Short Contribution

Essential role of insulin during early prepancreatic development of the frog *Microhyla ornata*#

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ABSTRACT We undertook the present study to examine the role of insulin during early development of frog when functional pancreas does not exist. Two approaches were adopted to achieve this objective. In the first approach, influence of exogenous insulin on the early embryonic development of the frog *Microhyla ornata* was studied. In the second approach, the effects of antiserum to insulin on embryonic development were studied. Exogenous insulin stimulated the embryonic development while immunoneutralization of endogenous insulin not only resulted in retardation of development but also induced developmental abnormalities. These results demonstrate the essential role of insulin during early embryonic development of this frog. To our knowledge, such findings have not been reported so far in any amphibian.

KEY WORDS: *insulin, amphibian development, neurulation, neural induction*

Hormones are believed to usually exert their influence on growth and differentiation only during late embryonic development. However, recent results suggest that insulin plays an important role during early embryonic development of chick and sea urchin (De Pablo and Roth, 1990; De Pablo *et al.*, 1990). We undertook the present study because similar information is not available for amphibian embryos (see De Pablo *et al.*, 1990). In order to assess the possible role of insulin during early development of the frog *Microhyla ornata*, effects of exogenous insulin and antiserum to insulin on its early embryonic development were studied. Preliminary results of this study have been recently reported in the form of an abstract (Ghaskadbi and Ghate, 1991).

Exogenous insulin in the dose range of 10-100 μg/ml stimulated the neural tube closure in a dose-dependent manner (Fig. 1). The action of insulin was quite swift as the effects became apparent immediately, even in embryos where the treatment was initiated at the late gastrula stage. This early stimulation of development due to excess insulin culminated in the formation of larger-than-normal tadpoles after 72 h (Fig. 2). Morphometric analysis of control and treated tadpoles showed an increase of more than 23% in the body length of tadpoles growing in the presence of excess insulin as compared to those growing in the normal medium (Table 1). Except for their larger size, these tadpoles were otherwise normal anatomically as well as behaviorally. The high doses of insulin required (microgram quantities) for the effects to become apparent are probably due to the intact vitelline membrane which is known to act as a barrier even for small molecules (Wadekar *et al.*, 1987). To confirm that insulin does play an essential role during early development, we neutralized the endogenous insulin pool in the developing embryos by using antiserum to porcine insulin and followed the subsequent development of such embryos. Presence of this antiserum in the medium resulted in severe retardation of development and led to various abnormalities in the tadpoles (Fig. 3). The abnormalities included incomplete utilization of yolk, reduced pigmentation, slight microcephaly, incomplete fusion of choroid fissure in the eye, shortening of body axis, curved tail, blistering, moderate ophthalmic edema, reduced fin and poor differentiation of somites. The average body length of abnormal tadpoles was reduced by more than 16% from that of the controls (Table 1). Control embryos developing in the presence of either goat or rabbit IgG developed in a normal fashion (Fig. 3, Table 1).

To check the specificity of the effect of antiserum to insulin, influence of exogenous insulin on the action of the antibody was studied. Simultaneous addition of insulin (10 μg/ml) not only reduced the number of abnormal tadpoles due to antibody treatment, but also reduced the extent of abnormality as seen from the measurement of body length of abnormal tadpoles (Table 2). This confirmed that the effects of antiserum to insulin are indeed due to selective neutralization of endogenous insulin.

Our results indicate that insulin is essential, probably as an early growth and differentiation signal for the early development of the frog, even at stages when functional pancreas is reported (Ortiz de

Abbreviations used in this paper: IgG, immunoglobulin; Con A, concanavalin A; TPA, phorbol 12-myristate 13-acetate.

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#This paper is dedicated to Professor Leela Mulherkar.

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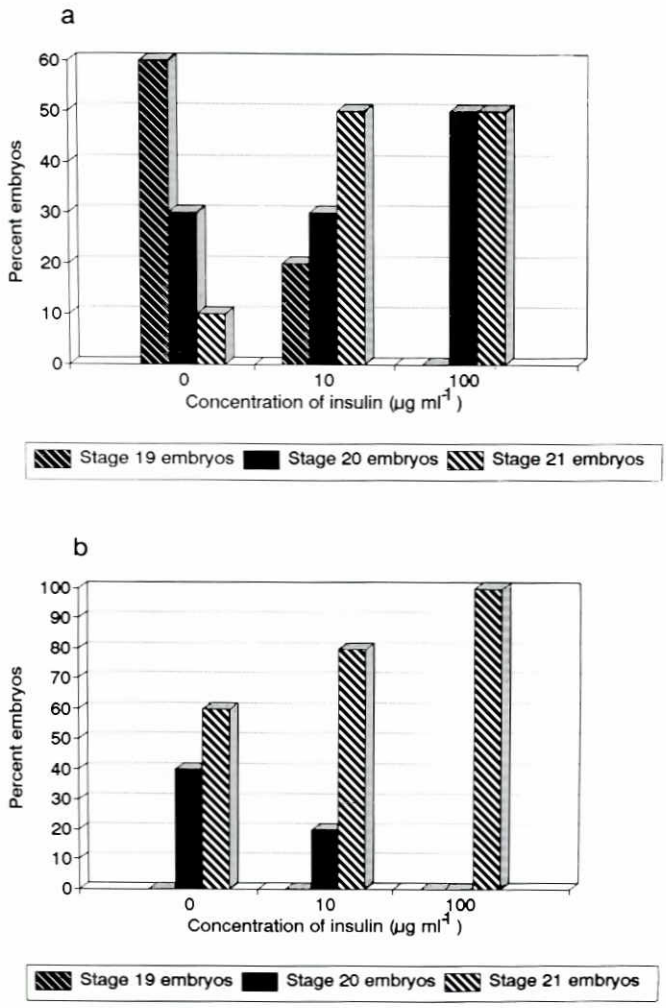

Fig. 1. Stimulation of a neural tube closure by insulin in frog embryos treated at early gastrula *(stage 10: Padhye and Ghate, 1989).* **(a)** *After 5 h 30 min.* **(b)** *After 5 h 45 min.*

Fig. 2. Stimulation of frog development by insulin in embryos treated at mid-gastrula (stage 13: Padhye and Ghate, 1989) and allowed to develop for 72 h (control at the top, experimental below).

Zarate *et al.*, 1991) to be absent. This contention is supported by the recent reports that at comparable embryonic stages of *Xenopus laevis*, genes encoding insulin (Shuldiner *et al.*, 1991) as well as those encoding receptors for insulin and insulin-like growth factor-I (Scavo *et al.*, 1991) are expressed.

Insulin is known to exert a variety of metabolic and growth-promoting effects in the target cells, both *in vitro* and *in vivo* (King and Kahn, 1985; Almas *et al.*, 1992). One of the early events in the mechanism of action of insulin is activation of the intrinsic tyrosine kinase activity of its plasma membrane receptor and receptor autophosphorylation (Kasuga *et al.*, 1982a,b). The well documented neuralizing effect of the plant lectin concanavalin A (Con A) in amphibian embryos (Takata *et al.*, 1981; Grunz, 1985; Gualandris *et al.*, 1987) is also deduced to be related to its mitogenic action involving activation of protein kinase C (Yamada, 1990). It is suggested that lectins exert their neuralizing effect by interacting with a plasma membrane receptor specific for an as yet unidentified natural inducer (Grunz, 1984). Furthermore, Con A is known to exhibit insulinomimetic properties after binding to the insulin receptors (Katzen *et al.*, 1981). It is an interesting possibility that the unexplained mechanism of neuralizing effect of Con A (Gualandris *et al.*, 1987) could be related to its insulinomimetic action through insulin receptors. Another artificial inducer is the tumor promoter phorbol 12-myristate 13-acetate (TPA), treatment with which leads to neural induction in *Triturus alpestris* (Davids *et al.*, 1987) and *Xenopus laevis* (Otte *et al.*, 1988). TPA also exerts its effects through direct activation of protein kinase C (Castagna *et al.*, 1982; Nishizuka, 1986). It is even more interesting to note in the present context that TPA, like insulin, brought about stimulation of neural tube closure in *Microhyla* embryos (S. Ghaskadbi, unpublished observations).

Stimulation of neural tube closure by insulin and demonstration of the essential role of insulin during early development in our studies along with the available information on inducing actions of Con A and TPA cited above suggests that insulin could have an important role in the phenomenon of neural induction in amphib-

TABLE 1

EFFECTS OF EXOGENOUS INSULIN AND ANTISERUM TO INSULIN ON THE DEVELOPMENT OF *MICROHYLA* EMBRYOS

Treatment	Developmental stage*	Duration (h)	Length of tadpole (mm) mean±SD**	% Increase/ decrease
Untreated control	13	72	4.46±0.09	–
Insulin (100 μg/ml)	13	72	5.50±0.16	23.32
Goat IgG	17	68	4.84±0.13	–
Antiserum to insulin (1:3000)	17	68	4.04±0.27	16.53

*According to Padhye and Ghate, 1989.
**Sample size= 10.

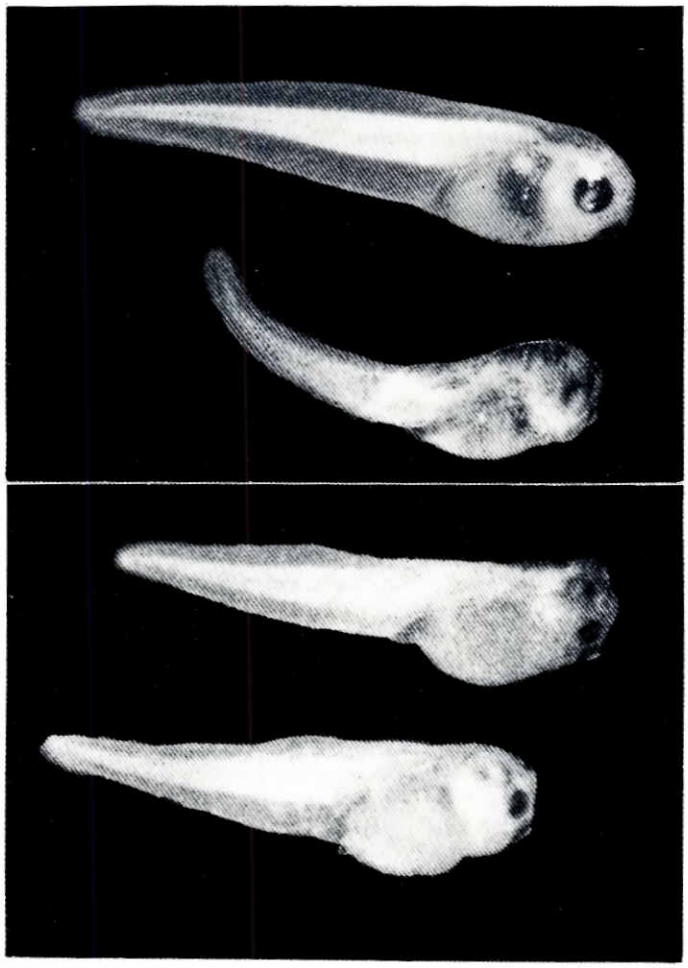

Fig. 3. Effects of antiserum to insulin on frog development (control at the top, experimental below). *The mid-neurula (stage 17: Padhye and Ghate, 1989) embryos were allowed to develop in sterile pond water containing antiserum to porcine insulin for 68 h. The control embryo was treated with goat IgG which exerted no apparent deleterious effect on the development.*

ians. How insulin can have its effect selectively on certain cells within the embryo and not on others is not clear but it could possibly be controlled through the availability of insulin receptors on target cells. Region-specific differences in the number of insulin receptors in gastrulating and neurulating chick embryos have indeed been reported where maximum receptors were detected in structures of ectodermal origin such as Hensen's node, neural folds, neural tube and optic vesicles (Girbau *et al.*, 1989). We plan to test this possibility in our laboratory.

Peptide growth factors have been very strongly implicated in amphibian mesoderm induction (Ruiz i Altaba and Melton, 1989; Smith, 1989; Slack *et al.*, 1990). Further, it has been suggested (Smith *et al.*, 1990) that regulatory molecules in the adult organism may perform important functions also during early development. The recent appreciation of the importance of insulin during early embryonic development of chick (Bassas *et al.*, 1987), sea urchin (De Pablo *et al.*, 1988), mouse (Rao *et al.*, 1990) and frog (present study) and the conserved nature of regulatory mechanisms of insulin (Blundell, 1980) similarly point to a more or less ubiquitous role for insulin during early embryonic development.

Experimental Procedures

Embryos

Freshly fertilized eggs of *M. ornata* were collected from natural ponds and allowed to develop in the laboratory (24-26°C) till the desired stage of development. The surrounding jelly was carefully removed with fine forceps before the beginning of an experiment and embryos were transferred to 5 cm-diameter glass Petri dishes containing sufficient amount (1 ml/embryo) of filtered and sterile pond water (Ghaskadbi, 1987).

Treatment and observations

Gastrulating embryos were allowed to develop in medium containing 10 or 100 μg/ml insulin (Sigma, USA). The control embryos developed in pond water alone. Progression of neural tube closure in both sets of embryos was carefully monitored, embryos were accordingly staged (stage 19, 20 or 21: Padhye and Ghate, 1989) and quantitated. The embryos were allowed to develop further for a period of 72 h in order to see if stimulation of neural tube closure due to insulin resulted in an increase in the overall growth of the tadpole. The medium was not replenished with insulin during this period. Each of the control and experimental set consisted of a minimum of 10 embryos and all experiments were repeated at least three times.

To confirm that insulin indeed plays an essential role during early development, effect of neutralization of endogenous insulin on development was studied. Gastrulating or neurulating embryos were transferred to a medium containing antiserum to porcine insulin (ICN Immunochemicals, USA; RIA titer: 1/80,000, final dilution 1:3000). The corresponding controls developed in the presence of either goat or rabbit IgG. The development of embryos from control and treated groups was monitored carefully up to 68 h and abnormalities, if any, were recorded.

The specificity of the effects of antiserum to insulin was examined by studying the influence of exogenous insulin on its effects. Insulin was added at a concentration of 10 μg/ml simultaneously with two different dilutions (1:1000, 1:3000) of the antibody and stage 17 embryos were allowed to grow in this medium for 24 h. At the end of the treatment, tadpoles were studied for abnormalities, if any, and the body length of normal and abnormal tadpoles was measured.

TABLE 2

INFLUENCE OF EXOGENOUS INSULIN ON THE EFFECTS OF ANTISERUM TO INSULIN ON THE DEVELOPMENT OF *MICROHYLA* EMBRYOS

Treatment*	Abnormal tadpoles (%)**	Length (mm)	
		normal tadpoles mean ± SD***	abnormal tadpoles mean ± SD***
Goat IgG	0(0)	3.46±0.12	–
Antiserum to insulin (1:1000)	60(24)	3.49±0.26	1.95±0.71
Antiserum to insulin (1:3000)	40(16)	3.17±0.10	1.95±0.08
Antiserum to insulin (1:1000) + insulin (10 μg/ml)	50(20)	3.16±0.48	2.02±0.42
Antiserum to insulin (1:3000) + insulin (10 μg/ml)	10(4)	3.30±0.21	2.58±0.01

*The treatment was started at stage 17 and continued for 24 h.
**Figures in parentheses indicate corresponding number of embryos.
***Sample size= 10.

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