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Effects of Bromo-Cyclic GMP and Bromo-Cyclic AMP on Embryonic Development of *Xenopus laevis*

Yuko Moroki[1], Shigeo Takeuchi[2], Kazuei Igarashi[3], Hiroshi Hara[4] and Koichiro Shiokawa[1]*

[1]*Laboratory of Molecular Embryology, Zoological Institute, Department of Biological Sciences, Graduate School of Science, University of Tokyo, Bunkyo-ku, Tokyo, 113-0033, Japan,*
[2]*Department of Biological Sciences, Faculty of Science, Kanagawa University, Hiratsuka, Kanagawa, 259-1205, Japan*
[3]*Faculty of Pharmaceutical Sciences, Chiba University, Inage-ku, Chiba, 268-8522, Japan and*
[4]*Deputy Senior Research Associate Molecular Biology Laboratory, Medicinal Research Laboratories Taisho Pharmaceutical Co., LTD., Yoshino-cho 1, Ohmiya-shi,Saitama, 330-0031, Japan*

ABSTRACT—Low molecular signalling molecules such as cAMP and cGMP are expected to have important functions in early morphogenetic processes in animal development. We examined the effect of 8-bromo-cyclic GMP (Br-cGMP) on *Xenopus* embryogenesis, using 8-bromo-cyclic AMP (Br-cAMP) as a reference. When *Xenopus* gastrulae were cultured in the medium which contained these analogues, their development was affected in specific and dosage-dependent manners: While Br-cAMP induced anomaly only in head part (swelling of myelencephalon with enlarged ventricle), Br-cGMP induced shortening in body length often accompanied by bending of the cephalo-caudal axis. In embryos treated with Br-cGMP at a high dose, cellular movement was inhibited as revealed by SEM and this resulted in the formation of tadpoles with unclosed yolk plug. Br-cGMP at lower doses induced severe inhibition of the development of notochord and muscles. Since HPLC analyses revealed that both analogues were uptaken into embryonic cells, we assumed that the morphological effects observed were induced by the interference of the normal functioning of cGMP and cAMP, respectively, by Br-cGMP and Br-cAMP. Based on the results obtained, we assume that while cGMP is involved mainly in the differentiation of mesodermal structures, especially in formation of notochord and muscles, cAMP is involved mainly in the differentiation of neural structures.

INTRODUCTION

In early amphibian embryogenesis, gastrulation and neurulation are most prominent morphogenetic events. Morphogenetic processes which take place during gastrulation are convergence and extension (Keller and Danilchik, 1988), involution (Vogt, 1922, 1929; Schechtman, 1942), epiboly (Vogt, 1929; Spemann, 1938), invagination (Holtfreter, 1942a, b), and migration (Holtfreter, 1944; Nakatsuji, 1974, 1975, 1986; Keller and Schoenwolf, 1977; Kubota and Durson, 1978). These rearrangements of cells are supposed to be driven by intercalation which produces large changes in the shape of cell population. Neural induction (Spemann and Mangold, 1924), another important inductive process, takes place between the invaginated prechordal plate and competent ectodermal cells, and while the former differentiate into notochord and induces muscle differentiation, the latter differentiates into neural tissues such as brain and spinal cord (Smith and Harland, 1992; Smith *et al*., 1993, Hemmati-Brivanlou *et al*., 1994; Sasai *et al*., 1994, 1995).

In both of these processes, various excreted growth factors are involved (Slack *et al*., 1987; Asashima *et al*., 1990). It has been assumed that intracellular signalling systems convey the exogenously given inductive stimuli into the nucleus of the competent cells. During the process of the signal transmission second messengers are expected to play important roles. Studies on this issue have been performed as with cAMP by Otte *et al*. (1989), who showed that the levels of both cAMP and adenylyl cyclase increase in gastrula ectoderm. In this study, Otte *et al*. (1989) used Br-cAMP and reported that this analogue does not have the neural inducing activity by itself, but expresses the activity when it was administered together with 12-tetradecanoyl phorbol-13-acetate (TPA). In spite of the increasing information about the possible functions of cAMP in embryonic development, little has been known about the roles played by another cyclic nucleotide, cGMP, although the changing level of cGMP as well as cAMP has been analyzed in early embryogenesis of *Xenopus laevis* (Schutter *et al*., 1975; Lϕvtrup-rein and Lϕvtrup, 1975) and starfish (Niitsu-Hosoya *et al*., 1987).

* Corresponding author: Tel. +81-3-5841-4431;
FAX. +81-3-5841-4410.
E-mail: Xshiokawa@biol.s.u-tokyo.ac.jp

We have been interested in the roles played by second messangers during *Xenopus* embryonic development. In the present study, we examined the effects of the treatment of *Xenopus laevis* embryos with 8-Br-cGMP (Br-cGMP), an analogue of cGMP and compared the effects with those of 8-Br-cAMP (Br-cAMP), an analogue of cyclic AMP. We report here that Br-cGMP as well as Br-cAMP induces specific but completely different types of malformation in the treated embryos in a dose dependent manner: While the former interferes with the formation of mesodermal structures, especially notochord and muscles, the latter interferes with the formation of hindbrain.

MATERIALS AND METHODS

Embryos

Eggs of *Xenopus laevis* were manually squeezed out from gravid females which had been injected with a human chorionic gonadotropic hormone, Gonatropin (Teikokuzoki). Eggs were artificially fertilized (Shibata *et al*., 1998), and after the first cleavage, their jelly layers were removed by the treatment with 2% cysteine-HCl (pH7.8). Dejellied embryos were reared in 10% Steinberg's solution (5.8 mM NaCl, 0.067 mM KCl, 0.034 mM Ca $(NO_3)_2$, 0.085 mM $MgSO_4$, 1 mM Hepes, pH 7.4) until the desired stages (Nieuwkoop and Faber, 1956).

Treatment of embryos with Br-cGMP or Br-cAMP

Br-cGMP (8-Bromo-guanosine cyclic 3', 5'-hydrogen phosphate monosodium) (SIGMA) or Br-cAMP (8-bromo-adenosine cyclic 3', 5'-hydrogen phosphate monosodium) (SIGMA) was dissolved at 1, 5, or 10 mM in 10% Steinberg's solution just before use. Embryos at stage 10^- or stage 14 were cultured in 10% Steinberg's solution which contained Br-cGMP or Br-cAMP at 23°C in the dark until stage 14 or stage 22. Embryos were then extensively washed and kept cultured in the fresh 10% Steinberg's solution without analogues. Embryos were examined under a binocular microscope when they reached the stage 37/38 (tadpole stage).

Scanning electron microscopy (SEM)

Embryos treated with the highest concentration (10 mM) of Br-cGMP at gastrula stage were sectioned sagittally with a stainless steel knife and fixed with 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) at 4°C for 24 hr. Sectioned materials were washed with 0.1M cacodylate buffer, dehydrated through a graded series of ethanol, treated with isoamylacetate, and dried at a critical point of carbon dioxide (JCPD-5, JEOL). Samples were coated with goldpalladium in an ion coater (JFC-1100E, JEOL), and observed under a scanning electron microscope (JSM-840, JEOL).

Histological examination

Embryos were fixed at tadpole stages (stage 38/39) in Bouin's fixative for 24 hr at room temperature. Embyos were dehydrated through a graded series of ethanol-butanol solution, embedded in paraffin, and sectioned at 5 μm in thickness. Sectioned materials were stained with hematoxylin and eosin and examined under a light microscope.

Determination of Br-cGMP and Br-cAMP

Embryos, with or without vitelline membranes, were homogenized in ice-cold 5% TCA (20 embryos/100 μl) in a glass homogenizer. The acid-soluble fractions were obtained by centrifugation at 12.000 rpm for 5 min. Aliquots (100 μl) of the extracts were analyzed by HPLC using a TSK gel ODS-80TM column (6.0×150 mm) (Tosho) at room temperature. Nonlinear KH_2PO_4 and acetonitrile gradient were used at a flow rate of 1.0 ml/min. Acetonitrile in the mobile phase used under the starting conditions was 5% and this was increased to 10% for the last 10 min. As references, commercially available cGMP, cAMP, Br-cGMP and Br-cAMP (Sigma, Yamasa, RBI) were chromatographed.

RESULT

Effects of Br-cGMP and Br-cAMP on embryonic development

We examined the effects of Br-cGMP and Br-cAMP by culturing embryos from the gastrula to tailbud stage (stage 10 to stage 22) in the medium which contained these analogues (1, 5, and 10 mM). As shown in Table 1, most of the embryos treated with 1 mM Br-cGMP developed into normal tadpoles, and percentage of abnormal embryos was only slightly higher (15%) than the controls (7–9%). One of the Br-cGMP-treated embryos is given in Fig. 1B, in which development in the trunk and tail regions was inhibited, and accordingly, the body length was shorter as compared with the control. When the dosage of Br-cGMP was increased to 5 mM, survival rate was unchanged. However, all the embryos treated at this dose showed the anomaly including shortening of body length (just like the one shown Fig. 1B) (this corresponded to "slight" in Table 1), inhibition of tail formation (Fig. 1C) (corresponding to "moderate" in Table 1) and shortening and curling of embryos accompanied by poor tail formation (Fig. 1D) (corresponding to "severe" in Table 1). Nevertheless, it appeared that head formation was not severely inhibited (Fig. 1C). Therefore, the effects of Br-cGMP observed at 1 and 5 mM were mainly on axis formation. At 10 mM of Br-cGMP, a large number of embryos (37%) stopped development at gastrula stage and died, and those survived (63% of the treated embryos) were all abnormal (severe) (Table 1). In the embryos treated with this dose, the blastpore was formed but was not closed, and thus, yolk plug remained even at the tailbud stage (not shown), although head part was still relatively normal in most of the embryos. When we compared the control embryos with those treated with 10 mM sodium bromide, no difference was observed (Table 1), indicating that bromine ion, which could be liberated when Br-cGMP was converted to cGMP, has no teratogenic effect.

When we performed similar experiments with 1 mM of Br-cAMP (Table 2), most of the embryos developed into tadpole (St. 38) with normal size and normal embryo axis. Even at this lowest dose, however, a small but significant percent (16%) of the treated embryos formed abnormal head, whose posterior part was enlarged as compared with the control. When the dose of Br-cAMP was increased to 5 mM and to 10 mM, the survival rate of treated embryos did not change, and only the percentage of tadpoles with the abnormal head increased greatly (to 47% and to 78%, respectively). A typical embryo with the head anomaly which was induced by the treatment at 10 mM Br-cAMP is shown in Fig. 1E. Since there was no other apparent abnormality, we concluded that the formation of abnormal posterior head part by the treatment with Br-cAMP was a quite specific effect. This result is comparable to

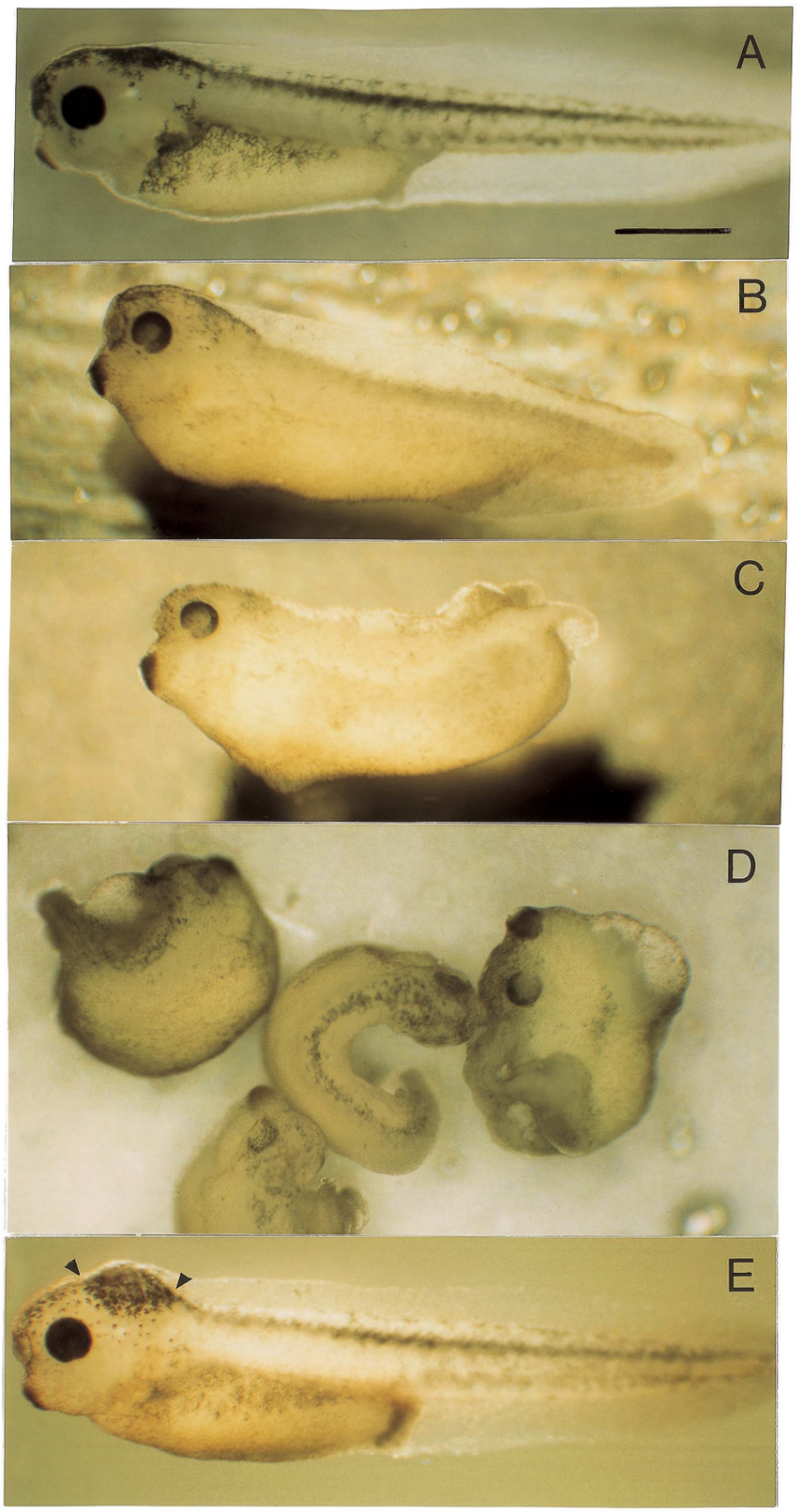

Fig. 1. Changes in morphology of embryos treated with Br-cGMP or Br-cAMP. Embryos were around stage 38. (A) Untreated control. (B) Treated with Br-cGMP (1 mM). (C) Treated with Br-cGMP (5 mM). (D) Treated with Br-cGMP (10 mM). (E) Treated with Br-cAMP (10 mM). This embryo showed hypertrophy of the hindbrain with an accumulation of pigment (arrow head). Scale bar: 1 mm.

Table 1. Effects on development of Br-cGMP treatment at different doses

Treatment	mM	Number of embryos used	Dead (%)	Total Number of survived, bu abnormal embryos (%)	Malfomation (%)				
					Body axis			Head formation	Other anomaly
					Severe	Moderate	Slight		
None		182	4 (2)	13 (7)	0 (0)	4 (2)	1 (1)	0 (0)	8 (4)
NaBar	10	104	7 (7)	9 (9)	0 (0)	7 (7)	1 (1)	0 (0)	1 (1)
8 Br-cGAMP	1	117	8 (7)	16 (15)	0 (0)	3 (3)	7 (6)	2 (2)	4 (4)
	5	116	8 (7)	108 (100)	67 (62)	37 (34)	4 (3)	0 (0)	0 (0)
	10	120	45 (37)	75 (100)	74 (62)	1 (1)	0 (0)	0 (0)	0 (0)

Embryos at gastrula stage (stage 10$^-$) were cultured until the tailbud stage (stage 22) in the medium which contained either Br-cGMP or NaBr at the indicated concentrations.

Table 2. Effects on development of Br-cAMP treatment at different doses

Treatment	mM	Number of embryos used	Dead (%)	Total Number of survived, bu abnormal embryos (%)	Malfomation (%)				
					Body axis			Head formation	Other anomaly
					Severe	Moderate	Slight		
None		182	4 (2)	13 (7)	0 (0)	4 (2)	1 (1)	0 (0)	8 (4)
None		130	4 (3)	9 (7)	0 (0)	1 (1)	4 (3)	1 (1)	3 (2)
NaBr	10	104	7 (7)	9 (9)	0 (0)	7 (7)	1 (1)	0 (0)	1 (1)
8-Br-cAMp	1	57	1 (2)	13 (23)	0 (0)	0 (0)	3 (5)	9 (16)	1 (2)
	5	76	3 (4)	42 (55)	1 (1)	1 (2)	4 (5)	36 (47)	0 (0)
	10	64	2 (3)	56 (90)	0 (0)	1 (2)	6 (10)	49 (78)	0 (0)

Embryos at gastrula stage (stage 10$^-$) were cultured until the tailbud stage (stage 22) in the medium which contained either Br-cAMP or NaBr at the indicated concentrations.

the finding by Otte *et al.* (1989) that Br-cAMP could have some important role in neural induction.

Since it was shown in the experiments in Tables 1 and 2 that Br-cGMP and Br-cAMP exert different effects on the development of *Xenopus embryos* when they were administered in the culture medium from the early gastrula to late neurula stage, we next tested if the sensitive step occurs during or after gastrulation. For this purpose, embryos were treated with the analogues either from stage 10 to stage 14 or from stage 14 to stage 22. We used here the dosage of 5 mM, since this dosage was quite effective in modifying development, yet it did not affect the survival rate. As shown in Table 3, the treatment of embryos with Br-cGMP during stage 10 to stage 14 exerted only weak effects on the axis formation, although in this case there appeared embryos with smaller eyes (9%) and slightly decreased pigmentation (11%), both of which were counted as “other anomaly” in Table 3. By contrast, the treatment of embryos from stage 14 to stage 22 gave the results which were comparable to those obtained by the treatment during the stage 10 to stage 22 (Table 1), although the effects observed here was weaker. These results indicate that the most sensitive step of embryogenesis to the treatment with Br-cGMP was after the gastrulation.

When Br-cAMP was tested in the similar way, it was found that the effect of this analogue was observed more or less similarly in the two different treatments (during the stage 10 to stage 14 and during the stage 14 to stage 22) (Table 4). The only anomaly observed was head formation here again. Thus, it appeared that the effect of Br-cAMP was not quite different depending on the stage of the embryos at which the treatment starts (stage 10 or stage 14). Therefore, we assumed that the most sensitive step would be the neurulation rather

Table 3. Effects on development of Br-cGMP treatment at different doses

Period of the treatment	Number of embryos used	Dead (%)	Total Number of survived, bu abnormal embryos (%)	Malfomation (%)				
				Body axis			Head formation	Other anomaly
				Severe	Moderate	Slight		
untreated	2	2 (1)	7 (3)	0 (0)	2 (1)	1 (1)	0 (0)	4 (2)
st. 10-st. 14	90	3 (3)	36 (40)	0 (0)	9 (10)	9 (10)	0 (0)	18 (20)
st. 14-st. 22	75	9 (12)	57 (76)	12 (16)	9 (12)	36 (48)	0 (0)	0 (0)

Embryos were cultured in the medium which contained 5 mM Br-cGMP during the indicated periods. In this series of experiment, those treated during stage 10 to stage 14 gave a high percentage of embryo with "other anomaly", which included small eyes (8.9%) and slightly decreaced pigmentation (10.11%).

Table 4. Effects on development of Br-cAMP treatment at different doses

Period of the treatment	Number of embryos used	Dead (%)	Total Number of survived, bu abnormal embryos (%)	Malfomation (%)				
				Body axis			Head formation	Other anomaly
				Severe	Moderate	Slight		
untreated	80	2 (1)	6 (3)	0 (0)	2 (1)	0 (0)	0 (0)	4 (2)
st. 10-st. 14	70	3 (4)	24 (34)	0 (0)	0 (0)	3 (4)	18 (26)	3 (4)
st. 14-st. 22	62	4 (6)	22 (35)	0 (0)	0 (0)	2 (3)	20 (32)	0 (0)

Embryos were cultured in the medium which contained 5 mM Br-cAMP during the indicated periods.

than the gastrulation for both Br-cGMP and Br-cAMP when dosage of the analogues was 5 mM.

Observation of gastrulae treated with 10 mM of Br-cGMP

In the above experiment in which 10 mM Br-cGMP was used, a large number of embryos arrested development at gastrulation. Even in those survived, blastopore was not closed completely. Therefore, using SEM, we examined cellular arrangement of the embryos treated with 10 mM Br-cGMP during gastrulation. As shown in Fig. 2A (arrow), most of the cells at the animal side in the control embryo were in two layers, whereas cells at the animal cap portion of Br-cGMP-treated embryos were in more than three layers (Fig. 2B, arrow). Further, the clumps of cells were observed in the marginal zone in the treated embryos. These results show that Br-cGMP at this high dose strongly affected gastrulation movement such as convergence and extension accompanied by intercalation, although it is not clear at this step if these effects have some physiological meaning in relation to signal transduction processes due to the too large dose of the analogue used.

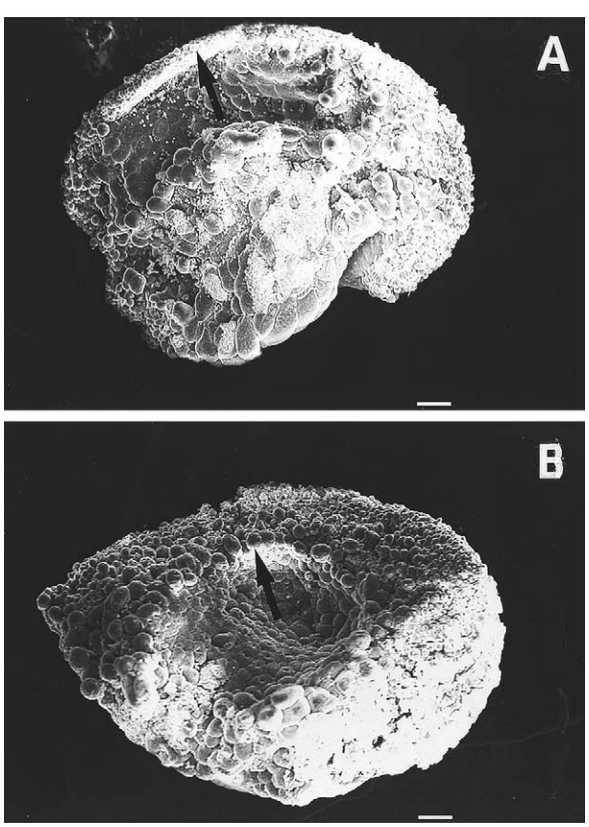

Fig. 2. Effect of 10 mM of Br-cGMP examined by SEM. (A) Untreated control embryo at the mid gastrula. Animal side is to the top. (B) An embryo treated with Br-cGMP (10 mM) from gastrula to mid gastrula stage. Cells at the animal side were in more than three layers (arrow) and the clumps of cells were observed at the marginal zone in relation to control embryo. Scale bars: 100 μm.

Histological examination of malformed embryos produced by the analogue treatments

When transverse sections of embryos treated with 5 mM of Br-cGMP were examined, brain was normal, although the diameter of notochord was much larger than the control (Fig. 3B). When sagittal sections were examined, the defect in the notochord became clearer. In Fig. 4B, it is shown that the notochord was not only thicker in size, but its sheath was thinner than the control, and vacuoles which normally observed in the notochord were quite abnormal. Thus, the size of each vacuole was much smaller, and the number of the vacuoles was much larger, as compared with the control (Fig. 4B). In addition to these abnormalities in notochord, spinal cord was also short in length, and furthermore, regionalization of brain appeared to have been disturbed.

We also examined the notochord of the embryos which were treated with 10 mM Br-cGMP. In the enlarged sagittal section of the embryo treated with10 mM Br-cGMP, a number of small eosinophilic granules which represented yolk granules (Fig. 5B, arrow head) were found in inter-vacuolar spaces. This suggests that utilization of yolk was not complete in these cells. It is quite clear here that the diameter of notochord was almost doubled, and the number of vacuoles being much larger, sizes of vacuoles being much smaller, and above all, the shape of vacuoles were quite different from those of the fully differentiated vacuoles in normal embryos. In these Br-cGMP-treated embryos, yolk granules were preserved also in muscle cells. Thus, differentiation of not only notochord but also muscles appeared to be much reduced as compared with the control at this high dose of Br-cGMP (Fig. 5).

Transverse sections of embryos treated with 10 mM Br-cAMP showed that myelencephalon was flattened and ventricle was deformed at the upper part (Figs. 3C) but notochord and other tissues were normal. Also in the sagittal section,

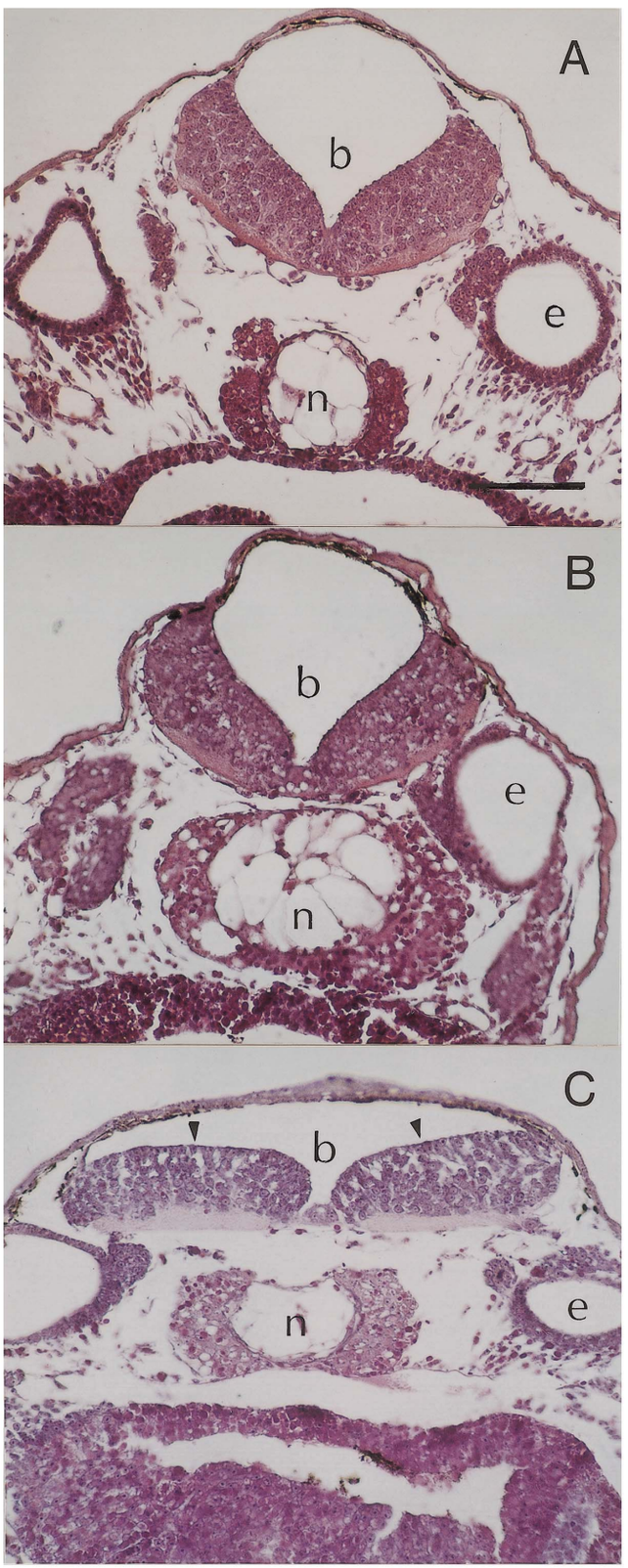

Fig. 3. Transverse sections through the ear placode region. Embryos were sectioned at stage 38. (A) Untreated. (B) Treated with Br-cGMP (5 mM). (C) Treated with Br-cAMP (10 mM). Myelencephalon was swollen and flattened (arrow head), but other structures seemed to be normal. Brain (b), ear placode (e), and notochord (n) are marked. Scale bar: 1 mm.

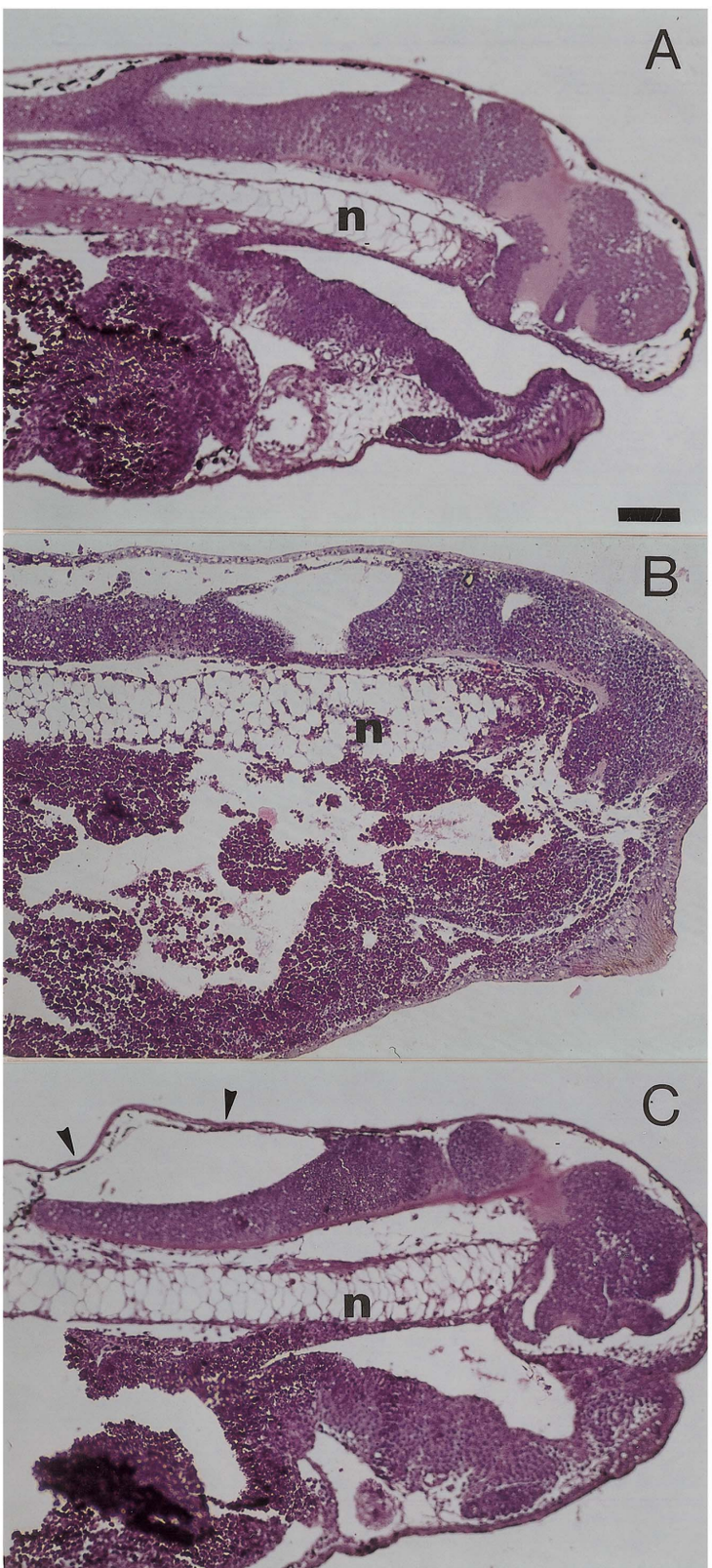

Fig. 4. Sagittal sections through the notochord. Embryos were sectioned at stage 38. (A) Untreated. (B) Treated with Br-cGMP (5 mM). The mesodermal differentiation, especially notochord, was poor. (C) Treated with Br-cAMP (10 mM). The hypertrophy of the hindbrain was observed and ventricle was expanded backward (arrow heads). Notochord (n) is marked. Scale bar: 1 mm.

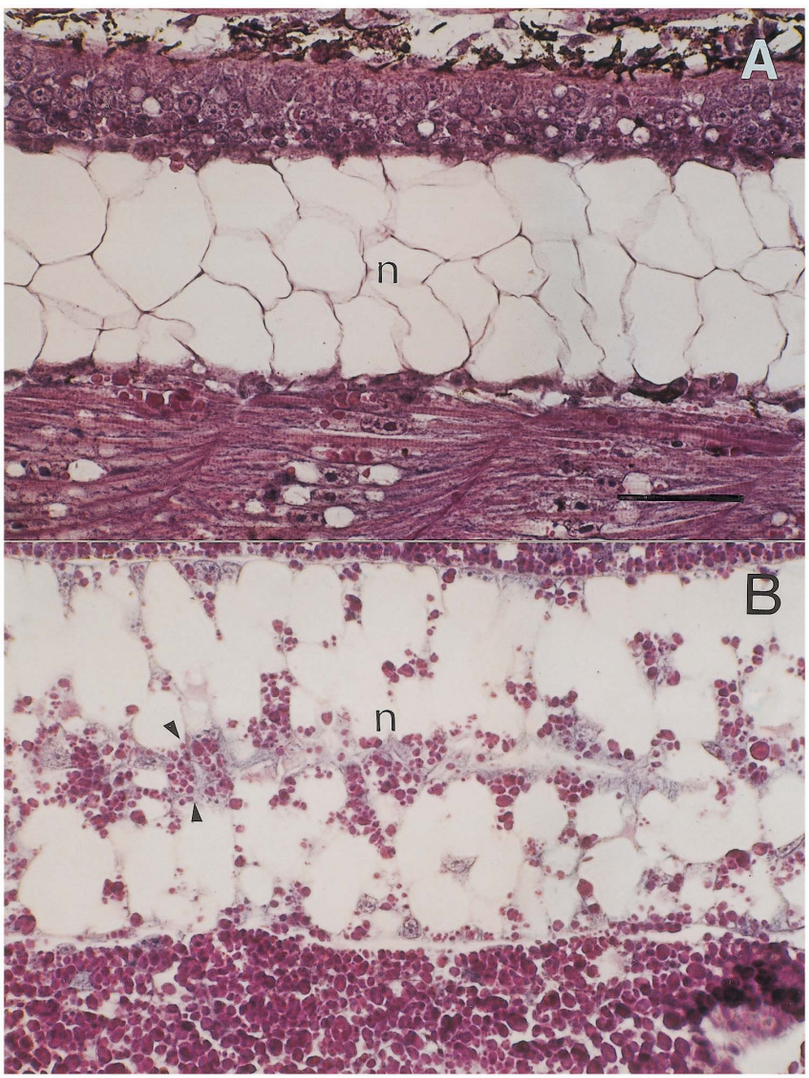

Fig. 5. Higher magnification of the notochord of the Br-cGMP-treated embryo. Embryos were sectioned at stage 38. (A) Untreated. Notochord shows well developed vacuolation. Striated muscles are also well developed. (B) Treated with Br-cGMP (10 mM). Vacuolation of cells in notochord and muscle differentiation was poor. Clumps of remnants of yolk granules were seen in inter-vacuolar spaces (arrow head). Notochord (n) and somite (s) are marked. Scale bar: 0.5 mm.

ventricle appeared to be larger and shifted backwards (Fig. 4 C). Interestingly, formation of forebrain and midbrain in these embros was not affected greatly (Fig. 4C). These results indicate that the swelling of the posterior part of the head by Br-cAMP treatment is a quite specific effect, and this is due mainly to the swelling of myelencephalon accompanied by the expansion of ventricle. Thus, the effect obtained with Br-cGMP was completely different from those obtained with Br-cAMP.

HPLC analysis of the uptake of Br-cGMP and Br-cAMP

Finally, we examined by HPLC the uptake of Br-cGMP and Br-cAMP within the embryo during the treatment. As shown in Fig. 6, the acid-soluble fraction of gastrulae treated with 5 mM of Br-cGMP or Br-cAMP for 30 min contained the component which was eluted from the column with the retention time of 16 min(B) or 23 min (C), respectively. Based on the results of the control and experimental runs of samples prepared from embryos with or without vitelline membranes, we calibrated that ca. 0.4 nmoles of Br-cGMP and ca. 0.4 nmoles of Br-cAMP were uptaken into the embryo. In Fig. 6, it was also seen that levels of endogeneous cGMP and cAMP were not increased to any detectable extent in the treated embryos. Since a *Xenopus* early embryo contains 0.1 pmoles of cGMP and 0.2 pmoles of cAMP (Lϕvtrup-Rein and Lϕvtrup, 1975), we roughly calculated that the intracellular level of the Br-cGMP and Br-cAMP here was approximately 4×10^3 and 2×10^3 -fold higher than that of their respective normal metabolite.

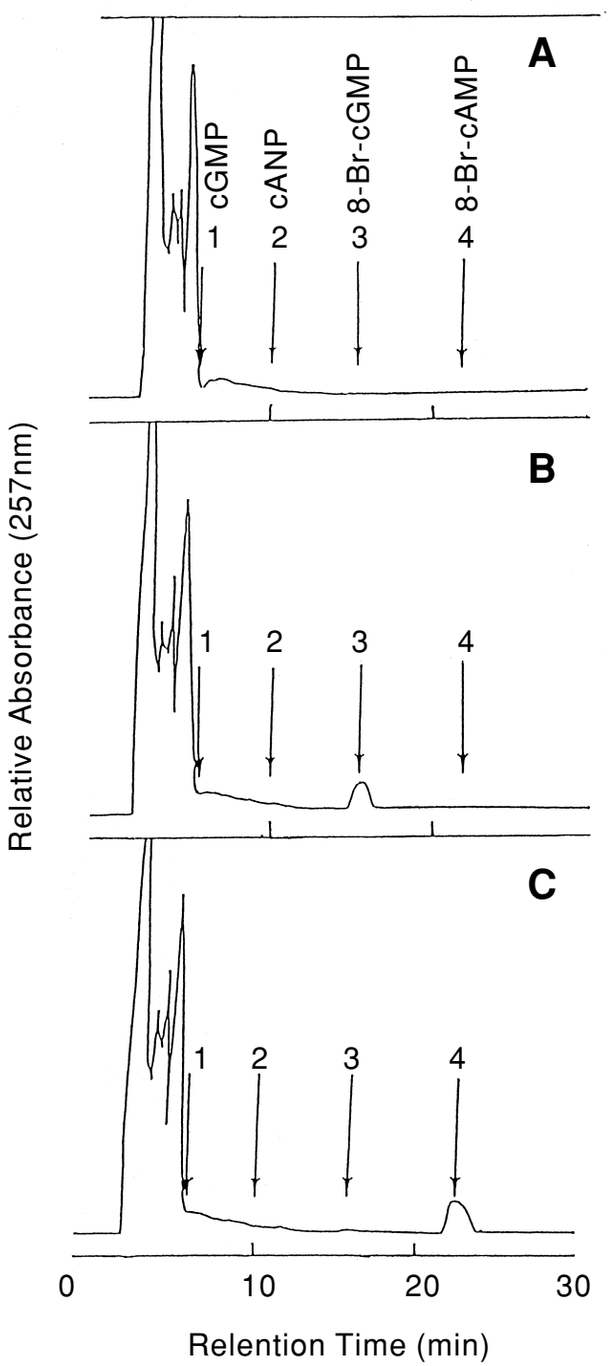

Fig. 6. HPLC analysis of the acid-soluble fraction of gastrule treated with Br-cGMP or Br-cAMP for 30 min. (A) Untreated. (B) Treated with 5 mM Br-cGMP. (C) Treated with 5 mM Br-cAMP. Extracts of embryos were subjected to HPLC using a nonlinear acetolitrile/KH_2PO_4 gradient. The compounds were detected by absorbancy at 257 nm. The retention time of cGMP (1), cAMP (2), Br-cGMP (3) and Br-cAMP (4) were 6 min, 10 min, 16 min and 23 min, respectively.

DISCUSSION

Based on the consideration that second messengers like cGMP and cAMP may play important roles in the developmental processes, we tested here the effects of Br-cGMP and Br-cAMP, analogues of cGMP or cAMP, respectively, on *Xenopus* embryogenesis. In HPLC analyses, considerable levels of these analogues were recovered from the embryos whose culture medium contained the analogues. Since the levels of the analogues recovered were the same irrespective of whether the vitelline membrane was removed or not, we assumed that the analogues recovered were not from the perivitelline space but from the treated embryos. Though we could not notice any measurble increase in the levels of endogenous cGMP and cAMP, we assumed that the Br-cGMP and Br-cAMP exerted their effects probably by directly affecting cGMP- or cAMP-dependent signalling pathway after being uptaken into cells.

From morphological examinations, while Br-cAMP specifically induced swelling of myelencephalon and expansion of ventricle, Br-cGMP consistently induced shortening of notochord with bending of the embryo axis. In Br-cGMP-treated embryos, the development of notochord was greatly inhibited. Vacuoles were not fully developed and their cellular arrangement was not normal. Therefore, cGMP appeared to strongly inhibit the morphogenesis of notochord. Also, muscle development was found to be severely inhibited: Muscle tissues were irregularly organized. Just like notochord, these undifferentiated muscle tissues contained yolk granules, probably as an indication of the delay of yolk utilization. Taken together, the shrinkage or bending of Br-cGMP-treated embryos appeared to have been caused by the lack of elongation or extension, and furthermore, delayed differentiation of the notochord (Jacobson, 1978).

When embryos were treated with Br-cGMP from stage 10 to stage 22 at 10 mM of Br-cGMP, a significantly large percentage of embryos arrested development at gastrulation and those survived did not close blastopore completely even at the tailbud stage. We assumed that the failure of the blastopore closure was due to the inhibition in the cellular movement during gastrulation, and this was suggested by SEM observation. It has been reported that during gastrulation and neurulation cells of presumptive notochord at the dorsal marginal zone undergo convergent extension along the midline and intercalate each other to elongate toward the blastopore (Keller *et al.*, 1989). It is possible that Br-cGMP at the higher concentration used caused the alteration of cellular movements, such as convergence and extension which is accompanied by intercalation to form a longer and narrower array of cells (Keller *et al.*, 1991), probably through stimulating the cGMP-dependent protein kinase (Macfarland, 1995), although there remained a possibility that Br-cGMP at the high concentration simply exerted the toxic effect rather than the physiologocal modification of the intracellular signalling processes.

Based on these results, we conclude that two cyclic nucle-

otide analogues used have completely different activities on *Xenopus* embryogenesis. We assume that cGMP has important functions in mesoderm development which results in the formation of notochord and somites, whereas cAMP contributes specifically to the formation of hindbrain.

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