RSK antiserum (Ab 8827, generous gift of Dr. Karol Bomsztyk, WA) diluted 1/1000 in TBS containing 2% BSA and 0.1% Tween-20. Blots were then revealed by chemiluminescence using goat anti-rabbit immunoglobulins coupled to horseradish peroxidase.

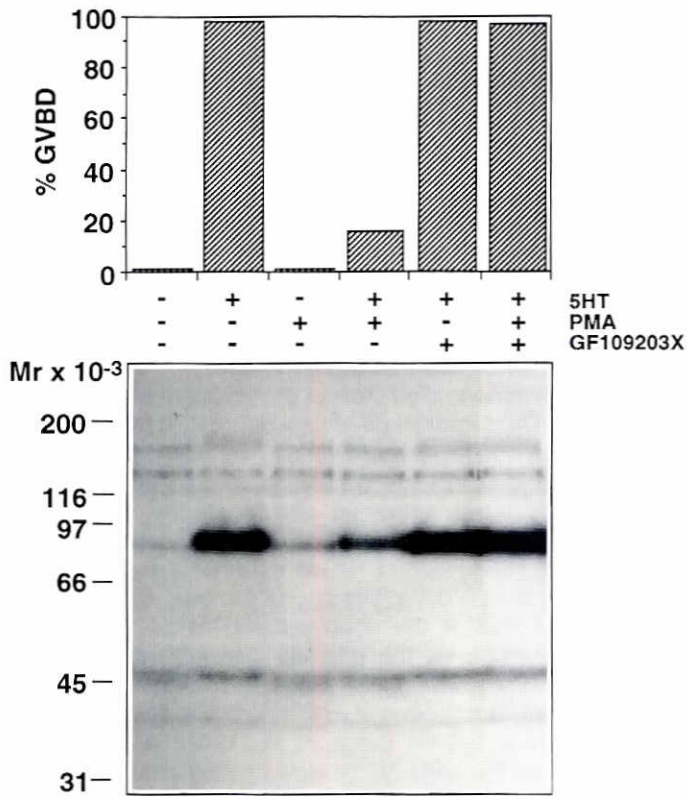

Fig. 9. Effect of PKC modulators on 5HT-induced PK85 activation. *Oocytes were treated as in Figure 8A with the indicated drugs. 10 min after 5HT addition, they were solubilized in SDS-sample buffer for in situ renaturation and phosphorylation* **(lower panel).** *GVBD was determined on aliquots 40 min after the addition of 5HT* **(upper panel).**

Phosphorylation of exogenous substrates

Partially purified PK85 or crude cytosolic extracts were incubated for 10 min in 20 µl of phosphorylation buffer containing 20 mM HEPES pH 7.4, 10 mM $MgCl_2$, 2 mM DTT, 50 µM (γ-^{32}P)ATP (0.5 µCi) and 0.5 mg/ml of protein substrate or 0.25 mg/ml peptide substrate. Phosphorylation was stopped by spotting an aliquot on P81 paper followed by several washes in 0.5% H_3PO_4. ^{32}P-incorporation was determined by liquid scintillation.

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