

bathing solution caused the normal initial increase in MEPP frequency ( $f_s/f_0$ ) but a subsequent decrease (as measured by  $f_{20}/f_0$ ), indicating that these sugars enter the nerve terminal in a stereospecific manner. Both D-glucose and 3-O-methyl-D-glucose entered nerve terminals in the diaphragm and extensor digitorum longus (EDL), but did not enter nerve terminals in the soleus. The lack of a difference in the rate of entry of D-glucose and 3-O-methyl-D-glucose shows that nerve terminal metabolism of glucose does not distort the estimate of glucose entry, since 3-O-methyl-D-glucose is not metabolized (7).

The proportion of type F nerve terminals is about 20 percent in the soleus muscle, 60 percent in the diaphragm, and 95 percent in the EDL (8, 9). The present experiments show that there is a positive correlation ( $r = .63$ ,  $P < .001$ ) between the proportion of type F nerve terminals in the three muscles and the rate of D-glucose entry, suggesting that glucose enters type F nerve terminals more rapidly than it enters type S terminals. Type F nerve terminals can be divided into those associated with fast oxidative-glycolytic muscle fibers and those associated with fast glycolytic ones (10). No fine distinction between the two type F nerve terminals can be made.

The functional significance of these findings is not known, but it is tempting to speculate about the relation between glucose transport and the rate of cell metabolism. Glucose transport is usually closely linked to the rate of cell metabolism (11). Cells with rapid glucose transport often do not regulate glucose entry into the cell, while cells with slow glucose transport do. I assume that the slower entry of glucose into type S nerve terminals means that glucose transport into these terminals is regulated. Since type S motor units are capable of sustained activity, slow entry of glucose suggests that the type S nerve terminal may have a large energy store, such as glycogen or lipid, or may be able to rapidly increase glucose transport as needed during activity. Type F motor units show brief bursts of activity and may be able to survive on the glucose provided by their more active transport systems. Synaptosomal preparations show evidence of two carriers that differ in their affinity for glucose (12). Hence it is conceivable that, depending on its metabolic requirements, a given neuron may only have carriers of high or low glucose affinity.

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## The Role of Zinc and Follicle Cells in Insulin-Initiated Meiotic Maturation of *Xenopus laevis* Oocytes

El-Etr *et al.* (1) showed that high concentrations of insulin (up to 400  $\mu\text{g/ml}$ ) initiated meiotic maturation in most full-grown *Xenopus laevis* oocytes and suggested that amphibian oocytes may thus serve "as a model system for the study of the poorly understood mechanism of insulin action." We have cultured growing *X. laevis* oocytes in the presence of 1  $\mu\text{g}$  of insulin per milliliter and have found maturation never occurs unless progesterone is added to the medium (2).

We therefore tested the effect on manually dissected, full-grown ( $> 1.2$  mm in diameter) oocytes of two insulin preparations (3) we have previously used for oocyte culture (4), but used the highest concentration (40  $\mu\text{g/ml}$ ; 1 U/ml; 7  $\mu\text{M}$ ) for which data were reported by El-Etr

*et al.* (1). The results (Table 1, experiment 1) indicated that the insulin preparation (lot 615-D63-10) we have routinely used for oocyte culture (2, 4) did indeed initiate germinal vesicle breakdown (GVBD) in some of the oocytes, thus confirming the results of El-Etr *et al.* (1). Spontaneous maturation did not occur in the absence of hormone, and progesterone initiated a 100 percent response, thus establishing appropriate controls for our experimental system. Our second insulin preparation (lot 615-1082B-108-1) appeared to be inactive (Table 1, experiment 1). The active insulin was a crystalline,  $\text{Zn}^{2+}$ -precipitated preparation [ $\text{Zn}^{2+}$  content = 0.7 percent by weight as determined by atomic absorption spectrometry (3)]; the inactive

Table 1. Initiation of meiotic maturation in full-grown *X. laevis* oocytes by insulin and  $\text{Zn}^{2+}$ . Results are expressed as the percentage incidence of germinal vesicle breakdown. For each test in experiments 1 and 3 we used 37 to 46 oocytes derived from three females; for each test in experiment 2 we used 26 to 30 oocytes derived from two females. Donor females were previously untreated by any hormone. In experiment 1, insulin was used at a concentration of 40  $\mu\text{g/ml}$  and the medium was solution O-R2 (13). In experiments 2 and 3, insulin was used at a concentration of 100  $\mu\text{g/ml}$ ; solution O-R2 was also modified by eliminating 1 mM  $\text{Na}_2\text{HPO}_4$  and replacing 5 mM Hepes-NaOH buffer with 5 mM tris-Cl buffer. This modification avoids precipitation of  $\text{Zn}^{2+}$  salts. Oocytes incubated at 21°C were observed for up to 24 hours for the appearance of a large white spot indicating germinal vesicle breakdown (9); scoring was routinely confirmed by piercing the animal pole with a fine needle and gently squeezing the equator with a forceps so as to extrude the germinal vesicle, if present (7, 14).

Addition	Experiment			
	1	2	3	
			Without Pronase	With Pronase
None	0	0	0	0
Progesterone (1 $\mu\text{g/ml}$ )	100	100	100	94
hCG (50 U/ml)			93	0
$\text{Zn}^{2+}$ -insulin (lot 615-D63-10)	21	69	83	26
$\text{Zn}^{2+}$ -insulin (lot 615-D63-10), dialyzed*		12	36	0
$\text{Zn}^{2+}$ -insulin (lot 615-07J-256)			68	17
Insulin (lot 615-1082B-108-1)	0	38		
Insulin (lot 050YB7)			32	2
$\text{ZnCl}_2$ ( $10^{-3}\text{M}$ )		100	100	93
$\text{ZnCl}_2$ ( $10^{-4}\text{M}$ )		41	38	46
$\text{ZnCl}_2$ ( $10^{-5}\text{M}$ )		7	10	16
$\text{ZnCl}_2$ ( $10^{-5}\text{M}$ ) + insulin (lot 615-1082B-108-1 or 050YB7)		50	63	11

\*Dialysis was for 24 hours at 4°C against 0.001N HCl, with Spectrapor 6 membranes (2000 molecular weight cutoff).

form was prepared from the former by multiple chromatographic steps and was not Zn<sup>2+</sup>-precipitated (that is, was Zn<sup>2+</sup>-free). Both had an identical potency of 25.4 U/mg as determined by U.S.P. rabbit bioassay (3) and both supported oocyte growth to an equal extent (4).

The activity of lot 615-D63-10 insulin thus appeared to be attributable either to a trace contaminant or to associated Zn<sup>2+</sup>. We therefore conducted a second set of experiments. When the insulin concentration was increased to 100 µg/ml, lot 615-D63-10 insulin initiated a better response (Table 1, experiment 2) but lot 615-1082B-108-I also promoted a positive, although more limited, response. The effect of lot 615-D63-10 was reduced, but not eliminated, by overnight dialysis against 1000 volumes of 0.001N HCl (or 10 mM EDTA, pH 7.0). Zn<sup>2+</sup> alone at a concentration of 10<sup>-3</sup> M was also a potent initiator of meiosis and a slight response occurred even with 10<sup>-5</sup> M Zn<sup>2+</sup>. The latter is approximately the concentration of Zn<sup>2+</sup> present in a 100 µg/ml solution of lot 615-D63-10.

The effect of "insulin" on full-grown oocytes thus seemed to be partially, but not completely, due to the presence of the Zn<sup>2+</sup> that is associated with most insulin preparations. The more limited effect of Zn<sup>2+</sup>-free insulin at a concentration of 100 µg/ml remained unexplained, however. Our manually dissected oocytes are surrounded by a layer of follicle cells that respond to gonadotropin by releasing a maturation-initiating steroid (5), presumably progesterone (6). We therefore tested whether the more limited effect of high concentrations of Zn<sup>2+</sup>-free insulin is mediated by the follicle cells. Because we had used all our Zn<sup>2+</sup>-free insulin we obtained two new preparations, both highly purified by multiple chromatographic steps: a Zn<sup>2+</sup>-containing preparation (lot 615-07J-256) and a Zn<sup>2+</sup>-free preparation (lot 050YB7). The former contained 0.6 percent Zn<sup>2+</sup> and the latter was a "sodium insulin preparation"; the relative potencies were 28.5 and 26.8 U/mg, respectively (3). We established both a minimal gonadotropin [human chorionic gonadotropin (hCG) (Sigma)] concentration (50 U/ml) that consistently initiated a maturation response in manually dissected oocytes and a treatment [Pronase (Calbiochem-Behring); 50 µg/ml for 10 to 12 minutes (7)] that abolished the effectiveness of hCG by removing a sufficient number of follicle cells from the oocyte. We then repeated our experiments using manually dissected oocytes that were either untreated or Pronase-treated. The results (Table 1, experiment

3) indicated that Pronase essentially abolished the response of oocytes to hCG, Zn<sup>2+</sup>-free insulin, or Zn<sup>2+</sup>-containing insulin that had been dialyzed. Pronase reduced the response of oocytes to nondialyzed, Zn<sup>2+</sup>-containing insulin, whereas it had little or no effect on the response to progesterone or Zn<sup>2+</sup> alone.

Thus the effect of normally available (Zn<sup>2+</sup>-containing) insulin preparations on *X. laevis* oocytes may be twofold: high concentrations of insulin may promote sufficient steroid production by follicle cells to initiate meiotic maturation, while insulin-associated Zn<sup>2+</sup> can also directly initiate meiotic maturation of amphibian oocytes. These two effects appear to be additive since in all cases the results obtained with 10<sup>-5</sup> M Zn<sup>2+</sup> plus Zn<sup>2+</sup>-free insulin were similar to those found for Zn<sup>2+</sup>-containing insulin (Table 1, experiments 2 and 3). Also lot 615-07J-256 initiated a similar, although slightly lower, response in *X. laevis* oocytes than that obtained with lot 615-D63-10 insulin; we believe this can be attributed to the somewhat lower Zn<sup>2+</sup> content rather than the removal of an active trace contaminant.

The role of the follicle cells remains ambiguous. Pronase treatment may also remove insulin receptors from the oocyte surface; if these could mediate GVBD and were not regenerated within 24 hours, the results in experiment 3 would be obtained. However, insulin promotes maximum oocyte growth at a concentration of 1 µg/ml (4), which is thus a saturating concentration for oocyte-associated insulin receptors; under these conditions, meiosis is never initiated (2). El-Etr *et al.* (1) claimed that follicle cell-free oocytes obtained from collagenase-treated ovaries were used for their experiments; however, published photomicrographs from the same laboratory have depicted follicle cells covering collagenase-obtained oocytes (8), and Merriam (9) has shown that oocytes from collagenase-treated follicles retain their "follicular epithelium" and remain responsive to gonadotropin. Thus part of the response to insulin and perhaps all of the more limited response to proinsulin (which is normally Zn<sup>2+</sup>-free) observed by El-Etr *et al.* (1) may have been mediated by follicle cells.

El-Etr *et al.* (1) also found that "denatured" insulin did not provoke meiotic maturation. Denaturation in this case was achieved by treatment with mercaptoethanol followed by dialysis, which would remove associated Zn<sup>2+</sup>. Thus, all activity would be lost. The direct effect of Zn<sup>2+</sup> on oocytes is less ambiguous than the insulin effect per se, since Pro-

nase treatment of oocytes does not interfere with its activity (Table 1, experiment 3). Furthermore, Zn<sup>2+</sup> appears to be 10 to 100 times more active than any other metal ion previously reported to initiate meiotic maturation in *X. laevis* oocytes (10), with one exception. In a recent report, Kofoid *et al.* (11) indicated that Co<sup>2+</sup> was the most active metal among several (Zn<sup>2+</sup> not included) compared in a normal, K<sup>+</sup>-containing medium: 0.5 × 10<sup>-4</sup> M initiated a 54 percent response. Since Co<sup>2+</sup> can replace Zn<sup>2+</sup> in most biochemical reactions, this finding is entirely consistent with our own observations. It appears, therefore, that Zn<sup>2+</sup> may have an effect on full-grown oocytes that may be related to its known mitogenic effect on lymphocytes (12).

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In response to Wallace and Misulovin (1), we now confirm and extend the data we described previously (2). We find that meiotic cell division can indeed be reinitiated by polypeptidic growth factors such as insulin (2), and also by multiplication-stimulating activity (MSA) (3) and insulin-like growth factor (IGF) (4), which are at least as active as insulin. The observation (1) that Zn<sup>2+</sup> can

## The role of zinc and follicle cells in insulin-initiated meiotic maturation of *Xenopus laevis* oocytes

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