membranes is probably more complex than a tyrosine kinase: PLC-γ1 interaction as enzyme-substrate, which would be expected to result in only a transient association. In fact, it has been reported that primarily nontyrosine phosphorylated PLC-γ1 molecules are associated with activated EGF receptors (*12*). When PLC-γ1 is immunoprecipitated from EGF or platelet-derived growth factor (PDGF) treatment of different cells, a similar spectrum of coprecipitating proteins has been noted (*6, 7, 13*). It is possible that these proteins may be involved in mediating the membrane association of PLC-γ1.

These results could suggest a role for the cellular redistribution of PLC-γ1 in the EGF stimulation of cellular PLC activity. EGF-induced membrane association of PLC-γ1 may stimulate the kinetics of phosphatidylinositide breakdown by increasing association of the enzyme with its substrate. This would suggest major differences in the mechanism by which G protein–dependent agonists such as bradykinin (which produces no relocalization or tyrosine phosphorylation of PLC) and growth factors (for which there is no evidence of G protein dependence) communicate with PLC to increase PIP_2 hydrolysis.

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15. Samples of each fraction were subjected to SDS–7.5% polyacrylamide gel electrophoresis (PAGE) (*16*) and electroblotted to nitrocellulose (Schleicher & Schuell, 0.45 μM) overnight at 25 V and blocked with BLOTTO (*17*). The filters were probed with a 1:500 mixture of PLC-γ1 MAbs (*18*) and then detected with rabbit antibodies to mouse immunoglobulin G (1:500, Sigma) and ^{125}I-labeled protein A (2 μCi/10 ml BLOTTO). Where indicated, filters were probed to detect EGF receptors with 1:500 rabbit antiserum 986 (*19*) and ^{125}I-labeled protein A. The radioactive bands were quantitated by cutting the filters and determining the amount of ^{125}I present. The amount of PLC-γ1 in each band was normalized according to the entire amount of PLC-γ1 analyzed, to give the relative percent of the total PLC-γ1 present in each fraction.
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Angiotensin II–Induced Calcium Mobilization in Oocytes by Signal Transfer Through Gap Junctions

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Angiotensin II (AII) stimulates rapid increases in the concentration of cytosolic calcium in follicular oocytes from *Xenopus laevis*. This calcium response was not present in denuded oocytes, indicating that it is mediated by AII receptors on the adherent follicular cells. The endogenous AII receptors differed in their binding properties from mammalian AII receptors expressed on the oocyte surface after injection of rat adrenal messenger RNA. Also, the calcium responses to activation of the amphibian AII receptor, but not the expressed mammalian AII receptor, were blocked reversibly by octanol and intracellular acidification, treatments that inhibit cell coupling through gap junctions. In addition, AII increased the rate of progesterone-induced maturation. Thus, an AII-induced calcium-mobilizing signal is transferred from follicle cells to the oocyte through gap junctions and may play a physiological role in oocyte maturation.

ANGIOTENSIN II EXERTS NUMEROUS physiological actions in addition to those related to the control of blood pressure and extracellular volume (*1*). The actions of AII are mediated by specific plasma membrane receptors that are coupled to phosphoinositide hydrolysis and Ca^{2+} mobilization in target tissues including smooth muscle, adrenal, liver, kidney, and brain. AII receptors from mammalian tissues have been expressed in *Xenopus laevis* oocytes (*2, 3*). Using albino frog oocytes injected with adrenal glomerulosa mRNA and the Ca^{2+} indicator aequorin, we observed that activation of AII receptors expressed from mammalian mRNA elevated intracellular Ca^{2+} concentrations (*3*). During these studies, we also detected endogenous amphibian AII receptors that could trigger Ca^{2+} mobilization in *Xenopus* oocytes. We have investigated the properties and location of this AII receptor.

Amphibian AII receptor–mediated Ca^{2+} responses were comparable to those reported (*3*) for adrenal mRNA-injected oocytes and were characterized by a rapid and transient rise in luminescence of the Ca^{2+} indicator aequorin (Fig. 1A). There was a large degree of variability in the incidence and magnitude of endogenous receptor activity that may reflect cyclical and seasonal variations in the endocrine control of receptor expression. However, in individual animals the peak light responses were uniform and dose-dependent, with a rank potency order of AII > AIII >> AI (Fig. 1A, inset). Endogenous AII responses were maximal in freshly isolated oocytes and decreased progressively during the next 4 days of incubation (Fig. 1B); most of the oocytes (81%) did not exhibit detectable light responses by day 3. This decrease was not due to differences in the content of aequorin, since oocytes injected on day 4 yielded similar light emission after lysing with 1% SDS as those injected with aequorin on day 1 (*4*). In contrast to the declining response mediated by the endogenous AII receptor, AII-induced light responses in oocytes injected with both aequorin and rat adrenal mRNA increased progressively with time and were maximal on day 3 (Fig. 1B). We therefore determined amphibian receptor-mediated responses to AII in freshly isolated oocytes 1 to 6 hours after injection of aequorin and mammalian AII receptor–mediated responses 3 days after coinjection with aequorin and rat adrenal mRNA.

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To determine if the cytosolic Ca^{2+} response in mRNA-injected oocytes was due to de novo AII receptors induced by mRNA injection or to potentiation of oocyte activation by endogenous AII receptors, we compared the mRNA dose-dependence of ^{125}I-labeled AII binding and AII-induced Ca^{2+} responses. ^{125}I-labeled AII binding and AII-induced Ca^{2+} responses were detectable at 5 ng mRNA and were maximal at 50 to 100 ng mRNA per oocyte (Fig. 1C). The correlation between ^{125}I-labeled AII binding and AII-induced light emission suggests that the number of expressed receptors determined the magnitude of the Ca^{2+} response elicited by the agonist. The binding of ^{125}I-labeled AII to the amphibian receptor also correlated with the magnitude of the AII-induced light responses in freshly isolated oocytes. Oocytes that responded 60-fold (±7, SEM; $n = 10$) exhibited ^{125}I-labeled AII binding of 114 amol per oocyte, whereas those that responded less than tenfold showed no detectable binding. (Error limits are ±SEM throughout this report.)

Several differences between the endogenous and exogenous AII receptor–mediated responses were revealed by more detailed analysis. For endogenous receptors, the recovery time required to reach basal light emission after the peak response was 4.6 ± 0.3 min ($n = 5$). In adrenal mRNA-injected oocytes from the same donor, the recovery time was significantly longer (11.7 ± 1.1 min) ($n = 10$). Calcium influx is primarily responsible for this delay in return to baseline (*3*). Omission of extracellular Ca^{2+} significantly shortened the recovery time, to 3.7 ± 0.5 min ($n = 5$), as in (*3*). In contrast, the recovery time of the light response mediated by the amphibian receptor (5.3 ± 0.4 min) ($n = 5$) and the shape of the light response were unaffected by the absence of extracellular Ca^{2+} (Fig. 1A). For both receptor types, the initial lag time before reaching maximum light emission was inversely related to the magnitude of the response (Fig. 1D). The lag time was significantly shorter for the mammalian (0.6 min) than the amphibian receptor (1.1 min) when similar response magnitudes (50:1; peak:basal) were compared. Also, the slope of the line relating lag time to response magnitude was steeper for the amphibian than the mammalian receptor.

The Ca^{2+} responses mediated by endogenous and exogenous AII receptors were rapidly desensitized; however, the mRNA-injected oocytes regained partial responsiveness to AII after washing and incubation for several hours. Sixteen hours after washout, the peak light intensity induced by a second application of AII (peak, 520 × 10^3 photons per second, ±80 × 10^3; $n = 10$) recovered by 30% when compared to oocytes not previously exposed to AII (peak, 2760 × 10^3 photons per second; ±350 × 10^3; $n = 10$). In contrast, the amphibian receptors did not regain responsiveness, even after 24 hours. The partial recovery in mRNA-injected oocytes may reflect the continuing synthesis and expression of new receptors at the cell surface. The reduced aequorin signal after AII treatment was not due to consumption of the photoprotein, since subsequent addition of 1% SDS caused 1000-fold increases in light emission over the subsequent 15 min in both AII-treated and untreated oocytes (*4*).

Evaluation of the actions of AII-related peptides in oocytes expressing mammalian adrenal AII receptors revealed that only [sarcosine^1]AII ([Sar^1]AII) and [Sar^1, Ile^8]AII elicited detectable light responses

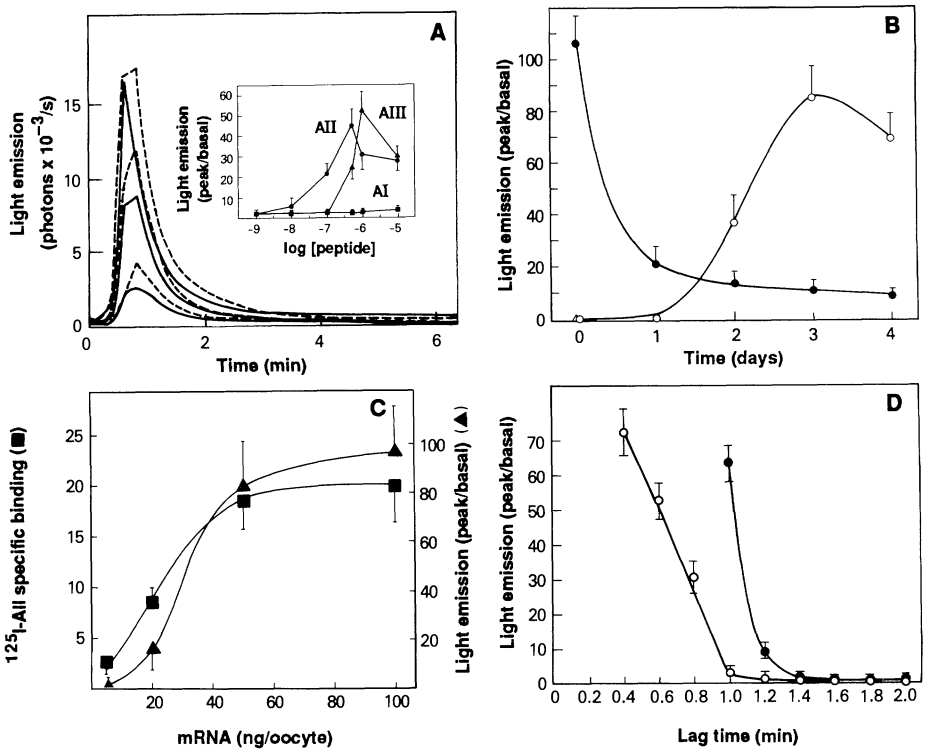


Fig. 1. (**A**) Time course of AII-evoked changes in light emission. Representative data from aequorin-injected albino *Xenopus* oocytes from three different frogs, stimulated with 500 nM AII in the presence (———) and absence (– – – –) of extracellular Ca^{2+} (*21*). (Inset) Relative potencies of angiotensins I, II, and III at the amphibian AII receptor. Each point represents the mean ± SEM of peak light emission from five to ten oocytes injected with aequorin. (**B**) Time course of endogenous and exogenous AII receptor expression. Amphibian (●) and mammalian (○) AII receptor–mediated light responses were measured as a function of time. Representative examples are shown of oocytes from three different donors; each point represents the mean ± SEM of data from five to ten oocytes. (**C**) Dependence of ^{125}I-labeled AII binding and AII-evoked light responses on mRNA dose. Three days after oocytes were coinjected with aequorin and increasing amounts of rat adrenal mRNA, ^{125}I-labeled AII binding (*22*) (■) and AII-induced Ca^{2+} responses (▲) were determined. Responses are expressed as the mean ± SEM of data from five to ten oocytes. Control oocytes (aequorin alone) did not specifically bind ^{125}I-labeled AII. (**D**) Relation between the magnitude of the peak and the delay of the AII-induced light response. The lag time, defined as the time elapsed between the onset of AII stimulation and the peak increase in light emission, was determined for the amphibian (●) and mammalian (○) AII receptors; each point represents the mean ± SEM of data from five to ten oocytes.

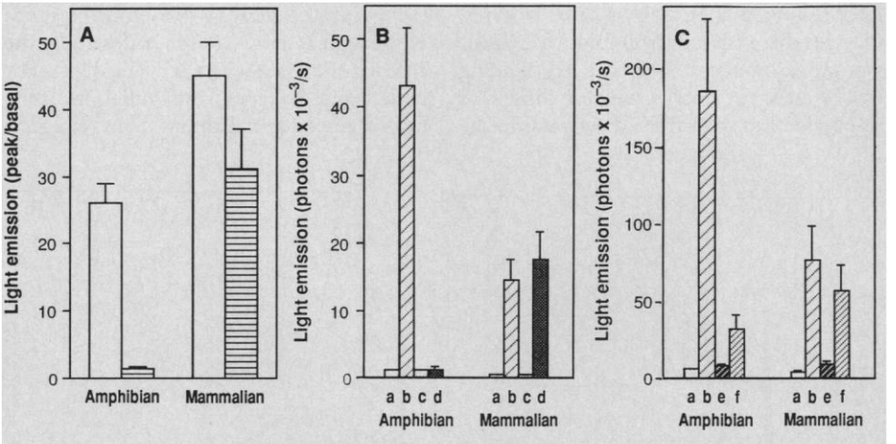
Table 1. Relative potencies of angiotensin-related peptides at amphibian and mammalian receptors. Data from aequorin-injected oocytes that showed 10- to 15-fold increases in Ca^{2+} to 500 nM AII were compared with those from oocytes coinjected with aequorin and rat adrenal mRNA that gave 10- to 15-fold responses to 5 μM AII. In this way, we controlled for the variability between response magnitudes and could directly compare the AII-related peptides (Peninsula Labs, Belmont, California) with each other and between both AII receptor types. Sar, Sarcosine; each value represents the mean ± SEM of data from three to six oocytes. ND, no detectable response.

Angiotensins	AII-induced peak light response (peak/basal)	
	Amphibian (500 nM)	Mammalian (5 μM)
AII	15 ± 3	14 ± 2
[Sar^1]AII	15 ± 2	10 ± 2
[Sar^1,Ile^8]AII	18 ± 3	2 ± 0.1
[Sar^1,Leu^8]AII	12 ± 3	ND
des-Asp^1-[Ile^8]AII	6 ± 1	ND
[Sar^1,Thr^8]AII	2 ± 0.2	ND
[Sar^1,Ala^8]AII	1.5 ± 0.2	ND
[Sar^1,Gly^8]AII	ND	ND
[Sar^1,Val^5,Ala^8]AII	ND	ND

(Table 1). In contrast, the endogenous AII receptors were also activated by [Sar^1, Thr^8] AII, des-Asp^1-[Ile^8]AII, and [Sar^1, Leu^8]-AII. The antagonist analog, [Sar^1,Ile^8]-AII, was more potent in evoking light emission at the amphibian receptor than at the mammalian AII receptor. The existence of molecular differences between mammalian and amphibian AII receptors has also been suggested by the inability of several AII antagonists ([Sar^1,Ala^8]AII, [Sar^1,Ile^8]AII, and [Ala^1,Ile^8]AII) to block vasoconstrictor responses to AII in the toad (*Bufo arenarum*), in contrast to their complete suppression of pressor responses to AII in the rat (*5*).

Gap junctions exist between follicle cell microvilli and oocytes in both amphibian and mammalian ovaries (*6*) and provide a structural pathway for cell-to-cell communication, by exchange of ions and small molecules (*7*). Follicle cells have been implicated in processes involved with amphibian oocyte growth and development, such as steroidogenesis, vitellogenin uptake, amino acid uptake, and protein synthesis (*8*). We therefore examined the possibility that the intrinsic AII receptor is located on the ovarian follicular cells and causes increased cytosolic Ca^{2+} concentrations in the oocyte by promoting the transfer of second messenger molecules through gap junctions. Studies on oocytes in which defolliculation was confirmed by histological examination (*4*) revealed that completely denuded oocytes lost their endogenous responses to AII (Fig. 2A), suggesting that the amphibian receptors reside on the follicular cells. In contrast, oocytes injected with rat adrenal mRNA 3 days before still retained their responsiveness to AII after defolliculation (Fig. 2A), indicating that mammalian AII receptors were located on the oocyte plasma membrane and were not damaged by the removal of follicle cells. We also obtained direct evidence for receptor localization from studies in membranes (*9*) from follicle-enclosed and denuded oocytes, and from isolated follicular cells. ^{125}I-labeled [Sar^1,Ile^8]AII bound specifically to follicular oocytes (118 ± 16 cpm per 100 μg of protein) and follicle cell membranes (678 ± 85 cpm per 100 μg of protein), but showed no specific binding to membranes of denuded oocytes.

Since aequorin is too large to pass through gap junctions (*7*), these results suggest that endogenous receptors located on follicular cells communicate with the oocyte by generating second messenger molecules that diffuse through gap junctions and mobilize stored Ca^{2+}. We therefore measured agonist-induced light responses mediated by amphibian and mammalian AII receptors after pretreating the oocytes with octanol, a known uncoupler of gap junctions (*10, 11*). Exposure to 1 mM octanol for 20 min inhibited the transfer of Lucifer yellow from the oocyte to the follicular cells, demonstrating that octanol had uncoupled gap junctions between the somatic and germinal cells (Fig. 2D). Under these conditions, the amphibian AII-evoked light response was completely inhibited (Fig. 2B), consistent with the ability of octanol to prevent AII-induced changes in membrane potential in oocytes (*12*). The AII-induced light response gradually recovered after washout of octanol and reached 50% of the original value after 2 hours. Another gap junction uncoupler (*11*), heptanol (3 mM), also completely inhibited the AII-induced light response of the amphibian receptor (*4*). In addition, exposure of oocytes for 1 min to modified Barth's solution (MBS) gassed with 100% CO_2, conditions that lower intracellular pH and uncouple gap junctions in other systems (*13*), caused 83% inhibition of the amphibian AII-evoked light response (Fig. 2C), followed by recovery to 73% of the original value after 5 min. The specificity of these actions for gap junction–mediated message transfer from follicle cells was shown by parallel studies on AII-induced light responses mediated by mammalian AII receptors expressed in the oocyte from adrenal mRNA. There was no significant effect of octanol or acidification on Ca^{2+} mobiliza-



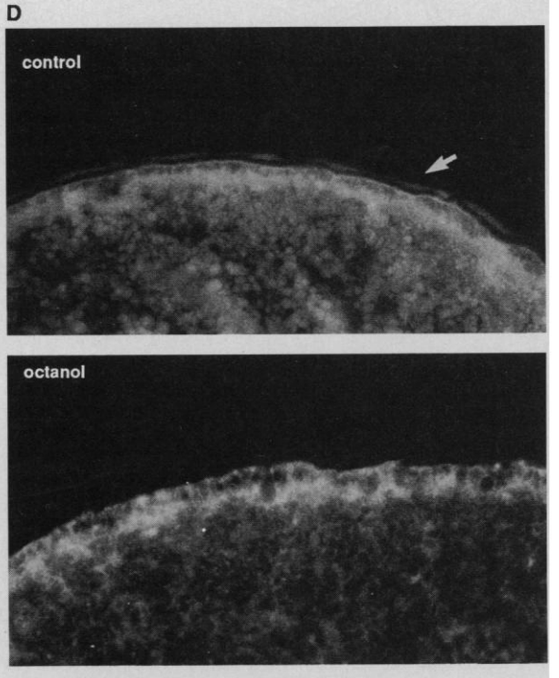


Fig. 2. (**A**) Cellular location of angiotensin II receptors in follicular oocytes. The magnitudes of the amphibian and mammalian AII-evoked light responses were determined in follicle-enclosed (open bar) and defolliculated (striped bar) oocytes (*21*). Oocytes were defolliculated 2 to 5 hours before the magnitude of the mammalian AII response was assessed. Responses are expressed as the mean ± SEM of data from eight to ten oocytes. (**B**) Inhibition of AII-evoked light responses by octanol. The magnitudes of the amphibian and mammalian AII receptor–mediated light responses were compared in untreated (a, control; b, AII) and octanol-treated (c, control; d, AII) oocytes. Responses are expressed as the mean ± SEM of data from eight to ten oocytes. (**C**) Inhibition of AII-evoked light responses by intracellular acidification. The magnitudes of the amphibian and mammalian AII receptor–mediated light responses were compared in control (a, control; b, AII untreated) and CO_2-treated oocytes (1 min, 100% CO_2) (e, control; f, AII). Responses are expressed as the mean ± SEM of data from eight to ten oocytes. (**D**) Octanol blockade of dye transfer in follicular oocytes. Transfer of Lucifer yellow into follicular cells (arrow) after injection into the oocyte (50 nl of 5% dye in 10 mM Hepes, pH 7.6) in control oocytes and inhibition by exposure to 1 mM octanol for 20 min. Each photograph (×125 magnification of 10-μm section) is representative of 20 to 30 oocytes fixed in 4% formaldehyde before embedding in OCT (Labtek, IL).

tion mediated by the exogenous receptors on the surface of the oocyte (Fig. 2, B and C). These data indicate that octanol and cytoplasmic acidification did not perturb signal generation by AII receptors located in the plasma membrane of the oocyte and that blockade of endogenous AII receptor–mediated responses is attributable to uncoupling of follicle cells from the oocyte.

Our results show that hormone-mediated signal transfer can occur through gap junctions between follicular cells and oocytes. This interpretation can also explain the differences in lag times of responses to endogenous and exogenous AII receptors, in that extra steps are required to elicit Ca^{2+} mobilization in the oocyte by the follicular AII receptor than by the exogenous receptor expressed directly on the oocyte. The nature of the molecules moving from the follicular cells to the oocyte is not yet clear, but candidates include inositol 1,4,5-trisphosphate and Ca^{2+} itself, which could diffuse into the oocyte through gap junctions (*14*). Ca^{2+}-activated Ca^{2+} release has been proposed to occur during the fertilization wave in the egg (*15*). Another possible mediator is inositol 1,3,4,5-tetrakisphosphate, which could explain the lack of dependence on extracellular Ca^{2+}, since it mobilizes intracellular Ca^{2+} in *Xenopus* oocytes without activating substantial Ca^{2+} entry (*16*).

The presence of functional AII receptors in the amphibian oocyte is consistent with recent reports that AII may regulate mammalian ovarian function. Autoradiographic studies have demonstrated the presence of high-affinity AII receptors in rat ovarian follicles before the first ovulation and an increase in their number after ovulation (*17*); also, AII promotes estrogen secretion in the rat ovary (*17*). The maturation of amphibian oocytes is controlled by hormonal factors through the mediation of the follicular cells, which maintain the arrest of stage 6 oocytes in prophase of the first meiotic division, and trigger their maturation (progression to the metaphase of the second meiotic division) by the release of progesterone (*18*). Progesterone-induced maturation of *Xenopus* oocytes is significantly accelerated by concomitant exposure to acetylcholine, by activation of muscarinic receptors in the oocyte (*19*). Since the cellular responses to muscarinic agonists include phosphoinositide hydrolysis and Ca^{2+} mobilization, we investigated the possibility that AII might influence progesterone-induced maturation by a similar mechanism.

In oocytes incubated with progesterone (3 μM), AII reduced the time in which 50% of the oocytes reached germinal vesicle breakdown ($GVBD_{50}$) by 45 ± 10 min (Fig. 3). AII alone did not induce oocyte maturation. The maturation-enhancing action of AII was dose-dependent (Fig. 3, inset) and was observed at concentrations consistent with its Ca^{2+}-mobilizing activity (Fig. 1A, inset). [Sar^1,Ala^8]AII blocked the potentiating effect of AII on maturation ($\Delta GVBD_{50}$, 8 min) at a concentration (1 μM) at which it also prevented the Ca^{2+}-mobilizing effect of AII (*4*). Collectively, these results suggest that the endogenous AII receptor plays a role in the regulation of oocyte maturation. Mobilization of cytosolic Ca^{2+} by AII could promote progesterone-induced maturation by amplifying the primary effect of progesterone (*18*) [namely, to decrease adenosine 3′,5′-monophosphate (cAMP) levels] by inhibiting adenylate cyclase activity or activating cAMP-specific phosphodiesterases. The finding that AII promotes oocyte maturation and Ca^{2+} mobilization by signal transfer through gap junctions is consistent with the proposal that modulation of gap junction communication may be involved in embryonic development and differentiation (*20*).

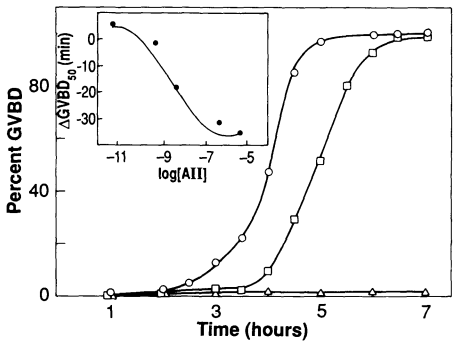


Fig. 3. Effect of AII on progesterone-induced maturation. Stage 6 follicular oocytes were exposed to AII (500 nM) alone (△), progesterone (1 μg/ml) alone (□), or AII for 1 hour before addition of progesterone (○). One hundred oocytes were incubated at room temperature in petri dishes (60 mm) in 10 ml of MBS. Maturation was scored (*19*) and expressed as the percentage of the maximum GVBD as a function of time. GVBD was normalized such that the total number of oocytes out of 100 that underwent GVBD after 24 hours was designated as 100%. A representative example of three similar experiments is shown. (Inset) AII dose-response curve. Stage 6 oocytes were exposed to increasing concentrations of AII for 1 hour before addition of progesterone.

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21. Oocytes from *Xenopus laevis* were isolated and injected with aequorin with or without mRNA extracted from rat adrenal zona glomerulosa tissue (*3*). Manual defolliculation was performed after treatment with collagenase (2 mg/ml in MBS) for 1 to 2 hours at room temperature.
22. Binding of ^{125}I-labeled AII to individual oocytes was measured after incubation for 3 hours in 600 μl of MBS containing 0.3% bovine serum albumin, ^{125}I-labeled AII (Du Pont Biotechnology Systems, 2200 Ci/mmol) (200,000 cpm per oocyte), and [^{14}C]sucrose (8.8 mCi/mmol) (10,000 cpm per oocyte) at room temperature. After incubation and washing, healthy oocytes were transferred to centrifuge tubes (1.5 ml) containing ice-cold dibutyryl phthalate. The oocytes were pelleted through the oil layer by centrifugation in an Eppendorf microfuge. The centrifuge tubes were frozen on dry ice, and the severed tips were analyzed for ^{125}I. [^{14}C]Sucrose incorporation was determined to assess oocyte integrity [E. Pure, A. D. Luster, J. C. Unkeless, *J. Exp. Med.* **160**, 606 (1984)]. Specific binding was defined as the total amount of ^{125}I-labeled AII bound minus the level bound in the presence of 1 μM unlabeled AII.

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