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NEURAL DIFFERENTIATION IN CLEAVAGE-ARRESTED ASCIDIAN BLASTOMERES INDUCED BY A PROTEOLYTIC ENZYME

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SUMMARY

1. As previously reported, ectodermal $a_{4\text{-}2}$ blastomeres isolated from 8-cell embryos of the ascidian, *Halocynthia roretzi* or *aurantium*, and cultured under conditions of cleavage arrest always differentiated into an epidermal phenotype, showing long-lasting Ca^{2+}-dependent action potentials and/or tunic on the cell surface. $a_{4\text{-}2}$ blastomeres contacted by a chordamesodermal blastomere, $A_{4\text{-}1}$, differentiated into a neural phenotype, characterized by fast Na^+-dependent spikes. Differentiation to a similar neural phenotype occurred when isolated $a_{4\text{-}2}$ blastomeres from *H. aurantium* embryos were treated with $> 0{\cdot}003\,\%$ subtilisin for 60 min at the 32-cell stage of the control embryo. Comparisons between induction by cell contact and induction by proteolytic enzymes were made and showed them to be similar in several respects.

2. When the serine protease, subtilisin, was used as the neural inducer, neural competence of $a_{4\text{-}2}$ blastomeres, measured as the percentage frequency of the induction of Na^+ spikes, increased after the 32-cell stage and decreased during the gastrula stage. The time course of the neural competence was the same as that for contact with the $A_{4\text{-}1}$ blastomere.

3. The neural competence of four different ectodermal blastomeres isolated from the 16-cell embryo was also examined using subtilisin as a neural inducer, and by contact with the $A_{4\text{-}1}$ blastomere from the 8-cell embryo. The competence was higher in anterior blastomeres than in posterior blastomeres for both types of induction. This regional difference in neural competence along the antero-posterior axis paralleled that expected from neural cell lineage during normal development, i.e. blastomeres with more cells of neural lineage among their derivatives showed higher competence.

4. *Streptomyces* subtilisin inhibitor, SSI ($0{\cdot}1\,\%$), a specific protease inhibitor for subtilisin-type serine proteases, significantly suppressed ($50\,\%$) neural induction of the ectodermal blastomere, $a_{4\text{-}2}$, by contact wtih the chordamesodermal blastomere, $A_{4\text{-}1}$.

5. Monensin, brefeldin A and bafilomycin A1, all of which affect secretory processes, suppressed the neural inducing ability of the chordamesodermal blastomere, $A_{4\text{-}1}$.

6. These results permit the hypothesis that a protease secreted from the

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chordamesoderm-generating blastomere induces the ectodermal blastomere to differentiate into neural cell type.

INTRODUCTION

A simple system of neural induction has been reconstituted using two cleavage-arrested blastomeres isolated from ascidian-8-cell embryos. The blastomeres of ascidian embryos are known to differentiate even in the cleavage-arrested condition (Whittaker, 1973). The anterior-animal or ectodermal blastomere, $a_{4\text{-}2}$, which gives rise to both epidermal and neural tissues in the normal development (Conklin, 1905; Nishida, 1987), generates sharp Na^+ spikes or long-lasting Ca^{2+} action potentials, following neural or epidermal differentiation respectively, in cleavage-arrested 8-cell embryos of *Halocynthiae* (Hirano, Takahashi & Yamashita, 1984). When the blastomere was isolated at the 8-cell stage and cultured under cleavage-arrested conditions, it was always found to differentiate into an epidermal phenotype when examined electrophysiologically and immunohistochemically, whereas, when it was cultured in contact with an anterior-vegetal chordamesodermal blastomere, $A_{4\text{-}1}$, it differentiated almost exclusively into a neural phenotype (Okado & Takahashi, 1988; 1990*a*, *b*, summarized in Fig. 8 of *b*). This phenomenon is comparable to neural induction, which has been studied for a long time in the early development of amphibian (Spemann, 1938; Gurdon, 1987; Hamburger, 1988) and ascidian embryos (Rose 1939; Reverberi, 1971; Whittaker, 1987; Nishida & Satoh, 1989). Recently, in addition, we found that pronase, a mixture of proteolytic enzymes, has neural inducing activity in the cleavage-arrested ascidian blastomere (Okado & Takahashi, 1990*b*).

There is some question as to whether the inducing activity of proteolytic enzymes is an essential process during induction by contact. To answer this, we have carried out three kinds of experiments. First, we tried to identify some purified proteases which have the same inducing activity as pronase, since pronase is a mixture of multiple proteases and therefore complicates analysis of the inductive processes involved in neural differentiation. We show here that a purified serine protease, subtilisin, has potent inducing activity. Second, since subtilisin in combination with its specific inhibitor, *Streptomyces* subtilisin inhibitor, provided us with a method of applying the inducer at precise times, the time sequence of changes in neural competence induced by subtilisin was compared with that induced by cell contact. Third, the regional difference of neural competence induced by subtilisin in four different ectodermal blastomeres from the 16-cell embryo was determined and compared with that induced by contact with $A_{4\text{-}1}$.

In addition, we have examined whether any protease inhibitors interfere with neural induction by cell contact, and whether substances that affect secretion, such as monensin, suppress induction. We suggest the inducer cell secretes a protease that acts on the competent cell to induce neural differentiation.

METHODS

Preparation

Embryos of *H. aurantium* were exclusively used except in the case of Fig. 2*A*, where *Halocynthia roretzi* embryos were also used. Fertilized eggs of *H. roretzi* were obtained by mixing eggs spawned from one animal with sperm from another. Fertilized eggs of *H. aurantium* were obtained by mixing

eggs dissected from one animal and sperm from another, as described previously (Okado & Takahashi, 1990*a*, *b*). The fertilized eggs were cultured in sea water at 9–10 °C until the 8- or 16-cell stage, and their chorionic membranes were stripped with fine tungsten needles. Each naked embryo was transferred into sea water containing 2 μg/ml cytochalasin B (Schroeder, 1978) to arrest cleavage. Blastomeres of dechorionated embryos at the 8-cell or 16-cell stage were separated mutually with a fine glass needle on a 0·4% agarose-coated surface, as described previously (Okado & Takahashi, 1990*a*). The identification of each blastomere at the 8-cell or 16-cell stage was made according to the description by Conklin (1905), as described previously (Hirano *et al.* 1984). The blastomeres isolated with fine glass needles were transferred into Millipore (0·22 μm)-filtered sea water containing cytochalasin B (2 μg/ml, Aldrich), streptomycin (20 μg/ml) and penicillin (20 μg/ml) in 0·4% agarose-coated microwells and cultured at 9–10 °C. The concentration of cytochalasin B was reduced from 2 to 0·2 μg/ml about 2·5–3·5 h after isolation.

Determination of differentiation type by membrane excitability

When the control intact embryo developed and became a tadpole larva after about 60 h, the differentiated phenotypes of the cleavage-arrested blastomeres were examined electrophysiologically. The external solution consisted of (mM): NaCl, 400; $SrCl_2$, 100; KCl, 10; Pipes-Na (piperazine-*N*-*N*′-bis(2-ethanesulphonic acid)), 5 (pH = 7·0); and 0·1% crystallized bovine serum albumin (Sigma). The blastomere was penetrated with a single microelectrode and the membrane potential held at −80 mV by applying constant current (Fig. 1). A depolarizing current pulse of 120 ms duration was applied to generate action potentials. Blastomeres that developed spikes (Fig. 1*A*; Okado & Takahashi, 1990*b*), composed of Na^+, Ca^{2+} and delayed K^+ channel currents, were regarded as having a neural phenotype. Blastomeres with long-lasting Sr^{2+} action potentials due to Ca^{2+} channels (Fig. 1*B*) or which were difficult to penetrate because of the presence of tunic membrane on the surface, which is an epidermal marker, were regarded as having an epidermal phenotype. The latter type of blastomere was found to express an epidermal antigen contained in tunic, while the former did not (Okado & Takahashi, 1990*a*, *b*). When no regenerative response was observed (Fig. 1*D*), blastomeres were regarded as being non-excitable, and were considered to have an incompletely developed epidermal phenotype or to have been injured during penetration. When evidence of an A-current was observed as a notch in the rising phase of a Ca^{2+} (Sr^{2+})-dependent regenerative response (Fig. 1*C*), the blastomere was regarded as being of the A-current type or type-II reported previously (Okado & Takahashi, 1990*a*). Only when the determination of type was obscure under current-clamp conditions, was voltage clamp applied by inserting another microelectrode as described previously (Okado & Takahashi, 1990*a*).

Treatment with proteases

As potential inducing agents for neural differentiation, various proteases were applied to the isolated and cleavage-arrested blastomere, $a_{4\cdot2}$, at the 32-cell stage of the intact embryo. Pronase was purchased from Kaken Chemical Co. Ltd (Tokyo, Japan) as 'Actinase E'. It was prepared as a 3% solution in sea water, dialysed against sea water at 3 °C and stored at −70 °C. It was diluted with sea water just before use. Trypsins type I (10000 BAEE unit/mg, chymotrypsin < 4 BAEE), type II (1000–2000 BAEE unit/mg, chymotrypsin 1000–2000 ATEE unit/mg), type III (10000–13000 BAEE unit/mg, chymotrypsin < 4 BAEE), type XI (diphenylcarbamyl chloride treated, 7500–9000 BAEE unit/mg chymotrypsin < 0·1 BAEE), type XIII (L-1-tosylamide-2-phenylethyl chloromethyl ketone treated, 10000–13000 BAEE unit/mg, chymotrypsin < 0·1 BAEE), chymotrypsins type II (40–60 unit/mg), type VII (*N*α-*p*-tosyl-L-lysine chloromethyl ketone treated, 40–60 unit/mg), collagenase type I and ficin (2·25% solution, 2·0 M NaCl and 0·03 M cistein) were all purchased from Sigma Chemical Co. (St Louis, MO, USA). Subtilisin and proteinase K were obtained from Boehringer-Mannheim Biochemicals. Ficin was dialysed against sea water at 4 °C and diluted to 2·0 or 0·5% before use. The others were dissolved in sea water before use.

Treatment with protease inhibitors

Streptomyces subtilisin inhibitor (SSI), was a gift from Professor Emeritus Ishii (Faculty of Pharmaceutical Sciences, Hokkaido University) and Professor Mitsui (Center for Physical Sciences, Nagaoka Institute of Technological Sciences). It was directly dissolved in sea water before use or a 0·2% stock solution in sea water, which was stored at −20 °C, was diluted before use. Phenylmethyl-sulphonyl fluoride (PMSF), α1-antitrypsin, α2-macroglobulin and pepstatin, were purchased from Boehringer-Mannheim Biochemicals, antipain, chymostatin and pepstatin, E-64 from

Peptide Institute Inc. (Osaka, Japan) and aprotinin and diisopropyl fluorophosphate (DFP) from Sigma. Pepstatin was dissolved at 2% in methanol and stored at −20 °C. E-64 was dissolved at 2% in 50% ethanol and stored at −20 °C. DFP was dissolved at 1 M in absolute isopropanol and stored at 4 °C. PMSF was dissolved at 1 M in absolute dimethylsulphoxide (DMSO). Chymostatin was dissolved at 0·1 M in absolute DMSO and stored at −20 °C. Other inhibitors were dissolved directly in sea water.

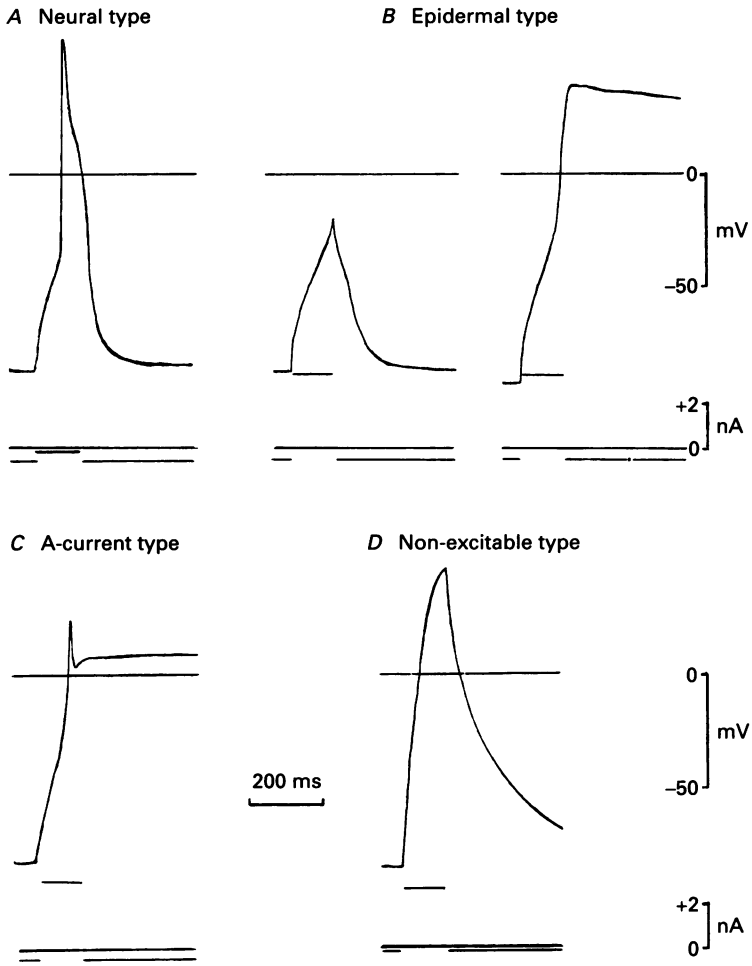

Fig. 1. Examples of the electrical responses observed in differentiated blastomeres which were isolated from 16-cell embryos, cleavage-arrested and cultured. *A*, neural type differentiation in an $a_{5\text{-}4}$ blastomere induced by contact with $A_{4\text{-}1}$. *B*, epidermal type differentiation observed in a $b_{5\text{-}4}$ blastomere by contact with $A_{4\text{-}1}$. *C*, A-current type differentiation in a $b_{5\text{-}4}$ blastomere by contact with $A_{4\text{-}1}$. *D*, non-excitable type observed in a $b_{5\text{-}4}$ blastomere induced by subtilisin at 0·005% for 60 min. All records made under current clamp. Upper and lower traces are potential and current records respectively.

PMSF- and DFP-treated pronase solutions were made as follows. Stock solution of PMSF or DFP was added to 0·3% pronase in sea water to give a final concentration of 1–10 mM. The mixture was incubated at 25 °C for 1 h and dialysed against sea water at 4 °C.

Treatment with monensin, brefeldin A or bafilomycin A1

Monensin (sodium salt, Sigma) was dissolved at 20 mM in ethanol and stored at −20 °C. For use it was diluted in sea water to 1·0 or 0·1 μM. Brefeldin A (Sandoz Company, Basel, Switzerland) was stored as a 10 mg/ml stock solution at −20 °C in absolute methanol. Bafilomycin A1 was kindly given by Dr Y. Moriyama (Osaka University) as a 1 mM solution in absolute ethanol.

RESULTS

Neural inducing ability of proteases

As reported previously (Okado & Takahashi, 1990*b*), the cleavage-arrested blastomere, $a_{4\text{-}2}$, isolated from ascidian embryos was induced to differentiate into a neural-type cell by treatment with pronase. The dose–response curve in Fig. 2*A* shows that the $a_{4\text{-}2}$ blastomere from *H. aurantium* was induced more readily than that from *H. roretzi*. For example, 0·02% pronase treatment for 15 min at the 32-cell stage induced 90% of the $a_{4\text{-}2}$ blastomeres of *H. aurantium*, but only 10% of *H. roretzi*. Subsequent experiments were performed using the more sensitive blastomeres, that is, those of *H. aurantium*.

Because pronase is a mixture of proteolytic enzymes produced by *Streptomyces griseus*, we tried to separate the individual proteases by chromatography on a CM-cellulose column according to Narahashi (1970) and to identify neural inducing proteases. When the neural inducing activity of each fraction was examined, it was found that neural inducing ability of proteases did not always reflect common protease activity measured with the casein–275 nm method (Narahashi, 1970). There were two vague peaks of inducing activity, corresponding to the neutral protease and alkali protease peaks reported previously by Narahashi (1970).

Since it is known that pronase consists for the most part of serine proteases, we examined whether the inducing ability of pronase was inhibited by treatment of serine protease inhibitors, such as DFP (not shown) or PMSF (Fig. 2*B*). We found that these inhibitors could reduce the inducing activity of pronase, but did not block it completely. For example, 10 mM PMSF inhibited the inducing activity of 0·2% pronase by 50%, although the percentage inhibition was greater at lower concentrations of pronase (Fig. 2*B*). This result suggested that not only serine proteases but also non-serine proteases in pronase had inducing ability.

Because it is difficult to interpret the effect of a mixture of proteases, as found in pronase, we examined the inducing activity of various purified proteases (Fig. 2*C*). Since we applied enzymes in the concentration range from 0·005 to 2% and for durations of 15 or 60 min, the dose of enzymes was represented by concentration (%) multiplied by duration (min) as a unit in the graph (Fig. 2*C*). One serine protease, subtilisin (shown as filled triangles), had similar inducing ability to pronase (shown as a dashed line). The other serine proteases, trypsin and chymotrypsin, had less activity. The trypsins without contamination of chymotrypsin (types XI and XIII) had less inducing activity than crude trypsin (type II), although the former had more trypsin activity (BAEE activity) than the latter, indicating that the specific proteolytic activity of trypsin has poor neural inducing activity. The crude chymotrypsin (type II) and that without trypsin (type VII) also had low activity. Even a mixture of purified trypsin (type XIII) and chymotrypsin (type VII) showed

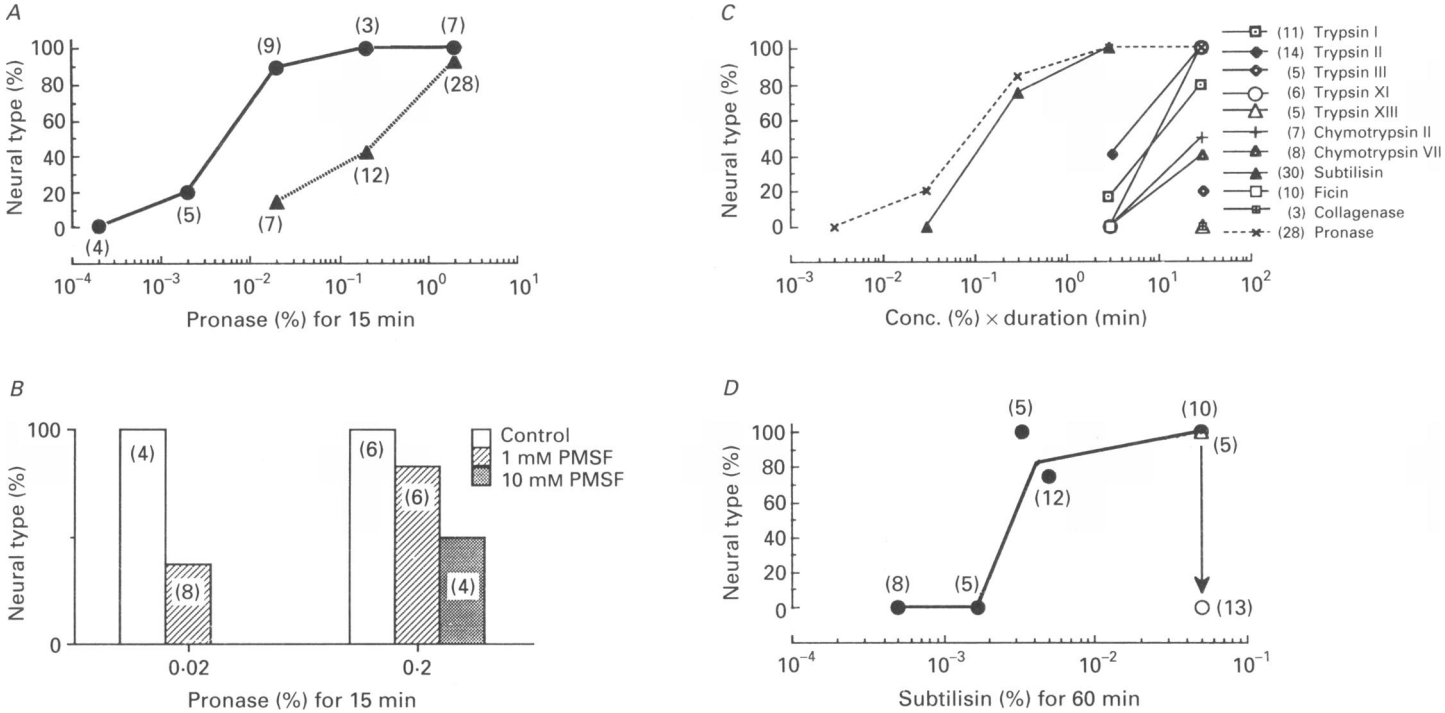

Fig. 2. *A*, dose–reponse curves of neural inducing activity of pronase (Actinase E, Kaken). Cleavage-arrested blastomeres, $a_{4\cdot2}$, isolated from 8-cell embryos of *Halocynthia roretzi* (▲) and *Halocynthia aurantum* (●) were treated with pronase at various concentrations for 15 min at the equivalent of the 32-cell stage of the control embryo, that is, 2·5 h after the 8-cell stage at 10 °C. *B*, effect of serine protease inhibitor (PMSF) upon the neural inducing activity of pronase. PMSF-treated pronase solution was prepared as described in the Methods. *C*, neural inducing activity of various proteases. The data for the dashed line (pronase) is derived from *A* (●). The abscissa represents dose of protease calculated as percentage concentration times duration of application (min). Figures in parentheses on right side are sample numbers for experiments of respective enzymes. *D*, dose–response curve of neural inducing activity of subtilisin (●). ○, 0·05% SSI blocked neural induction produced by 0·05% subtilisin. △, 0·005% SSI had no effect on induction. Cleavage-arrested blastomeres, $a_{4\cdot2}$, isolated from 8-cell embryos of *Halocynthia aurantium* were treated with subtilisin at various concentrations for 60 min at the 32-cell stage; data are combined from embryos of four batches. In *A*, *B* and *D* the figures in parentheses are the numbers of samples used to calculate the respective percentage frequencies for plotting or drawing columns.

much less effect, indicating that neither trypsin nor chymotrypsin are candidates for the potent inducing protease. The thiol protease, ficin, could induce neural differentiation of blastomeres only when used at a higher concentration (0·5%) for 60 min, which was 100-fold higher than the amount of purified subtilisin (0·005% for 60 min) required for neural induction. The fact that proteases other than serine proteases had inducing ability may be consistent with the observed partial inhibition of neural inducing activity of pronase by the serine protease inhibitors, DFP, or PMSF (Fig. 2*B*). The metal protease, collagenase, had no inducing activity even at 0·5% for 60 min. Another dose–response curve of subtilisin was separately obtained as shown in Fig. 2*D*; in this experiment subtilisin was applied for 60 min at the 32-cell stage of the control embryo. The threshold contration for Na^+ spike induction was about 0·003% or 1·15 μM. The effect of subtilisin (0·05% or 19 μM) was blocked completely by the same concentration of *Streptomyces* subtilisin inhibitor (SSI, 0·05% or 44 μM as monomer), while SSI at the one-tenth of this concentration (0·005% or 4·4 μM) could not suppress the effect at all (Fig. 2*D*, open circle, open triangle and arrow; Tonomura, Sato, Miwa & Komiyama, 1985). The stoichiometry of the inhibition is known to be two molecules of subtilisin bound to the dimeric SSI (Tonomura *et al.* 1985). Therefore the neural inducing activity is due to the proteolytic activity of subtilisin itself. SSI could not inhibit the inducing ability of pronase at the same concentration. Hereafter, all experiments were done with subtilisin as the inducing protease.

Time sequence of changes in neural competence of $a_{4\text{-}2}$

In a previous study (Okado & Takahashi, 1990*b*), we showed that the receptiveness of the $a_{4\text{-}2}$ to pronase as well as competence to induction by contact was lost during the gastrula stage. We found, however, that sensitivity to pronase (2%, 15 min) existed as early as the 8-cell stage, while competence to induction by contact appeared only after the 32-cell stage. Thus, this discrepancy must be explained before we can determine the mechanisms underlying the two modes of induction. In the present study, by using various concentrations of subtilisin and SSI (Tonomura *et al.* 1985), the precise time sequence of changes in neural competence was examined. In the case of subtilisin at 0·05% for 60 min, the blastomere $a_{4\text{-}2}$ was induced at the 8-cell stage to differentiate into a neural-type cell (filled circles) (Fig. 3*A*). This result with subtilisin (0·05%, 60 min) is consistent with the previous result with pronase (2%, 15 min). In Fig. 3 the dashed lines represent neural competence of $a_{4\text{-}2}$ with induction by contact, which was reported previously (Okado & Takahashi, 1990*b*). It was possible that the discrepancy between the time courses of neural competence by protease and by cell contact might result from an overdose of subtilisin which could cause a saturating effect even in a slightly competent phase. To test this possibility, the concentration of subtilisin was reduced to 0·005%. In this situation, at the 8-cell stage the $a_{4\text{-}2}$ did not differentiate neurally, but epidermally (Fig. 3*B*). Therefore, the discrepancy comes at least partially from the overdose of subtilisin. Another possibility is that some protease remained after washout with sea water and that this remaining protease showed residual activity subsequent to the time of treatment. In order to test this possibility, the blastomere was washed, after subtilisin treatment, with sea water containing SSI for 60 min (Fig. 3*C*). This

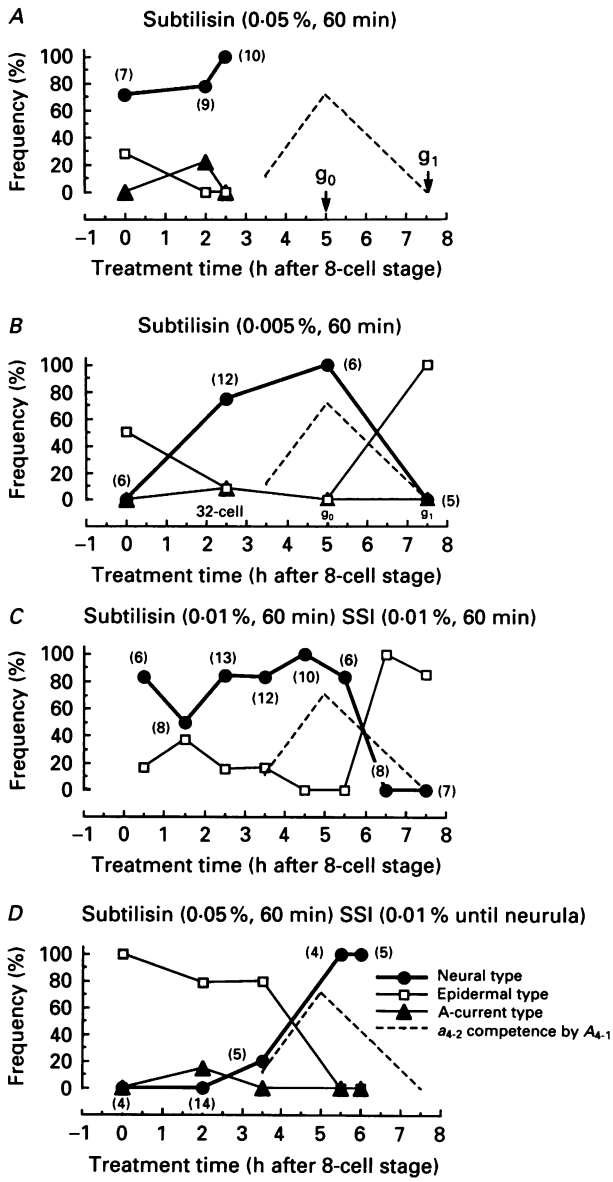

Fig. 3. Time sequence of changes in neural competence of $a_{4\cdot2}$ blastomeres isolated from 8-cell embryos of *H. aurantium* induced by treatment with subtilisin. The dashed lines represent the time sequence of neural competence of $a_{4\cdot2}$ induced by contact with $A_{4\cdot1}$, which was reported previously (g_0 and g_1 are early and middle substages within the gastrula stage; Okado & Takahashi, 1990*b*). ●, neural development. ▲, A-current type (previously called type-II; Okado & Takahashi, 1990*b*) development. □, epidermal type development. Temperature was 9–10 °C in *A*, 10 °C in *B* and *C*, and 9 °C in *D*. *A*, the cleavage-arrested and isolated $a_{4\cdot2}$ cells were treated with 0·05 % subtilisin for 60 min at various stages. *B*, the cleavage-arrested and isolated $a_{4\cdot2}$ cells were treated with 0·005 % subtilisin for 60 min at various stages. *C*, the cleavage-arrested and isolated $a_{4\cdot2}$ cells were treated with 0·01 % subtilisin for 60 min at various stages, and then they were immersed in sea water containing 0·01 % SSI for 60 min to wash out the remaining subtilisin. *D*, the cleavage-arrested and isolated $a_{4\cdot2}$ cells were treated with 0·05 % subtilisin for 60 min at

procedure had little effect. In order to suppress the remaining protease activity completely after washing, the blastomere was kept in sea water containing SSI until about the neurula stage (Fig. 3*D*). This experiment showed that the rising phase of neural competence induced by subtilisin agreed with that induced by contact with $A_{4\text{-}1}$. These results indicate that treatment with subtilisin mimicked contact with $A_{4\text{-}1}$ well with regard to the time sequence of neural competence, both in the rising and fading phases. The applied subtilisin was difficult to eliminate by washing in sea water alone or in sea water containing SSI for a limited time, such as 60 min.

Regional specificity of ectodermal blastomeres

In addition to the anterior ectodermal blastomere, $a_{4\text{-}2}$, the posterior $b_{4\text{-}2}$, isolated from the 8-cell embryo, was also neurally induced by contact with the anterior-vegetal blastomere $A_{4\text{-}1}$, though less frequently, as described previously (Okado & Takahashi, 1990*b*). Thus, there was clear regional specificity of ectodermal blastomeres with respect to neural competence. It is important to determine whether this regional difference in competence was also observed by induction with protease. For this purpose, four ectodermal blastomeres isolated from 16-cell embryos, anterior $a_{5\text{-}3}$, $a_{5\text{-}4}$, posterior $b_{5\text{-}3}$ and $b_{5\text{-}4}$, located in order on the antero-posterior axis, were studied to examine more precisely the regional differences in neural competence along this axis and the results obtained from two modes of induction were compared. The isolated blastomeres from 16-cell embryos were cultured and placed in contact with the same inducing blastomere $A_{4\text{-}1}$ (isolated from the 8-cell stage) at the 64-cell stage of the control embryo. Two main differences were observed among the blastomeres. First, $a_{5\text{-}3}$ and $a_{5\text{-}4}$ were induced to differentiate neurally with high frequency ($> 80\%$), while $b_{5\text{-}3}$ and $b_{5\text{-}4}$ were induced with only low frequency ($< 40\%$) (Fig. 4). Second, A-current-type spikes developed characteristically in $b_{5\text{-}3}$ and $b_{5\text{-}4}$. These results indicated that the neural competence of ectoderm is already specified at the 16-cell stage. In fact, a previous study with pronase (Okado & Takahashi, 1990*b*) indicated that as early as the 8-cell stage neural competence of the isolated $a_{4\text{-}2}$, which is the parent cell of $a_{5\text{-}3}$ and $a_{5\text{-}4}$, is higher than that of the isolated $b_{4\text{-}2}$, which is the parent cell of $b_{5\text{-}3}$ and $b_{5\text{-}4}$. In $b_{4\text{-}2}$ the probability of Na^+ spike induction was relatively low, while the A-current-type spikes were observed relatively frequently (Okado & Takahashi, 1990*b*). Since A-current-type spikes were generally observed in $A_{4\text{-}1}$ blastomeres from the 8-cell embryo (Okado & Takahashi, 1990*a*) and $b_{4\text{-}2}$ and $A_{4\text{-}1}$ both include presumptive spinal cord, the caudal portion of neural tissue of the tadpole larva (Nishida, 1987), it is inferred that A-current type spikes might be a cell membrane marker of spinal cord and/or caudal neural tissue.

various stages, and then they were immersed in sea water containing 0·1% SSI until the neurula stage of intact embryos to inhibit activity of subtilisin completely. Cytochalasin B was reduced from 2 to 0·2 μg/ml at the neurula stage. Abscissa, hours after the isolation of blastomeres at 8-cell stage. Thus, 2·5 h corresponds to 32-cell stage and plus 5 h corresponds to the transition from the 64-cell to the 110-cell stage of the control embryo. Data from embryos of six batches were combined. Ordinate, percentage frequencies of respective differentiation types indicated by the induction of Na^+ spikes, Ca^{2+} (Sr^{2+})-dependent epidermal type action potential or A-current type spikes. The figures in parentheses are sample numbers used for calculation of percentage frequencies at respective developmental times.

Actually the A-current type spikes were also observed in $a_{4\text{-}2}$, although exceptionally, when the enzyme was applied at earlier stages such as the 8-cell or 16-cell stage, as shown in Fig. 3*A*, *B* and *D* (filled triangles; Okado & Takahashi, 1990*a*).

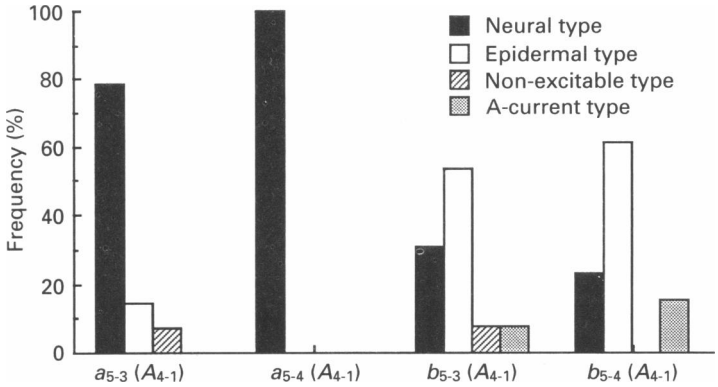

Fig. 4. Neural competence of isolated and cleavage-arrested ectodermal blastomeres of 16-cell embryos of *H. aurantium* induced by contact with the $A_{4\text{-}1}$ blastomere from the 8-cell embryo. The ectodermal blastomeres, $a_{5\text{-}3}$ (14 cells), $a_{5\text{-}4}$ (10), $b_{5\text{-}3}$ (13) and $b_{5\text{-}4}$ (13) isolated from 16-cell embryos were placed in contact with $A_{4\text{-}1}$ blastomeres isolated from 8-cell embryos at about the 64-cell stage of the control embryo, i.e. 2 h after the 16-cell stage. Data from embryos of three batches were combined.

Since we did not know the real inducing activity of $A_{4\text{-}1}$ in cell contact, it was necessary to analyse competence with subtilisin as an inducer at various levels of enzyme concentration (Fig. 5). The results were summarized as follows. First, regional differences in neural competence were revealed at subtilisin concentrations greater than 0·003 %, which is the threshold concentration for Na^+ spike development for all of the blastomeres except the $b_{5\text{-}4}$. Second, the order of neural competence of the four blastomeres studied was $a_{5\text{-}3} > a_{5\text{-}4} > b_{5\text{-}3} > = b_{5\text{-}4}$. In blastomeres $b_{5\text{-}3}$ and $b_{5\text{-}4}$ the frequency of neural development was saturated at a level of less than 50 % even when subtilisin was applied at concentration greater than 0·01 %. Third, in $b_{5\text{-}3}$ and $b_{5\text{-}4}$ A-current-type spikes developed following treatment with subtilisin (Fig. 5, $b_{5\text{-}3}$, $b_{5\text{-}4}$) as in the case of induction by contact with $A_{4\text{-}1}$, but such spikes did not appear in $a_{5\text{-}3}$ and $a_{5\text{-}4}$. In addition to the epidermal type definitely identified by the presence of $Ca^{2+}(Sr^{2+})$ action potentials and/or tunic, the non-excitable type was frequently observed in the $b_{5\text{-}3}$ and $b_{5\text{-}4}$ following treatment with subtilisin at more than 0·003 %, but we considered that this type may represent an incomplete form of epidermal development. A type of differentiation with only delayed outward current was observed in the case of $b_{5\text{-}3}$ and $b_{5\text{-}4}$ treated with 0·2 % subtilisin. We considered that a part of this type is an incomplete form of A-current-type spikes because the outward current was frequently associated with fast inactivation. Some injurious effects of the high dose treatment with subtilisin may be one of the reasons why incomplete forms appeared in significant numbers. However, it should be noted that Na^+ spikes induced in $a_{5\text{-}3}$ and $a_{5\text{-}4}$ with 0·2 % subtilisin were well developed, comprising large Na^+ and delayed K^+ currents as reported previously (Okado & Takahashi, 1990*b*).

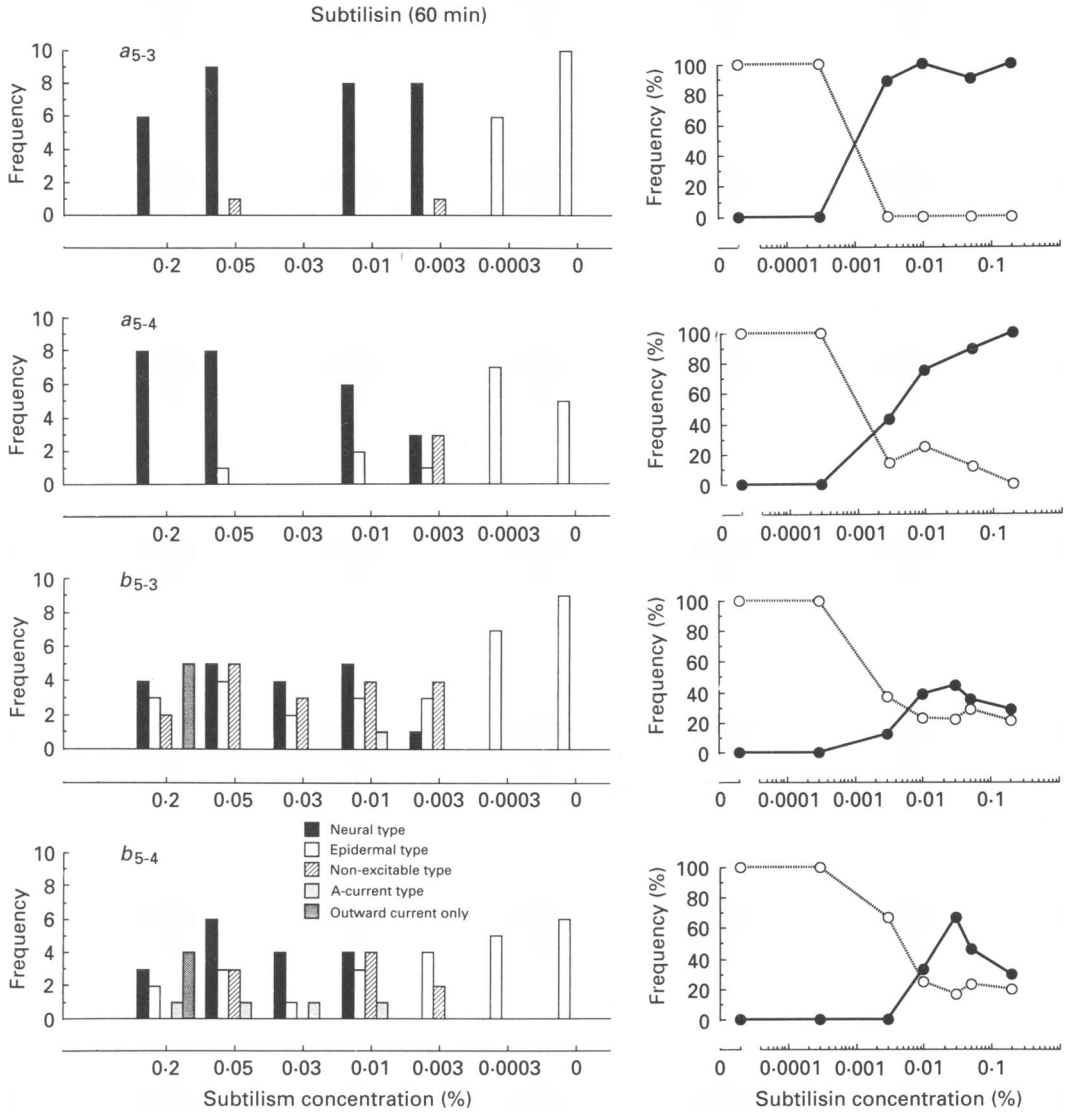

Fig. 5. Neural competence of cleavage-arrested ectodermal blastomeres isolated from 16-cell embryos of *H. aurantium* and induced by subtilisin. The ectodermal blastomeres, $a_{5\text{-}3}$ (49 cells), $a_{5\text{-}4}$ (44), $b_{5\text{-}3}$ (74) and $b_{5\text{-}4}$ (58) isolated from 16-cell embryos were cleavage-arrested and treated with subtilisin at various concentrations from 0 to 0·2% for 60 min, at about the 64-cell stage of control embryos, i.e. 2 h after the 16-cell stage. Data from embryos of seven batches were combined. On the left side, are frequency histograms of the various differentiated phenotypes induced at various subtilisin concentrations. In the graphs on the right side, percentage frequencies of neural-type (●) and epidermal-type development (○) shown in the histograms were plotted against the dose of subtilisin on a semilogarithmic scale.

The percentage frequencies of defined neural- and epidermal-type development shown in Figs 4 and 5 are plotted according to the location of the blastomeres along the antero-posterior axis in Fig. 6*A* and *B* both for induction by subtilisin and by contact. Here, the percentage frequencies were considered to represent potencies of

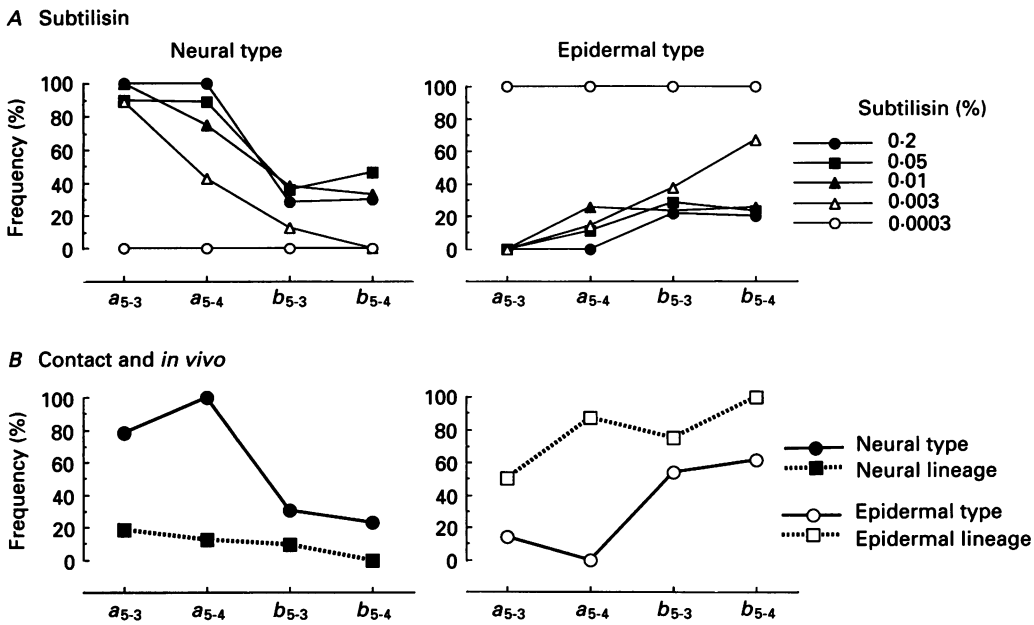

Fig. 6. Regional differences in neural competence in ectodermal blastomeres isolated from 16-cell embryos. Percentage frequencies of neural and epidermal phenotypes are plotted according to the order of blastomeres along anterior–posterior axis. *A*, percentage frequency of neural and epidermal development induced by subtilisin was plotted against the location of blastomeres, calculated from the data in Fig. 5. *B*, percentage frequency of neural- and epidermal-type development induced by contact with $A_{4\text{-}1}$ was plotted similarly (calculated from the data shown in Fig. 4). The dotted lines with filled and open squares represent percentage ratios of cell numbers of presumptive neural and epidermal lineages respectively at the 110-cell stage derived from each ectodermal blastomere of the control 16-cell embryo for comparison with the regional differences in neural competence. The ratios were calculated tentatively, based on the differentiation type of derivatives from respective blastomeres according to the data reported by Nishida (1987). Brain, brainstem and pigment cells were regarded as neural tissues in the calculation. It is known that at the 110-cell stage most of the blastomeres are restricted to express a particular tissue (Nishida, 1987). However, where two or three kinds of tissue are expressed after development in the derivatives from a blastomere at the 110-cell stage, the number for the respective lineage was counted as a half or a third.

neural or epidermal competence of the respective blastomeres. Dotted lines in Fig. 6*B* illustrate the inferred ratio of presumptive neural and epidermal lineages at the 110-cell stage, which are derived from respective blastomeres of the 16-cell embryos according to cell lineage studies in *H. roretzi* by Nishida (1987) obtained with HRP (horseradish peroxidase) as a tracer. The number of cells neural lineage derived from ectodermal blastomeres decreases in the order $a_{5\text{-}3}$, $a_{5\text{-}4}$, $b_{5\text{-}3}$, $b_{5\text{-}4}$, assuming that brain,

brainstem and pigment cell are regarded as neural tissues (Nishida, 1987). The results shown in Fig. 6 led us to the following two conclusions. First, the regional difference of neural competence by subtilisin was similar to that by contact with $A_{4\text{-}1}$, and, thus, the effect of subtilisin closely mimicked that of contact with $A_{4\text{-}1}$, which

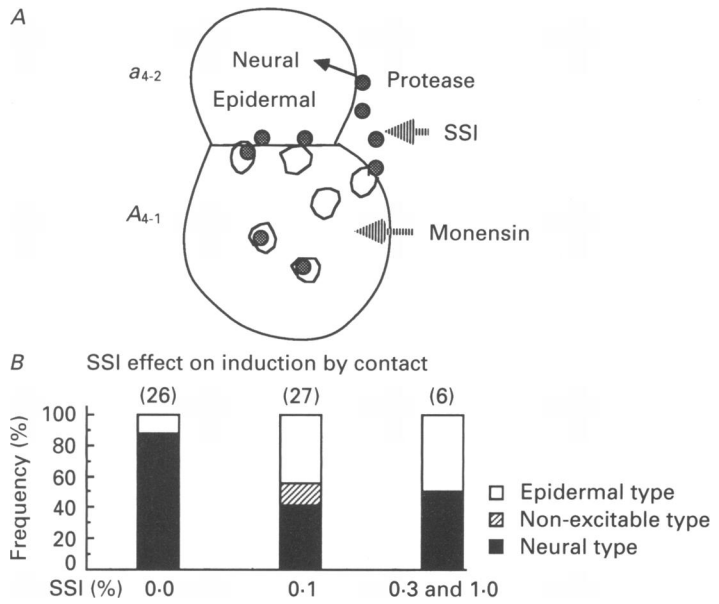

Fig. 7. *A*, hypothesis for neural induction by secreted proteases and schematic illustration for effects of externally applied SSI or monensin. *B*, effect of SSI on neural differentiation of $a_{4\text{-}2}$ blastomeres induced by contact with $A_{4\text{-}1}$. The figures in parentheses are sample numbers for respective concentrations of SSI. The samples for 0·3 and 1·0% were combined because there were only three samples for each concentration.

was regarded as the natural inducing stimulus *in vivo*. Second, the regional specificity for neural differentiation in normal development, as indicated by the difference in neural lineage populations derived from respective blastomeres (Fig. 6*B*), was largely paralleled by the regional difference of neural competence examined by either the foreign inducer, subtilisin, (Fig. 6*A*) or the natural inducing stimulus, contact with $A_{4\text{-}1}$ (Fig. 6*B*). This result suggested that neural competence was predetermined ahead of induction, probably by cytoplasmic segregation among blastomeres as early as at the 16-cell stage; that is likely also to be the case for neural development *in vivo*. However, Nishida (1987) showed in his cell lineage studies that $b_{5\text{-}4}$ generates no neural tissue *in vivo*, while the present study clearly showed that $b_{5\text{-}4}$ was as neurally competent as $b_{5\text{-}3}$. This point will be discussed later.

Effect of various protease inhibitors

The above results led us to hypothesize that the inducing substance is a protease that it is secreted from $A_{4\text{-}1}$, and that it induces the $a_{4\text{-}2}$ cell to differentiate into neural cell type (Fig. 7*A*). To examine whether proteases really are involved in the

neural induction of $a_{4\cdot2}$ by $A_{4\cdot1}$, various protease inhibitors were added to sea water in microwells, each of which contained an $a_{4\cdot2}$ and an $A_{4\cdot1}$ blastomere. The results obtained are illustrated in Table 1. They indicate that one of the specific serine protease inhibitors, SSI (Tonomura *et al.* 1985), and a wide-spectrum inhbiitor, α2-macroglobulin (Harpel, 1976), significantly inhibited neural induction. At 0·1 % SSI

TABLE 1. Effect of various protease inhibitors on neural induction by contact

Protease inhibitor	Mol. wt.	Concentration (% (mM))	Neural type (frequency (%))	Epidermal type (frequency (%))
Control			23/26 (88)	3/26 (12)
Serine protease inhibitors				
Aprotinin	6511	0·1 (0·15)	2/2 (100)	0/2 (0)
α1-antitrypsin	54000	0·4 (0·07)	3/4 (75)	0/ 4 (0)*
SSI	23000 as dimer	1·0 (0·440)	1/3 (33)	2/3 (67)
		0·3 (0·132)	2/3 (67)	1/3 (33)
		0·1 (0·044)	11/27 (41)	12/27 (44)*
DFP		(10)	5/5 (100)	0/5 (0)
		(1·0)	3/3 (100)	0/3 (0)
Chymostatin	*ca.* 600	0·03 (0·5)	5/5 (100)	0/5 (0)
		0·006 (0·1)	1/2 (50)	0/2 (0)*
Serine–thiol protease inhibitors				
Antipain	604	0·02 (0·3)	4/4 (100)	0/4 (0)
Leupeptin	460	0·5 (10)	2/2 (100)	0/2 (0)
		0·05 (1·0)	8/10 (80)	0/10 (0)*
Thiol protease inhibitor				
E-64	357	0·02 (0·5)	2/2 (100)	0/2 (0)
Aspartyl protease inhibitor				
Pepstatin	685	0·02 (0·3)	4/4 (100)	0/4 (0)
Wide-spectrum inhibitor				
α2-macroglobulin	*ca.* 800000 as tetramer	1·0 (0·0125)	1/7 (14)	2/7 (29)*
		0·3 (0·00375)	2/3 (67)	1/3 (33)
		0·1 (0·00125)	2/4 (50)	2/4 (50)

The $a_{4\cdot2}$ and $A_{4\cdot1}$ blastomeres were isolated from 8-cell embryos of *H. aurantium*, cleavage-arrested and cultured separately with 2 μg/cytochalasin B. Two hours after isolation the $A_{4\cdot1}$ cells were immersed in sea water containing the protease inhibitor. Two and half hours after isolation $a_{4\cdot2}$ cells were put in contact with the treated $A_{4\cdot1}$ cell, kept in sea water containing various protease inhibitors and cultured. At the neurula stage of the control embryo the $a_{4\cdot2}$ cell in contact with the $A_{4\cdot1}$ cell was transferred into protease inhibitor-free sea water. The concentration of cytochalasin B was reduced from 2 to 0·2 μg/ml at the time of contact, i.e. 2·5 h after isolation. Reduction of cytochalasin B facilitates the contact (Okado & Takahashi, 1990*b*). Asterisks in the 'epidermal type' column indicate that blastomeres other than neural or epidermal type in respective experiments were of the non-excitable type which was likely to be an incomplete form of the epidermal phenotype.

inhibited neural induction in 50 % of cases, but even higher concentrations of SSI, 0·3 or 1 %, did not increase this level of inhibition (Fig. 7*B*; Table 1). α2-Macroglobulin at high concentration (1 %) inhibited neural development significantly, but definite epidermal type differentiation was relatively rare and a high percentage (57 %) of the blastomeres became non-excitable (Table 1). The other serine protease inhibitors,

thiol protease inhibitors and aspartyl protease inhibitors had no effect (Table 1). Incompleteness of inhibition with SSI could be explained in the following two ways: first, other proteases which were insensitive to the above inhibitors also contribute to neural induction; second, SSI may not have easy access to the intercellular space between the contacting membranes of $a_{4\text{-}2}$ and $A_{4\text{-}1}$ during the induction phase (Fig. 7*A*).

Effect of substances affecting secretion; monensin, brefeldin A and bafilomycin A1

Monensin, a carboxylic Na^+–H^+ ionophore isolated from *Streptomyces cinnamonensis*, is known to interfere with the intracellular pathway through the Golgi apparatus that is responsible for processing secretory proteins or assembling integral proteins into the membrane. It is also known that monensin causes a rapid swelling of the Golgi apparatus (Tartakoff, 1983). To test whether a secretory process is involved in neural induction, the effects of monensin on neural induction were examined. The $a_{4\text{-}2}$ and $A_{4\text{-}1}$ blastomeres isolated at the 8-cell stage were cultured separately and placed in contact with each other for 6 h from the 32-cell stage to the late gastrula stage, during which 0·1 μM monensin was applied (Fig. 8*A*). In all six cases examined monensin inhibited neural induction in $a_{4\text{-}2}$ blastomeres and directed differentiation towards an epidermal phenotype. In five control experiments $a_{4\text{-}2}$ blastomeres from the same batch were induced to differentiate neurally in all cases. To exclude the possibility that monensin interferes with neural development itself after induction, we showed that $a_{4\text{-}2}$ blastomeres treated with subtilisin (0·05% for 60 min) developed a neural phenotype even when kept in sea water containing 0·1 μM monensin for 6 h after the treatment ($n = 6$, Fig. 8*B*). To determine whether monensin affected $a_{4\text{-}2}$ or $A_{4\text{-}1}$, either blastomere was kept in sea water containing monensin (1 μM for 60 min) and washed just before contact (Fig. 8*C* and *D*). Only when the inducing blastomere, $A_{4\text{-}1}$, was treated with monensin was neural induction significantly suppressed, suggesting that some secretory process in the $A_{4\text{-}1}$ was necessary for neural induction. Although monensin affected inductive activity of $A_{4\text{-}1}$ it did not significantly alter the frequency of occurrence of the various phenotypes of $A_{4\text{-}1}$ after differentiation, the blastomere showing A-current type spikes or epidermal type action potentials or becoming non-excitable, similar to those described previously (Okado & Takahashi, 1990*a*, *b*).

We also examined the effects of other secretion blocking agents: brefeldin A (Misumi, Misumi, Miki, Takatsuki, Tamura & Ikehara, 1986), which provokes dynamic resorption of most of the membranes of the Golgi apparatus into the endoplasmic reticulum and results in blocking transport of secretory proteins to post-Golgi compartments (Misumi *et al.* 1986; Lippincott-Schwartz, Yuan, Bonifacino & Klausner, 1989), and bafilomycin A1 (Umata, Moriyama, Futai & Medkada, 1990), which is a specific inhibitor of vacuolar-type H^+-ATPases and possibly blocks transport processes in the Golgi apparatus. Both brefeldin A (10 μg/ml) and bafilomycin A1 (1 μM) inhibited the neural inducing ability of $A_{4\text{-}1}$. When $A_{4\text{-}1}$ blastomeres were pre-treated with brefeldin A for 60 min and washed before being placed in contact with $a_{4\text{-}2}$ blastomeres, no $a_{4\text{-}2}$ blastomeres differentiated neurally; they all developed an epidermal phenotype ($n = 5$). Treatment of $A_{4\text{-}1}$ blastomeres with a lower concentration (1 μg/ml) of brefeldin A showed a much less

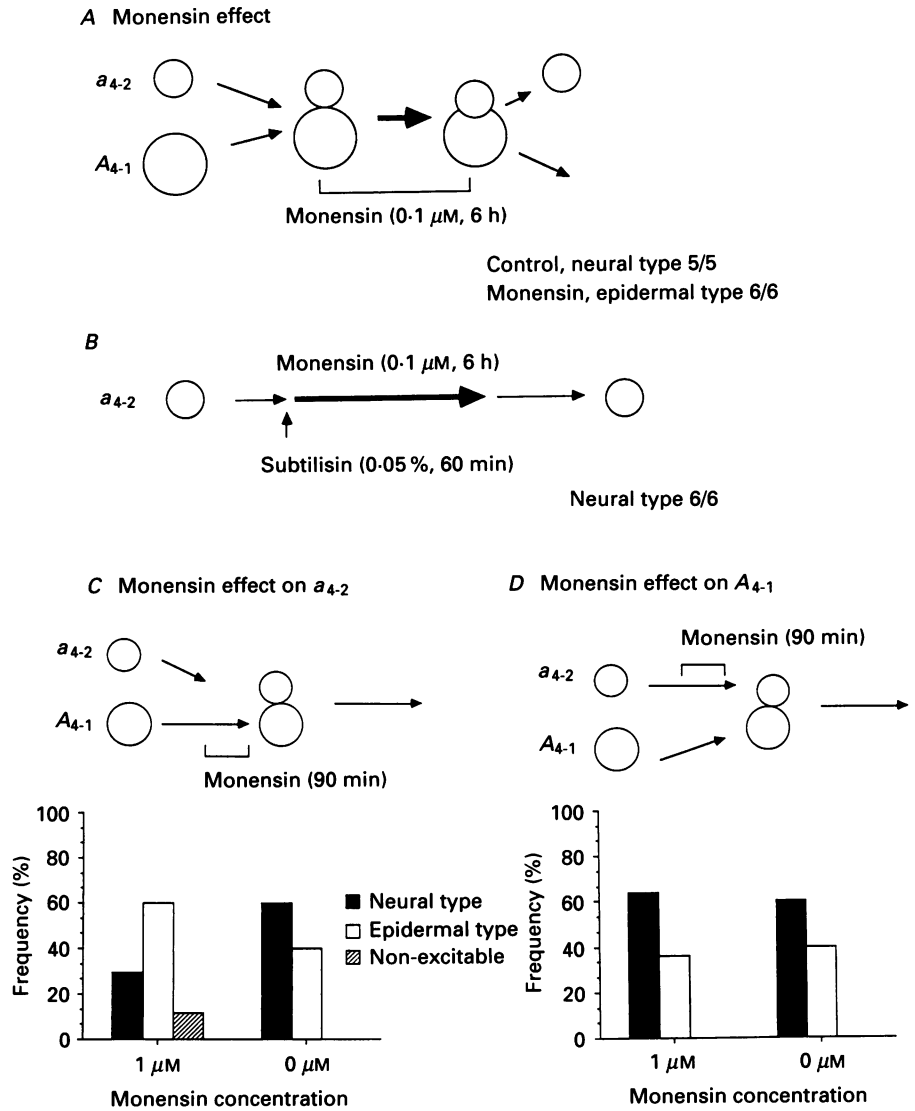

Fig. 8. Effect of monensin on $a_{4\text{-}2}$ and $A_{4\text{-}1}$ of *H. aurantium* embryo during neural induction. *A*, the cleavage-arrested and isolated $a_{4\text{-}2}$ blastomere was contacted by the $A_{4\text{-}1}$ blastomere at the 32-cell stage of control embryos in sea water containing 0·1 μM monensin for 6 h. They were then washed, separated and cultured without monensin. All of six cases failed to differentiate neurally, but differentiated epidermally. Control experiments without monensin were similarly performed with blastomeres from the same batch of embryos. All of five cases differentiated neurally. *B*, the $a_{4\text{-}2}$ was treated with 0·05% subtilisin for 60 min at the 32-cell stage of control embryos, and transferred into sea water containing 0·1 μM monensin for 6 h. In all of six cases $a_{4\text{-}2}$ differentiated neurally. *C*, the $A_{4\text{-}1}$ was immersed in sea water containing 1 μM monensin for 90 min about 1 h after the 8-cell stage before contacting with $a_{4\text{-}2}$. The lower part shows a histogram of percentage frequencies of differentiation type; $n = 17$ for the experiment and $n = 15$ for the control (0 μM). *D*, the $a_{4\text{-}2}$ was treated before contact. The procedure was similar to that in *C*; $n = 11$ for the experiment and $n = 15$ for the control (0 μM). The data from three batches of embryos were combined for Fig. 8*C* and *D*. In all cases, the

significant effect, with two out of four contacted $a_{4\cdot2}$ blastomeres adopting a neural phenotype. To rule out direct epidermalizing effects on $a_{4\cdot2}$ by an brefeldin A that might remain on the $A_{4\cdot1}$ after washing, four $a_{4\cdot2}$ blastomeres neurally induced by subtilisin (0·05% for 60 min) were treated directly by applying brefeldin A at a concentration of 10 μg/ml for 10 h. Three (two with Na^+ spikes and one with delayed K^+ current only) out of four became neural in type and one became non-excitable. This shows that direct application of brefeldin A slightly suppressed neural development. However, it seems unlikely that the amount of brefeldin A that might remain on $A_{4\cdot1}$ blastomeres would have direct effects on $a_{4\cdot2}$. The suppressing effect of bafilomycin A1 (1 μM) on $A_{4\cdot1}$ was relatively weak. Three out of eleven $a_{4\cdot2}$ blastomeres, which were in contact with $A_{4\cdot1}$ blastomeres treated by bafilomycin A1 (1 μM for 60 min) before contact, differentiated into epidermal type, three differentiated neurally and the remaining five showed small regenerative responses possibly due to Ca^{2+} (Sr^{2+}) currents. When four $a_{4\cdot2}$ blastomeres induced by subtilisin (0·05% for 60 min) were treated with the same concentration of bafilomycin A1, they all differentiated neurally, indicating no direct effect of the drug on $a_{4\cdot2}$. All the above results suggest that neural induction requires some secretory processes or transport processes through the Golgi apparatus in the inducer blastomere, $A_{4\cdot1}$.

DISCUSSION

Protease as a neural inducer

In the present experiments we found that subtilisin, a serine protease, had potent neural inducing activity on the ascidian presumptive neural blastomere, $a_{4\cdot2}$, and mimicked the *in vivo* inducing activity of $A_{4\cdot1}$ blastomeres by showing both a similar time sequence of changes in neural competence of $a_{4\cdot2}$ blastomeres, and the same regional difference in neural competence among ectodermal blastomeres of 8- or 16-cell embryos. Further, it was found that natural induction by contact with $A_{4\cdot1}$ could be partially suppressed by the subtilisin inhibitor, SSI.

In 1979, it was reported that trypsin, another serine protease, can induce some neural structures, namely pigment spots and a tissue-specific enzyme, brain pigment cell tyrosinase, in animal quartets isolated from 8-cell embryos of *Ascidia maldia* and *Phallusia mamilata*, and cultured independently of the natural inducer in the anterior-vegetal quartet (Ortolani, Patricolo & Mansueto, 1979). Since, in our case, the inducing ability of subtilisin was greater than that of trypsin, it is safe to conclude that some proteases, possibly serine proteases, can have neural inducing activity on ascidian ectoderms. In embryos of other species, although proteases are not regarded as candidates for the neural inducer, a few papers suggest the involvement of proteolysis in neural development. For example, it is reported that the dorsal lip has a higher catheptic activity than ventral tissue in the early gastrula of *Discoglossus pictus* (D'Amelio & Ceas, 1957) or *Xenopus laevis* (Deuchar, 1958), and that the injection of endopeptidase inhibitors into the dorsal region of *Xenopus*

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concentration of cytochalasin B was reduced from 2 to 0·2 μg/ml at the time of contact, that is, 2·5 h after isolation. Reduction of cytochalasin B facilitates contact (Okado & Takahashi, 1990*b*).

embryos causes abnormal gastrulation and defects of the eye and neural tube formation (Miyata & Kihara, 1987). However, in these reports they have stressed roles for proteolysis mainly in new protein synthesis during gastrulation and have not discussed the possibility of an involvement in neural inducing activity.

Possible mechanisms of neural induction by proteases

Since subtilisin mimicked the *in vivo* inducing activity and secretory process inhibitors, monensin, brefeldin A and bafilomycin A1, affected induction by contact, it is natural to have a working hypothesis that some inducing protease is secreted from the inducing $A_{4\text{-}1}$ blastomere and that the released protease interacts with some receptor on the membrane of neurally competent blastomeres, producing intracellular signals to switch the cell fate (Fig. 7*A*).

Recently, genetic studies on *Drosophila melanogaster* development have disclosed that eleven maternal genes are involved in the specification of dorsal–ventral pattern at the beginning of development, i.e. the non-cellular or cellular blastoderm stage (DeLotto & Spierer, 1986). Of the eleven genes, *dorsal*, *snake*, *easter* and *toll* have been cloned and the primary structures of their encoded proteins have been clarified (Hashimoto, Hudson & Anderson, 1988; Steward, Zusman, Huang & Schedl, 1988; Chasan & Anderson, 1989). The embryos from mutant mothers, such as *dl/dl* females, develop in such a way that normal ventro-lateral pattern elements, which include presumptive neuroepithelial region, are displaced or absent and replaced by dorsal pattern elements (DeLotto & Spierer, 1986). Intensive molecular biological studies have shown that the *toll* gene appears to encode a transmembrane protein (Hashimoto *et al.* 1988) and both *snake* and *easter* genes seems to encode extracytoplasmic serine proteases (DeLotto & Spierer, 1986; Chasan & Anderson, 1989). These studies suggest that *toll* and *snake* and/or *easter* may interact each other to produce intracellular signals which will be transmitted to the *dorsal* protein, possibly one of the DNA binding proteins closely related to the avian cellular oncogene c-*rel* (Steward *et al.* 1988), and to direct the cells in the *dorsal*-activated part of the blastoderm to form ventral elements (Hashimoto *et al.* 1988). Thus, by analogy, it is highly probable that some serine protease, which is released, interacts with a membrane receptor, activating the receptor and resulting in neural induction of ascidian ectodermal blastomeres.

There are, however, many other possibilities which could explain the induction of ascidian blastomeres by proteases. Recently, Grunz & Tacke (1989) have shown that when *Xenopus laevis* blastula or early gastrula ectoderm is dissociated and the cells kept dispersed for up to 5 h prior to reaggregation, the resulting spheres of ectodermal cells differentiate autonomously into large neural structures independently of the neural inducer, while dissociated and immediately reaggregated ectoderm will only differentiate into ciliated epidermis, in accordance with the classical experiments. This autoneutralization can be prevented by cell supernatant from dissociated ectoderm, which contains extracellular matrix components (ECM) (Grunz & Tacke, 1990). The inhibiting substances contain a substantial amount of glyco-conjugated proteins, because the inhibitory effects are inactivated by phenol extraction (Grunz & Tacke, 1990). Although possible proteolysis of this inhibitory ECM was not discussed by the authors, limited degradation of the ECM around competent blastomeres of the ascidian embryo might be provoked by the applied

proteases, resulting in differentiation of the ectoderm blastomeres into neural derivatives. Since our way of isolating blastomeres with a glass needle was likely to keep the ECM intact, it is also possible that the release of degrading proteases occurs during induction by contact and that those proteases specifically destroy a local extracellular structure which normally suppresses the autonomous neural differentiation of competent ectodermal cells.

In the present experiments, the secretory process inhibitors, monensin, brefeldin A and bafilomycin A1, affected neural induction, allowing us to hypothesize that an inducer protease is secreted by inducing blastomeres (Fig. 7*A*). In fact, the secretion of serine proteases from the same *Halocynthia* eggs into the perivitelline space has been suggested to occur during fertilization or after activation by the ionophore A23187, since expansion of the perivitelline space just after egg activation is blocked by various serine protease inhibitors. Furthermore, the ranking of inhibitory potencies among inhibitors correlates well with those of the trypsin-like proteases purified from eggs (Sawada, Kawahigashi, Yokosawa & Ishii, 1985). This type of protease secretion has also been demonstrated in mouse eggs in the case of the breakdown of the zona *pellucida* glycoprotein (Moller & Wasserman, 1989). Thus, the blastomeres from the early 8-cell *Halocynthia* embryo may retain a mechanism for releasing proteases.

The roles of extracellularly released proteinases in cellular invasiveness, such as angiogenesis, tumour metastasis, wound healing and migration of cells during development, have been well documented (Mullins & Rohrlich, 1983), the importance being further demonstrated in the case of eversion of the insect imaginal disc (Pino-Heiss & Schubiger, 1989) and neurite growth of cultured neurones (Pittman, 1985). If a protease is an inducer in contact-mediated induction, the regulated proteolysis may be required to localize at the site of cell–cell contact. This could be achieved partly through membrane bound proteases and partly through a ubiquitous presence of protease inhibitors. The appearance of membrane-bound proteases has been reported during transformation of an embryonic fibroblast line (Chen & Chen, 1987), and also during degradation of serum amyloid protein A by mononuclear leukocytes (Zucker-Franklin, Lavie & Franklin, 1981). Recently, the physiological significance of membrane-bound subtilisin-type serine proteases has been well documented in the processing of prohormones, the proteases being defined as dibasic processing endopeptidase (Barr, 1991). Subtilisin and sublisin-like serine proteases have long been considered to be only of prokaryotic origin, but since the discovery of the KEX2 endoprotease as a subtilisin-like enzyme that cleaves the pro-α-factor, a mating hormone, in *Saccharomyces cerevisiae* (Mizuno, Nakamura, Oshima, Tanaka & Matsuo, 1988; Fuller, Brake & Thorner, 1989), several homologues of this enzyme have been cloned in mammals, including furin/PACE, which is known as a receptor-like protein, and the gene of which is located just ahead of a proto-oncogene (Bresnahan, Leduc, Thomas, Thorner, Gibson, Brake, Barr & Thomas, 1990). Although the subtilisin-like membrane-bound proteases are all suggested to be located in rough endoplasmic membranes or Golgi apparatus membranes (Barr, 1991), an interesting question arises as to whether the fact that subtilisin is much more effective than other proteases in the *Halocynthia* embryo is related to the activation of pro-receptors by dibasic processing endopeptidases bound to the surface membrane of the inducing blastomere. It should also be noticed as one of the

possible ways of activating receptors with proteolytic enzymes that Vu *et al.* (Vu, Hung, Wheaton & Coughlin, 1991) have demonstrated that a new amino-terminal of thrombin receptor created by cleavage with thrombin works as a tethered ligand and activates the receptor.

In order to prove the working hypothesis presented in the present paper, it is, however, necessary to identify the protease on the surface membrane of the inducing blastomere, $A_{4\text{-}1}$, at the time of induction and/or to identify a receptive element or elements on the membrane of the competent blastomere, $a_{4\text{-}2}$, that is common to both induction by proteases and induction by contact.

Early determination of neural competence

The anterior half of the ectoderm has a larger presumptive region for neural tissues than the posterior half (Conklin, 1905; Nishida, 1987). It is questioned whether this regional specificity in neural development depends entirely on the regional difference in competence of the blastomeres to be induced. In our previous report, a regional difference of neural competence was demonstrated between anterior and posterior ectodermal blastomeres in 8-cell embryos (Okado & Takahashi, 1990*b*) and, in the present experiment, among four ectodermal blastomeres in the 16-cell embryo. These studies revealed that anterior blastomeres in ectoderm were neurally induced with higher frequency than posterior blastomeres (Fig. 6*A* and *B*). This tendency is consistent with the more neural lineage in the anterior half of the ectoderm reported by Nishida (1987) in normal development, as shown in Fig. 6*C*. Therefore, the regional specificity of neural development *in vivo* is largely derived from the regional difference of neural competence among ectodermal blastomeres. Since the ascidian egg has long been considered as a typical mosaic one (Conklin, 1905), it is claimed that the apparent inductive formation of neural tissues is not a real one but simply results from evocation of a predetermined neural lineage (Reverberi, 1971). Our present results appear to confirm this claim by demonstrating the importance of predetermination of the neural competence ahead of induction. However, in order to discuss the issue completely, the regional difference of inducing activity among inducing blastomeres is also required to be examined. Further, although the $b_{5\text{-}4}$ blastomere has no neural fates *in vivo* (Nishida, 1987), it was induced to become a neural-type cell by treatment with subtilisin or by contact with the $A_{4\text{-}1}$ blastomere, switching the developmental fate from epidermal to neural. This discrepancy is most probably due to inaccessibility of $b_{5\text{-}4}$ derivatives to the derivatives of $A_{4\text{-}1}$ in the normal embryo during the gastrula stage. In this case the morphogenetic movement of inducer blastomeres relative to competent blastomeres *in vivo* could be considered to be the cause of regional specificity. It is thus concluded that induction actually occurs in the ascidian embryo and that the difference in predetermined competence is not the only factor underlying regional specificity in neural development, as shown in other vertebrate embryos.

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