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Short Contribution

An appraisal of the developmental importance of polyamine changes in early *Xenopus* embryos

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ABSTRACT The biological importance of the various changes in polyamine metabolism that occur during early *Xenopus* development have been investigated. Incubation of embryos in high salt medium was observed to cause a precocious fall in ornithine decarboxylase activity without affecting development. Similarly, inhibiting ornithine decarboxylase activity with specific inhibitors did not affect development. Injecting spermidine, within physiologically relevant limits, caused a dose-dependant inhibition of mitotic divisions in the injected blastomere. Increasing the intracellular putrescine did not affect cell division or development. Co-injection of both spermidine and putrescine, so that the original molar ratio of these two polyamines was conserved, abrogated the inhibition of cell division observed when spermidine was injected alone. Therefore, in *Xenopus* embryos the intracellular spermidine concentration must be retained within certain limits relative to that of putrescine to allow normal development.

KEY WORDS: *development, polyamines, Xenopus laevis*

The natural polyamines, putrescine, spermidine and spermine, are ubiquitous molecules and many observations show that the enzyme activities associated with polyamine biosynthesis must be critically regulated in proliferating cells. A minimum intracellular polyamine concentration is essential for cell proliferation (Steglich and Scheffler, 1982; Stoscheck *et al.*, 1982; McConolgue *et al.*, 1983; Casero *et al.*, 1984; Pohjanpelto *et al.*, 1985) whereas an experimentally produced over-expression of ornithine decarboxylase (ODC) activity, at least in certain cultured cell lines, can lead to a transformed phenotype (Auvinen *et al.*, 1992).

Fertilization of the *Xenopus* egg triggers completion of the meiotic divisions and the embryo then undergoes 12 rapid quasi-synchronous mitotic divisions (for review see Kirschner *et al.*, 1985). Zygotic gene transcription is first clearly detected after the twelfth mitotic division, about 7 h after fertilization. At this time, called the mid-blastula transition (MBT) (Signoret and Lefresne, 1971), the cell cycle slows and the blastomeres cease to divide synchronously.

We have previously shown (Osborne *et al.*, 1991) that in *Xenopus* embryos, from the 2-cell stage up to the MBT, ODC activity increases with a concomitant increase in intracellular putrescine and spermidine. Then, after the MBT, ODC activity decreases rapidly and the intracellular putrescine and spermidine concentrations cease to increase. Contrary to the situation normally observed in cultured cells, the intracellular putrescine concentration is almost twice that of spermidine (Osborne *et al.*, 1991). Also the *Xenopus* embryos produced from our breeding stock do not contain any detectable spermine.

We have evaluated here the biological relevance of two of these particular characteristics of polyamine metabolism in *Xenopus* embryos, asking the following questions: is an increase in ODC activity and the consequential rise in intracellular putrescine required for the rapid mitotic divisions which follow the fertilization and/or, is the limitation in intracellular spermidine a necessity for the correct development of the *Xenopus* embryo?

An initial observation, suggesting that an increase in ODC activity and intracellular putrescine was not an absolute requirement for normal development, was made while analyzing the changes in polyamine biosynthesis in embryos cultured in media of different ionic strengths. Fig. 1 shows the effects, on the ODC activity and intracellular putrescine concentration, of increasing the media NaCl concentration from 33 mM NaCl (standard conditions, F1 medium) to 90 mM. Embryos were fertilized in standard conditions (F1 medium) and at 2 h post-fertilization (2-4 cell stage) half were transferred into F1 medium supplemented with NaCl to 90 mM. The data in Fig. 1A shows that one hour after the transfer the ODC activity in the embryos in media with 90 mM NaCl was already less than that in the embryos in F1 medium (33 mM NaCl) and it steadily

Abbreviations used in this paper: MBT, mid-blastula transition; ODC, ornithine decarboxylase.

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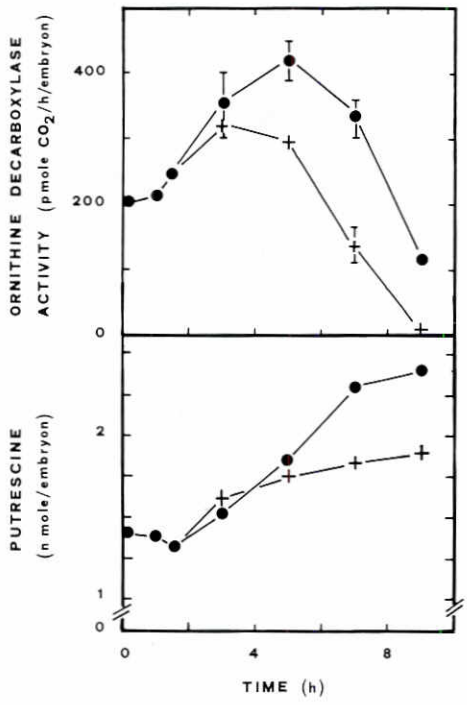

Fig. 1. Effect of 90 mM NaCl on the temporal changes in ornithine decarboxylase activity. *Embryos fertilized in F1 medium (33 mM NaCl) were divided into two batches. One was transferred into F1 medium supplemented with NaCl to a final concentration of 90 mM (+), and the other remained in F1 medium (●). At the indicated times 10 embryos were removed from each culture for ODC activity measurements and 5 embryos were taken for polyamine analyses. Similar results were obtained in two independent experiments.*

decreased in the embryos in 90 mM NaCl from then on. For the batch of embryos continually cultured in F1 medium the ODC activity increased for 3 h after the transfer (5 h post-fertilization). The precocious fall of ODC activity in embryos cultured in 90 mM NaCl, relative to those in F1 medium, was accompanied by a reduction in the accumulation of putrescine (Fig. 1B). The data given in Table 1 shows that there is a dose-dependant effect of ionic strength on the decrease in ODC activity and the lesser accumulation of intracellular putrescine.

The rate at which the blastomeres of the pre-MBT embryo divide is unaffected by the NaCl concentration of the external medium. This implies that a continual increase in intracellular polyamines is not required for the maintenance of the rapid cleavage rate of the blastomeres. This was substantiated by injecting embryos with one of the irreversible inhibitors of ODC: α-difluoromethyl ornithine or (2R,5R)-6-heptyne-2,5-diamine (Metcalf *et al.*, 1978; Danzin *et al.*, 1983). When injected (final concentration 100 μM) into one of the cells of a 2-cell embryo, both of these inhibitors efficiently reduced the ODC activity in the injected embryos. At the 512-cell stage (6 h post-fertilization; 4 h post-injection), ODC activity in embryos injected with α-difluoromethyl ornithine or (2R,5R)-6-heptyne-2,5-diamine was respectively 9% and 7% of that in mock injected embryos. Despite this inhibition of ODC activity during early development, neither the rate of development nor the external morphology of the embryos, at least up to the tadpole stage, were affected. Therefore, an increase in ODC activity is not an absolute requirement for early *Xenopus* development.

To ascertain whether the limitation in intracellular spermidine was of importance for the developing embryos, one blastomere of 2-cell embryos was injected with increasing amounts of spermidine. For 250 pmoles injected, which increased the spermidine content of the injected blastomere from about 350 pmoles (the spermidine content of *Xenopus* eggs (Osborne *et al.*, 1991)) to 600 pmoles (the amount in *Xenopus* embryos at the MBT), a dramatic blastomere disorganization occurred (Fig. 2), starting with a delayed division of the injected blastomere (see also Fig. 3). Cleavage arrest was less evident when smaller amounts of spermidine were injected and embryos injected with 50 pmoles and 25 pmoles of spermidine developed normally. Almost identical results were obtained if the same amounts of spermine were injected, although no endogenous spermine is present in the eggs laid by our breeding stock of *Xenopus* females (Osborne *et al.*, 1991).

Embryos injected with 400 pmoles of putrescine, which increased the intracellular amount of this polyamine from about 600 pmoles (the amount of this polyamine in eggs), to 1 nmole (the amount in the embryos at the MBT), developed normally at least up to the gastrula stage. Embryos injected with buffer also developed normally.

In *Xenopus* eggs the amount of putrescine exceeds that of spermidine ([putrescine]/[spermidine]= 1.7), and this ratio does not significantly change during the cleavage stage of early develop-

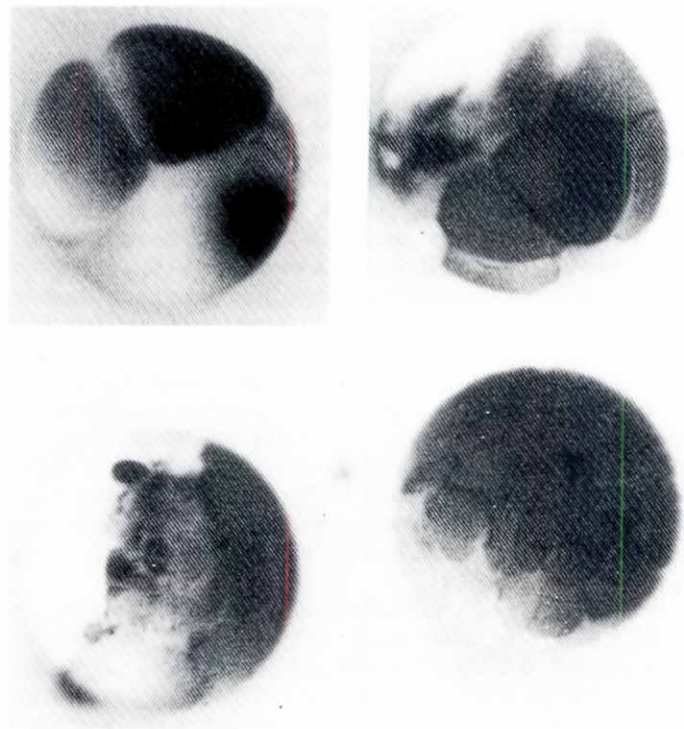

Fig. 2. Effect of spermidine microinjection on early embryos. *250 pmoles of spermidine was injected into one blastomere of a 2-cell embryo. Top left: about 30 min after injection, division in the injected blastomere is blocked. Top right: about 1 h after injection, only the non-injected blastomere continues to divide, horizontal cleavage clearly visible. Bottom: several hours after injection, comparison of a spermidine-injected embryo (left) and a buffer-injected embryo (right).*

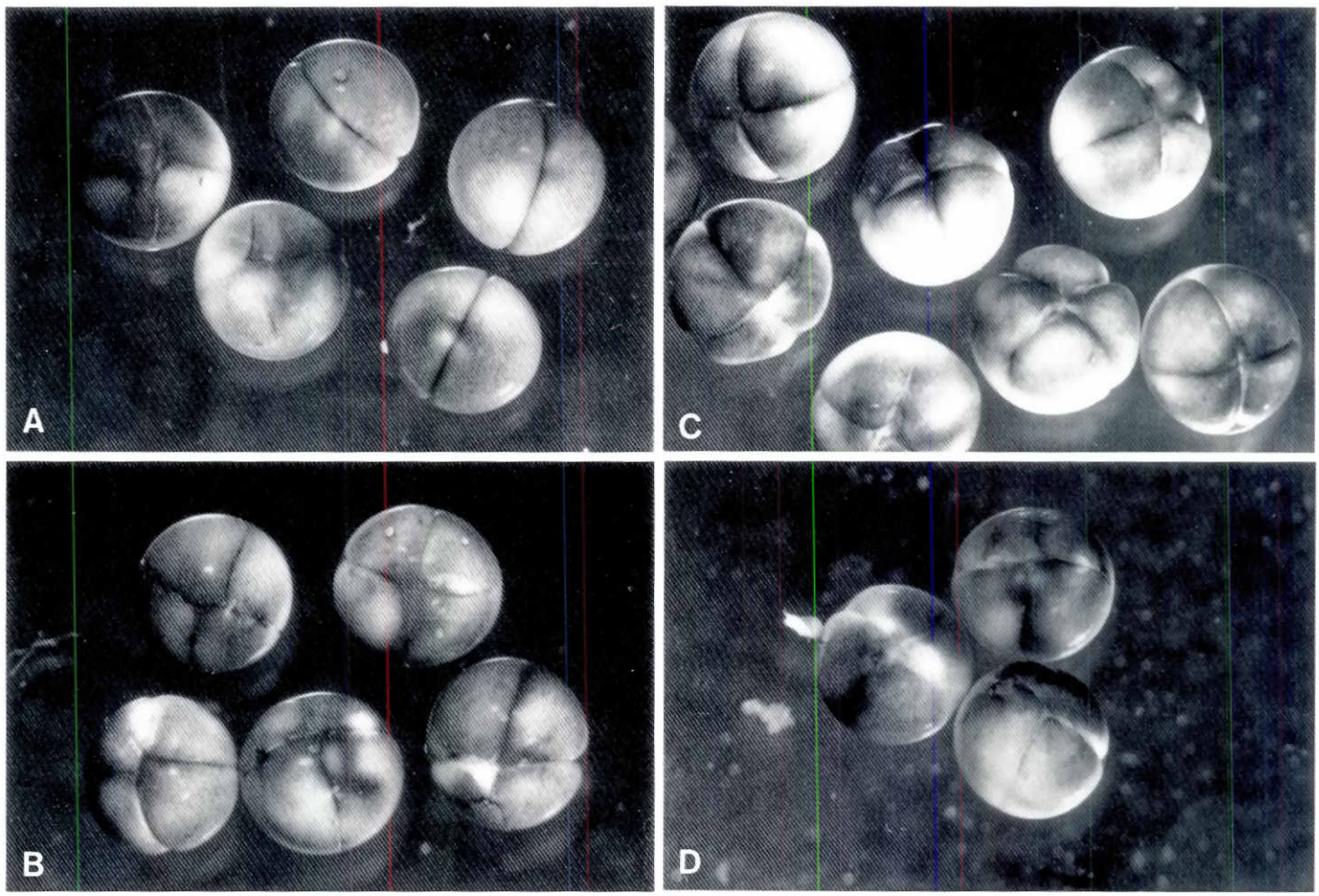

Fig.3. Effect of spermidine and putrescine co-injection on early embryo cleavage. *Two-cell embryos were injected with 250 pmoles of spermidine* **(A)**, *250 pmoles of spermidine with 400 pmoles of putrescine* **(B)**, *or 250 pmoles of spermidine with 300 pmoles of putrescine* **(D)**. *The injected embryos were fixed in 5% trichloroacetic acid when the control non-injected embryos* **(C)** *were half-way through the 4-cell stage (about 15 min after the second cleavage).*

ment (Osborne *et al.*, 1991). In the experiments described above cleavage arrest was most evident when the amount of spermidine injected brought the total spermidine content of the early embryo close to that of the putrescine. To evaluate the effect of this change in the putrescine to spermidine molar ratio, 2-cell embryos were injected with either 250 pmoles of spermidine alone or with 250 pmoles of spermidine and either 400 pmoles or 300 pmoles of putrescine. All the embryos were fixed when the control (non-injected) embryos were about half-way through the 4-cell stage (Fig. 3)

Cleavage arrest was clearly observed for the embryos injected with 250 pmoles of spermidine alone (Fig. 3A). In this particular experiment cleavage of both blastomeres was arrested probably because the spermidine was injected just after the first cleavage furrow appeared, thereby allowing the injected molecules to diffuse into the non-injected blastomere. In a few instances, two of which are shown, the beginning of a second cleavage furrow was observed, but it was never as pronounced as in the control embryos (Fig. 3C). Injection of 250 pmoles of spermidine with 400 pmoles of putrescine (Fig. 3B), which maintained the normal ratio of these two polyamines, completely abrogated the cleavage arrest observed when this amount of spermidine alone was injected. Abrogation of cleavage arrest was also obtained when 300 pmoles of putrescine was injected with the spermidine (Fig. 3D). However, in this case, where the final putrescine to spermidine molar ratio was 1.5, the second cleavage furrows that were formed showed a lesser degree of invagination.

Therefore, artificially increasing the spermidine to putrescine ratio in the 2-cell *Xenopus* embryos causes cleavage arrest and eventually disorganization of the injected blastomere, whereas concomitantly increasing the total amount of spermidine and putrescine does not affect blastomere cleavage. This implies that the limitation of the spermidine concentration in *Xenopus* embryos, relative to that of putrescine, is of biological importance. It is not clear, at present, what biological process in *Xenopus* embryos is sensitive to the spermidine/putrescine ratio. As most studies concerned with the physico-chemical aspects of polyamine function have been performed in somatic cells, where the molar ratio of spermidine to putrescine normally exceeds ten, a prognosis is difficult to make.

A corollary to the data presented here is that a correctly regulated expression of the different genes involved in polyamine biosynthesis,

TABLE 1

EFFECT OF NACL CONCENTRATION ON ORNITHINE DECARBOXYLASE ACTIVITY AND INTRACELLULAR PUTRESCINE

NaCl (mM)	ODC activity (pmole CO_2/h/embryo)	intracellular putrescine (nmole/embryo)
30	340±10	2.3±0.1
50	330±20	2.4±0.1
70	250±20	2.0±0.1
90	135±30	1.8±0.1

Embryos were fertilized and transferred into F1 medium supplemented with NaCl so as to achieve the indicated final concentrations, as described in the legend to Fig. 1. The embryos were cultured until 7 h after fertilization, 5 h after transfer into the different media, and then samples were taken for determination of the ODC activity in cytosol extracts and the amount of intracellular putrescine.

which leads to the controlled limitation in the increase in intracellular spermidine, is required for the normal development of the *Xenopus* embryo. Spermidine is synthesized from putrescine and decarboxylated S-adenosyl methionine. Attempts to measure S-adenosyl methionine decarboxylase activity in *Xenopus* embryos were unsuccessful; the activity in the extracts was not significantly different from background values (Duval and Osborne, unpublished data). Therefore, the limitation in intracellular spermidine may be due to the low S-adenosyl methionine decarboxylase activity in *Xenopus* embryos.

Experimental Procedures

Eggs obtained from laboratory-reared *Xenopus laevis* females were fertilized and the embryos were allowed to develop in F1 medium (Hollinger and Corton, 1980) as previously described (Paris *et al.*, 1988). Where indicated F1 medium was supplemented with NaCl to the required concentration. Embryos were microinjected with 10 nl or 25 nl in the equatorial region (Bellé *et al.*, 1986). Where indicated embryos were fixed in 5% trichloroacetic acid before photography. ODC activity in duplicate cytosol extracts was determined by measuring the release of [$^{14}CO_2$] from 1-^{14}C-ornithine and intracellular polyamines were quantified by high pressure liquid chromatography as previously described (Osborne *et al.*, 1989). ODC measurements were performed in triplicate and the polyamine analyses were repeated at least twice.

Acknowledgments

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