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Coupling of inositol phospholipid hydrolysis to peptide hormone receptors expressed from adrenal and pituitary mRNA in *Xenopus laevis* oocytes

(angiotensin II receptor/thyrotropin-releasing hormone receptor/inositol phosphates)

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ABSTRACT **The expression of several neurotransmitter and drug receptors from injected exogenous mRNA in *Xenopus laevis* oocytes has been demonstrated by electrophysiological measurements of ion channel activation. The expression of specific receptors for peptide hormones in such a translation system would facilitate studies on the structure and regulation of cell-surface receptors as well as their coupling to membrane transduction mechanisms. The expression of receptors for calcium-mobilizing hormones in *Xenopus* oocytes was sought by analysis of phospholipid turnover in hormone-stimulated oocytes. For this purpose, *Xenopus* oocytes were injected with mRNA extracted from bovine adrenal and pituitary glands and incubated with *myo*-[^{3}H]inositol to label plasma-membrane phosphatidylinositol phosphates. The expression of functionally active receptors for angiotensin II (AII) and thyrotropin-releasing hormone (TRH) was demonstrated by the stimulation of [^{3}H]inositol phosphate production by AII and TRH in the mRNA-injected, [^{3}H]inositol-prelabeled oocytes. The ability of AII and TRH to act by way of newly synthesized receptors from mammalian endocrine tissues to stimulate phosphatidylinositol polyphosphate hydrolysis in *Xenopus* oocytes suggests a generalized and conserved mechanism of receptor coupling to the transduction mechanism responsible for activation of phospholipase C in the plasma membrane.**

Receptors for peptide and protein hormones are present in relatively low abundance in the plasma membrane, where they interact with extracellular ligands that exert specific regulatory actions on cellular function. The rarity and hydrophobicity of plasma-membrane receptors contribute to the difficulty of their isolation and structural characterization as well as the analysis of their regulation and activation mechanisms. The corresponding rarity of mRNAs coding for peptide hormone receptors has, with few exceptions (1–3), impeded their characterization by molecular biological approaches.

The *Xenopus* oocyte has provided a sensitive assay system for translation of many exogenous eukaryotic mRNAs (4, 5). The oocyte enzymes have been shown to correctly synthesize and modify or mature numerous exogenous proteins (reviewed in ref. 5), which are then placed in their appropriate cellular locations. Several functional receptors for neurotransmitter ligands have been expressed in *Xenopus* oocytes from injected mRNA extracted from various neural tissues. These include receptors activated by acetylcholine (6–8), γ-aminobutyric acid (9, 10), glycine (10), glutamate (11, 12), serotonin, and kainate (10) (reviewed in ref. 13). RNA transcribed from cloned cDNA has also been shown to produce functional acetylcholine receptors in *Xenopus* oocytes (14). In the case of the nicotinic acetylcholine receptor, expression in oocytes can be demonstrated by binding of radioactive bungerotoxin (4) as well as by activation of ion transport. However, detection of less abundant receptors has usually required sensitive electrophysiological techniques to measure their introduced or coupled ion channel activity in the oocyte plasma membrane. It would clearly be of value to be able to detect the presence of expressed hormone receptors in the oocyte plasma membrane by amplifying their activation signals for analysis by direct biochemical means.

A major mechanism for transduction of hormone signals involves the activation of phospholipase C upon binding of hormones to their cell-surface receptors, with hydrolysis of phosphatidylinositol bisphosphate to form inositol trisphosphate and diacylglycerol (15). The inositol 1,4,5-trisphosphate (Ins-1,4,5-P_3) thus produced acts on intracellular receptors (16) to mobilize calcium, a second messenger of hormone action in many cell types. In a recent report, the *Xenopus* oocyte was found to possess an endogenous inositol phospholipid-linked muscarinic receptor system. Stimulation of the oocyte with acetylcholine increased the accumulation of inositol phosphates, and injection of Ins-1,4,5-P_3 was shown to mimic the muscarinic depolarizing chloride current (17). In the present study, the products of the endogenous phospholipase C-linked transduction mechanism in *Xenopus laevis* oocytes were analyzed and partially characterized by anion-exchange HPLC, and expressed receptors from two mammalian endocrine glands were shown to be capable of coupling to this mechanism. Such a functional assay for detecting and characterizing receptor mRNAs, based on amplification of receptor–hormone interaction by the effector enzyme of the plasma-membrane transduction mechanism, should be of general application to receptors for peptide and protein hormones.

MATERIALS AND METHODS

Stimulation of Endogenous Acetylcholine Receptors. Oocytes at stages 5 and 6 (18) with retained follicular cells were separated from *X. laevis* ovaries and incubated overnight at room temperature with gentle shaking in 0.3 ml of OR-2 buffer (19) [82.5 mM NaCl/2.5 mM KCl/1 mM $MgCl_2$/1 mM $CaCl_2$/5 mM Hepes/1 mM sodium phosphate, pH (final) 7.8] per 10 oocytes. Inositol phospholipids were labeled by incubation with 40 μCi of *myo*-[^{3}H]inositol (56 Ci/mmol; 1 Ci = 37 GBq). The cells were then washed and resuspended for

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Abbreviations: Ins-1,4-P_2, inositol 1,4-bisphosphate; Ins-3,4-P_2, inositol 3,4-bisphosphate; Ins-1,4,5-P_3, inositol 1,4,5-trisphosphate; AII, angiotensin II; TRH, thyrotropin-releasing hormone.

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15 min in 0.2 ml of OR-2 buffer with 10 mM LiCl substituted for NaCl before addition of 200 μM acetylcholine.

Measurement of Inositol Phosphate Accumulation. Incubations were terminated by addition of perchloric acid to 5% (wt/vol) to the oocytes and freezing in dry ice. After thawing, the oocytes were disrupted with a glass rod and supernatants were extracted and neutralized using a 1:1 trichlorotrifluoroethane/tri-*n*-octylamine mixture (20). Inositol phosphates were then separated by HPLC as described (21, 22) on a strong anion-exchange column [4.6 × 250 mm Adsorbosphere (5 μm), Alltech/Applied Science, Deerfield, IL] using a linear gradient of aqueous ammonium phosphate (pH 3.35), 0–0.6 M, over 65 min. Peaks were identified by their elution times coincident with or relative to tritiated inositol phosphate standards (DuPont–New England Nuclear) and were quantitated by an integration program.

Preparation of mRNA. Bovine adrenal glands were conveyed on ice from the slaughterhouse, bovine anterior pituitary glands were frozen in liquid nitrogen at the slaughterhouse, and rat adrenals and rat brain parietal cortex were dissected and frozen in isopentane on dry ice immediately after death. RNA was extracted by homogenizing tissue in 4 M guanidinium isothiocyanate/50 mM Tris, pH 7.5/10 mM EDTA/2% sarcosyl/2% 2-mercaptoethanol; this was followed by addition of 0.1 g of CsCl per ml, ultracentrifugation through a cushion of 5.7 M CsCl/0.1 M EDTA, pH 7.5, for 20 hr at 130,000 × *g*, 20°C, and phenol/chloroform extraction. Poly(A)$^+$ mRNAs were prepared by column chromatography on oligo(dT)-cellulose (23).

Expression of Exogenous mRNA. Oocytes were injected (24) with ≈70 ng of total mRNA in 50 nl of H_2O into the cytoplasm. Injected and control oocytes were incubated at room temperature in groups of 20 in 0.5 ml of OR-2 medium containing 60 μCi of *myo*-[^{3}H]inositol (56 Ci/mmol) and additives—1 mM sodium pyruvate, 0.2% bovine serum albumin, fraction 5 (Boehringer Mannheim), 50 units of nystatin per ml, and 100 Kallikrein units of aprotinin per ml. After 30 and 40 hr, healthy oocytes were selected, washed, and stimulated with appropriate hormones for 90 min in the presence of 10 mM Li$^+$ in OR-2 medium with the same additives. Extracted inositol phosphates were separated and analyzed by HPLC as described above.

RESULTS

The presence of Li$^+$ in the incubation medium augmented the accumulation of inositol phosphates during muscarinic stimulation of *Xenopus* oocytes (17) by inhibiting several of the steps in Ins-1,4,5-P_3 degradation (25). The increasing accumulation of inositol phosphates for up to 90 min during stimulation by acetylcholine is shown in Fig. 1. Labeled peaks corresponding to Ins-1,4-P_2 and Ins-1,4,5-P_3 were identified by comparison with standard labeled inositol phosphates during elution by an ammonium phosphate gradient from a HPLC anion-exchange column (21, 22). The peak presumed to be Ins-3,4-P_2 was tentatively identified by its position relative to inositol 4-monophosphate and Ins-1,4-P_2. Many samples, both stimulated and unstimulated, also showed a peak eluting at the expected position of inositol 1,3,4-trisphosphate (see Fig. 3).

The relative amounts of the two inositol bisphosphate isomers and inositol trisphosphates varied with batch of oocytes but were consistent between samples prepared from individual toads. In some batches of oocytes (see Fig. 3), activation was accompanied also by the appearance of small peaks eluting at positions expected of the higher inositol polyphosphates containing four, five, and six phosphate groups. Increases in inositol monophosphate were also apparent in stimulated oocytes, but variability between samples made this peak less useful for detecting responses to hormonal stimulation. This variability was related to high uptake of labeled inositol by tissue surrounding the undenuded oocytes, with metabolism to inositol monophosphate that was unaffected by stimulation of the receptors on the oocyte itself. By comparison with undenuded oocytes, those treated for 2 hr with collagenase (Sigma, type 1A; 2 mg/ml), followed by removal of outer cell layers with forceps, absorbed 17% of total cpm, metabolized the inositol to 17% of inositol monophosphate, but produced ≈60% labeled inositol bisphosphate and inositol trisphosphate on stimulation with acetylcholine. The stimulatory effect of acetylcholine was completely blocked by atropine. Denuded oocytes were also more fragile on microinjection and had a lower survival rate during incubation.

Figs. 2 and 3 show some results of microinjection of oocytes with exogenous mRNA that was followed by incubation with *myo*-[^{3}H]inositol for 30 hr and stimulation with appropriate hormones in the presence of 10 mM Li$^+$. On addition of 0.1 μM AII, oocytes injected with bovine adrenal mRNA showed increased inositol phosphate accumulation. Samples injected with bovine pituitary mRNA were likewise stimulated by AII or 1 μM TRH. No stimulation was observed with negative controls from noninjected nonstimulated oocytes, noninjected oocytes stimulated with AII or

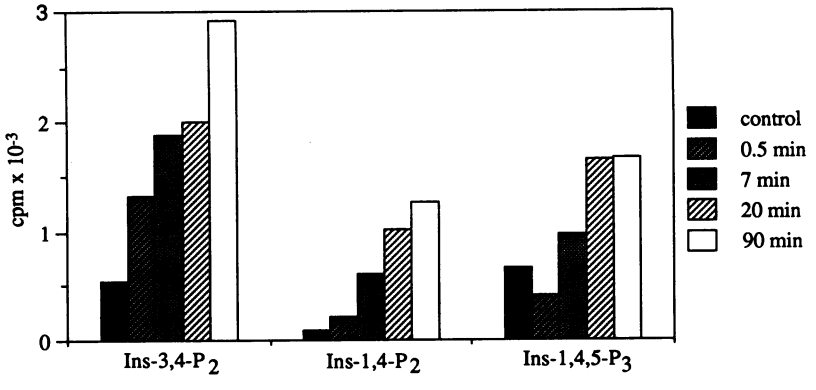

FIG. 1. Kinetics of inositol phosphate accumulation in *Xenopus* oocytes during stimulation by acetylcholine in the presence of Li$^+$. Ten oocytes per sample with retained follicular cells were incubated overnight in OR-2 buffer at room temperature with 40 μCi of *myo*-[^{3}H]inositol. Samples were stimulated with 0.2 mM acetylcholine in the presence of 10 mM Li$^+$ for the times indicated (control for 90 min). Extracted inositol phosphates were separated by HPLC and peaks were identified by their elution times coincident with tritiated inositol phosphate standards [inositol 1,4-bisphosphate (Ins-1,4-P_2) and Ins-1,4,5-P_3] or relative to such standards [inositol 3,4-bisphosphate (Ins-3,4-P_2), tentatively]. Mean total label absorbed per 10 oocytes was 190,000 cpm (12% coefficient of variation). Mean tritiated monophosphate was 37,000 cpm (11% coefficient of variation).

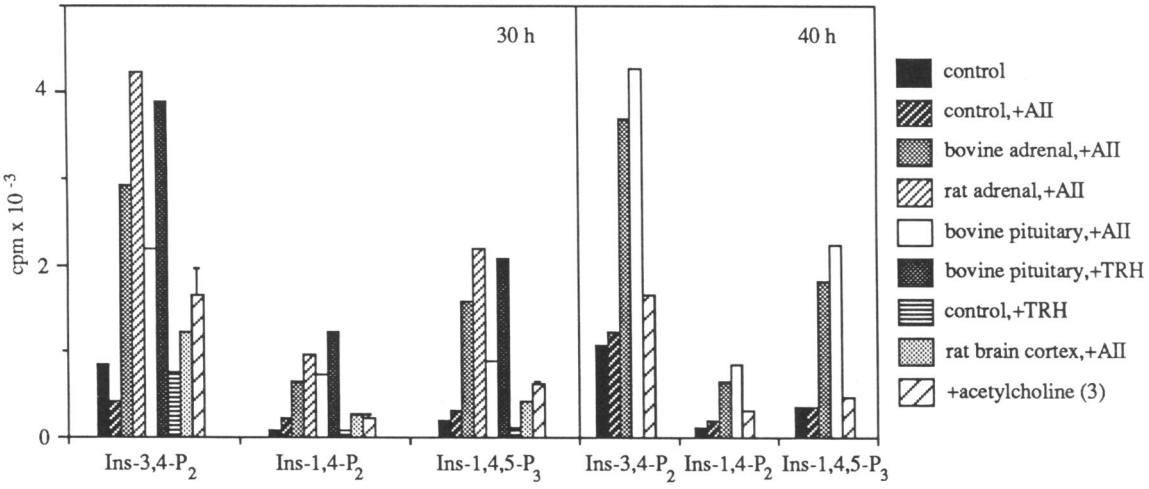

FIG. 2. Production of inositol phosphates by oocytes expressing exogenous receptors for angiotensin II (AII) and thyrotropin-releasing hormone (TRH). Oocytes were injected into the cytoplasm with ≈70 ng of total mRNA extracted from the tissues shown in the legend. Control and acetylcholine-stimulated oocytes were not injected. All oocytes were incubated at room temperature in groups of 20 in OR-2 medium containing 60 μCi of *myo*-[^{3}H]inositol and additives. After 30 and 40 hr, healthy oocytes were selected (9–14 per sample), washed, and stimulated as appropriate with hormones (AII, 0.1 μM; TRH, 1 μM; or acetylcholine, 0.2 mM) for 90 min in the presence of Li^+ in OR-2 medium with the same additives. Tritiated inositol phosphates were then extracted and measured by HPLC. Data shown have been normalized to 300,000 total cpm in each sample. HPLC elution patterns for some of these data are shown in Fig. 3. Error bars indicate the SEM for three samples stimulated with acetylcholine. +, Samples were stimulated with the hormone shown.

TRH, oocytes injected with bovine adrenal mRNA and not stimulated with AII, and oocytes injected with rat parietal cortex mRNA and stimulated with AII [parietal cortex has been shown to contain very few, if any, AII receptors (26) but has an active inositol phospholipid cycle (27)]. Although the positive control stimulation by acetylcholine (0.3 mM) was prominent in most experiments, the oocytes from this particular ovary gave unusually low responses to the agonist. Similar responses were obtained from the same batch of oocytes 40 hr after injection of the same bovine adrenal and pituitary mRNA preparations and in controls, except that the response to AII of pituitary mRNA-injected oocytes was relatively enhanced (Fig. 2).

In initial experiments in which inositol phosphates were separated by step elution from anion-exchange chromatography (25), oocytes injected with other preparations of bovine adrenal mRNA and bovine pituitary mRNA responded positively to stimulation with a mixture of AII and TRH in comparison with negative controls, both 24 and 48 hr after injection of the messages. Injection of 50 nl of H_2O or 4 mM Tris buffer (pH 7.5) did not cause accumulation of inositol phosphates in unstimulated oocytes and did not change the response to acetylcholine stimulation. Oocytes injected with bovine pituitary mRNA also secreted proteins reacting positively in radioimmunoassays for bovine prolactin (25 ng per 10 oocytes over 30 hr) and bovine growth hormone (6 ng per 10 oocytes), whereas noninjected oocytes and oocytes injected with adrenal and rat brain mRNA gave negative results (<0.3 ng).

DISCUSSION

In oocytes injected with bovine adrenal or anterior pituitary mRNA, addition of AII promoted the accumulation of inositol phosphates in a manner characteristic of agonist-stimulated cells. Such responses were absent in the uninjected cells and those injected with mRNA from a tissue not known to contain AII receptors. Similarly, oocytes injected with bovine pituitary mRNA showed a stimulatory response to TRH. Thus, new receptors that are functionally responsive to their specific hormones appear to have been translated from the exogenous mRNAs extracted from mammalian endocrine tissues, and their occupancy leads to activation of phosphatidylinositol polyphosphate hydrolysis.

In addition to the intrinsic interest of this observation, the use of an assay system based on the induction of receptor-coupled inositol phospholipid hydrolysis in *Xenopus* oocytes should facilitate the isolation and identification of specific receptor mRNAs and their corresponding cDNAs. It should also be of value in studies on the control of receptor expression, and investigation of receptor mutations and abnormalities. Recently, expression of TRH receptor mRNA in oocytes has been detected by activation of chloride channels, providing an assay to monitor transcriptional regulation of receptors in down-regulated pituitary tumor cells (28). The present results demonstrate that another concomitant of intracellular calcium mobilization—in this case the presumed initiating step of phosphatidylinositol polyphosphate hydrolysis—can be triggered by occupancy of two functional hormone receptors (for TRH and AII) expressed from pituitary and adrenal mRNA. The same techniques used with mRNA transcribed from modified receptor cDNA should allow study of receptor structure–function relationships, including detection of receptor domains for hormone binding and coupling to specific membrane-transduction mechanisms.

Most receptors are extremely rare proteins, and their corresponding mRNA levels probably account for <0.01% of total tissue mRNA (29). The amplification of receptor activation by enzymes of the transduction mechanism after occupancy by the homologous hormone should be applicable to other receptors that stimulate inositol phospholipid hydrolysis. By implication, receptors linked to the adenylate and guanylate cyclase transduction mechanisms would similarly be amenable to study by assaying for cyclic AMP and cyclic GMP production after hormonal activation of the expressed receptors in the oocyte.

It is of interest that newly generated foreign receptors from different species activate the *Xenopus* phosphatidylinositol phosphate transduction mechanism after occupancy by their homologous hormones, which are normally ineffective in oocytes. This may indicate a generalized and conserved mechanism of interaction of peptide hormone receptors with the guanine nucleotide regulatory proteins (30–32) that participate in this transduction mechanism, which also mediates the actions of TRH and AII in pituitary and adrenal target cells.

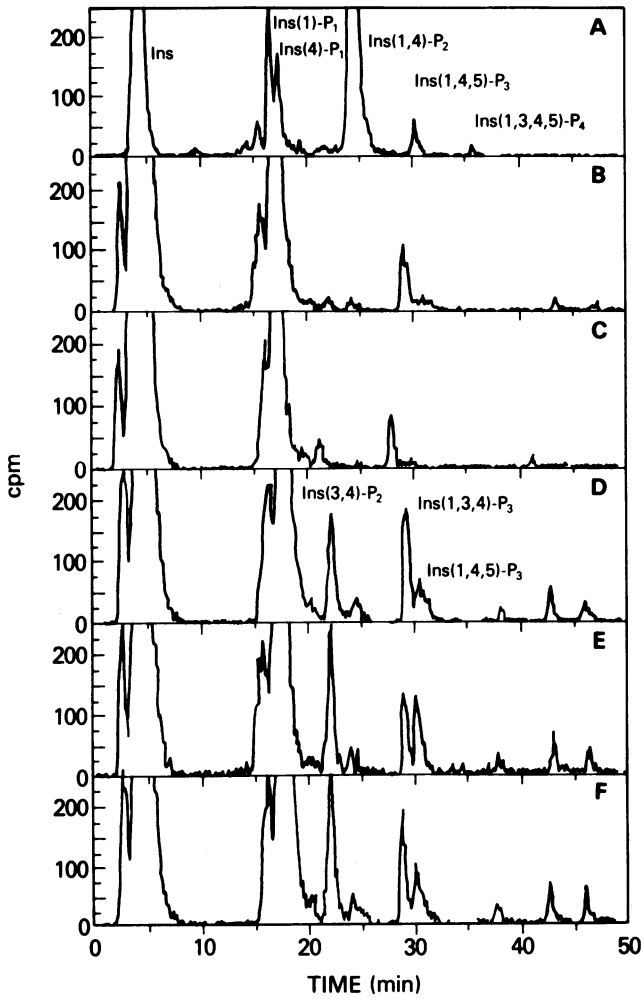

FIG. 3. HPLC elution patterns of inositol phosphates from hormone-stimulated oocytes injected with mRNA and their controls as analyzed in Fig. 2. (*A*) Mixture of standard tritiated inositol phosphates. (*B*) AII-stimulated noninjected oocytes. (*C*) TRH-stimulated noninjected oocytes. (*D*) AII-stimulated oocytes injected with bovine adrenal mRNA. (*E*) AII-stimulated oocytes injected with rat adrenal mRNA. (*F*) TRH-stimulated oocytes injected with bovine pituitary mRNA. Ins, inositol; Ins(1)-P_1, inositol 1-monophosphate; Ins(4)-P_1, inositol 4-monophosphate; Ins(1,4)-P_2, inositol 1,4-bisphosphate; Ins(1,4,5)-P_3, inositol 1,4,5-trisphosphate; Ins(1,3,4,5)-P_4, inositol 1,3,4,5-tetrakisphosphate. The positions of peaks migrating in positions corresponding to inositol 3,4-bisphosphate [Ins(3,4)-P_2] and inositol 1,3,4-trisphosphate [Ins(1,3,4)-P_3] are also indicated.

These studies also indicate that the *Xenopus* oocyte system should be useful for investigation of the control of the phosphatidylinositol polyphosphate transduction process itself. This mechanism is active not only in endocrine cells but also in the control of cell growth and differentiation and in cell transformation by certain oncogenes (33). Expression of the regulatory and enzymatic components of phospholipase C (and adenylate cyclase) from mRNAs for the native and mutant proteins should facilitate the genetic analysis of these moieties as well as their mode of coupling to endogenous or foreign receptors in the oocyte plasma membrane.

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