

# Intercellular Communication between Follicular Angiotensin Receptors and *Xenopus laevis* Oocytes: Mediation by an Inositol 1,4,5-Trisphosphate-dependent Mechanism

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**Abstract.** In *Xenopus laevis* oocytes, activation of angiotensin II (AII) receptors on the surrounding follicular cells sends a signal through gap junctions to elevate cytoplasmic calcium concentration ( $[Ca^{2+}]_i$ ) within the oocyte. The two major candidates for signal transfer through gap junctions into the oocyte during AII receptor stimulation are  $Ins(1,4,5)P_3$  and  $Ca^{2+}$ . In  $[^3H]$ inositol-injected follicular oocytes, AII stimulated two- to fourfold increases in phosphoinositide hydrolysis and production of inositol phosphates. Injection of

the glycosaminoglycan, heparin, which selectively blocks  $Ins(1,4,5)P_3$  receptors, prevented both AII-stimulated and  $Ins(1,4,5)P_3$ -induced  $Ca^{2+}$  mobilization in *Xenopus* follicular oocytes but did not affect mobilization of  $Ca^{2+}$  by ionomycin or GTP. These results indicate that the AII-regulated process of gap junction communication between follicular cells and the oocyte operates through an  $Ins(1,4,5)P_3$ -dependent mechanism rather than through transfer of  $Ca^{2+}$  into the ooplasm and subsequent  $Ca^{2+}$ -induced  $Ca^{2+}$  release.

ANGIOTENSIN II (AII)<sup>1</sup> stimulates a variety of physiological responses in different tissues and is a major regulator of blood pressure and extracellular volume (8, 9). Specific plasma membrane receptors for AII are coupled to phosphoinositide hydrolysis and  $Ca^{2+}$  mobilization in smooth muscle, adrenal, liver, heart, kidney, and brain. We recently demonstrated that the follicle cells surrounding *Xenopus laevis* oocytes possess AII receptors which, upon activation, elevate the intraoocyte  $Ca^{2+}$  concentration ( $[Ca^{2+}]_i$ ) (45). These  $[Ca^{2+}]_i$  responses were blocked by octanol treatment and intracellular acidification, maneuvers that inhibit cell coupling through gap junctions. Cell-cell communication through gap junctions between follicle cell microvilli and the oocyte has been suggested to occur in both the amphibian and mammalian ovary. Such a mechanism would permit significant exchange of molecules between the follicle cells and the ovum during oocyte maturation and development (7, 23, 30, 49).

Gap junctions are permeable to several cytoplasmic ions and molecules including nucleotides (49), sugars (43), amino acids, and small peptides (28, 50). Recently, gap junctions between hepatocytes were shown to be permeable to physiological concentrations of  $Ca^{2+}$  and inositol 1,4,5-trisphosphate ( $Ins[1,4,5]P_3$ ) (46). Since AII receptors in other tissues are coupled to the phosphoinositide signaling pathway, two candidates for transfer from follicular cells to the oocyte through gap junctions during AII-receptor stimu-

lation are  $Ins(1,4,5)P_3$  and  $Ca^{2+}$ . The glycosaminoglycan, heparin sulfate, is a potent and selective inhibitor of  $[^3H]Ins(1,4,5)P_3$  binding to high affinity sites in rat cerebellar membranes (59) and in the bovine adrenal cortex (22). Heparin also inhibits  $Ins(1,4,5)P_3$ -induced  $Ca^{2+}$  release in several microsomal preparations (20, 26) and permeabilized cells (20, 24, 38). In the present study, we demonstrate that activation of AII receptors is coupled to phosphoinositide hydrolysis and production of inositol phosphates in follicular oocytes. Heparin specifically blocks AII- and  $Ins(1,4,5)P_3$ -evoked  $Ca^{2+}$  release in follicular oocytes, but not that elicited by ionomycin and GTP. These findings indicate that the communication through gap junctions between AII-stimulated follicular cells and the oocyte operates through an  $Ins(1,4,5)P_3$ -dependent mechanism.

## Materials and Methods

### Materials and Buffers

$[Val^5]$ angiotensin (AII) was obtained from Peninsula Laboratories Inc. (Belmont, CA), and m-aminobenzoate, heparins, chondroitin sulfate, hyaluronic acid, and GTP were obtained from Sigma Chemical Co. (St. Louis, MO).  $Ins(1,4,5)P_3$ ,  $Ins(2,4,5)P_3$ ,  $Ins(1,3,4,5)P_4$ , fura-2, dextran sulfate, and ionomycin were purchased from Calbiochem-Behring Corp. (San Diego, CA). Aprotinin was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN) and collagenase was from Collaborative Research (Waltham, MA).  $[^3H]$ inositol (60 Ci/mmol) and  $[^3H]$ inositol phosphate standards were obtained from Amersham Corp. (Arlington Heights, IL). Albino *Xenopus laevis* frogs were purchased from Xenopus 1 (Ann Arbor, MI). Modified Barth's solution (MBS) was composed of 82.5 mM NaCl, 2.5 mM KCl, 1 mM  $MgCl_2$ , 1 mM  $CaCl_2$ , 5 mM Hepes, pH

1. **Abbreviations used in this paper:** AII, angiotensin II;  $[Ca^{2+}]_i$ , cytoplasmic calcium concentration; MBS, modified Barth's solution.

8, and 1 mM Na<sub>2</sub>PO<sub>4</sub>, pH 7.8. Ca<sup>2+</sup>-free MBS was prepared by substituting MnCl<sub>2</sub> for CaCl<sub>2</sub>. All other chemicals were of reagent grade. Aequorin was obtained from Dr. John R. Blinks (Department of Pharmacology, Mayo Foundation, Rochester, MN).

### Xenopus Oocyte Preparation and Injection

Ovarian lobes were obtained from anesthetized (0.2% m-aminobenzoate) *Xenopus laevis* frogs. Individual (stage V and VI) follicular oocytes were microinjected and subsequently incubated in MBS supplemented with aprotinin (100 kallikrein U/ml). Oocytes were manually defolliculated after incubation with collagenase (2 mg/ml) for 1–2 h; we have previously shown that such treatment does not impair the activation of AII receptors expressed in oocytes from rat adrenal mRNA (45). Microinjections were performed with a computerized pressure-controlled system (Atto Instrument Co., Potomac, MD) in which the injection volume was controlled in conjunction with a television camera that monitored the meniscus on the injection pipette. A Flaming/Brown micropipette puller (model P-87; Sutter Instrument Co., San Rafael, CA) was used to construct micropipettes (R6 custom glass tubing) (Drummond Scientific Co., Broomall, PA) with internal tip diameter of 5–10 μm. The injected oocytes were kept in MBS at 18°C for the desired time, and healthy oocytes were transferred to fresh MBS daily. For dose-response determinations, intraoocyte concentrations are based on the assumption that the free intracellular volume equals 450 nl (11) and the injection material is evenly distributed throughout the available cell volume.

### Extraction and Separation of Inositol Phosphates

[<sup>3</sup>H]inositol (5 × 10<sup>5</sup> cpm/oocyte), [<sup>3</sup>H]Ins(1,4,5)P<sub>3</sub> (500 cpm/oocyte), and [<sup>3</sup>H]Ins(1,3,4,5)P<sub>4</sub> (500 cpm/oocyte) purified by HPLC (25) to ensure <5% contamination, were injected into oocytes and reactions were terminated at selected times by adding an equal volume of 10% perchloric acid to samples containing 25 oocytes. After freezing, homogenization, and centrifugation, supernatants were extracted with a 1:1 mixture of Freon and tri-*n*-octylamine (25). After neutralization, samples were applied to an HPLC column (Adsorbosphere SAX, 5 μm, 250 mm, Alltech Associates, Inc., Deerfield, IL) and eluted with a linear gradient (10 mM/min) of ammonium phosphate (1).

### Aequorin Measurements

Aequorin was dissolved in 50 μM Hepes, 1 mM EDTA, and microinjected into the oocyte cytoplasm (33 ng/oocyte) alone or in combination with heparin and related compounds. Injected oocytes were individually transferred to 20-ml glass scintillation vials containing 0.5 ml MBS and photon emission was measured at 6-s intervals for 2 min in a liquid scintillation counter (model LS250; Beckman Instruments, Inc., Fullerton, CA) with the coinci-

dence gate switched off (44). These data were expressed as photons emitted per second or as fold (i.e., peak to basal) increases. In experiments involving measurements directly after microinjection, light emission was measured in a custom designed photon counter and was expressed in counts per volt (Dr. Alec Eidsath, Department of Biomedical Engineering, National Institutes of Health, Bethesda, MD) as previously described (45).

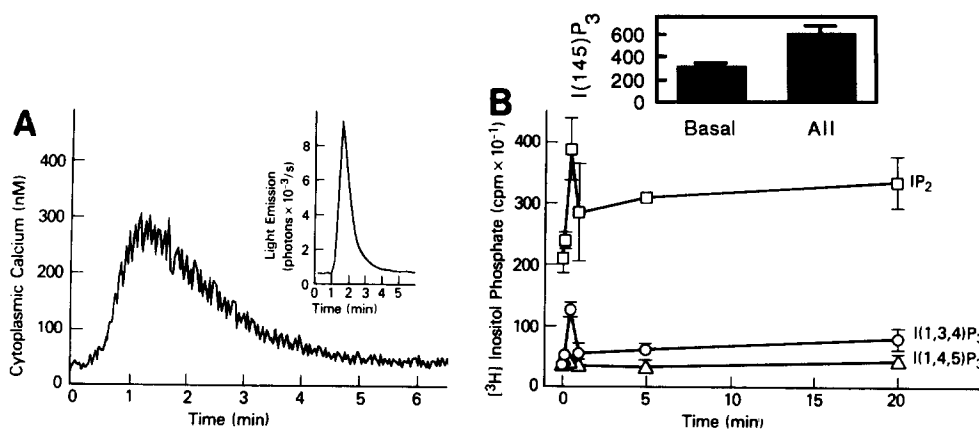
### Fluorescence Imaging

Oocytes were injected with 33 nl of 2 mM fura-2 0.5–4 h before imaging with an Atto-fluor digital microscopy system (Atto Instruments, Rockville, MD). The system used an inverted epifluorescence microscope model IM-35; Carl Zeiss, Inc., Thornwood, NY) and is described in detail elsewhere (6). A 100 W mercury burner served as the source for excitation. Fura-2 was excited at 340 and 380 nm alternately, using 10-nm band pass interference filters. Emission was selected by using a 510-nm-long pass filter and was monitored with an intensified charged-coupled device (CCD) camera. Oocytes were microinjected with 10 nl of specified compounds using a micromanipulator and examined under a 6× objective lens. A calibration standard curve was constructed with oocytes injected with known amounts of 1 mM EGTA and 1 mM CaCl<sub>2</sub> (6). The autofluorescence of uninjected oocytes was determined and subtracted from the basal levels of fluorescence from fura-2-injected oocytes.

### Results

#### AII Stimulates Ca<sup>2+</sup> Mobilization and Inositol Phosphate Production

In follicular oocytes injected with the Ca<sup>2+</sup>-specific photoprotein, aequorin (45), agonist stimulation of AII receptors evokes [Ca<sup>2+</sup>]<sub>i</sub> mobilization as evidenced by rapid and transient increases in light emission (Fig. 1 A, inset). Changes in [Ca<sup>2+</sup>]<sub>i</sub> levels are easy to detect but difficult to quantitate with aequorin, since the amplitude of the signal is proportional to the cube of the increase in [Ca<sup>2+</sup>]<sub>i</sub> (4). To determine the actual changes in [Ca<sup>2+</sup>]<sub>i</sub>, follicular oocytes were injected with fura-2 and imaged with an Atto-fluor digital microscopy system. A maximal stimulatory concentration of AII (500 nM) increased [Ca<sup>2+</sup>]<sub>i</sub> from 40 to 300 nM (Fig. 1 A). Measurements performed on 3–6 oocytes from three different frogs indicated that a 116 ± 19-fold light re-



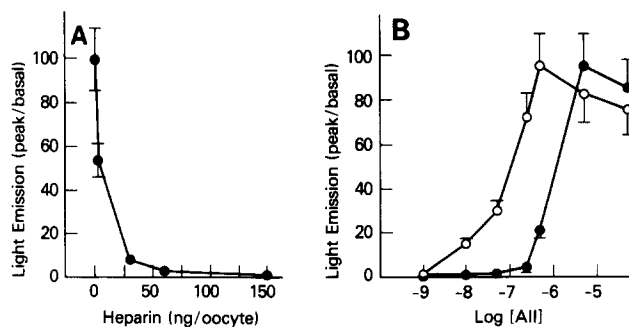
**Figure 1.** (A) Time course of AII-evoked changes in [Ca<sup>2+</sup>]<sub>i</sub>. The data are from a single oocyte which is representative of results from five oocytes injected with fura-2 (2 mM) 1–3 h before AII (500 nM) stimulation, and are expressed as the levels of intracellular [Ca<sup>2+</sup>]<sub>i</sub>. (Inset) Time course of AII-evoked changes in light emission. The data are from a single oocyte which is representative of results from five oocytes injected with aequorin and [<sup>3</sup>H]inositol, and are expressed as photons emitted per second. (B) Time

course of AII-induced inositol phosphate production. Oocytes from the same frog as in Fig. 1 A, inset were injected with [<sup>3</sup>H]inositol 24 h before AII stimulation. The data are expressed as the production of [<sup>3</sup>H]InsP<sub>2</sub>, [<sup>3</sup>H]Ins(1,3,4)P<sub>3</sub>, and [<sup>3</sup>H]Ins(1,4,5)P<sub>3</sub> over 20 min of stimulation and are representative of six experiments performed in duplicate. (Inset) Stimulation of Ins(1,4,5)P<sub>3</sub> production by AII. Ins(1,4,5)P<sub>3</sub> levels were measured in oocytes stimulated for 10 s with 500 nM AII. Bars indicate the mean ± standard error (SE) of data from six independent experiments.

sponse to AII corresponded to a  $7.5 \pm 0.8$ -fold increase in  $[Ca^{2+}]_i$ . The  $[Ca^{2+}]_i$  response to AII is similar to that previously observed in fura-2 injected oocytes (6) except that our basal levels of  $[Ca^{2+}]_i$  are significantly lower; this difference is attributable to our practice of subtracting oocyte autofluorescence. The fura-2 assay is more cumbersome (4–5 oocytes/h) compared with the aequorin method (20–30 oocytes/h) and is 15-fold less sensitive. Subsequent studies were performed with the aequorin assay, which permitted the generation of full-dose response curves on follicular oocytes from the same frog within a 12-h period.

Because AII-induced  $[Ca^{2+}]_i$  signals vary markedly between frogs (45), we screened follicular oocytes from several animals for high  $[Ca^{2+}]_i$  responses to AII (>20-fold increases in light emission) before evaluating inositol phosphate production during AII stimulation. Such follicular oocytes were incubated for 24 h after microinjection with  $[^3H]$ inositol and then stimulated with 500 nM AII for up to 20 min. Before AII stimulation, follicular oocytes were preincubated for 2 h with LiCl (40 mM) to inhibit 5-phosphatase activity and augment the agonist-stimulated levels of  $Ins(1,4)P_2$  and  $Ins(1,3,4)P_3$  (16, 48). There was a rapid rise in the levels of  $InsP_2$  and  $Ins(1,3,4)P_3$  after stimulation by AII, to peak values at 30 s (Fig. 1 B). In comparison,  $Ins(1,4,5)P_3$  levels were significantly lower (Fig. 1 B, inset). From the average of six separate experiments,  $Ins(1,4,5)P_3$  increased from background levels of  $302 \pm 52$  cpm to peak levels of  $605 \pm 74$  cpm ( $n = 6$ ,  $P < 0.05$ ) at 10 s. At later times (>1 min), the levels of  $InsP_2$  and  $Ins(1,3,4)P_3$  showed a secondary rise that was slightly but consistently higher than basal. This two- to fourfold increase in inositol phosphate turnover correlated with the 12-fold increase in light emission observed in the same oocytes injected with aequorin 24 h earlier (Fig. 1 A, inset).

We (45) and others (18) have previously demonstrated that the majority of the AII receptors in follicular oocytes are located in the follicle cells surrounding the oocyte. Over the last two years, we examined over 650 oocytes from 34 frogs and found that defolliculation caused loss of the AII-evoked  $Ca^{2+}$  responses in oocytes from 87% of the frog population, and that oocytes from 13% of frogs retained >30% of their AII-induced  $Ca^{2+}$  responses after defolliculation. These findings suggest that a small population of frogs possess AII receptors located on the oocyte membrane as well as the follicular cells, analogous to the two types of muscarinic receptors in *Xenopus* oocytes (13, 32, 33). Lupu-Meir et al. (32) reported that the majority (90%) of frogs possessing muscarinic receptors (termed “common” donors) lose their responses after defolliculation while a small population of frogs (termed “variant” donors) retain them. The authors also state that different populations of frogs apparently contain different proportions of common versus variant donors which could explain the discrepancies in percentages of common and variant populations between their work (32) and that of others (29). Such an explanation could also account for the differences between our results showing that oocytes from 29 out of 34 frogs (>20 oocytes/frog) lost their AII-induced  $Ca^{2+}$  responses and a previous report (58) demonstrating that oocytes from three frogs retained 30–100% of their AII-induced  $Ca^{2+}$ -dependent chloride currents after defolliculation by collagenase treatment. These findings suggest that AII receptors located on the oocyte sur-



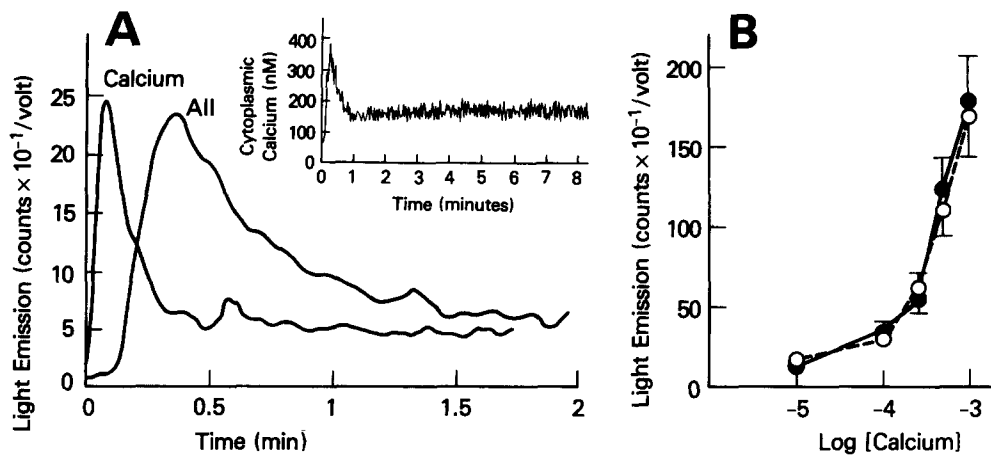
**Figure 2.** (A) Dose-dependent inhibition of AII-induced  $Ca^{2+}$  mobilization by heparin. Oocytes were coinjected with aequorin (33 ng/oocyte) and increasing concentrations of heparin 1–4 h before AII (500 nM) stimulation. The data are expressed as percent of control light emission ( $27 \pm 2.4$ -fold stimulation) from aequorin-injected oocytes. Each point represents the mean  $\pm$  S.E. of data from three to six oocytes. (B) Dose dependence of AII-induced  $[Ca^{2+}]_i$  responses in the presence and absence of heparin. Oocytes were injected with aequorin (33 ng/oocyte) (○) or coinjected with aequorin and heparin (2 ng/oocyte) (●) and stimulated with increasing concentrations of AII. Each point represents the mean  $\pm$  SE of data from three to six oocytes and is expressed as the fold stimulation (*peak/basal*) of light emission.

face may contribute to AII-induced  $Ca^{2+}$  responses to varying degrees depending on the frog population, and indicate the need for appropriate controls for each population of oocytes. However, our experiments were performed in frogs whose oocytes completely (>99%) lost both their AII-induced  $Ca^{2+}$  responses and inositol phosphate turnover upon defolliculation, indicating that under these conditions the contribution of AII receptors in the oocyte membrane to intraoocyte  $Ca^{2+}$  responses would be minimal in this population of frogs.

#### Heparin Inhibits AII-induced $Ca^{2+}$ Mobilization

The glycosaminoglycan, heparin, interacts directly with the  $Ins(1,4,5)P_3$  receptor and inhibits  $[Ca^{2+}]_i$  responses to  $Ins(1,4,5)P_3$  in permeabilized cells and microsomal preparations (20, 22, 24, 26, 38, 55, 59). To determine whether  $Ins(1,4,5)P_3$  mediates the AII-induced increase in  $[Ca^{2+}]_i$ , follicular oocytes were coinjected with aequorin and increasing amounts of heparin ( $M_r = 6,000$ ) 1–4 h before AII stimulation. Under these conditions, AII-induced light responses were completely inhibited by heparin at concentrations above 70 ng/oocyte (Fig. 2 A). Half-maximal inhibition occurred at 7 ng/oocyte, and assuming an even distribution of heparin and a cytoplasm volume of 450 nl, the  $IC_{50}$  was  $\sim 15 \mu g/ml$ . This is similar to the  $IC_{50}$  values reported for heparin's inhibition of  $Ins(1,4,5)P_3$ -dependent  $[Ca^{2+}]_i$  release in other cell types (20, 24, 38). Furthermore, low concentrations of heparin (2 ng/oocyte) shifted the AII dose-response curve to the right (Fig. 2 B), indicating that heparin inhibits  $Ins(1,4,5)P_3$  binding to its receptor in a competitive manner.

The inhibitory effect of heparin is not related to interference in the aequorin assay, since similar light responses were elicited by lysis (in 0.1% SDS) of follicular oocytes injected with aequorin alone ( $523 \pm 63$ ) and follicular oocytes coinjected with heparin and aequorin ( $499 \pm 54$ ). Direct injection of  $CaCl_2$  evoked a more rapid ( $t_{peak} = 4$  s) and transient



**Figure 3.** (A) Time courses of AII- and  $\text{Ca}^{2+}$ -induced light emission. Oocytes were injected with aequorin (33 ng/oocyte) 1–4 h before AII (500 nM) stimulation or  $\text{Ca}^{2+}$  (100  $\mu\text{M}$ ) injection. The data are from single oocytes which are representative of data from five oocytes each and expressed as the number of counts per volt emitted per second. (Inset) Time course of evoked changes in  $[\text{Ca}^{2+}]_i$  after  $\text{Ca}^{2+}$  injection. The results are from a single oocyte which is representative of data from 5 oocytes injected with

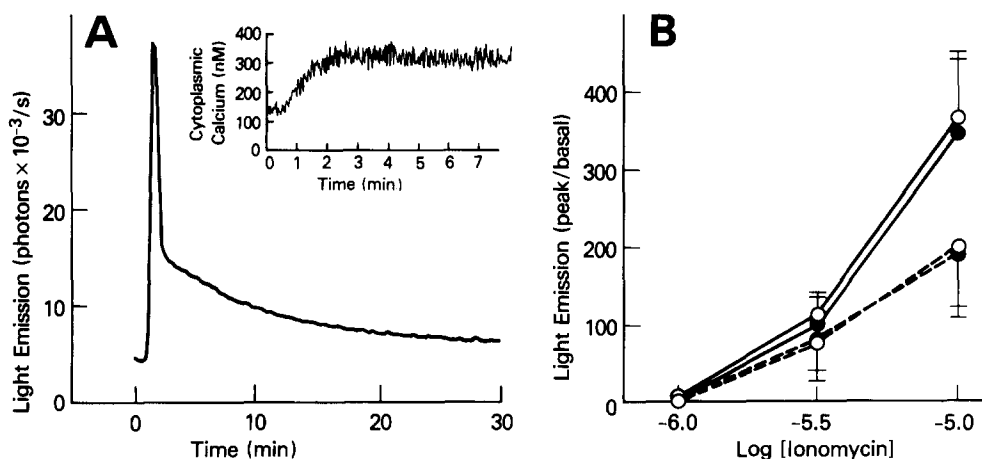
fura-2 (2 mM) 1–4 h before direct  $\text{Ca}^{2+}$  (1.5 mM) injection. The data are expressed as the levels of  $[\text{Ca}^{2+}]_i$ . (B) Dose dependence of  $\text{Ca}^{2+}$ -induced light emission in the presence and absence of heparin.  $[\text{Ca}^{2+}]_i$ -induced light emission was measured in control oocytes (aequorin alone) (○) and in oocytes coinjected with heparin (330 ng/oocyte) and aequorin (●) 1–4 h previously. Such oocytes were injected with 33 nl of  $\text{CaCl}_2$  solutions of the concentrations shown on the abscissa. Each point represents the mean  $\pm$  SE of data from 3–6 oocytes and is expressed as the fold stimulation (peak/basal).

emission of light than AII ( $t_{\text{peak}} = 22$  s), reflecting the additional steps involved in receptor-mediated  $\text{Ins}(1,4,5)\text{P}_3$ -induced  $[\text{Ca}^{2+}]_i$  mobilization (Fig. 3 A). Large concentrations of injected  $\text{Ca}^{2+}$  (>1 mM) were required to evoke measurable increases in  $[\text{Ca}^{2+}]_i$  in fura-2 injected follicular oocytes, suggesting that injected  $\text{Ca}^{2+}$  was rapidly sequestered, bound, or extruded (Fig. 3 A, inset). Heparin did not inhibit light emission evoked by direct injection of  $\text{Ca}^{2+}$  (Fig. 3 B), and did not affect the time course of  $\text{Ca}^{2+}$ -induced light emission (data not shown). These findings confirm that heparin does not interfere with  $\text{Ca}^{2+}$  binding to aequorin in the oocyte.

We also examined heparin's effects on  $\text{Ca}^{2+}$  mobilization evoked by the  $\text{Ca}^{2+}$  ionophore, ionomycin. Ionomycin induced a rapid increase in light emission which was more sustained (Fig. 4 A) than that observed with AII stimulation

(Fig. 1 A, inset). The maximum light responses to ionomycin were equivalent to twofold increases in  $[\text{Ca}^{2+}]_i$  (basal,  $110 \pm 24$  nM; ionomycin,  $240 \pm 52$  nM;  $n = 6$ ); an example is shown in Fig. 4 A, inset. Comparison of the signals from the  $\text{Ca}^{2+}$  indicators, fura-2 and aequorin, after ionomycin and AII stimulation demonstrated that aequorin is an order of magnitude more sensitive to  $[\text{Ca}^{2+}]_i$  changes than fura-2. Aequorin responses also show a different profile and are larger with increasing stimulus concentrations. These findings are consistent with previous reports comparing aequorin and fura-2  $\text{Ca}^{2+}$  signals (60) and probably result from the ability of aequorin to detect relatively large but spatially localized  $\text{Ca}^{2+}$  increases (4) which are not reflected in the average  $[\text{Ca}^{2+}]_i$  that is measured by fluorescent indicators.

Light responses were observed from 500 nM to a maximum at 10  $\mu\text{M}$  ionomycin, and ionomycin-induced light



**Figure 4.** (A) Time course of ionomycin-induced light emission. Ionomycin (10  $\mu\text{M}$ )-induced light emission was measured in oocytes injected with aequorin 1–4 h previously. The data are from a single oocyte which is representative of 5 oocytes and are expressed as the number of photons emitted per second. (Inset) Time course of ionomycin-evoked changes in intracellular  $[\text{Ca}^{2+}]_i$ . The data are from a single oocyte which is representative of results from five oocytes injected with fura-2 (2 mM) 1–4 h

before ionomycin (10  $\mu\text{M}$ ) stimulation, and are expressed as levels of intracellular  $\text{Ca}^{2+}$ . (B) Dose-dependence of ionomycin-induced  $[\text{Ca}^{2+}]_i$  responses. Light emission responses to increasing concentrations of ionomycin were measured in the absence (dotted lines) and presence (solid line) of extracellular  $[\text{Ca}^{2+}]_i$  in control oocytes injected with aequorin alone (○) and in oocytes coinjected with heparin (330 ng/oocyte) and aequorin (●) 1–4 h previously. Each point represents the mean  $\pm$  SE fold stimulation of peak light responses in three to six oocytes.

**Table I. Specificity of Heparin Inhibition of AII-induced  $Ca^{2+}$  Mobilization**

Heparin and related compounds	IC <sub>50</sub> (pg/oocyte)
Heparin ( $M_r = 3,000$ )	43
Heparin ( $M_r = 6,000$ )	86
Heparin ( $M_r = 20,000$ )	218
Heparin (desulfated)	429
Chondroitin sulfate*	>3300
Hyaluronic acid*	>3300

\* Concentrations >200  $\mu\text{g/ml}$  were not tested because of limitations in solubility and injection volume.

Oocytes were coinjected with aequorin (33 ng/oocyte), and increasing concentrations of heparin and related compounds, 1–4 h before addition of AII (500 nM). Data are expressed as percent of control light responses (peak/basal:  $31 \pm 3.5$ ,  $n = 30$ ) from aequorin-injected oocytes. IC<sub>50</sub> values are defined as the concentration of inhibitor that reduced control responses by 50%.

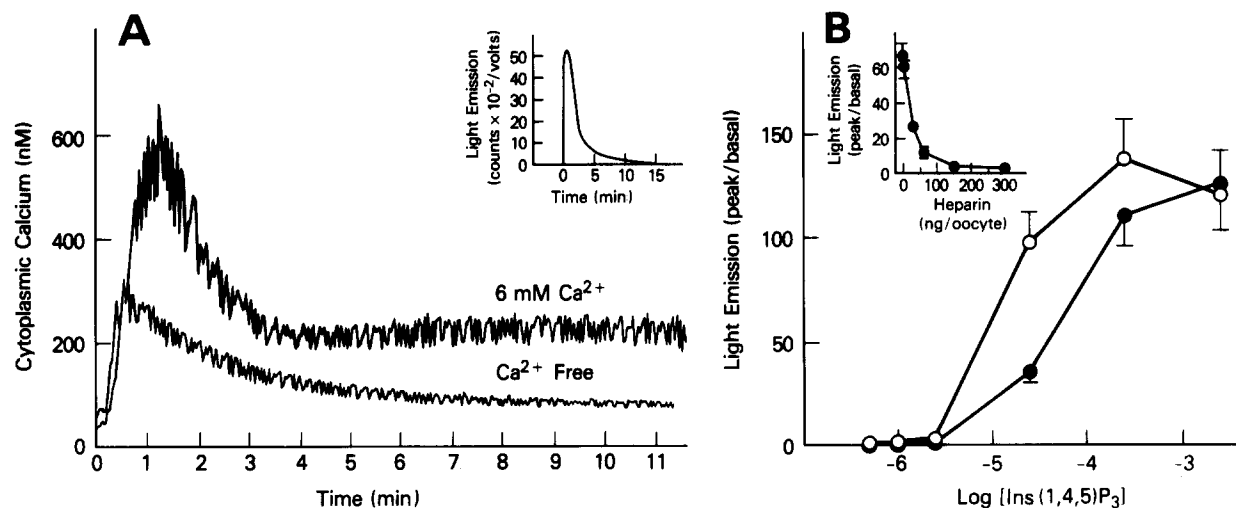
emission considerably exceeded that elicited during maximum responses to AII. Both light responses and  $Ca^{2+}$  levels returned to basal levels after 30 min at maximal levels of ionomycin. The ionophore concentration required for maximal  $[Ca^{2+}]_i$  increases was fivefold higher than observed in other tissues (39), probably reflecting the enclosure of the oocyte by surrounding follicular cells. In  $Ca^{2+}$ -free MBS, ionomycin elicited a prominent albeit reduced increase in  $[Ca^{2+}]_i$  (Fig. 4 B). Heparin did not influence the time course of ionomycin-induced light emission (data not shown), nor did it affect the ionomycin-induced  $[Ca^{2+}]_i$  mobilization in the presence and absence of extracellular  $[Ca^{2+}]_i$  (Fig. 4 B). This finding reflects the ability of the ionophore to promote  $Ca^{2+}$  release from intracellular stores

in addition to the agonist-sensitive pools, as well as  $Ca^{2+}$  influx from the extracellular fluid (39).

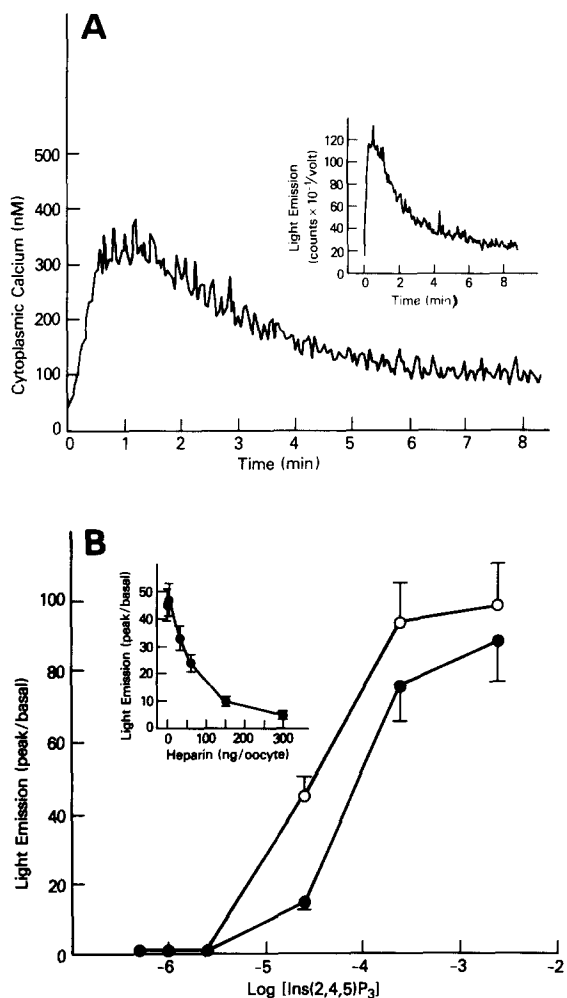
Heparin is a heterogeneous mixture of linear polysaccharide chains of varying lengths, saccharide composition, and degree of *N*-acetylation and *N*-sulfation. Its binding to  $\text{Ins}(1,4,5)\text{P}_3$  receptors is highly dependent on sulfation and chain length, and decreases markedly with desulfation but increases as the size of the heparin chain is reduced below 8–24 monosaccharide units (57, 59). In the follicular oocyte, desulfated heparin was significantly less effective as an inhibitor of AII responses than heparin sulfate. We also observed a clear dependence on chain length; the lower molecular weight heparins were significantly more potent inhibitors than the high molecular weight heparins on a mass basis (Table I), although the molar IC<sub>50</sub> values were similar ( $\sim 26$  nM). Two other glycosaminoglycans, chondroitin sulfate and hyaluronic acid, were without effect at concentrations as high as 100  $\mu\text{g/ml}$ . This is consistent with their inability to inhibit  $\text{Ins}(1,4,5)\text{P}_3$  binding and  $\text{Ins}(1,4,5)\text{P}_3$  induced  $Ca^{2+}$  release in other tissues (20, 21, 24, 38, 59).

### Heparin Competitively Inhibits $\text{Ins}(1,4,5)\text{P}_3$ -induced $Ca^{2+}$ Mobilization

Heparin is known to inhibit  $\text{Ins}(1,4,5)\text{P}_3$ -induced  $Ca^{2+}$  release from permeabilized cells (20, 24, 38) and isolated microsomes (20, 26, 57). We examined  $\text{Ins}(1,4,5)\text{P}_3$ -induced  $[Ca^{2+}]_i$  release in follicular oocytes coinjected 1–4 h previously with aequorin and heparin. In control oocytes (aequorin alone), injection of  $\text{Ins}(1,4,5)\text{P}_3$  (330 nM intraoocyte concentration) induced rapid increases in light emission which peaked within 1 min (Fig. 5 A, inset) and



**Figure 5.** (A) Time course of  $\text{Ins}(1,4,5)\text{P}_3$ -evoked changes in  $[Ca^{2+}]_i$ . The data are representative of  $[Ca^{2+}]_i$  levels in five oocytes injected with fura-2 (2 mM) 1–4 h before injection of  $\text{Ins}(1,4,5)\text{P}_3$  (10  $\mu\text{M}$ ) in the presence of 6 mM  $Ca^{2+}$  and also in  $Ca^{2+}$ -free medium. (Inset) Time course of  $\text{Ins}(1,4,5)\text{P}_3$ -induced light emission. The time course of  $\text{Ins}(1,4,5)\text{P}_3$  (10  $\mu\text{M}$ )-induced light emission is representative of five oocytes and is expressed as the number of counts per volt emitted per second. (B) Dose dependence of  $\text{Ins}(1,4,5)\text{P}_3$ -induced  $[Ca^{2+}]_i$  mobilization in the absence and presence of heparin. Light emission was measured immediately after increasing concentrations of  $\text{Ins}(1,4,5)\text{P}_3$  were injected into control oocytes (aequorin alone) (○) and oocytes coinjected with aequorin and heparin (330 ng/oocyte) (●) 1–4 h previously. Each point represents the mean fold stimulation of peak light responses  $\pm$  SE of data from three to six oocytes. (Inset) Dose dependence of heparin inhibition of  $\text{Ins}(1,4,5)\text{P}_3$ -induced  $[Ca^{2+}]_i$  mobilization. Light emission was measured immediately after  $\text{Ins}(1,4,5)\text{P}_3$  (5  $\mu\text{M}$ ) was injected into oocytes coinjected with aequorin and increasing concentrations of heparin 1–4 h previously. Each point represents the mean fold stimulation of peak light responses  $\pm$  SE of data from three to six oocytes.



**Figure 6.** (A) Time course of Ins(2,4,5)P<sub>3</sub>-evoked changes in [Ca<sup>2+</sup>]<sub>i</sub>. The data are representative of results from five oocytes injected with fura-2 (2 mM) 1–4 h before Ins(2,4,5)P<sub>3</sub> (10 μM) stimulation and are expressed as the levels of intracellular [Ca<sup>2+</sup>]<sub>i</sub>. (*Inset*) Time course of Ins(2,4,5)P<sub>3</sub>-induced light emission. The time course of Ins(2,4,5)P<sub>3</sub> (10 μM)-induced light emission is representative of results from five oocytes and is expressed as the number of counts per volt emitted per second. (B) Dose dependence of Ins(2,4,5)P<sub>3</sub>-induced [Ca<sup>2+</sup>]<sub>i</sub> release in the absence and presence of heparin. Light emission was measured immediately after increasing concentrations of Ins(2,4,5)P<sub>3</sub> were injected into control oocytes (aequorin above) (○) and oocytes coinjected with aequorin and heparin (330 ng/oocyte) (●) 1–4 h previously. Each point represents the mean fold stimulation of peak light responses ± SE of data from three to six oocytes. (*Inset*) Dose dependence of heparin inhibition of Ins(2,4,5)P<sub>3</sub>-induced [Ca<sup>2+</sup>]<sub>i</sub> release. Light emission was measured immediately after Ins(2,4,5)P<sub>3</sub> (10 μM) was injected into oocytes coinjected with aequorin and increasing concentrations of heparin 1–4 h previously. Each point represents the mean fold stimulation of peak light responses ± SE of data from three to six oocytes.

were paralleled by increases in intracellular [Ca<sup>2+</sup>]<sub>i</sub> in fura-2-injected oocytes (Fig. 5 A). These responses occurred in the absence of extracellular Ca<sup>2+</sup>, and were magnified (×2) in high Ca<sup>2+</sup> (6 mM) medium (Fig. 5 A). Measurements of light emission in follicular oocytes injected with Ins(1,4,5)P<sub>3</sub> showed that the magnitude of the [Ca<sup>2+</sup>]<sub>i</sub> response and the kinetics of peak formation were highly variable between

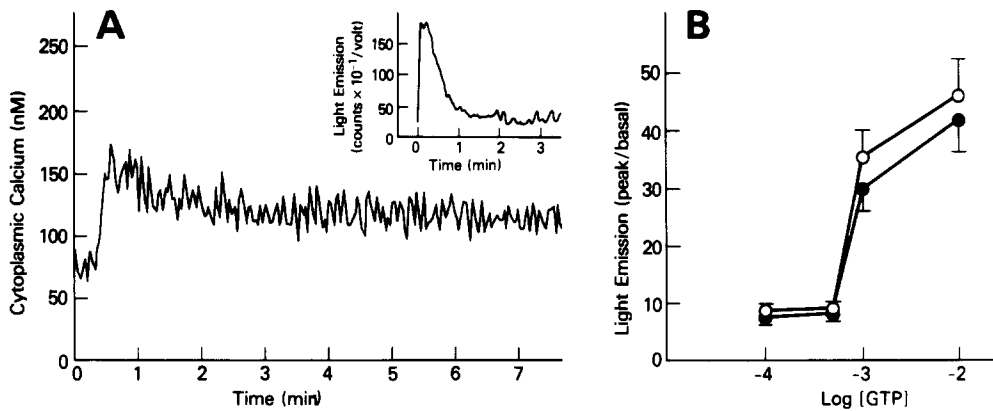
frogs and individual oocytes, which prevented statistical analysis of the data. Ins(1,4,5)P<sub>3</sub>-induced chloride currents are also known to be highly variable and to depend upon the site of injection, since most of the [Ca<sup>2+</sup>]<sub>i</sub>-regulated chloride channels are located in the animal pole (31). The concentration of Ins(1,4,5)P<sub>3</sub> required for a half-maximal aequorin response was 20 μM (=1.5 μmol/oocyte) (Fig. 5 B). It is noteworthy that μM concentrations of Ins(1,4,5)P<sub>3</sub> evoked detectable increases in [Ca<sup>2+</sup>]<sub>i</sub>, whereas much higher (mM) concentrations of Ca<sup>2+</sup> itself were required for similar responses (Fig. 3). In fura-2-injected follicular oocytes, Ins(1,4,5)P<sub>3</sub> elicited dose-dependent increases in [Ca<sup>2+</sup>]<sub>i</sub> with half-maximal effects at 50 μM, at which Ins(1,4,5)P<sub>3</sub> concentrations evoked 17-fold increases in [Ca<sup>2+</sup>]<sub>i</sub> (data not shown).

Heparin (M<sub>r</sub> = 6,000) reduced Ins(1,4,5)P<sub>3</sub> (5 μM)-induced [Ca<sup>2+</sup>]<sub>i</sub> mobilization in a dose-dependent manner (Fig. 5 B, *inset*) with maximal inhibition at 170 ng/oocyte. The relatively high sensitivity of the Ins(1,4,5)P<sub>3</sub> site to heparin in oocytes is similar to that reported for inhibition of Ins(1,4,5)P<sub>3</sub>-mediated Ca<sup>2+</sup> release in microsomal cell fractions (20, 26). In the presence of heparin (330 ng/oocyte), the Ins(1,4,5)P<sub>3</sub> dose-response curve was shifted to the right by approximately one order of magnitude. The inhibitory effect of heparin could be overcome upon addition of high doses of Ins(1,4,5)P<sub>3</sub> (200 μM), reflecting a competitive process (Fig. 5 B).

#### Heparin Selectively Inhibits Ins(1,4,5)P<sub>3</sub> Receptor-mediated Ca<sup>2+</sup> Mobilization

The specificity of heparin's interaction with the amphibian Ins(1,4,5)P<sub>3</sub> receptor is indicated by its ability to inhibit [Ca<sup>2+</sup>]<sub>i</sub> responses induced by Ins(2,4,5)P<sub>3</sub>, which releases Ca<sup>2+</sup> by binding to the Ins(1,4,5)P<sub>3</sub> receptor (15, 53). Injections of Ins(2,4,5)P<sub>3</sub> evoked dose-dependent increases in light emission in aequorin-injected follicular oocytes (Fig. 6 A, *inset*) and parallel increases in [Ca<sup>2+</sup>]<sub>i</sub> (Fig. 6 A), with more sustained responses than those elicited by Ins(1,4,5)P<sub>3</sub> in fura-2-injected follicular oocytes (Fig. 5 A). Ins(2,4,5)P<sub>3</sub> elicited a smaller maximal response than Ins(1,4,5)P<sub>3</sub> and the increases in light emission were shifted to the right (Fig. 6 B) compared with Ins(1,4,5)P<sub>3</sub> (Fig. 5 B). Heparin blocked this effect (Fig. 6 B, *inset*) in a less potent manner than Ins(1,4,5)P<sub>3</sub> (Fig. 5 B, *inset*), probably reflecting the slower hydrolysis of Ins(2,4,5)P<sub>3</sub> by the 5'-phosphatase that degrades Ins(1,4,5)P<sub>3</sub> (36). The inhibition by heparin could be overcome by increasing concentrations of Ins(2,4,5)P<sub>3</sub> (Fig. 6 B).

It is well established that Ins(1,4,5)P<sub>3</sub> releases Ca<sup>2+</sup> from a subpopulation of intracellular Ca<sup>2+</sup> sequestering compartments (21). Recent studies have indicated that distinct compartments of releasable Ca<sup>2+</sup> exist within permeabilized cells, and that translocation of Ca<sup>2+</sup> between them is mediated by a GTP-activated process (37). GTP or nonhydrolyzable GTP analogues such as guanosine 5'-O-(3-thiotrisphosphate) (GTPγS) have been found to release Ca<sup>2+</sup> in a number of cells, including rat liver and vascular smooth muscle cells (37, 38), in a manner distinct from Ins(1,4,5)P<sub>3</sub>-induced [Ca<sup>2+</sup>]<sub>i</sub> release (10). In follicular oocytes, GTP-induced light responses (Fig. 7 A, *inset*) paralleled the rapid increases in [Ca<sup>2+</sup>]<sub>i</sub> (Fig. 7 A) which were more sustained than observed with inositol phosphates. In these cells, GTP-



**Figure 7.** (A) Time course of GTP-evoked changes in intracellular  $[Ca^{2+}]_i$ . The data are from a single oocyte which is representative of results from five oocytes injected with fura-2 (2 mM) 1–4 h before GTP (1 mM) stimulation, and are expressed as the levels of  $[Ca^{2+}]_i$ . (Inset) Time course of GTP-evoked changes in light emission. Light emission was measured immediately after GTP (1 mM) was injected into oocytes injected with aequorin 1–4 h previ-

ously. The data are from a single oocyte which is representative of five oocytes and expressed as the number of counts per volt emitted per second. (B) Dose dependence of GTP-induced  $[Ca^{2+}]_i$  release in the absence and presence of heparin. Light emission was measured immediately after increasing concentrations of GTP were injected into control oocytes (aequorin alone) (○) and in oocytes coinjected with aequorin and heparin (330 ng/oocyte) (●) 1–4 h previously. Each point represents the mean  $\pm$  SE of data from three to six oocytes and is expressed as the fold stimulation above basal.

induced  $Ca^{2+}$  mobilization was dose-dependent and resistant to inhibition by heparin (Fig. 7 B).

### *Ins(1,3,4,5)P<sub>4</sub>-Induced Ca<sup>2+</sup> Mobilization*

Metabolism of  $Ins(1,4,5)P_3$  is known to proceed via two alternative pathways involving either hydrolysis of the 5'-phosphate to generate  $Ins(1,4)P_2$  or phosphorylation by a 3-kinase to yield  $Ins(1,3,4,5)P_4$  (3). The formation of  $Ins(1,3,4,5)P_4$  in agonist-stimulated cells has led to the proposal that this molecule could also have a messenger role in  $Ca^{2+}$  mobilization (42). Microinjection of  $Ins(1,3,4,5)P_4$  in follicular oocytes elicited a  $Ca^{2+}$ -dependent chloride current in the absence of extracellular  $Ca^{2+}$  (14, 41, 52, 54). These data and observations in sea urchin eggs (12), suggest that  $Ins(1,3,4,5)P_4$  is capable of mobilizing intracellular  $Ca^{2+}$  stores.

Consistent with the electrophysiological data in *Xenopus* oocytes, we found that  $Ins(1,3,4,5)P_4$  evoked a light response (Fig. 8 A, inset) which paralleled the increases in  $[Ca^{2+}]_i$  (Fig. 8 A). In comparison with  $Ins(1,4,5)P_3$  (Fig. 5 B), a fivefold greater concentration of  $Ins(1,3,4,5)P_4$  was required to evoke half-maximal  $[Ca^{2+}]_i$  release (Fig. 8 B). The  $Ca^{2+}$  signals generated by  $Ins(1,4,5)P_3$  (Fig. 5 A) and  $Ins(1,3,4,5)P_4$  (Fig. 8 A) exhibited different response kinetics. Typically, the response to  $Ins(1,4,5)P_3$  was a prompt rise to a peak  $[Ca^{2+}]_i$  level followed by a relatively rapid decay back to baseline levels (Fig. 5 A). In comparison,  $Ins(1,3,4,5)P_4$  evoked broader  $[Ca^{2+}]_i$  peaks. Furthermore, high  $Ca^{2+}$  (6 mM) did not double the peak  $[Ca^{2+}]_i$  levels observed in follicular oocytes injected with minimal doses of  $Ins(1,3,4,5)P_4$  in  $Ca^{2+}$ -free MBS (Fig. 8 A), in contrast to the effects of extracellular  $Ca^{2+}$  on  $Ins(1,4,5)P_3$ -induced responses of comparable magnitude (Fig. 5 A).

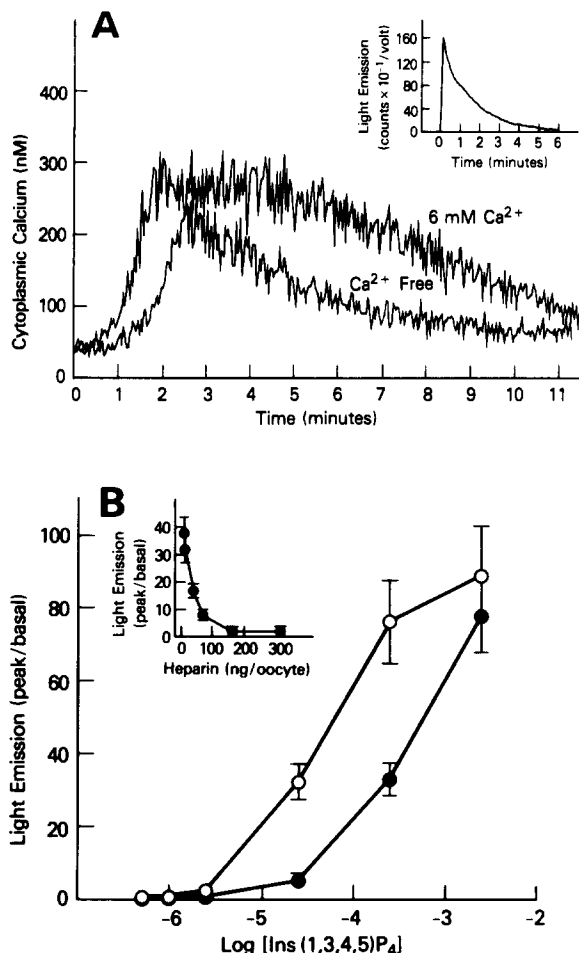
This difference between the two inositol phosphates in their dependencies upon extracellular  $Ca^{2+}$  can be compared to the effect of extracellular  $Ca^{2+}$  on their respective evoked chloride currents (14, 41, 52, 54). Electrophysiological responses to  $Ins(1,4,5)P_3$  usually comprise two or more components in which a transient current ( $I_1$ ) forms immediately followed by a subsequent phase characterized by oscillations ( $I_2$ ). Injection of  $Ins(1,3,4,5)P_4$  in contrast, usually

results in only the  $I_2$  oscillatory phase; the rapid  $I_1$  phase is either absent or very small. Electrophysiological data from oocytes injected with  $Ins(1,4,5)P_3$  show that  $Ca^{2+}$ -free medium abolishes the  $Ins(1,4,5)P_3$ -induced  $I_1$  current while leaving the  $I_2$  current intact. In comparison, extracellular  $Ca^{2+}$  removal has little effect on the  $I_2$  current evoked by injection of  $Ins(1,3,4,5)P_4$ . These reports together with our results suggest that  $Ins(1,4,5)P_3$  plays a role in  $Ca^{2+}$  entry whereas  $Ins(1,3,4,5)P_4$  does not. This effect of  $Ins(1,4,5)P_3$  is only observed at elevated extracellular  $Ca^{2+}$  levels ( $>3$  mM) and is consistent with the report that  $Ins(1,4,5)P_3$  activates a  $Ca^{2+}$  channel by a ligand binding mechanism (51).

Heparin inhibited  $Ins(1,3,4,5)P_4$ -induced  $Ca^{2+}$  mobilization (Fig. 8 B) with similar potency to that observed for inhibition of  $Ins(1,4,5)P_4$ -induced  $Ca^{2+}$  release (Fig. 5 B). Increasing concentrations of  $Ins(1,3,4,5)P_4$  could overcome the heparin inhibition (Fig. 8 B), suggesting that heparin is acting competitively. These data can be accounted for by: (a) major contamination of  $Ins(1,3,4,5)P_4$  with  $Ins(1,4,5)P_3$ , however, phosphate and nuclear magnetic resonance (NMR) analysis indicate that the  $Ins(1,3,4,5)P_4$  preparation does not contain other inositol phosphates (Calbiochem-Behring Corp.); (b) extensive metabolism of  $Ins(1,3,4,5)P_4$  to  $Ins(1,4,5)P_3$  which then elicits  $Ca^{2+}$  release; (c) mobilization of  $Ca^{2+}$  by binding of  $Ins(1,3,4,5)P_4$  to the  $Ins(1,4,5)P_3$  receptor; or (d) direct interaction of  $Ins(1,3,4,5)P_4$  with its own receptor. Specific  $Ins(1,3,4,5)P_4$  binding sites that are apparently distinct from the  $Ins(1,4,5)P_3$  receptor site have been identified in HL-60 cells (5), rat brain (56), and adrenal cortex (15). Heparin has been reported to be a more potent inhibitor of  $Ins(1,3,4,5)P_4$  than  $Ins(1,4,5)P_3$ -mediated  $Ca^{2+}$  release from cerebellar microsomes (27), but appears to be a relatively specific inhibitor of  $Ins(1,4,5)P_3$ -induced  $Ca^{2+}$  release from adrenal microsomes (15).

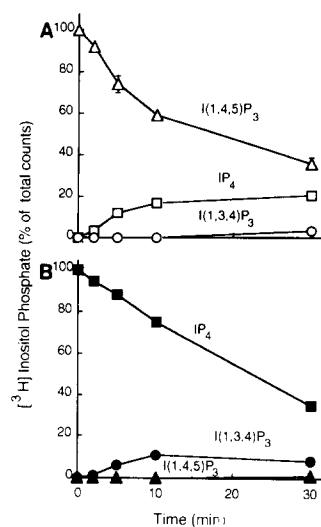
### *Metabolism of [<sup>3</sup>H]Ins(1,3,4,5)P<sub>4</sub> and [<sup>3</sup>H]Ins(1,4,5)P<sub>3</sub>*

To investigate the first two possibilities, we injected [<sup>3</sup>H]- $Ins(1,4,5)P_3$  and followed its metabolism by HPLC analysis under the conditions used in fura-2 and aequorin experiments. During the 2-min period in which we observed AII-mediated  $[Ca^{2+}]_i$  release, 3.5% of the [<sup>3</sup>H] $Ins(1,4,5)P_3$  was



**Figure 8.** (A) Time course of  $\text{Ins}(1,3,4,5)\text{P}_3$ -evoked changes in  $[\text{Ca}^{2+}]_i$  in the absence and presence of extracellular  $\text{Ca}^{2+}$ . The results are from single oocytes and are representative of data from five oocytes injected with fura-2 (2 mM) 1–4 h before  $\text{Ins}(1,3,4,5)\text{P}_3$  (50  $\mu\text{M}$ ) stimulation in the presence of 6 mM  $\text{Ca}^{2+}$  and also in  $\text{Ca}^{2+}$ -free medium. The data are expressed as the levels of  $[\text{Ca}^{2+}]_i$ . (Inset) Time course of  $\text{Ins}(1,3,4,5)\text{P}_3$ -induced light emission. The time course of  $\text{Ins}(1,3,4,5)\text{P}_3$  (50  $\mu\text{M}$ )-induced light emission is from a single oocyte which is representative of five oocytes each and is expressed as the number of counts per volt emitted per second. (B) Dose dependence of  $\text{Ins}(1,3,4,5)\text{P}_3$ -induced  $\text{Ca}^{2+}$  release in the absence and presence of heparin. Light emission was measured immediately after increasing concentrations of  $\text{Ins}(1,3,4,5)\text{P}_3$  were injected into control oocytes (aequorin alone) (○) and in oocytes coinjected with aequorin and heparin (330 ng/oocyte) (●) 1–4 h previously. Each point represents the mean fold stimulation of peak light responses  $\pm$  SE of data from three to six oocytes. (Inset) Dose dependence of heparin inhibition of  $\text{Ins}(1,3,4,5)\text{P}_3$ -induced  $[\text{Ca}^{2+}]_i$  release. Light emission was measured immediately after  $\text{Ins}(1,3,4,5)\text{P}_3$  (50  $\mu\text{M}$ ) was injected into oocytes coinjected with aequorin and increasing concentrations of heparin 1–4 h previously. Each point represents the mean  $\pm$  SE of data from three to six oocytes and is expressed as the fold stimulation (*peak/basal*).

phosphorylated to  $[\text{^3H}]\text{Ins}(1,3,4,5)\text{P}_4$  (Fig. 9 A). By 30 min, 20% of the  $\text{Ins}(1,4,5)\text{P}_3$  was phosphorylated to  $\text{Ins}(1,3,4,5)\text{P}_4$  and the remainder of the counts accumulated in degradation products of  $\text{Ins}(1,4,5)\text{P}_3$ , namely  $6.0 \pm 0.6\%$  in  $\text{InsP}_2$ ,  $15 \pm 1\%$  in  $\text{InsP}_1$ , and  $6.2 \pm 0.7\%$  in  $\text{Ins}$ . This is in contrast to studies reported in *Xenopus* oocyte homogenates in which 30% of the added  $[\text{^3H}]\text{Ins}(1,4,5)\text{P}_3$  was metabolized to  $[\text{^3H}]\text{Ins}(1,3,4,5)\text{P}_4$  after 8 min (35).



**Figure 9.** (A) Time course of  $\text{Ins}(1,4,5)\text{P}_3$  metabolism. Oocytes were injected with  $[\text{^3H}]\text{Ins}(1,4,5)\text{P}_3$  (500 cpm/oocyte) and incubated in MBS for 0–20 min. At the indicated times, the oocytes (25/sample) were frozen on dry ice after the addition of 10% perchloric acid, then extracted and analyzed by HPLC. The data are expressed as the percent of the total counts and are representative of three experiments each performed in duplicate. (B) Time course of  $\text{Ins}(1,3,4,5)\text{P}_4$  metabolism. Oocytes were injected with  $[\text{^3H}]\text{Ins}(1,3,4,5)\text{P}_4$  (500 cpm/oocyte) and incubated in MBS for 0–20 min. At the indicated times, the oocytes (25/sample) were frozen on dry ice after the addition of 10% perchloric acid, then extracted and analyzed by HPLC. The data are expressed as the percent of the total counts and are representative of three experiments each performed in duplicate.

To determine whether  $\text{Ins}(1,3,4,5)\text{P}_4$ -induced  $\text{Ca}^{2+}$  responses could be attributed to  $\text{Ins}(1,4,5)\text{P}_3$  formation, we injected  $[\text{^3H}]\text{Ins}(1,3,4,5)\text{P}_4$  into follicular oocytes and followed its metabolism by HPLC analysis. The metabolic products formed from  $[\text{^3H}]\text{Ins}(1,3,4,5)\text{P}_4$  were identified by an HPLC method that fully resolves both isomers of inositol trisphosphate (1). Follicular oocytes metabolized  $[\text{^3H}]\text{Ins}(1,3,4,5)\text{P}_4$  primarily to  $[\text{^3H}]\text{Ins}(1,3,4)\text{P}_3$ , with no detectable formation of  $[\text{^3H}]\text{Ins}(1,4,5)\text{P}_3$  for up to 10 min (Fig. 9 B), which is considerably longer than the 2-min time span over which we observed  $\text{Ins}(1,3,4,5)\text{P}_4$ -induced  $\text{Ca}^{2+}$  mobilization. After 30 min, 10% of the  $[\text{^3H}]\text{Ins}(1,3,4,5)\text{P}_4$  was metabolized to  $\text{Ins}(1,3,4)\text{P}_3$ , which subsequently underwent further dephosphorylation to  $\text{InsP}_2$  ( $26 \pm 0.1\%$ ),  $\text{InsP}_1$  ( $16.2 \pm 1.0\%$ ), and  $\text{Ins}$  ( $7.2 \pm 0.1\%$ ) with only 1.6% converted to  $[\text{^3H}]\text{Ins}(1,4,5)\text{P}_3$ . This is in contrast to studies reported in *Xenopus* oocyte homogenates, in which 8.4% of the  $[\text{^3H}]\text{Ins}(1,3,4,5)\text{P}_4$  was metabolized to  $[\text{^3H}]\text{Ins}(1,4,5)\text{P}_3$  after 3 min (35). These differences between inositol phosphate metabolism in injected oocytes compared with homogenates is probably attributable to the loss of membrane integrity and intracellular milieu of the intact oocyte upon homogenization.

The finding that  $\text{Ins}(1,4,5)\text{P}_3$  was a very minor product of  $\text{Ins}(1,3,4,5)\text{P}_4$  metabolism is in agreement with previous findings in permeabilized adrenal cells, that  $\text{Ins}(1,3,4,5)\text{P}_4$  is predominantly dephosphorylated to  $\text{Ins}(1,3,4)\text{P}_3$  (1, 15), which itself has no  $[\text{Ca}^{2+}]_i$ -mobilizing activity in the oocyte (data not shown). Because of the marked difference in the effect of extracellular  $\text{Ca}^{2+}$  and the minute conversion of  $[\text{^3H}]\text{Ins}(1,3,4,5)\text{P}_4$  to  $[\text{^3H}]\text{Ins}(1,4,5)\text{P}_3$ ,  $\text{Ins}(1,3,4,5)\text{P}_4$  is likely to exert its effects by interacting with an intracellular receptor and not as a result of its metabolism to  $\text{Ins}(1,4,5)\text{P}_3$ .

## Discussion

These findings have provided direct evidence that AII receptors in *Xenopus* follicular oocytes are coupled to phos-



phoinositide hydrolysis. The relatively small increase in  $\text{Ins}(1,4,5)\text{P}_3$  production (100%) probably reflects its rapid production and metabolism, while the inhibition of 5-phosphatase activity by lithium permits larger accumulations (by 100–300%) of  $\text{IP}_2$  and  $\text{Ins}(1,3,4)\text{P}_3$  (Fig. 1). These findings are generally similar to the characteristics of inositol phosphate production observed during AII stimulation of other cell types (2). In a recent report, McIntosh and McIntosh (34) were unable to detect inositol phosphate production upon stimulation of endogenous AII receptors in *Xenopus* oocytes. This apparent discrepancy with our results probably reflects our selection of oocytes from frogs that exhibit high AII responsiveness; we did not detect increases in inositol phosphates in oocytes which responded less than eightfold in the aequorin assay. Furthermore, we found that injecting [ $^3\text{H}$ ]inositol rather than incubating oocytes with [ $^3\text{H}$ ]inositol, and preincubating for 2 h with 40 mM lithium chloride, were both necessary conditions for observing AII-mediated inositol phosphate production.

It is interesting that the AII-induced  $\text{Ca}^{2+}$  signal is far more robust than the corresponding  $\text{Ins}(1,4,5)\text{P}_3$  response, at least as revealed by the formation of [ $^3\text{H}$ ]  $\text{Ins}(1,4,5)\text{P}_3$  (Fig. 1). We observed 10–100-fold changes in AII-induced aequorin responses compared with only two- to fourfold increases in inositol phosphate production, which suggests that very little  $\text{Ins}(1,4,5)\text{P}_3$  is required to release large amounts of intracellular  $\text{Ca}^{2+}$  or that individual compartments of  $\text{Ins}(1,4,5)\text{P}_3$ -sensitive  $\text{Ca}^{2+}$  pools exist within the oocyte. This latter suggestion is supported by the recent report of Shapira et al. (47) which demonstrated that *Xenopus* oocytes possess distinct subcellular  $\text{Ca}^{2+}$  pools that couple selectively to two different populations of membrane receptors. The fact that considerable amounts of injected  $\text{CaCl}_2$  are required to evoke a detectable aequorin response (in contrast to the large light responses to small amounts of  $\text{Ins}[1,4,5]\text{P}_3$ ) could reflect extensive binding or sequestration of microinjected  $\text{Ca}^{2+}$  that impedes contact with the  $\text{Ca}^{2+}$  indicators. In contrast,  $[\text{Ca}^{2+}]_i$  mobilization by  $\text{Ins}(1,4,5)\text{P}_3$  may occur in regions that are more accessible to the indicators. These findings are in agreement with electrophysiological data in *Xenopus* oocytes in that  $\text{Ins}(1,4,5)\text{P}_3$  evokes far greater chloride responses than 100-fold higher concentrations of  $\text{CaCl}_2$  (14, 40, 52, 54). These findings are relevant to the observation that the ER network of the  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release system is not present in the oocyte, but develops during its maturation to the egg (19).

Our results indicate that heparin inhibits AII-induced  $\text{Ca}^{2+}$  release in follicular oocytes by interacting specifically with the amphibian  $\text{Ins}(1,4,5)\text{P}_3$  receptor. The dose response curves of  $\text{Ca}^{2+}$  mobilization induced by AII (Fig. 2 B),  $\text{Ins}(1,4,5)\text{P}_3$  (Fig. 5 B), and  $\text{Ins}(2,4,5)\text{P}_3$  (Fig. 6 B) were all shifted to the right in the presence of heparin, indicating that heparin inhibits the *Xenopus*  $\text{Ins}(1,4,5)\text{P}_3$  receptor in a competitive manner. Heparin does not interfere with  $\text{Ca}^{2+}$  homeostasis per se, since it has no effect on ionomycin-induced  $\text{Ca}^{2+}$  influx and mobilization (Fig. 4) at concentrations which inhibit AII- (Fig. 2),  $\text{Ins}(1,4,5)\text{P}_3$ - (Fig. 5), and  $\text{Ins}(2,4,5)\text{P}_3$ - (Fig. 6) induced  $\text{Ca}^{2+}$  release. The ability of GTP to evoke heparin-insensitive  $\text{Ca}^{2+}$  release (Fig. 7) supports the view that heparin's action is on the  $\text{Ins}(1,4,5)\text{P}_3$ -activated  $\text{Ca}^{2+}$  release mechanism, especially since GTP and  $\text{Ins}(1,4,5)\text{P}_3$  have been shown to release

$\text{Ca}^{2+}$  from the same pool (10, 21). These data are consistent with observations that heparin does not alter other  $\text{Ca}^{2+}$  transport activities including the GTP-activated  $\text{Ca}^{2+}$  translocation process or any undefined passive  $\text{Ca}^{2+}$  fluxes that may contribute to the attainment of  $\text{Ca}^{2+}$  equilibrium in microsomes (20). Taken together, these results suggest that heparin inhibits agonist-induced  $\text{Ca}^{2+}$  mobilization in a manner similar to its interaction with the mammalian  $\text{Ins}(1,4,5)\text{P}_3$  receptor, suggesting conservation between the two receptors. Furthermore, this is the first demonstration that *Xenopus* oocytes possess a GTP-sensitive  $\text{Ca}^{2+}$  pool from which  $\text{Ca}^{2+}$  release can be triggered independently of  $\text{Ins}(1,4,5)\text{P}_3$  and indicates that the *Xenopus* oocyte may serve as a valuable model for studying the regulation of GTP and  $\text{Ins}(1,4,5)\text{P}_3$ -sensitive  $\text{Ca}^{2+}$  pools. The advantage of the *Xenopus* oocyte system lies in the ability to inject agents into oocytes and follow agonist-induced  $\text{Ca}^{2+}$  mobilization in an intact cell, compared with studies using permeabilized cells which could lead to artifactual alteration of intracellular constituents resulting from the permeabilization process.

The ability of heparin to abolish AII-induced  $\text{Ca}^{2+}$  release indicates that the  $[\text{Ca}^{2+}]_i$  response is completely dependent upon  $\text{Ins}(1,4,5)\text{P}_3$  production. We have previously demonstrated that AII does not induce significant  $\text{Ca}^{2+}$  entry in *Xenopus* follicular oocytes even in the presence of high extracellular  $\text{Ca}^{2+}$  (45). This was confirmed in the present study with both aequorin and fura-2 (not shown) and is consistent with the ability of heparin to block all of the detectable AII-induced light emission. It is possible that heparin exerts other effects in addition to inhibiting the  $\text{Ins}(1,4,5)\text{P}_3$  receptor, since it is known to interfere with a variety of physiological processes. However, the findings that heparin competitively inhibits  $\text{Ins}(1,4,5)\text{P}_3$  (Fig. 5 B) and  $\text{Ins}(2,4,5)\text{P}_3$ - (Fig. 6 B) induced  $\text{Ca}^{2+}$  release, but not  $\text{Ca}^{2+}$  (Fig. 3 B), ionomycin (Fig. 4 B), or GTP- (Fig. 7 B) induced  $\text{Ca}^{2+}$  release, at concentrations that completely inhibit AII responses (Fig. 2), are consistent with its selective inhibition of the *Xenopus* oocyte  $\text{Ins}(1,4,5)\text{P}_3$  receptor.

We recently demonstrated that AII receptors are located on the surrounding follicular cells of *Xenopus* oocytes and transfer signals to the oocyte via gap junctions, leading to elevations of intraoocyte  $[\text{Ca}^{2+}]_i$  (45). Two potential candidates for this signal are  $\text{Ins}(1,4,5)\text{P}_3$  and  $\text{Ca}^{2+}$ , both of which can be transmitted through gap junctions in rat liver hepatocytes (46). The fact that heparin completely inhibits AII-induced  $\text{Ca}^{2+}$  release (Fig. 2 B) at concentrations we have shown to selectively inhibit the oocyte  $\text{Ins}(1,4,5)\text{P}_3$  receptor (Fig. 5 B), coupled with the demonstration that heparin does not interfere with light emission evoked by the  $\text{Ca}^{2+}$  ionophore, ionomycin (Fig. 4 B), or by direct injection of  $\text{Ca}^{2+}$  into the oocyte cytoplasm (Fig. 3 B), indicates that the  $\text{Ca}^{2+}$ -mobilizing signal transferred from follicular cells to the oocyte is not  $\text{Ca}^{2+}$  alone. However, our results do not rule out the possibility that transfer of  $\text{Ca}^{2+}$  through gap junctions could regulate the  $\text{Ins}(1,4,5)\text{P}_3$ -evoked  $\text{Ca}^{2+}$  mobilization. Recently, Finch et al. (17) have demonstrated that  $\text{Ca}^{2+}$  can induce sequential positive and negative feedback regulation of  $\text{Ins}(1,4,5)\text{P}_3$ -induced  $\text{Ca}^{2+}$  release. Our results also do not rule out the possibility that  $\text{Ins}(1,3,4,5)\text{P}_4$  could regulate  $\text{Ins}(1,4,5)\text{P}_3$ -evoked  $\text{Ca}^{2+}$  mobilization as a result of the capability of the oocyte to metabolize  $\text{Ins}(1,4,5)\text{P}_3$  to  $\text{Ins}(1,3,4,5)\text{P}_3$  (Fig. 9). The metabolism of

Ins(1,4,5)P<sub>3</sub> could lead to Ins(1,3,4,5)P<sub>4</sub> transfer through gap junctions and subsequent [Ca<sup>2+</sup>]<sub>i</sub> mobilization in addition to Ins(1,4,5)P<sub>3</sub>-induced Ca<sup>2+</sup> release, either through Ins(1,3,4,5)P<sub>4</sub> binding and activating the Ins(1,4,5)P<sub>3</sub> receptor or by activating its own receptor.

In conclusion, the endogenous AII receptors of the follicle cells surrounding *Xenopus* oocytes are coupled to activation of phospholipase C, as demonstrated by the ability of AII to stimulate inositol phosphate production in follicular oocytes. The ability of heparin to block AII-induced elevations of intra-oocyte Ca<sup>2+</sup>, in a manner specific for the Ins(1,4,5)P<sub>3</sub> receptor, indicates that [Ca<sup>2+</sup>]<sub>i</sub> responses in the oocyte are mediated by an Ins(1,4,5)P<sub>3</sub>-dependent mechanism. This probably involves the transfer from follicular cells of Ins(1,4,5)P<sub>3</sub>, rather than Ca<sup>2+</sup> or another signal molecule that is independent of Ins(1,4,5)P<sub>3</sub> production.

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