bathing solution caused the normal initial increase in MEPP frequency (f_5/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.

JACKSON B. PICKETT

Department of Neurology, University of California, San Francisco 94143

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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 μg/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 μg 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 μg/ml; 1 U/ml; 7 μ*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, Zn^{2+}-precipitated preparation [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 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 μg/ml and the medium was solution O-R2 (*13*). In experiments 2 and 3, insulin was used at a concentration of 100 μg/ml; solution O-R2 was also modified by eliminating 1 m*M* Na_2HPO_4 and replacing 5 m*M* Hepes-NaOH buffer with 5 m*M* tris-Cl buffer. This modification avoids precipitation of 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 μg/ml)	100	100	100	94
hCG (50 U/ml)			93	0
Zn^{2+}-insulin (lot 615-D63-10)	21	69	83	26
Zn_{2+}-insulin (lot 615-D63-10), dialyzed*		12	36	0
Zn_{2+}-insulin (lot 615-07J-256)			68	17
Insulin (lot 615-1082B-108-I)	0	38		
Insulin (lot 050YB7)			32	2
$ZnCl_2$ (10^{-3}*M*)		100	100	93
$ZnCl_2$ (10^{-4}*M*)		41	38	46
$ZnCl_2$ (10^{-5}*M*)		7	10	16
$ZnCl_2$ (10^{-5}*M*) + insulin (lot 615-1082B-108-I or 050YB7)		50	63	11

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

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form was prepared from the former by multiple chromatographic steps and was not Zn^{2+}-precipitated (that is, was Zn^{2+}-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^{2+}. 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 0.001*N* HCl (or 10 m*M* EDTA, *p*H 7.0). Zn^{2+} alone at a concentration of 10^{-3} *M* was also a potent initiator of meiosis and a slight response occurred even with 10^{-5}*M* Zn^{2+}. The latter is approximately the concentration of Zn^{2+} 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^{2+} that is associated with most insulin preparations. The more limited effect of Zn^{2+}-free insulin at a concentration of 100 μg/ml remained unexplained, however. Our manually dissected oocytes are surrrounded 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^{2+}-free insulin is mediated by the follicle cells. Because we had used all our Zn^{2+}-free insulin we obtained two new preparations, both highly purified by multiple chromatographic steps: a Zn^{2+}-containing preparation (lot 615-07J-256) and a Zn^{2+}-free preparation (lot 050YB7). The former contained 0.6 percent Zn^{2+} 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^{2+}-free insulin, or Zn^{2+}-containing insulin that had been dialyzed. Pronase reduced the response of oocytes to nondialyzed, Zn^{2+}-containing insulin, whereas it had little or no effect on the response to progesterone or Zn^{2+} alone.

Thus the effect of normally available (Zn^{2+}-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^{2+} 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^{-5}*M* Zn^{2+} plus Zn^{2+}-free insulin were similar to those found for Zn^{2+}-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^{2+} 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^{2+}-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^{2+}. Thus, all activity would be lost. The direct effect of Zn^{2+} on oocytes is less ambiguous than the insulin effect per se, since Pronase treatment of oocytes does not interfere with its activity (Table 1, experiment 3). Furthermore, Zn^{2+} 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^{2+} was the most active metal among several (Zn^{2+} not included) compared in a normal, K^+-containing medium: 0.5×10^{-4}*M* initiated a 54 percent response. Since Co^{2+} can replace Zn^{2+} in most biochemical reactions, this finding is entirely consistent with our own observations. It appears, therefore, that Zn^{2+} may have an effect on full-grown oocytes that may be related to its known mitogenic effect on lymphocytes (*12*).

ROBIN A. WALLACE
ZIVA MISULOVIN

Biology Division,
Oak Ridge National Laboratory,
Oak Ridge, Tennessee 37830

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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^{2+} can

promote meiosis confirms our data (*5*).

Wallace and Misulovin suggest that the insulin effect "seemed to be partially, but not completely, due to the presence of the Zn^{2+} that is associated with most insulin preparations" (*1*). However, using proinsulin (*2*) and MSA and IGF (Table 1), we found that Zn^{2+}-free polypeptides can reinitiate meiosis in *Xenopus laevis* oocytes. It is of interest that insulin and MSA have recently been shown to bind strongly to distinct specific receptors on somatic cells, and weakly to each other's receptor (*6*). These results support the concept of a "metabolic receptor" for insulin, with which MSA and IGF interact weakly, and of a "growth factor receptor" which mediates MSA and IGF effects on cell division but with which insulin interacts weakly. That MSA on a molar basis appears to be more active than insulin in reinitiating *Xenopus laevis* meiosis suggests that in this case we are dealing with such a growth factor receptor. This is also compatible with the efficacy of proinsulin compared to insulin (approximately one-third as active) (*2*) and to the lack of effect of relaxin (*6, 7*). Antibodies to insulin receptors (*8*) can neither mimic nor abolish insulin's effect on meiosis.

Wallace and Misulovin indicate that insulin at a concentration of 1 μg/ml is a "saturating concentration for oocyte-associated insulin receptors" (*1*). In fact they refer for that statement to "dormant" 4*N* chromosome-oocyte development, clearly more related to metabolic events than to cell division processes. Moreover, we are not surprised that, under their experimental conditions, "maturation never occurs" with insulin at 1 μg/ml, since we have also found that this concentration is barely active, and they indicated elsewhere (*9*) that often their cultured oocytes respond relatively weakly to progesterone itself.

The concept that the "effect of high concentrations of Zn^{2+}-free insulin is mediated by the follicle cells," which would be the target for insulin and which would in turn release progesterone, was assessed in experiments where insulin was no longer active, and with pronase-treated oocytes (*1*). However, this negative result may be not be entirely convincing since membrane receptors, as suggested (*1*), may be injured by such treatment. Indeed, we have found that the insulin effect on our usual oocyte preparations was never abolished by cyano-ketone, an inhibitor of 3β-hydroxysteroid dehydrogenase activity, and therefore of progesterone synthesis (*10*). We ascertained that cyano-ketone did not change the effect of progesterone on meiosis (Table 2). Moreover, since our defolliculated oocytes did not respond to human chorionic gonadotropin (hCG) (*11*), we prepared non-defolliculated oocytes; these underwent meiotic maturation with hCG treatment, but no such maturation occurred if cyano-ketone was added, thus confirming other results (*12*) (data not shown). These experiments indicate that even if there are a few follicle cells remaining on oocytes prepared in the usual way, it would be surprising if insulin, proinsulin, MSA, or IGF promoted enough progesterone biosynthesis to establish the rather high concentration of steroid necessary for meiotic maturation (*13*). In other experiments (*5*), we have observed that insulin and progesterone display different effects on the concentration of adenosine 3′,5′-monophosphate in oocytes treated with cholera toxin. If the effects of insulin were mediated by progesterone, these results would be difficult to explain.

We conclude that (i) polypeptidic growth factors, including insulin, participate directly in reinitiating meiosis in *Xenopus laevis* oocytes, probably by way of a growth factor receptor, (ii) Zn^{2+} is also an inducer, and (iii) neither Zn^{2+} nor follicle cells explain insulin action.

Table 1. Meiotic maturation of *Xenopus laevis* oocytes exposed to progesterone, insulin, MSA, and IGF. Meiotic maturation was assessed as usual by germinal vesicle breakdown (*13*) and scored for groups of 30 oocytes, all from a single female in each experiment.

Treatment	Germinal vesicle breakdown (%)	
	Experiment 1*	Experiment 2†
None	0	0
Progesterone (1 μ*M*)	92	100
Progesterone (0.1 μ*M*)	74	66
Insulin (7 μ*M*)	72	24
Insulin (3.5 μ*M*)	42	42
MSA (0.5 μ*M*)	70	
MSA (0.4 μ*M*)	60	
MSA (0.25 μ*M*)	48	
MSA (0.1 μ*M*)	12	
IGF (200 μg/ml)		30
IGF (125 μg/ml)		30
IGF (75 μg/ml)		10

*Exposure for 19 hours. †Exposure for 30 hours.

Table 2. Meiotic maturation of *Xenopus laevis* oocytes exposed to progesterone, insulin, and cyano-ketone. Meiotic maturation was assessed as in Table 1. Abbreviations: DF, oocytes were defolliculated as usual; F, the oocytes were not defolliculated; C, cyano-ketone. Note that non-defolliculated oocytes respond to a lesser extent to all agents.

Treatment	Experiment 3*		Experiment 4†	
	DF	F	DF	F
None	0	0	0	0
Progesterone (5 μ*M*)	80	20		
Progesterone (5 μ*M*) plus C	84	16		
Progesterone (0.5 μ*M*)			100	43
Progesterone (0.5 μ*M*) plus C			100	50
Insulin (7 μ*M*)	24	0	55	25
Insulin (7 μ*M*) plus C	44	0	80	35

*Exposure for 19 hours. †Exposure for 20 hours.

M. EL-ETR
Lab-Hormones-U. 33 Inserm, Université Paris Sud, 94270 Bicêtre, France
S. SCHORDERET-SLATKINE
Département de Gynécologie et d' Obstétrique Hôpital Cantonal, Genève, Suisse
E. E. BAULIEU
Lab Hormones-U. 33 Inserm, Université Paris Sud

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