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 (All) receptors on the surrounding follicular cells sends a signal through gap junctions to elevate cytoplasmic calcium concentration ([Ca$^{2+}$]) 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, All 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 All-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 All-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.

**Materials and Methods**

**Materials and Buffers**

Angiotensin II (All) was obtained from Peninsula Laboratories Inc. (Belmont, CA), and m-aminobenzoate, heparin, 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 I (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 7.4.
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 (60 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

[3H]Insitol (5 × 10⁴ cpm/oocyte), [3H]Ins(1,4,5)P₃ (500 cpm/oocyte), and [3H]Ins(1,3,4,5)P₄ (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 nm, Alltech Associates, 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 coincidence 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 Eidiath, 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 CaCl₂ (6). The autofluorescence of uninjected oocytes was determined and subtracted from the basal levels of fluorescence from fura-2–injected oocytes.

Results

All Stimulates Ca²⁺ Mobilization and Inositol Phosphate Production

In follicular oocytes injected with the Ca²⁺-specific photoprotein, aequorin (45), agonist stimulation of All receptors evokes [Ca²⁺]i mobilization as evidenced by rapid and transient increases in light emission (Fig. 1 A, inset). Changes in [Ca²⁺]i 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 [Ca²⁺]i (4). To determine the actual changes in [Ca²⁺]i, follicular oocytes were injected with fura-2 and imaged with an Atto-fluor digital microscopy system. A maximal stimulatory concentration of All (500 nM) increased [Ca²⁺]i 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-

course of All-induced inositol phosphate production. Oocytes from the same frog as in Fig. 1 A, inset were injected with [3H]inositol 24 h before All stimulation. The data are expressed as the production of [3H]InsP₁, [3H]Ins(1,3,4)P₃, and [3H]Ins(1,4,5)P₃ over 20 min of stimulation and are representative of six experiments performed in duplicate. (Inset) Stimulation of Ins(1,4,5)P₃ production by All. Ins(1,4,5)P₃ levels were measured in oocytes stimulated for 10 s with 500 nM All. Bars indicate the mean ± standard error (SE) of data from six independent experiments.
response to AII corresponded to a 7.5 ± 0.8-fold increase in [Ca^{2+}]. The [Ca^{2+}] response to AII is similar to that previously observed in fura-2 injected oocytes (6) except that our basal levels of [Ca^{2+}] 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+}] signals vary markedly between frogs (45), we screened follicular oocytes from several animals for high [Ca^{2+}] 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(1,4,5)P_{3} 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 ± 52 cpm to peak levels of 605 ± 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- Meiri 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](https://example.com/figure2.png)

**Figure 2.** (A) Dose-dependent inhibition of AII-induced Ca^{2+} mobilization by heparin. Oocytes were co-injected 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 ± 2.4-fold stimulation) from aequorin-injected oocytes. Each point represents the mean ± S.E. of data from three to six oocytes. (B) Dose dependence of AII-induced [Ca^{2+}], responses in the presence and absence of heparin. Oocytes were injected with aequorin (33 ng/oocyte) (○) or co-injected with aequorin and heparin (2 ng/oocyte) (●) and stimulated with increasing concentrations of AII. Each point represents the mean ± S.E. 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+}], 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+}], follicular oocytes were co-injected with aequorin and increasing amounts of heparin (M, = 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 ~15 μg/ml. This is similar to the IC_{50} values reported for heparin's inhibition of Ins(1,4,5)P_{3}-dependent [Ca^{2+}], 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 ± 63) and follicular oocytes co-injected with heparin and aequorin (499 ± 54). Direct injection of CaCl_{2} evoked a more rapid (t_{max} = 4 s) and transient
fura-2 (2 mM) 1–4 h before direct Ca²⁺ (1.5 mM) injection. The data are expressed as the levels of [Ca²⁺],. (B) Dose dependence of Ca²⁺-induced light emission in the presence and absence of heparin. [Ca²⁺]-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 CaCl₂ solutions of the concentrations shown on the abscissa. Each point represents the mean ± SE of data from 3–6 oocytes and is expressed as the fold stimulation (peak/basal).

emission of light than All (peak = 22 s), reflecting the additional steps involved in receptor-mediated Ins(1,4,5)P₃-induced [Ca²⁺] mobilization (Fig. 3 A). Large concentrations of injected Ca²⁺ (>1 mM) were required to evoke measurable increases in [Ca²⁺], in fura-2 injected follicular oocytes, suggesting that injected Ca²⁺ was rapidly sequestered, bound, or extruded (Fig. 3 A, inset). Heparin did not inhibit light emission evoked by direct injection of Ca²⁺ (Fig. 3 B), and did not affect the time course of Ca²⁺-induced light emission (data not shown). These findings confirm that heparin does not interfere with Ca²⁺ binding to aequorin in the oocyte.

We also examined heparin’s effects on Ca²⁺ mobilization evoked by the Ca²⁺ ionophore, ionomycin. Ionomycin induced a rapid increase in light emission which was more sustained (Fig. 4 A) than that observed with All stimulation (Fig. 1 A, inset). The maximum light responses to ionomycin were equivalent to twofold increases in [Ca²⁺], (basal, 110 ± 24 nM; ionomycin, 240 ± 52 nM; n = 6); an example is shown in Fig. 4 A, inset. Comparison of the signals from the Ca²⁺ indicators, fura-2 and aequorin, after ionomycin and All stimulation demonstrated that aequorin is an order of magnitude more sensitive to [Ca²⁺] 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 Ca²⁺ signals (60) and probably result from the ability of aequorin to detect relatively large but spatially localized Ca²⁺ increases (4) which are not reflected in the average [Ca²⁺], that is measured by fluorescent indicators.

Light responses were observed from 500 nM to a maximum at 10 μM ionomycin, and ionomycin-induced light before ionomycin (10 μM) stimulation, and are expressed as levels of intracellular Ca²⁺. (B) Dose-dependence of ionomycin-induced [Ca²⁺], responses. Light emission responses to increasing concentrations of ionomycin were measured in the absence (dotted lines) and presence (solid line) of extracellular Ca²⁺, 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 ± SE fold stimulation of peak light responses in three to six oocytes.
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 Ins(1,4,5)P₃ 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 all 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 IC₅₀ values were similar (~26 nM). Two other glycosaminoglycans, chondroitin sulfate and hyaluronic acid, were without effect at concentrations as high as 100 μg/ml. This is consistent with their inability to inhibit Ins(1,4,5)P₃ binding and Ins(1,4,5)P₃-induced Ca²⁺ release in other tissues (20, 21, 24, 38, 39).

Heparin Competitively Inhibits Ins(1,4,5)P₃-induced Ca²⁺ Mobilization

Heparin is known to inhibit Ins(1,4,5)P₃-induced Ca²⁺ release from permeabilized cells (20, 24, 38) and isolated microsomes (20, 26, 57). We examined Ins(1,4,5)P₃-induced [Ca²⁺] mobilization in the presence and absence of extracellular [Ca²⁺] (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 [Ca²⁺] mobilization in the presence and absence of extracellular [Ca²⁺] (Fig. 4 B). This finding reflects the ability of the ionophore to promote Ca²⁺ release from intracellular stores in addition to the agonist-sensitive pools, as well as Ca²⁺ influx from the extracellular fluid (39).

Heparin and related compounds IC₅₀ (pg/oocyte)

| Heparin (M = 3,000) | 43 |
| Heparin (M = 6,000) | 86 |
| Heparin (M = 20,000) | 218 |
| Heparin (desulfated) | 429 |
| Chondroitin sulfate | >3300 |
| Hyaluronic acid | >3300 |

* Concentrations >200 μg/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 ± 3.5, n = 30) from aequorin-injected oocytes. IC₅₀ 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 Ca²⁺ levels returned to basal levels after 30 min at maximal levels of ionomycin. The ionophore concentration required for maximal [Ca²⁺] increases was fivefold higher than observed in other tissues (39), probably reflecting the enclosure of the oocyte by surrounding follicular cells. In Ca²⁺-free MBS, ionomycin elicited a prominent albeit reduced increase in [Ca²⁺] (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 [Ca²⁺] mobilization in the presence and absence of extracellular [Ca²⁺] (Fig. 4 B). This finding reflects the ability of the ionophore to promote Ca²⁺ release from intracellular stores in addition to the agonist-sensitive pools, as well as Ca²⁺ influx from the extracellular fluid (39).

Table I. Specificity of Heparin Inhibition of AII-induced Ca²⁺ Mobilization

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| Heparin (desulfated)          | 429             |
| Chondroitin sulfate           | >3300           |
| Hyaluronic acid               | >3300           |

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Heparin Competitively Inhibits Ins(1,4,5)P₃-induced Ca²⁺ Mobilization

Heparin is known to inhibit Ins(1,4,5)P₃-induced Ca²⁺ release from permeabilized cells (20, 24, 38) and isolated microsomes (20, 26, 57). We examined Ins(1,4,5)P₃-induced [Ca²⁺] release in follicular oocytes coinjected 1-4 h previously with aequorin and heparin. In control oocytes (aequorin alone), injection of Ins(1,4,5)P₃ (330 nM intraocytoe concentration) induced rapid increases in light emission which peaked within 1 min (Fig. 5 A, inset) and

![Figure 5](image-url)
were paralleled by increases in intracellular [Ca2+]i, in fura-
2-injected oocytes (Fig. 5 A). These responses occurred in
the absence of extracellular Ca2+, and were magnified (×2)
in high Ca2+ (6 mM) medium (Fig. 5 A). Measurements of
light emission in follicular oocytes injected with Ins(1,4,5)P3
showed that the magnitude of the [Ca2+]i 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)P3-induced chloride currents are
also known to be highly variable and to depend upon the site
of injection, since most of the [Ca2+]i-regulated chloride
channels are located in the animal pole (31). The concentra-
tion of Ins(1,4,5)P3 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)P3 evoked
detectable increases in [Ca2+]i, whereas much higher (mM)
concentrations of Ca2+ itself were required for similar re-
sponses (Fig. 3). In fura-2-injected follicular oocytes, Ins-
(1,4,5)P3 elicited dose-dependent increases in [Ca2+]i, with
half-maximal effects at 50 μM, at which Ins(1,4,5)P3 concen-
trations evoked 17-fold increases in [Ca2+]i (data not shown).

Heparin (M, = 6,000) reduced Ins(1,4,5)P3 (5 μM)-
induced [Ca2+]i 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)P3 site to
heparin in oocytes is similar to that reported for inhibition
of Ins(1,4,5)P3-mediated Ca2+ release in mesosom al cell
fractions (20, 26). In the presence of heparin (330 ng/oocyte),
the Ins(1,4,5)P3 dose–response curve was shifted to the
right by approximately one order of magnitude. The inhibi-
tory effect of heparin could be overcome upon addition of
high doses of Ins(1,4,5)P3 (200 μM), reflecting a competi-
tive process (Fig. 5 B).

**Heparin Selectively Inhibits Ins(1,4,5)P3,
Receptor-mediated Ca2+ Mobilization**

The specificity of heparin's interaction with the amphibian
Ins(1,4,5)P3 receptor is indicated by its ability to inhibit
[Ca2+]i responses induced by Ins(2,4,5)P3, which releases
Ca2+ by binding to the Ins(1,4,5)P3 receptor (15, 53). Injec-
tions of Ins(2,4,5)P3 evoked dose-dependent increases in
light emission in aequorin-injected follicular oocytes (Fig.
6 A, inset) and parallel increases in [Ca2+]i (Fig. 6 A), with
more sustained responses than those elicited by insulinP3,
in fura-2-injected follicular oocytes (Fig. 5 A). Ins(2,4,5)P3
elicited a smaller maximal response than Ins(1,4,5)P3, and
the increases in light emission were shifted to the right (Fig.
6 B) compared with Ins(1,4,5)P3 (Fig. 5 B). Heparin blocked
this effect (Fig. 6 B, inset) in a less potent manner than
Ins(1,4,5)P3 (Fig. 5 B, inset), probably reflecting the slower
hydrolysis of Ins(2,4,5)P3 by the S'-phosphatase that de-
grades Ins(1,4,5)P3 (36). The inhibition by heparin could
be overcome by increasing concentrations of Ins(2,4,5)P3
(Fig. 6 B).

It is well established that Ins(1,4,5)P3 releases Ca2+ from
a subpopulation of intracellular Ca2+ sequestering com-
partments (21). Recent studies have indicated that distinct com-
partments of releasable Ca2+ exist within permeabilized
cells, and that translocation of Ca2+ between them is medi-
ated by a GTP-activated process (37). GTP or nonhydrolyza-
table GTP analogues such as guanosine 5'-0-(3-thiotriphos-
phate) (GTPgammaS) have been found to release Ca2+ in
a number of cells, including rat liver and vascular smooth
muscle cells (37, 38), in a manner distinct from Ins(1,4,5)P3-
induced [Ca2+]i release (10). In follicular oocytes, GTP-
induced light responses (Fig. 7 A, inset) paralleled the rapid
increases in [Ca2+]i (Fig. 7 A) which were more sustained
than observed with inositol phosphates. In these cells, GTP-

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**Figure 6.** (A) Time course of Ins(2,4,5)P3-evoked changes in
[Ca2+]i. The data are representative of results from five oocytes in-
jected with fura-2 (2 mM) 1–4 h before Ins(2,4,5)P3 (10 μM) stim-
ulation and are expressed as the levels of intracellular [Ca2+]i. (Inset)
Time course of Ins(2,4,5)P3-induced light emission. The time course
of Ins(2,4,5)P3 (10 μM)-induced light emission is representa-
tive 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)P3-induced [Ca2+]i release in the absence and presence
of heparin. Light emission was measured immediately after
increasing concentrations of Ins(2,4,5)P3 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 hepa-
rin inhibition of Ins(2,4,5)P3-induced [Ca2+]i release. Light emis-
ion was measured immediately after Ins(2,4,5)P3 (10 μM) was in-
jected into oocytes coinjected with aequorin and increasing concen-
trations 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.
induced Ca\(^{2+}\) mobilization was dose-dependent and resistant to inhibition by heparin (Fig. 7B).

**Ins(1,3,4,5)P\(_4\)-Induced Ca\(^{2+}\) 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\(_4\). 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. 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\(^{2+}\). These data and observations in sea urchin eggs 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. 8A, inset) which paralleled the increases in [Ca\(^{2+}\)]. In comparison with Ins(1,4,5)P\(_3\) (Fig. 5B), a fivefold greater concentration of Ins(1,3,4,5)P\(_4\) was required to evoke half-maximal [Ca\(^{2+}\)] release (Fig. 8B). The Ca\(^{2+}\) signals generated by Ins(1,4,5)P\(_3\) (Fig. 5A) and Ins(1,3,4,5)P\(_4\) (Fig. 8A) exhibited different response kinetics. Typically, the response to Ins(1,4,5)P\(_3\) was a prompt rise to a peak [Ca\(^{2+}\)] level followed by a relatively rapid decay back to baseline levels (Fig. 5A). In comparison, Ins(1,3,4,5)P\(_4\) evoked broader [Ca\(^{2+}\)] peaks. Furthermore, high Ca\(^{2+}\) (6 mM) did not double the peak [Ca\(^{2+}\)] levels observed in follicular oocytes injected with minimal doses of Ins(1,3,4,5)P\(_4\) in Ca\(^{2+}\)-free MBS (Fig. 8A), in contrast to the effects of extracellular Ca\(^{2+}\) on Ins(1,4,5)P\(_3\)-induced responses of comparable magnitude (Fig. 5A).

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. Electrophysiological responses to Ins(1,4,5)P\(_3\), usually comprise two or more components in which a transient current (I\(_t\)) forms immediately followed by a subsequent phase characterized by oscillations (I\(_o\)). Injection of Ins(1,3,4,5)P\(_4\) in contrast, usually results in only the I\(_t\) oscillatory phase; the rapid I\(_o\) 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 current while leaving the I\(_o\) current intact. In preparation does not contain other inositol phosphates (Calbiochem-Behring Corp.), 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, rat brain, and adrenal cortex. Heparin has been reported to be a more potent inhibitor of Ins(1,3,4,5)P\(_4\)-mediated Ca\(^{2+}\) release from cerebellar microsomes, but appears to be a relatively specific inhibitor of Ins(1,4,5)P\(_3\)-induced Ca\(^{2+}\) release from adrenal microsomes.

**Metabolism of [H]Ins(1,3,4,5)P\(_4\) and [H]Ins(1,4,5)P\(_3\)**

To investigate the first two possibilities, we injected [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\(^{2+}\)] release, 3.5% of the [H]Ins(1,4,5)P\(_3\) was
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Figure 8. (A) Time course of Ins(1,3,4,5)P3-evoked changes in [Ca2+], in the absence and presence of extracellular Ca2+. The results are from single oocytes and are representative of data from five oocytes injected with fura-2 (2 mM) 1-4 h before Ins(1,3,4,5)P3 (50 μM) stimulation in the presence of 6 mM Ca2+ and also in Ca2+-free medium. The data are expressed as the levels of [Ca2+]. (Inset) Time course of Ins(1,3,4,5)P3-induced light emission. The time course of Ins(1,3,4,5)P3 (50 μM)-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 Ins(1,3,4,5)P3-induced Ca2+ release in the absence and presence of heparin. Light emission was measured immediately after increasing concentrations of Ins(1,3,4,5)P3 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 ± SE of data from three to six oocytes. (Inset) Dose dependence of heparin inhibition of Ins(1,3,4,5)P3-induced [Ca2+], release. Light emission was measured immediately after Ins(1,3,4,5)P3 (50 μM) was injected into oocytes coinjected with aequorin and increasing concentrations of heparin 1-4 h previously. Each point represents the mean ± SE of data from three to six oocytes and is expressed as the fold stimulation (peak/basal).

phosphorylated to [3H]Ins(1,3,4,5)P4 (Fig. 9A). By 30 min, 20% of the Ins(1,4,5)P3 was phosphorylated to Ins(1,3,4,5)P4, and the remainder of the counts accumulated in degradation products of Ins(1,4,5)P3, namely 6.0 ± 0.6% in InsP3, 15 ± 1% in InsP1, and 6.2 ± 0.7% in Ins. This is in contrast to studies reported in *Xenopus* oocyte homogenates in which 30% of the added [3H]Ins(1,4,5)P3 was metabolized to [3H]Ins(1,3,4,5)P4 after 8 min (35). To determine whether Ins(1,3,4,5)P4-induced Ca2+ responses could be attributed to Ins(1,4,5)P3 formation, we injected [3H]Ins(1,3,4,5)P4 into follicular oocytes and followed its metabolism by HPLC analysis. The metabolic products formed from [3H]Ins(1,3,4,5)P4, were identified by an HPLC method that fully resolves both isomers of inositol trisphosphate (I). Follicular oocytes metabolized [3H]Ins(1,3,4,5)P4 primarily to [3H]Ins(1,3,4)P3, with no detectable formation of [3H]Ins(1,4,5)P3, for up to 10 min (Fig. 9B), which is considerably longer than the 2-min time span over which we observed Ins(1,3,4,5)P3-induced Ca2+ mobilization. After 30 min, 10% of the [3H]Ins(1,3,4,5)P4 was metabolized to Ins(1,3,4)P3, which subsequently underwent further dephosphorylation to InsP3 (26 ± 0.1%), InsP1 (16.2 ± 1.0%), and Ins (7.2 ± 0.1%) with only 1.6% converted to [3H]Ins(1,4,5)P3. This is in contrast to studies reported in *Xenopus* oocyte homogenates, in which 8.4% of the [3H]Ins(1,3,4,5)P4 was metabolized to [3H]Ins(1,4,5)P3 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 Ins(1,4,5)P3 was a very minor product of Ins(1,3,4,5)P4 metabolism is in agreement with previous findings in permeabilized adrenal cells, that Ins(1,3,4,5)P4 is predominantly dephosphorylated to Ins(1,3,4)P3 (1, 15), which itself has no [Ca2+]-mobilizing activity in the oocyte (data not shown). Because of the marked difference in the effect of extracellular Ca2+ and the minute conversion of [3H]Ins(1,3,4,5)P4 to [3H]Ins(1,4,5)P3, Ins(1,3,4,5)P4 is likely to exert its effects by interacting with an intracellular receptor and not as a result of its metabolism to Ins(1,4,5)P4.

**Discussion**

These findings have provided direct evidence that AII receptors in *Xenopus* follicular oocytes are coupled to phos-
phosphoinositide hydrolysis. The relatively small increase in Ins(1,4,5)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 IP$_3$ and Ins(1,3,4)P$_4$ (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 [H]inositol rather than incubating oocytes with [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 Ca$^2+$ signal is far more robust than the corresponding Ins(1,4,5)P$_3$ response, at least as revealed by the formation of [H]Ins(1,4,5)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 Ins(1,4,5)P$_3$ is required to release large amounts of intracellular Ca$^2+$ and that individual compartments of Ins(1,4,5)P$_3$-sensitive 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 Ca$^2+$ pools that couple selectively to two different populations of membrane receptors. The fact that considerable amounts of injected CaCl$_2$ are required to evoke a detectable aequorin response (in contrast to the large light responses to small amounts of Ins(1,4,5)-IP$_3$) could reflect extensive binding or sequestration of microinjected Ca$^2+$ that impedes contact with the Ca$^2+$ indicators. In contrast, [Ca$^{2+}$], mobilization by Ins(1,4,5)P$_3$ may occur in regions that are more accessible to the indicators. These findings are in agreement with electrophysiologival data in Xenopus oocytes in that Ins(1,4,5)P$_3$ evokes far greater chloride responses than 100-fold higher concentrations of CaCl$_2$ (14, 40, 52, 54). These findings are relevant to the observation that the ER network of the Ca$^{2+}$-induced 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 Ca$^{2+}$ release in follicular oocytes by interacting specifically with the amphibian Ins(1,4,5)P$_3$ receptor. The dose response curves of Ca$^{2+}$ mobilization induced by AII (Fig. 2 B), Ins(1,4,5)P$_3$ (Fig. 5 B), and Ins(2,4,5)P$_3$ (Fig. 6 B) were all shifted to the right in the presence of heparin, indicating that heparin inhibits the Xenopus Ins(1,4,5)P$_3$ receptor in a competitive manner. Heparin does not interfere with Ca$^{2+}$ homeostasis per se, since it has no effect on ionomycin-induced Ca$^{2+}$ influx and mobilization (Fig. 4) at concentrations which inhibit AII- (Fig. 2), Ins(1,4,5)P$_3$-(Fig. 5), and Ins(2,4,5)P$_3$- (Fig. 6) induced Ca$^{2+}$ release. The ability of GTP to evoke heparin-insensitive Ca$^{2+}$ release (Fig. 7) supports the view that heparin's action is on the Ins(1,4,5)P$_3$-activated Ca$^{2+}$ release mechanism, especially since GTP and Ins(1,4,5)P$_3$ have been shown to release Ca$^{2+}$ from the same pool (10, 21). These data are consistent with observations that heparin does not alter other Ca$^{2+}$ transport activities including the GTP-activated Ca$^{2+}$ translocation process or any undefined passive Ca$^{2+}$ fluxes that may contribute to the attainment of Ca$^{2+}$ equilibrium in microsomes (20). Taken together, these results suggest that heparin inhibits agonist-induced Ca$^{2+}$ mobilization in a manner similar to its interaction with the mammalian Ins(1,4,5)P$_3$ receptor, suggesting conservation between the two receptors. Furthermore, this is the first demonstration that Xenopus oocytes possess a GTP-sensitive Ca$^{2+}$ pool from which Ca$^{2+}$ release can be triggered independently of Ins(1,4,5)P$_3$ and indicates that the Xenopus oocyte may serve as a valuable model for studying the regulation of GTP and Ins(1,4,5)P$_3$-sensitive Ca$^{2+}$ pools. The advantage of the Xenopus oocyte system lies in the ability to inject agents into oocytes and follow agonist-induced Ca$^{2+}$ mobilization in an intact cell, compared with studies using permeabilized cells which could lead to artificial alteration of intracellular constituents resulting from the permeabilization process.

The ability of heparin to abolish AII-induced Ca$^{2+}$ release indicates that the [Ca$^{2+}$]$_j$ response is completely dependent upon Ins(1,4,5)P$_3$ production. We have previously demonstrated that AII does not induce significant Ca$^{2+}$ entry in Xenopus follicular oocytes even in the presence of high extracellular 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 Ins(1,4,5)P$_3$ receptor, since it is known to interfere with a variety of physiological processes. However, the findings that heparin competitively inhibits Ins(1,4,5)P$_3$ (Fig. 5 B) and Ins(2,4,5)P$_3$- (Fig. 6 B) induced Ca$^{2+}$ release, but not Ca$^{2+}$ (Fig. 3 B), ionomycin (Fig. 4 B), or GTP- (Fig. 7 B) induced Ca$^{2+}$ release, at concentrations that completely inhibit AII responses (Fig. 2), are consistent with its selective inhibition of the Xenopus oocyte Ins(1,4,5)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 [Ca$^{2+}$]$_j$ (45). Two potential candidates for this signal are Ins(1,4,5)P$_3$ and Ca$^{2+}$, both of which can be transmitted through gap junctions in rat liver hepatocytes (46). The fact that heparin completely inhibits AII-induced Ca$^{2+}$ release (Fig. 2 B) at concentrations we have shown to selectively inhibit the oocyte Ins(1,4,5)P$_3$ receptor (Fig. 5 B), coupled with the demonstration that heparin does not interfere with light emission evoked by the Ca$^{2+}$ ionophore, ionomycin (Fig. 4 B), or by direct injection of Ca$^{2+}$ into the oocyte cytoplasm (Fig. 3 B), indicates that the Ca$^{2+}$-mobilizing signal transferred from follicular cells to the oocyte is not Ca$^{2+}$ alone. However, our results do not rule out the possibility that transfer of Ca$^{2+}$ through gap junctions could regulate the Ins(1,4,5)P$_3$-evoked Ca$^{2+}$ mobilization. Recently, Finch et al. (17) have demonstrated that Ca$^{2+}$ can induce sequential positive and negative feedback regulation of Ins(1,4,5)P$_3$-induced Ca$^{2+}$ release. Our results also do not rule out the possibility that Ins(1,3,4,5)P$_4$ could regulate Ins(1,4,5)P$_3$-evoked Ca$^{2+}$ mobilization as a result of the capability of the oocyte to metabolize Ins(1,4,5)P$_3$ to Ins(1,3,4,5)P$_4$ (Fig. 9). The metabolism of

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Ins(1,4,5)P₃ could lead to Ins(1,3,4,5)P₄ transfer through gap junctions and subsequent [Ca²⁺] mobilization in addition to Ins(1,4,5)P₃-induced Ca²⁺ release, either through Ins(1,3,4,5)P₃ binding and activating the Ins(1,4,5)P₃ 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 Ca²⁺, in a manner specific for the Ins(1,4,5)P₃ receptor, indicates that [Ca²⁺], responses in the oocyte are mediated by an Ins(1,4,5)P₃-dependent mechanism. This probably involves the transfer from follicular cells of Ins(1,4,5)P₃, rather than Ca²⁺ or another signal molecule that is independent of Ins(1,4,5)P₃ production.

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