J. Physiol. (1984), **347**, *pp.* 301–325
With 1 *plate and* 13 *text-figures*
Printed in Great Britain

DETERMINATION OF EXCITABILITY TYPES IN BLASTOMERES OF THE CLEAVAGE-ARRESTED BUT DIFFERENTIATED EMBRYOS OF AN ASCIDIAN

BY TOMOO HIRANO, KUNITARO TAKAHASHI AND NAOHIDE YAMASHITA

From the Department of Neurobiology, Institute of Brain Research, Faculty of Medicine, University of Tokyo, Tokyo, Japan

(*Received 19 October 1982*)

SUMMARY

1. Cleavage of the embryo of *Halocynthia roretzi* was arrested with cytochalasin B at 1- to 32-cell stages and the embryo was cultured in sea water containing cytochalsin B until a developmental time equivalent to the hatching of the control larva. Membrane properties of the blastomeres were studied with constant-current and voltage-clamp techniques.

2. Four types of membrane response – neural, epidermal, muscular and non-excitable – were identified on the basis of the shapes and ionic dependence of action potentials in the blastomeres of 8- to 32-cell embryos. Only the epidermal type of response was found in the blastomeres of 1- to 4-cell embryos.

3. The blastomeres with responses of neural type had Na, Ca, delayed K rectifier, anomalous K rectifier and Ca-induced K channels. Those of epidermal type had Ca, anomalous K rectifier and Ca-induced K channels. Those of muscular type had Ca, delayed K rectifier, anomalous K rectifier and possibly Ca-induced K channels. Those of non-excitable type had almost none or small amounts of outward- and inward-going rectifier channels.

4. The characteristic responses of neural type were found in small blastomeres in the animal hemisphere, which included some presumptive neural regions. The responses of muscular type were found in large blastomeres of the vegetal hemisphere, which included some presumptive regions for muscle. Those of epidermal type were found in the blastomeres of the animal hemisphere which did not differentiate into the neural type. Those of non-excitable type were found in some blastomeres of the vegetal hemisphere.

5. Blasomeres of 1- to 32-cell cleavage-arrested embryos, which were presumed to possess more than one possible developmental fate, did not develop mosaic membrane properties but differentiated into one of the four types, with a probability dependent upon a gradient of ooplasmic segregation at the time of arrest.

INTRODUCTION

In a previous study (Takahashi & Yoshii, 1981) it was reported that membrane differentiation, such as electrical excitability, occurs in cells of the ascidian embryo whose cleavage is arrested with cytochalasin B at various stages during early development and cultured for a certain period afterwards. Development of electrical excitability up to 25 h after fertilization at 15 °C was quantitatively analysed over the whole surface membrane of the cleavage-arrested 16-cell embryo, because during this period there is tight electrotonic coupling between blastomeres and the embryo behaves electrically as a single cell (Takahashi & Yoshii, 1981). After 25 h, when the cells in the control embryo fully differentiate and the embryo becomes ready to hatch, three types of electrical excitability, i.e. ectodermal (neural or epidermal), muscular and non-excitable, are found in the blastomeres of the cleavage-arrested 64-cell embryo (Takahashi & Yoshii, 1981). Presumptive muscle blastomers identified electrophysiologically corresponded to those described classically (Conklin, 1905) and histochemically (Whittaker, 1973).

Cytoplasmic localization of certain factors in an oocyte or in a developing embryo has been considered as the basis for differential gene activation in different blastomeres during development (Davidson, 1976). In the ascidians, the arrest of cell cleavage prevents the segregation of cytoplasmic factors at various stages (Whittaker, 1973). It is known that the developmental fate of each blastomere in the ascidian embryo is uniquely determined after the 64-cell stage, possibly because cytoplasmic segregation is completed at this stage (Conklin, 1905; Reverberi, 1961). The developmental fate of individual blastomeres of 32-cell or smaller cell-numbered embryos is not yet uniquely determined. Therefore, the question arises as to whether in cleavage-arrested 32-cell or smaller cell-numbered embryos a single type of differentiation is uniquely expressed, or whether two or more mixed types appear in mosaic fashion in a single blastomere. In the present experiments we aimed to determine types of membrane excitability quantitatively by analysis of current components under voltage-clamp conditions in individual blastomeres of 1- to 32-cell cleavage-arrested embryos at the time when the control larvae hatched. We also attempted to analyse properties of current components in the differentiated blastomeres, and to compare them with those in the undifferentiated oocyte.

METHODS

Materials

Adult ascidians (*Halocynthia roretzi*) which had mature eggs and sperm were kept in a cold aquarium at 2·5–3·0 °C. Eggs and sperm were collected from spawning animals after 24 h in a warm bath at 10 °C. The eggs were fertilized by adding sperm suspensions taken from another animal. Fertilized eggs were cultured in Petri dishes placed on a slowly moving table in a water-bath kept at a constant temperature of 7–9 °C. When the cell cleavage was to be arrested, developing embryos were transferred to another Petri dish containing 1–2 μg/ml cytochalasin B (Aldrich) in sea water. Without slow movement of the Petri dishes (0·2–0·5 cycles/s), arrested embryos were deformed. Cleavage-arrested embryos were cultured until the control tadpole larvae hatched. In some experiments the Ca ionophore A23187 (20 μM, Calbiochem) was added to the sea water of the culturing bath after 32 h of development at 8 °C (which corresponds to the late neurula stage of the control larva) in order to inhibit differentiation of the arrested embryo. Just before electrical

measurements were made, the chorion and follicular envelope of embryos were removed by digestion with 10 mg/ml pronase in sea water for 20 min at 15 °C, and then the embryos washed with high-protein sea water (bovine serum albumin, BSA 10 mg/ml) and kept in it at 4 °C, to prevent further digestion. After at most 2 h in the BSA sea water, the denuded embryo was placed in the experimental bath at 10 °C for electrical measurements. Methods for exchanging solutions and keeping bath temperature constant were as described previously (Miyazaki, Takahashi & Tsuda, 1974). Compositions of solutions used are listed in Table 1. All experimental solutions contained 10 mg/ml BSA to prevent further digestion and 2 μg/ml cytochalasin B to prevent irregular cell cleavage. For most of the experiments Na–Sr solution was used as a control solution because in Sr solution the inward current through Ca channels was the largest in the *Halocynthia* egg (Okamoto, Takahashi & Yoshii, 1976*b*) and the Ca-activated K conductance was relatively suppressed.

TABLE 1. Ionic composition of solutions

Solutions†	NaCl	Choline-Cl	TEA-Cl	$SrCl_2$	$MnCl_2$	$MgCl_2$	KCl	PIPES-Na*
Na–Sr (standard)	400	—	—	100	—	—	10	5 (Na 7·5)
Na–Mn	452·5	—	—	—	15‡	50‡	10	5
Choline–Sr	—	400	—	100	—	—	10	5
Choline–Mn	—	452·5	—	—	15	50	10	5
TEA–Sr	—	—	400	100	—	—	10	5
100 K–15 Mn	362·5	—	—	—	15	50	100	5

* The pH was adjusted to 7·0.

† All solutions included 10 mg/ml bovine serum albumin (BSA) and 1–2 μg/ml cytochalasin B.

‡ This combination of divalent salt was necessary for keeping the same threshold potential for Na current as in Na–Sr solution.

Electrical recordings

A conventional two-micropipette technique was employed for both voltage clamps and current clamps. The experimental techniques were as described previously (Miyazaki *et al.* 1974; Okamoto, Takahashi & Yoshii, 1976*a*). Electrode resistances were less than 5 MΩ. The holding potential in voltage-clamp experiments was always −75 mV. Potental and current recordings were stored in two transient recorders and reproduced on a d.c. pen recorder. These methods of measurement were as described previously (Takahashi & Yoshii, 1981). The input capacity (C) of the penetrated blastomere was measured from the initial slope of the electrotonic potential, $(dV/dt)_{t=0}$, produced by the rectangular pulse current (I) using the equation: $C = I/(dV/dt)_{t=0}$. The coupling with other blastomeres was checked by measuring the spread of electrotonic potential.

Acetylcholinesterase staining

The embryos examined were fixed and stained for acetylcholinesterase when necessary by Karnovsky's acetylthiocholine method (Karnovsky & Roots, 1964). Details of this staining procedure were previously described by Ohmori & Sasaki (1977).

RESULTS

Types of electrical excitability in the cleavage-arrested embryo

Identification of blastomeres in the 16-cell embryo. All blastomeres in the early ascidian embryo from 1- to 128-cell stages are named after Conklin's description (1905). Developmental fates of these blastomeres (see Table 4) are given by Conklin (1905), Hirai (1941) and Reverberi (1961). In the cleavage-arrested 16-cell embryo, eight relatively small blastomeres, pairs of $a_{5\text{-}3}$, $a_{5\text{-}4}$, $b_{5\text{-}3}$ and $b_{5\text{-}4}$ blastomeres, constitute the animal hemisphere, as shown in Pl. 1*A*. Two pairs of blastomeres (the

$a_{5\text{-}3}$ and $a_{5\text{-}4}$ blastomeres) located anteriorly are the ancestors of three pairs of presumptive brain-vesicle blastomeres (the $a_{7\text{-}9}$, $a_{7\text{-}10}$ and $a_{7\text{-}13}$ blastomeres) and five pairs of presumptive epidermal blastomeres (the $a_{7\text{-}11}$, $a_{7\text{-}12}$, $a_{7\text{-}14}$, $a_{7\text{-}15}$ and $a_{7\text{-}16}$ blastomeres) in the 64-cell embryo. While fates of individual blastomeres in the 64-cell embryo are uniquely determined, the $a_{5\text{-}3}$ and $a_{5\text{-}4}$ blastomeres in the 16-cell embryo will give rise to both neural and epidermal cells. Two pairs of small blastomeres (the $b_{5\text{-}3}$ and $b_{5\text{-}4}$ blastomeres) located posteriorly in the animal hemisphere are exclusively presumptive epidermal cells. Plate 1 *B* shows the eight large blastomeres in the vegetal hemisphere of the cleavage-arrested 16-cell embryo. The four round cells lined up anteriorly are two pairs of blastomeres (the $A_{5\text{-}1}$ and $A_{5\text{-}2}$ blastomeres) which are the ancestors of three pairs of endodermal blastomeres, three pairs of presumptive notochordal blastomeres and two pairs of presumptive neural tube blastomeres in the 64-cell embryo. Thus, both the $A_{5\text{-}1}$ and $A_{5\text{-}2}$ blastomeres will give rise to endodermal, notochordal and neural cells. Two large blastomeres in the middle zone of the vegetal hemisphere are $B_{5\text{-}1}$ blastomeres, the ancestors of two pairs of endodermal, a pair of mesenchymal and a pair of presumptive muscular blastomeres in the 64-cell embryo. Finally a pair of small blastomeres at the posterior end of the vegetal hemisphere are two $B_{5\text{-}2}$ blastomeres, which are the ancestors of three pairs of presumptive muscular and a pair of mesenchymal blastomeres in the 64-cell embryo.

Types of electrical excitability in the cleavage-arrested 16-cell embryo. In a previous study (Takahashi & Yoshii, 1981), an Na- and Ca-dependent action potential appeared in the cleavage-arrested 16-cell embryo after 17–25 h of culture at 15 °C. In the present study some experiments were done after 60 h of culture at 8 °C (which corresponds to 25 h at 15 °C) but most were done after 75 h of culture at 8 °C when the control larvae hatched. The nomenclature and morphological features of various stages during normal development of *Halocynthia roretzi*, with corresponding developmental times at 9 °C, have been given in a previous paper (Ohmori & Sasaki, 1977). (In the present experiments we reduced the temperature of the culture bath to 8 °C, which increased the developmental time by about 10% relative to that at 9 °C.) Blastomeres in the 16-cell embryo fully differentiated and showed one of four types of membrane response: neural, epidermal, musclar or non-excitable.

Fig. 1 illustrates examples of three types of action potential evoked by constant-current stimulation in 100 mM-$SrCl_2$, 400 mM-NaCl, 10 mM-KCl solution (Na–Sr solution). The action potential of the first type (Fig. 1 *A*) had a plateau and the cells having this action potential maintained a resting potential of −40 to −50 mV. For the measurement the membrane potential was held below −70 mV by applying d.c. current to remove inactivation of the Na channel. The threshold of the action potential was around −35 mV and it overshot more than +35 mV. The action potential remained after replacement of 100 mM-Sr with 15 mM-Mn and 50 mM-Mg, though the duration of the plateau was diminished. Thus, the action potential was both Na- and Sr-dependent. As described later, this type of action potential appeared exclusively in the blastomeres which include presumptive brain vesicle region (see Table 4). We therefore designated the action potential as neural type.

The action potential of the second type showed a very long-lasting plateau (Fig. 1 *B*). The duration was more than 2 s at 10 °C. The resting potential of the cells which showed this type of response was more negative than −60 mV. The threshold

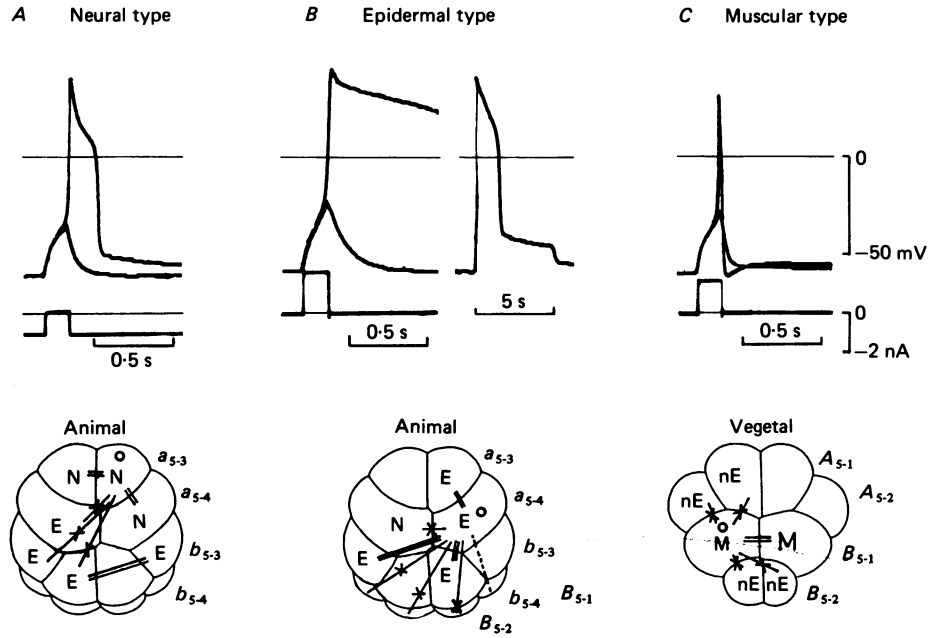


Fig. 1. Three types of action potential obtained in cleavage-arrested 16-cell embryos and coupling patterns of penetrated embryos. *A*, an action potential of neural type obtained in the right $a_{5\text{-}3}$ blastomere of a 16-cell embryo, which was electrically coupled to left $a_{5\text{-}3}$ and right $a_{5\text{-}4}$, but isolated from left $a_{5\text{-}4}$ and all *b* blastomeres. The input capacity was 2·55 nF. The resting potential of this neural-type blastomere was about −40 mV and a steady hyperpolarizing current was applied to keep the membrane potential at −70 mV. The right $b_{5\text{-}3}$ blastomere and left $b_{5\text{-}4}$ blastomere were coupled to each other and had an input capacity of 7·14 nF, showing an action potential of epidermal type. In all diagrams, double lines indicate electrotonic connexion between two blastomeres and a single line with a cross indicates no connexion. Open circles indicate the blastomeres in which action potentials were recorded. N, E, M and nE are neural, epidermal, muscular and non-excitable type, respectively.

B, an action potential of epidermal type obtained in the right $a_{5\text{-}4}$ blastomere in another 16-cell embryo, which was electrically coupled to right $a_{5\text{-}3}$, right $b_{5\text{-}4}$ and left $b_{5\text{-}3}$, but isolated from left $a_{5\text{-}4}$, which showed an action potential of neural type. The input capacity was 6·45 nF. In the potential records, a slower sweep record (ten times) is also illustrated on the right side. $B_{5\text{-}1}$ and $B_{5\text{-}2}$ were on the vegetal side. The broken line indicates slight residual coupling between $a_{5\text{-}4}$ and $B_{5\text{-}1}$.

C, spike potential of muscular type was obtained from a $B_{5\text{-}1}$ in the same 16-cell embryo illustrated in Fig. 1*B* and on the same side of the right $a_{5\text{-}4}$. The $B_{5\text{-}1}$ blastomere was coupled to the contralateral $B_{5\text{-}1}$, but isolated from both the $B_{5\text{-}2}$ blastomeres. Input capacity was 2·64 nF. The $B_{5\text{-}2}$ blastomeres were both non-excitable.

potential was −25 mV and the overshoot was more than 45 mV. The action potential was abolished when 100 mM-Sr was replaced with 15 mM-Mn and 50 mM-Mg. Thus, in contrast to the neural type, the action potential was purely Sr-dependent. As shown later, this type of action potential was obtained mainly in blastomeres which include presumptive epidermal region (see Table 4). We therefore designated the action potential as epidermal type.

The third type was a spike potential with duration less than 50 ms at half-amplitude

at 10 °C. The threshold for the spike was −30 mV in Na–Sr solution. The spike potential was completely abolished when 100 mM-Sr was replaced with 15 mM-Mn and 50 mM-Mg. It was identical with the Ca spike found in muscle cells of the control larvae or in presumptive muscle blastomeres of the cleavage-arrested 64-cell embryo (Miyazaki, Takahashi & Tsuda, 1972; Takahashi & Yoshii, 1981), because the spike was preserved in Na–Ca solution. Therefore, it was classified as an action potential of muscular type. The resting potential of the cell which showed the muscular-type action potential was more negative than −60 mV and in the same range as the epidermal type.

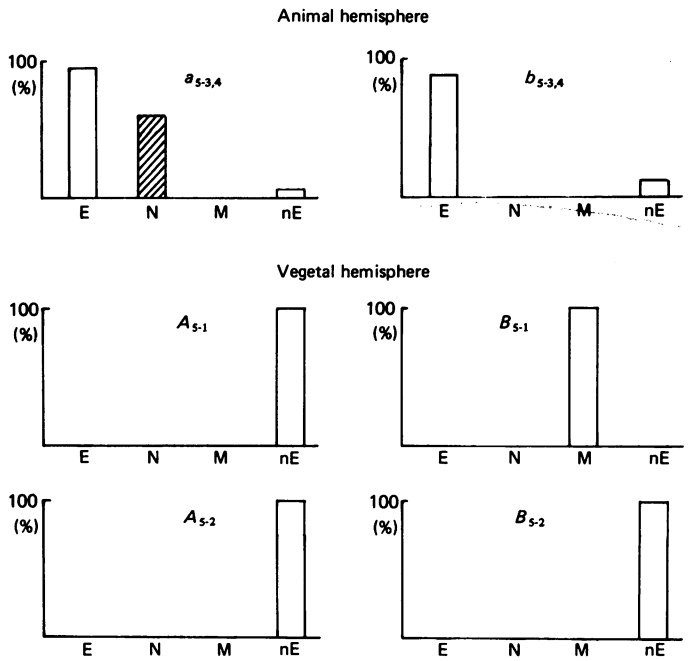


Fig. 2. Percentages of differentiation to a cell of each type in respective blastomeres of a cleavage-arrested 16-cell embryo. Sample numbers are given in the text.

In a previous paper (Takahashi & Yoshii, 1981) the discrimination between epidermal and neural or muscular types was not always clear because in the Na–Ca solution used the epidermal action potential showed a relatively rapid decay due to Ca-induced K outward current (Hirano & Takahashi, 1984) and was apparently similar to the action potentials of neural or muscular type. However, the Na–Sr solution in the present experiment allowed us clear-cut identification of three types of action potential.

Each identified type of the membrane response appeared in specified blastomeres of the 16-cell embryo. In 59 % of the 16-cell embryos examined (17/29), the neural-type action potential appeared in either $a_{5\text{-}3}$ or $a_{5\text{-}4}$, which were presumptive neural and epidermal blastomeres as described above. The distribution pattern of neural-type blastomeres in an embryo is shown in Fig. 1*A*, lower diagram. In this embryo the

neural-type action potential was obtained in the right $a_{5\text{-}3}$ of the animal hemisphere. The penetrations of the second electrode in six other blastomers revealed that the right $a_{5\text{-}4}$ and the left $a_{5\text{-}3}$ also produced the neural-type action potential and that both blastomeres were tightly coupled to the right $a_{5\text{-}3}$. The left $a_{5\text{-}4}$, the left $b_{5\text{-}3}$ and the left $b_{5\text{-}4}$ were not coupled with the right $a_{5\text{-}3}$ and produced the epidermal-type action potential. The number of neural-type blastomeres in an embryo ranged from one to three, and in 41 % (12/29) of embryos examined, no blastomeres of neural type were found. When the action potential of neural type was found by penetrating $a_{5\text{-}3}$ or $a_{5\text{-}4}$, the input capacity was $1{\cdot}62 \pm 1{\cdot}07$ nF ($n = 15$, mean $\pm$S.D.), which was more than three times as large as those measured in single isolated blastomeres of non-excitable type in the animal hemisphere (0·43–0·57 nF) or in single large blastomeres of the vegetal hemisphere whose differentiation was pharmacologically inhibited by Ca ionophore ($0{\cdot}45 \pm 0{\cdot}04$ nF, $n = 4$; see below). This may correspond to the fact that, when blastomeres in *a* group differentiated to neural type, they formed a group of two or three blastomeres which were coupled together, as described above.

In most cases, $b_{5\text{-}3}$ and $b_{5\text{-}4}$ blastomeres in the animal hemisphere, which were exclusively presumptive epidermal blastomeres, generated the epidermal-type action potential (87·5 %, 14/16). The embryo illustrated in Fig. 1*B* showed the epidermal-type action potential in the right $a_{5\text{-}4}$ blastomere. The recording from the second electrode revealed that the right $a_{5\text{-}3}$, the left $b_{5\text{-}3}$ and the right $b_{5\text{-}4}$ produced the epidermal-type action potential and were coupled with the right $a_{5\text{-}4}$, but that the left $a_{5\text{-}4}$ generated the neural-type action potential and was not connected to the blastomeres which showed the epidermal-type action potential. Thus, the epidermal-type action potential was also found in some *a* group blastomere which did not show the neural-type action potential in addition to being found in *b* group blastomeres, and all epidermal-type blastomeres seemed to be electrotonically coupled in an embryo. Correspondingly, the input capacity was 5–7 nF, which is more than ten times as large as that of an isolated blastomere in the animal hemisphere. There were occasionally a few non-excitable blastomeres of *a* or *b* group, which were found to be electrically isolated from the other blastomeres.

$A_{5\text{-}1}$ and $A_{5\text{-}2}$ blastomeres, located anteriorly in the vegetal hemisphere, never produced a regenerative response ($A_{5\text{-}1}$, 6/6; $A_{5\text{-}2}$, 5/5) and were classified as non-excitable, although $A_{5\text{-}1}$ and $A_{5\text{-}2}$ included the presumptive region for neural tube in the larval tail. The resting potential of the non-excitable blastomeres was around -10 mV in Sr–Na solution. The input capacity of $A_{5\text{-}1}$ or $A_{5\text{-}2}$ blastomeres varied from 0·5 to 1·6 nF, suggesting that *A* group blastomeres were frequently coupled with each other and that the degree of coupling within *A* group might be variable. An example of embryos which showed non-excitable *A* group blastomeres is shown in the diagram of Fig. 1*C*, which illustrates the vegetal hemisphere of the same embryo as shown in Fig. 1*B*. The *A* group blastomeres were not coupled with *B* group blastomeres located posteriorly in the vegetal hemisphere.

In 97 % (27/28) of embryos examined, $B_{5\text{-}1}$, which is located in the middle zone of the vegetal hemisphere, showed the muscular-type spike potential. Thus, $B_{5\text{-}1}$ always differentiated to a cell of muscular type in the cleavage-arrested embryo, although this blastomere included not only the presumptive muscular region but also endo-

dermal and mesenchymal regions. The diagram in Fig. 1*C* illustrates a coupling pattern of the vegetal hemisphere. The two muscular type $B_{5\text{-}1}$ blastomeres were frequently coupled and separated from the small $B_{5\text{-}2}$ blastomeres and from *A* group blastomeres. The input capacity of the $B_{5\text{-}1}$ blastomere was $3{\cdot}35 \pm 0{\cdot}88$ nF ($n = 18$) when the $B_{5\text{-}1}$'s on both sides were coupled or $1{\cdot}46 \pm 0{\cdot}32$ nF ($n = 7$) when the penetrated blastomere was isolated. Thus, even a single $B_{5\text{-}1}$ could have a large input capacity when it differentiated to a cell of muscular type, compared with the capacity of the isolated blastomere in an undifferentiated state (0·45 nF). The $B_{5\text{-}1}$ blastomeres on both sides showed strongly positive and uniform staining of acetylcholinesterase by the Karnovsky method in 96% (24/25) of the cleavage-arrested 16-cell embryos. Staining in one embryo is shown in Pl. 1*B*.

The relatively small blastomere, $B_{5\text{-}2}$, showed no spike potential (4/4), though it included the presumptive muscular region. Thus, $B_{5\text{-}2}$ in the 16-cell embryo was non-excitable. Correspondingly, in 96% of the cleavage-arrested 16-cell embryos (24/25) acetylcholinesterase was not demonstrated in $B_{5\text{-}2}$. A summary of the differentiation types in the 16-cell embryo is given in Fig. 2.

Types of electrical excitability in the cleavage-arrested 8-cell embryo. In the 8-cell embryo of the ascidian (see Fig. 4*C*) two pairs of relatively small blastomeres, the $a_{4\text{-}2}$ and $b_{4\text{-}2}$ blastomeres, lie in the animal hemisphere. The $a_{4\text{-}2}$ blastomere is located anteriorly, is the ancestor of $a_{5\text{-}3}$ and $a_{5\text{-}4}$ in the 16-cell embryo, and is the presumptive blastomere for neurones and epidermis. The $b_{4\text{-}2}$ blastomere is located posteriorly and is the ancestor of $b_{5\text{-}3}$ and $b_{5\text{-}4}$ in the 16-cell embryo. Thus, $b_{4\text{-}2}$ is exclusively a presumptive epidermal blastomere. In the vegetal hemisphere there are two pairs of large blastomeres, the $A_{4\text{-}1}$ and $B_{4\text{-}1}$ blastomeres. $B_{4\text{-}1}$ is the ancestor of the presumptive muscular blastomeres in the 64-cell embryo.

In cleavage-arrested 8-cell embryos which had been cultured for 75 h at 8 °C (i.e. until the time the control larvae hatched), the three types of action potential already described were also observed, as shown in Fig. 3. A neural-type action potential was found in one blastomere of the animal hemisphere in 20% of embryos examined (13/66). Since only $a_{4\text{-}2}$ is the ancestor of presumptive neural cells in the 8-cell embryo, the differentiation into neural type probably occurs in one of the two $a_{4\text{-}2}$ blastomeres. However, in the cleavage-arrested 8-cell embryo the relative locations of each blastomere were somewhat distorted, and it was often difficult to determine the anteroposterior direction. Consequently in most cases we could not distinguish the *a* from the *b* blastomeres. Two blastomeres in the animal hemisphere differentiated to cells of neural type in only one embryo out of sixty-six cases. The blastomeres in the animal hemisphere which did not generate neural-type action potentials produced epidermal-type action potentials. These blastomeres were tightly coupled, as in the 16-cell embryo. Thus, blastomeres other than the one which differentiated to a cell of neural type were coupled in the animal hemisphere and showed an epidermal-type action potential.

In the vegetal hemisphere, one of the four large blastomeres generated spike potentials of muscular type in 34% of the embryos examined (20/58). Two large blastomeres in a single embryo differentiated simultaneously in 5/58 cases (9%). More than two large blastomeres never differentiated at the same time in any embryo. Since only $B_{4\text{-}1}$ is the ancestor of the presumptive muscle cells, this suggests that

differentiation into muscular type occurs in either one or both of the $B_{4\text{-}1}$ blastomeres with a certain probability. In contrast all $B_{5\text{-}1}$ blastomeres in the 16-cell embryo produced an action potential of muscular type. Large blastomeres which did not show a spike potential of muscular type were either electrically isolated from other blastomeres and remained inexcitable or were coupled to the small blastomeres in the animal hemisphere and developed an epidermal-type action potential. In 12% of the embryos examined (7/58) all blastomeres in both animal and vegetal hemispheres were coupled and produced an action potential of epidermal type. When the large blastomeres produced a spike potential of muscular type, the staining for acetylcholinesterase was positive, as shown in Pl. 1*C*.

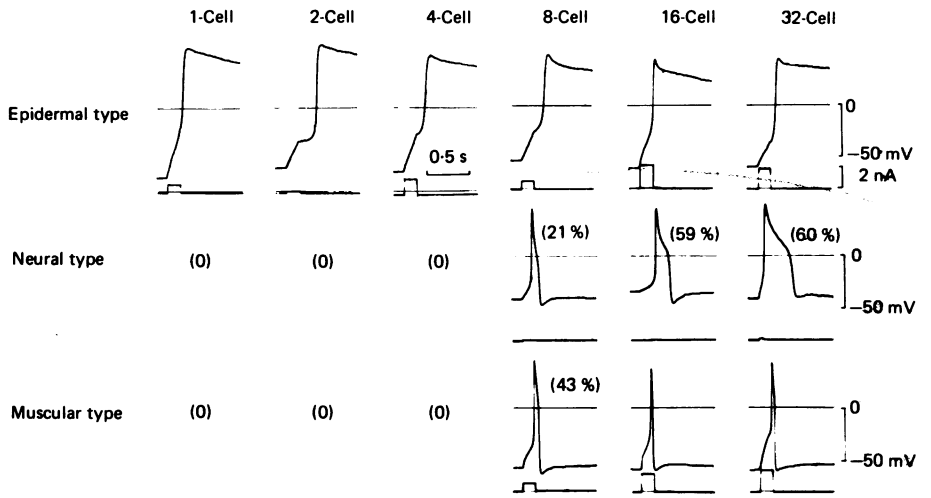


Fig. 3. Types of action potentials obtained in embryos whose cleavage was arrested at various stages from 1-cell to 32-cell. Upper and lower traces give potential and current recordings respectively. For 1-cell, 2-cell and 4-cell embryos, a slight steady hyperpolarizing current was applied to keep membrane potential more negative than −70 mV. For explanation of percentages see text.

The uncertainty in anteroposterior direction in the arrested 8-cell embryo made it difficult to distinguish $A_{4\text{-}1}$ from $B_{4\text{-}1}$ because both $A_{4\text{-}1}$ and $B_{4\text{-}1}$ are large blastomeres of similar size in the vegetal hemisphere. Assuming that only $B_{4\text{-}1}$ could differentiate to a cell of muscular type, the $A_{4\text{-}1}$ blastomeres seem either to become a non-excitable cell, or to be coupled with the animal hemisphere showing the action potential of epidermal type. In one embryo, the dislocation of blastomeres was small and the anterior small blastomere in the animal hemisphere, i.e. $a_{4\text{-}2}$, developed a neural-type action potential, while the posterior large blastomere in the vegetal hemisphere, i.e. $B_{4\text{-}1}$, generated a spike potential of muscular type.

In Fig. 4*A*, membrane currents at four or five levels of membrane potential in Na–Sr solution are shown for four types of blastomeres in the cleavage-arrested 8-cell embryo. In the neural-type blastomere (one of two $a_{4\text{-}2}$ blastomeres) inward current was clearly shown at −19, 0 and +18 mV, and a long-lasting tail appeared at −19

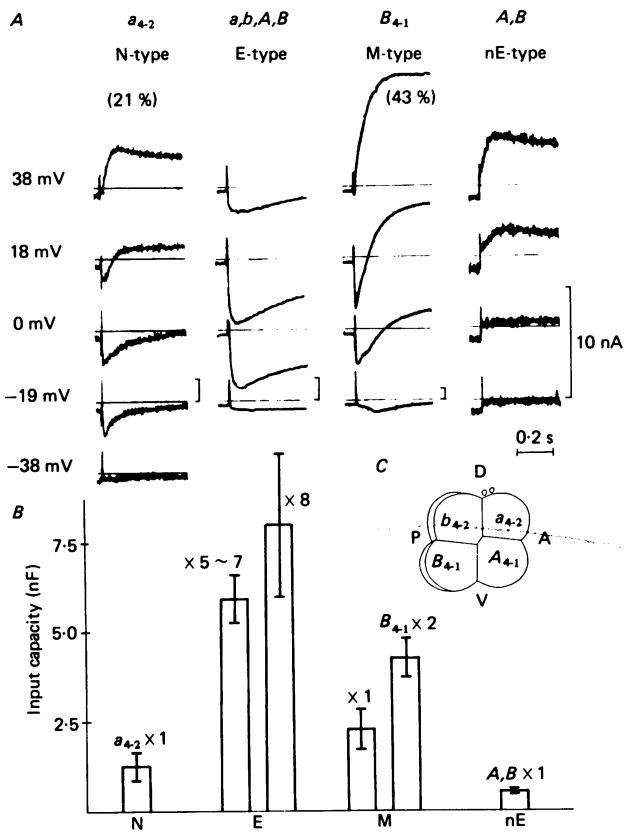


Fig. 4. Types of differentiation in the cleavage-arrested 8-cell embryo. *A*, membrane currents obtained under voltage-clamp conditions in Na–Sr solution. A neural-type (N-type) response was obtained in an electrically isolated blastomere located in the animal hemisphere with an input capacity of 1·37 nF. An epidermal-type (E-type) response was obtained in an embryo in which all eight blastomeres were coupled together, with an input capacity of 7·20 nF. A muscular-type (M-type) response was obtained from a large blastomere in the vegetal hemisphere, input capacity 2·37 nF. A non-excitable-type (nE-type) response was obtained from a spherical blastomere in the vegetal hemisphere, input capacity 0·65 nF. Time scale is given at the lower end of non-excitable-type traces. Current scales at bottom of each column of traces indicate 10 nA. *B*, input capacity of blastomeres. Neural type: twelve embryos. Epidermal type: seven totally coupled 8-cell embryos and twenty-nine partially coupled embryos (five to seven coupled blastomeres). Muscular type: seventeen embryos in which a single $B_{4\cdot1}$ differentiated and four in which both $B_{4\cdot1}$ blastomeres differentiated. Non-excitable type: thirteen electrically isolated blastomeres in the vegetal hemisphere. Columns and bars indicate mean ± S.D. *C*, diagram of 8-cell embryo (lateral view). D, V, A and P indicate dorsal, ventral, anterior and posterior.

and 0 mV. The initial fast inward current was Na current and the tail was due to Sr current, as will be described later. Beyond 0 mV a delayed outward current was clearly observed. The input capacity was 1·26 ± 0·40 nF (n = 12) and the examination of electrotonic spread showed that the differentiated $a_{4\cdot2}$ blastomere in the 8-cell embryo was uncoupled from its neighbours except for the case in which two $a_{4\cdot2}$

blastomeres differentiated into neural type. Comparison of the capacity value with that of the electrically isolated inexcitable large blastomeres $A_{4\text{-}1}$ or $B_{4\text{-}1}$ ($0{\cdot}53 \pm 0{\cdot}07$ nF; $n = 13$) indicates that differentiation to a cell of neural type is accompanied by an enlargement of input capacity.

Fig. 4*A* (E-type) illustrates inward current of slow rise and slow decay in an embryo in which all eight blastomeres were coupled together and developed an epidermal-type action potential. Recording and current electrodes were separately inserted into two small blastomeres in the animal hemisphere. Delayed rectification was hardly observed in Na–Sr solution. The range of electrotonic coupling of the blastomeres of epidermal type was not confined within the animal hemisphere but included also some blastomeres in the vegetal hemisphere. When all blastomeres in an embryo were coupled together and generated an action potential of epidermal type, the input capacity was $8{\cdot}04 \pm 2{\cdot}00$ nF ($n = 5$). When one to three large blastomeres were separated from epidermal-type blastomeres, the input capacity was $5{\cdot}93 \pm 0{\cdot}68$ nF ($n = 29$), as shown in Fig. 4*B*.

When $B_{4\text{-}1}$ differentiated to a cell of muscular type, it showed an inward current with threshold potential of -30 mV and a large delayed outward current (Fig. 4*A*, M-type). The input capacity was $2{\cdot}28 \pm 0{\cdot}56$ nF ($n = 17$) when only one $B_{4\text{-}1}$ differentiated to a cell of muscular type and $4{\cdot}27 \pm 0{\cdot}54$ nF ($n = 4$) when two $B_{4\text{-}1}$ blastomeres differentiated and were coupled with each other.

When the electrically isolated large blastomere, either $A_{4\text{-}1}$ or $B_{4\text{-}1}$, was non-excitable, the outward current increased slightly more than would be expected for a linear current–voltage (I–V) relation (Fig. 4*A*, E-type); there was slight outward rectification. However, this was small compared with that found in the muscular or neural type blastomeres (note different scales for currents in Fig. 4*A*). The input capacity of the non-excitable blastomeres was $0{\cdot}53 \pm 0{\cdot}07$ nF ($n = 13$).

Types of electrical excitability in the cleavage-arrested 32-cell embryo. The above-mentioned three types of action potential were also found in the cleavage-arrested 32-cell embryo in Na–Sr solution. The epidermal-type action potential was always found in blastomeres in the animal hemisphere (7/7). In 60% of embryos examined (6/10) the neural-type action potential was found in either $a_{6\text{-}5}$ or $a_{6\text{-}7}$, which was the descendent of $a_{5\text{-}3}$ or $a_{5\text{-}4}$ in the 16-cell embryo. The spike potential of muscular type was always found in the $B_{6\text{-}2}$ blastomere, which was the descendent of $B_{5\text{-}1}$ in the 16-cell embryo (5/5).

Electrical excitability in the differentiated cleavage-arrested 1-cell, 2-cell and 4-cell embryo. As described above, action potential of epidermal, neural and muscular type were commonly found in 8-cell, 16-cell and 32-cell embryos (Fig. 3). However, in cleavage-arrested 1-cell, 2-cell and 4-cell embryos (59, 6, and 9 respectively) all the blastomeres developed epidermal-type action potentials (Fig. 3). As will be described later, the membrane current in the uncleaved but differentiated 1-cell embryo under voltage clamp was found to be identical with that in the blastomeres of epidermal type in 8-cell or 16-cell embryos. The blastomeres in 2-cell or 4-cell embryos were coupled to each other, so that the whole embryo behaved electrically as a single cell. The capacity of the uncleaved and differentiated 1-cell embryo was $2{\cdot}50 \pm 0{\cdot}58$ nF ($n = 33$), which was about 1·5 times greater than that of the unfertilized egg ($1{\cdot}73 \pm 0{\cdot}13$ nF; $n = 6$). Therefore, an increase in the capacity accompanied differen-

tiation of epidermal type. It was previously reported that fertilization or activation itself does not increase capacity in the ascidian egg (Kozuka & Takahashi, 1982). Acetylcholinesterase staining was never positive in the 1-cell (18), 2-cell (48) or 4-cell (18) embryos, in agreement with Satoh (1979). Plate 1*D* shows lack of staining for acetylcholinesterase in a 1-cell embryo.

Comparison of properties of ion channels in neural-, muscular- and epidermal-type blastomeres with those in the egg

Neural-type responses. Membrane currents recorded under voltage clamp for the $a_{5\text{-}3}$ or $a_{5\text{-}4}$ blastomeres, which gave neural-type action potentials in the cleavage-arrested 16-cell embryo, are shown in Fig. 5. Fast and slow components of the inward current existed as in the egg cell membrane (Okamoto *et al.* 1976*b*). Replacement of 100 mM-Sr with 15 mM-Mn and 50 mM-Mg abolished the slow component while the threshold potential of the fast component was unchanged. On replacement of Na with equimolar choline the fast component was abolished and the slow component remained unchanged. Replacement of Sr with equimolar Ca retained the slow component (not shown). In choline–Mn solution both components were abolished and only the outward current remained. Thus, as in the egg cell membrane, the fast and slow components were identified as Na and Sr currents through Na and Ca channels respectively.

In Fig. 6*B*, the *I–V* relations for both Na and Sr current of neural type are illustrated. The threshold potential for Na current in 16-cell embryos was −35 to −30 mV in both Na–Sr and Na–Mn solutions and 15–20 mV more positive than that in the egg (−50 mV: Fig. 6*A*). The potential level for the maximum peak inward current (V_p) and that for half-maximum ($V_{\frac{1}{2}}$) were also shifted in the positive direction by 15–20 mV. The $V_{\frac{1}{2}}$ and V_p values for eggs and 16-cell embryos are listed in Table 2. The kinetic properties and voltage dependence of Na current were essentially identical with those of the egg except for the positive shift of the voltage dependence (Fig. 5, Na−Mn; Table 2). In the case of blastomere $a_{4\text{-}2}$ of the 8-cell embryo, which differentiated to a cell of neural type, the shift of the potential dependence was 5–10 mV less than that in $a_{5\text{-}3}$ or $a_{5\text{-}4}$ of 16-cell embryos (Table 2, 8-cell stage). By contrast, the threshold potential for Sr current in the blastomere of neural type was shifted in the negative direction by about 10 mV compared with that in the egg (see Fig. 11*C*, egg-type; Table 2).

The delayed outward current existed in Na–Sr, Na–Mn, choline–Mn and possibly choline–Sr solutions, but it might be slightly suppressed in choline–Mn solution (Fig. 5). In Na–Mn solution the outward current was analysed by measuring the reversal potential in 10 mM and 100 mM-extracellular K (Fig. 7*B*). Dashed lines indicate *I–V* relations for the outward current at the steady state or the peak amplitude, when currents showed slow inactivation (Fig. 7*A*), in 10 mM-K (○) and 100 mM-K (△). The threshold potential for activation of the outward current was −10 mV in both 10 mM-K and 100 mM-K solutions. This relatively positive level for activation was partly responsible for the plateau in the neural-type action potential (Fig. 1*A*). Continuous lines indicate instantaneous *I–V* relations measured by applying test pulses immediately after a conditioning depolarization to +30 mV for 150 ms. Na

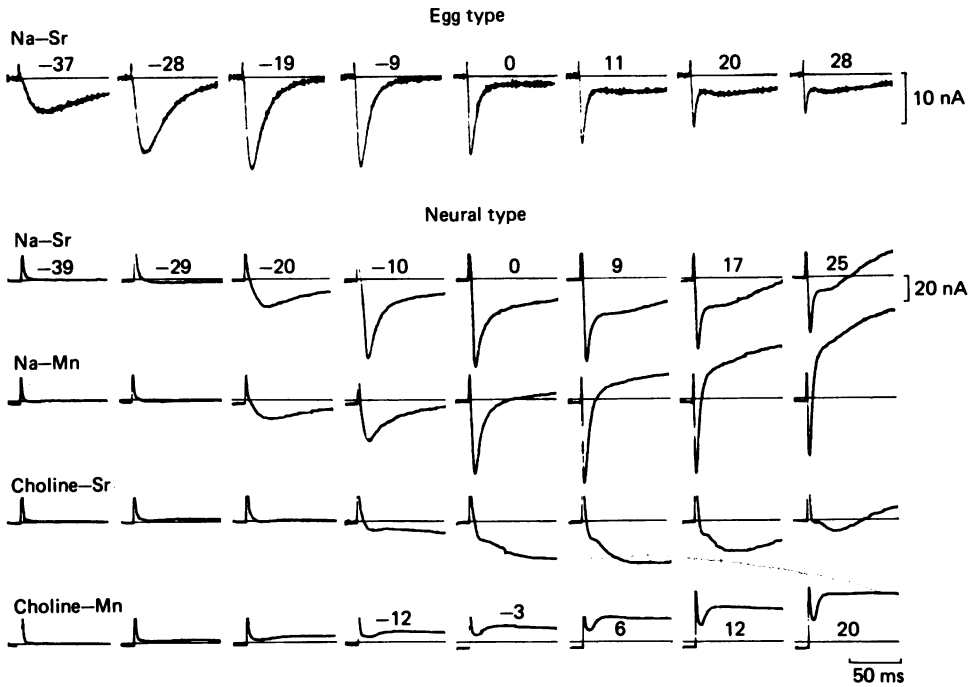


Fig. 5. The current traces in the top line were obtained from an unfertilized egg in Na–Sr solution for comparison (current scale at right side) with the traces from blastomeres. The other current traces were from an $a_{5\cdot3}$ blastomere of neural type (current scale at the right of the second line) in four different solutions. The embryo was the same as that illustrated in Fig. 1*A*. The $a_{5\cdot3}$ was coupled to the ipsilateral $a_{5\cdot4}$ and contralateral $a_{5\cdot3}$ and had an input capacity of 2·55 nF. Small figures on traces in this and subsequent Figures give membrane potential during command pulse. Thin horizontal line on each trace gives zero current level in this and successive Figures. Time scale for all traces is given in the right-hand lower corner.

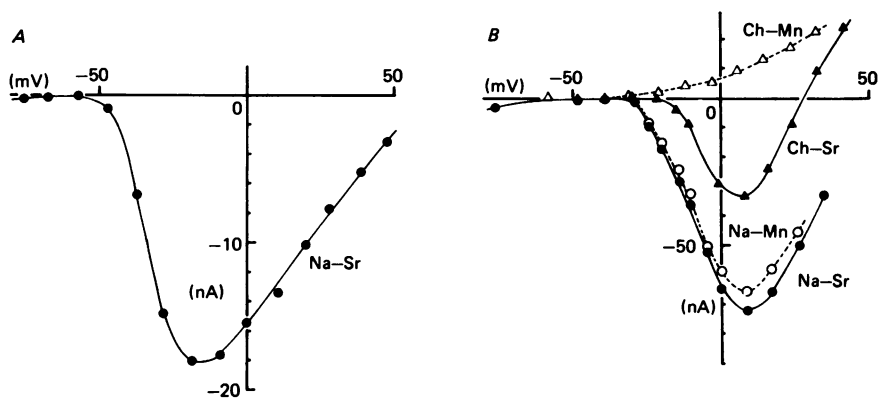


Fig. 6. *A*, egg-type *I–V* relation for Na channels taken from data of Fig. 5 (egg-type, Na–Sr). *B*, *I–V* relations for a blastomere of neural type at the peak of inward current in Na–Sr, Na–Mn, choline–Sr and choline–Mn solutions, also from data in Fig. 5.

current was completely inactivated when the test pulse was applied. The outward current reversal potential was −75 mV in an external K concentration of 10 mM and shifted to −33 mV in 100 mM-K, i.e. 42 mV per tenfold change in K concentration. Although this shift is smaller than that expected from the Nernst equation for a pure K electrode, it is clear that K ions are the main ions contributing to the outward current. Thus, the existence of K channels was confirmed. These K channels were mostly delayed K rectifier channels as commonly observed in other neurones, but in

TABLE 2. Voltage-dependence of Na and Sr currents*

Cell type	Stage of cleavage arrest	V_i (mV)	V_p (mV)	Number of sample
		Na current		
Egg cell		−35·4±2·3†	−17·9±2·4	12
Neural type				
	8-cell stage ($a_{4\text{-}2}$)	−27·5±2·3	−8·5±3·2	4
	16-cell stage ($a_{5\text{-}3,4}$)	−19·9±2·2	−1·7±3·1	7
		Sr current		
Egg cell		−0·8±2·0	15·3±2·4	12
Neural type				
	8-cell stage	—	—	—
	16-cell stage‡	−7·0	9·0	1
Epidermal type				
	1-cell stage	−9·0±2·4	10·7±2·8	27
	2- and 4-cell stage	−7·7±2·7	14·5±2·5	6
	8-cell stage	−9·4±2·2	9·5±2·7	4
	16-cell stage	−10·6±3·0	6·8±2·5	5
Muscle type				
	8-cell stage ($B_{4\text{-}1}$)	−5·6±3·0	14·3±2·9	8
	16-cell stage ($B_{5\text{-}1}$)	−7·0±3·2	12·9±3·8	11

* All values measured in the standard solution of Na–Sr, except for ‡ (see below).
† The values are mean ±S.D.
‡ The values were measured in the Sr–choline solution.

addition the existence of Ca-induced K channels was suggested by the enhancement of outward current in Na–Ca solution (not illustrated). As shown by the I–V relation for the steady state in 100 mM-K, marked anomalous rectification was found with hyperpolarization beyond the reversal level. An increase in the number of anomalous K rectifier channels has been reported during development of excitability in the cleavage-arrested 16-cell embryo (Takahashi & Yoshii, 1981).

Muscular-type responses. Muscular-type action potentials were observed in the $B_{4\text{-}1}$ blastomeres in the 8-cell embryo and the $B_{5\text{-}1}$ blastomeres in the 16-cell embryo. Membrane currents in the $B_{4\text{-}1}$ blastomere of the vegetal hemisphere in the 8-cell embryo are illustrated in Fig. 8*A*. Both inward and marked delayed outward currents existed. The I–V relations at the peak of inward current (continuous lines) and at the steady state or the peak of outward current (dashed lines) are plotted in Fig. 8*B*. In the records shown, the threshold potentials for inward and outward currents were −30 and −25 mV respectively, and in other embryos they ranged from −30 to −20 mV. The maximum peak amplitude for the inward current was obtained

at +15 mV (V_p). The mean and S.D. values of $V_{\frac{1}{2}}$ and V_p for $B_{4\text{-}1}$ and $B_{5\text{-}1}$ are listed in Table 2. The maximum inward current ranged from 30 to 100 nA. Replacement of 100 mM-Sr with 15 mM-Mn and 50 mM-Mg abolished the inward current completely, while it left the outward current with a slightly positively shifted threshold potential and a decrease in the slope conductance (Fig. 8*A* and *B*). The inward current remained after replacement of Sr with Ca, though it showed a gradual reduction after a few minutes. Inward current, therefore, probably flows through Ca channels, with

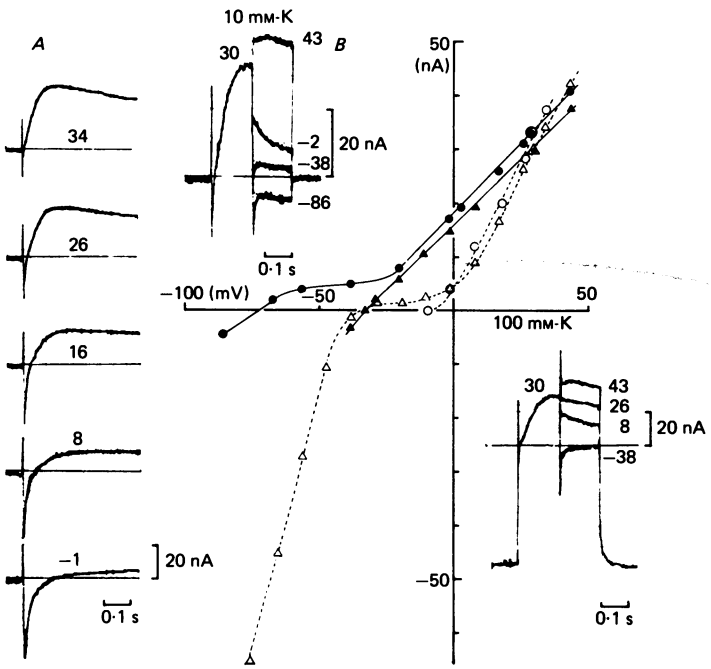


Fig. 7. *A*, outward currents in 10 mM-K Na–Mn solution obtained from an $a_{5\text{-}3}$ or $a_{5\text{-}4}$ blastomere which differentiated to neural type, with input capacity of 4·55 nF. *B*, the steady-state *I–V* relations (dashed lines) and instantaneous *I–V* relations (continuous lines) after a 150 ms, +30 mV conditioning pulse in 10 mM-K (○) and 100 mM-K (△) Na–Mn solutions. Data from the same blastomere as shown in *A*. Insets show the membrane currents in response to double-step pulses.

the same potential dependence as in blastomeres of neural type or epidermal type. The threshold potential for the outward current in 15 mM-Mn solution was about −25 mV, as in Sr solution (Fig. 8*B*). An increase in K concentration from 10 to 100 mM shifted the reversal potential of the outward current from −70 to −30 mV (Fig. 9). The outward current was suppressed when tetraethylammonium (TEA) was substituted for Na in the Na–Sr solution, while the long-lasting inward Sr current remained (Fig. 10). The intracellular injection of Cs abolished almost completely the outward current in TEA-Sr solution, as reported in the following paper (Hirano & Takahashi, 1984). Thus, it was concluded that the outward current flowed through

delayed K rectifier channels. In addition, there must be Ca-induced K channels because replacement of Sr with Mn decreased the slope conductance of the steady-state *I–V* curve (Fig. 8*B*) and replacement of Sr with Ca increased the outward current (not illustrated). Anomalous K rectifier channels with activation and inactivation kinetics were also found (Fig. 8*C*).

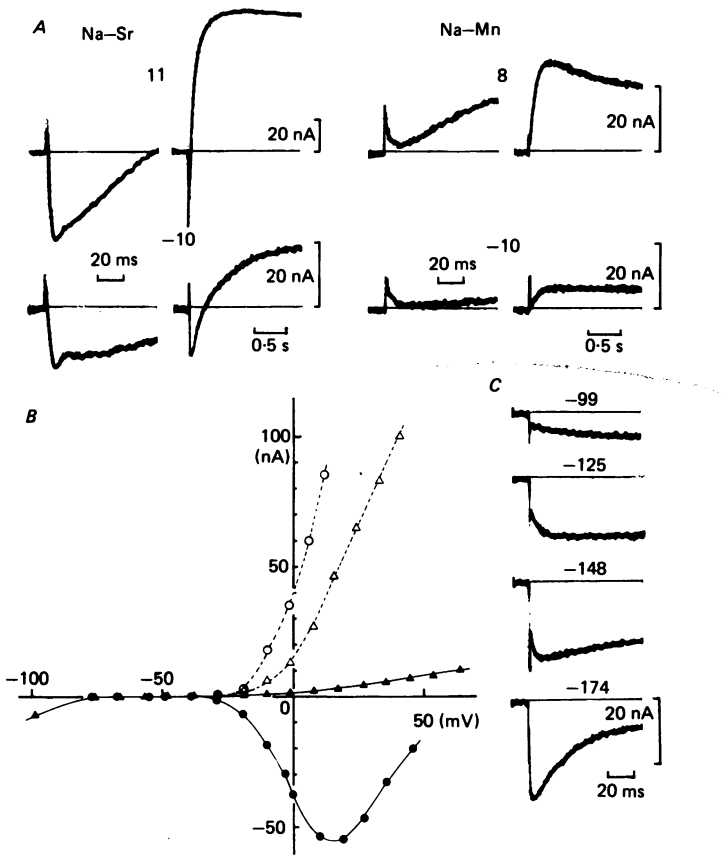


Fig. 8. Membrane currents in a blastomere of muscular type. *A*, left two pairs of traces, Na–Sr solution; right two pairs of traces, Na–Mn solution. Left and right traces in each pair were recorded at fast and slow sweeps respectively. This large blastomere $B_{4\cdot1}$ lay in the vegetal hemisphere of a cleavage-arrested 8-cell embryo, and was electrically isolated with an input capacity of 1·89 nF. *B*, *I–V* relations at the peak of inward current (filled symbols) and at steady state or the peak of outward current (open symbols) in Na–Sr (circles) and Na–Mn (triangles) solutions. Same blastomere as in *A*. *C*, anomalous rectification in the same blastomere as shown in *A*. Four traces give membrane currents at four levels of potential in Na–Mn solution.

Epidermal-type responses. An epidermal-type action potential was found in some blastomeres of the 8-cell and 16-cell embryos and in the 1-cell, 2-cell and 4-cell embryos. Membrane current was most easily analysed in the uncleaved but differentiated 1-cell embryo. The inward current in the 1-cell embryo was immediately abolished by replacement of Sr with Mn plus Mg (Fig. 11*A*, Na–Mn). The remaining

outward current was almost instantaneous and was probably mostly leakage current. The inward current was markedly suppressed by 2 mM-La (Fig. 11*B*), as found for other Ca-channel currents (Hagiwara & Byerly, 1981). Inward current remained after replacement of Sr with Ca concomitantly with a marked increase in outward current. Thus, it was concluded that the inward current flowed through Ca channels. However, the Sr current through the Ca channel had a different time course for that in the egg.

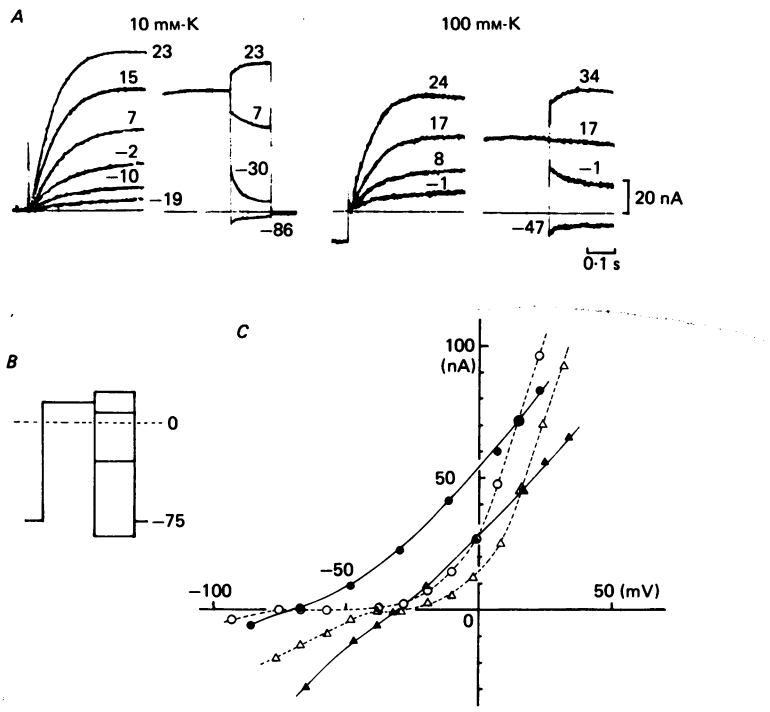


Fig. 9. *A*, two sets of outward currents in bilaterally coupled $B_{5\text{-}1}$ blastomeres which differentiated to muscular type cells (input capacity, 2·17 nF). Left and right traces in each set had single- and double-step pulses respectively. The conditioning pulses were +15 mV, 800 ms and +17 mV, 800 ms in 10 mM-K and 100 mM-K respectively. *B*, schematic illustration of double-step command pulses. *C*, *I–V* relations of outward current at the steady state (open symbols and dashed lines) or instantaneous *I–V* relations after 800 ms conditioning pulses (filled symbols and continuous lines) to +15 and +17 mV in 10 mM-K (circles) and 100 mM-K (triangles) solutions respectively. Double circle and triangle indicate the potentials of conditioning pulses. Same blastomere as in *A*.

The decay time course was more than four times as slow as that in the egg at comparable potential levels (Fig. 11*A*; note different time scales for the egg-type and epidermal-type traces). The *I–V* relation for the Sr current had a threshold potential of about −25 mV and a maximum inward current at +10 to +15 mV. The means and S.D. values of $V_{\frac{1}{2}}$ and V_p for epidermal-type blastomeres in 1-cell, 2-cell, 4-cell, 8-cell and 16-cell embryos are listed in Table 2. The $V_{\frac{1}{2}}$'s were almost in the same range as those for Ca channels of neural and muscular type (Fig. 7*C*, epidermal-type), and were more negative by about 10 mV than that in the egg membrane (Table 2).

As shown in Fig. 11*C* the steady-state *I–V* relation of the blastomeres of epidermal type in Na–Mn solution was N-shaped, having a maximum at −45 mV and a minimum at 0 mV. An increase in outward current above 0 mV may partly be attributed to the time-dependent outward current, but this was much smaller than that in blastomeres of neural or muscular type. The non-linearity below 0 mV was mainly due to the anomalous K rectifier channels in the blastomere of epidermal type. The current through anomalous K rectifier channels was markedly enhanced in Na–Mn solution as compared with that in Sr solution, because of slower inactivation in Na–Mn solution, as found in the egg membrane (Ohmori, 1978).

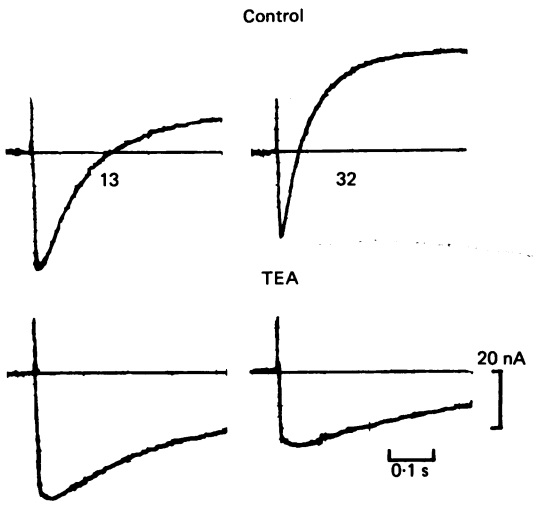


Fig. 10. Effect of replacement of Na in Na–Sr solution with tetraethylammonium (TEA) upon the outward current of bilaterally coupled $B_{4\text{-}1}$ blastomeres which differentiated to muscular-type cells (input capacity 4·84 nF). Left pair of traces, voltage steps to +13 mV; right pair, +32 mV.

Inhibition of differentiation by Ca ionophore. As described above, the $B_{5\text{-}1}$ blastomere in the cleavage-arrested 16-cell embryo always differentiated into a cell of muscular type. When the Ca ionophore A23187 (20 μM) was added to the culture sea water after the late neural stage of the control embryo (32 h at 8 °C), the differentiation of $B_{5\text{-}1}$ was completely inhibited. Under voltage clamp the membrane current was very similar to that of non-excitable cells, as shown in Fig. 12*A*. The input capacity was $0{\cdot}45 \pm 0{\cdot}04$ nF ($n = 4$) and examination of electrotonic spread revealed that the blastomere was electrically isolated. The value was only one-third of that of a single differentiated $B_{5\text{-}1}$ blastomere (Fig. 12*B*). Acetylcholinesterase was not stained in $B_{5\text{-}1}$ in 91 % of A23187-treated embryos (10/11), while that of the control embryo was stained in both $B_{5\text{-}1}$ blastomeres (96 %, 24/25) (Fig. 12*C*). Other blastomeres, such as $A_{5\text{-}1}$, $a_{5\text{-}4}$ and *b* group, were also examined in A23187-treated embryos and there was no sign of differentiation into excitable cells. Although the site of the drug action has not been analysed yet, it is interesting to infer that the excess of intracellular Ca ions produced by A23187 can induce a general inhibition of cellular differentiation at a critical period during development.

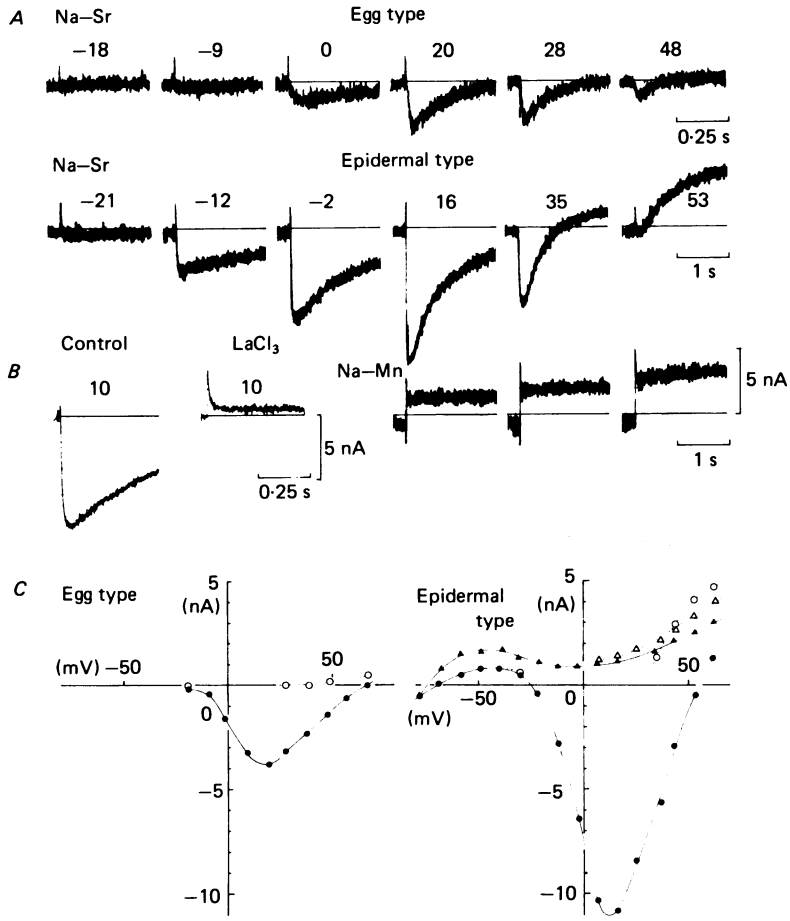


Fig. 11. Membrane currents in the differentiated cleavage-arrested cell embryo. *A*, top traces show inward currents of an unfertilized egg, after a 1 s, −39 mV conditioning pulse to inactivate Na current. The isolated Sr current through Ca channels was recorded. Middle traces are currents in Na–Sr solution, and bottom traces those in Na–Mn solutions obtained from a 1-cell embryo differentiated to an epidermal-type cell. *B*, effect of 2 mM-$LaCl_3$ upon Sr current of the 1-cell embryo. Left, control; right, Na–Sr solutions containing 2 mM-$LaCl_3$. *C*, *I–V* relations of the peak inward current in the same egg (left) and 1-cell embryo (right) as shown in *A*. For the 1-cell embryo, currents in Na–Sr and Na–Mn solutions are illustrated by circles and triangles respectively. Dashed lines and open symbols were for the peak of outward currents.

DISCUSSION

In the present experiments, blastomeres of ascidian embryos, whose cleavage had been arrested at the 1- to 32-cell stages showed four types of differentiation with respect to membrane excitability: epidermal, neural, muscular and non-excitable. These types of differentiation were characterized by the species of ion channels existing in the cell membranes of the respective blastomeres, as summarized in Table

3. Additional means for determination of cell type were histochemical staining of acetylcholinesterase and myofibrils observed with the electron microscope for muscle cells, and a thin transparent sheet of tunica for epidermal cells. An increase in the input capacity indicates surface expansion and/or an increase in the specific

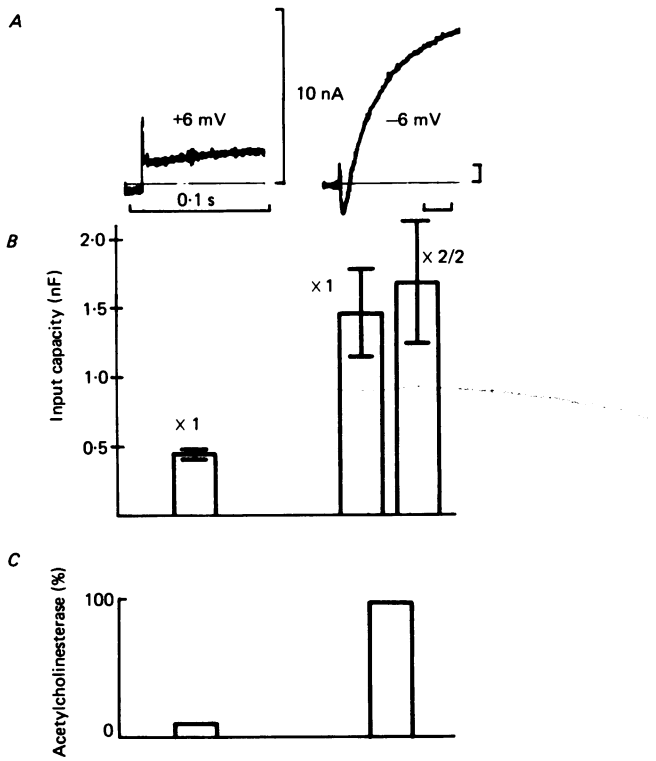


Fig. 12. Effects of 20 μM-A23187 upon the differentiation of the $B_{5\text{-}1}$ blastomere. *A* (left), membrane current at 6 mV of a $B_{5\text{-}1}$ blastomere in a cleavage-arrested 16-cell embryo which was kept in sea water containing both A23187 and cytochalasin B from the late neurula stage of the control larvae (32 h at 8 °C) and examined in standard Na–Sr solution (without A23187) at the developmental time equivalent to hatching of the control larvae (75 h at 8 °C). The blastomere was electrically isolated and had an input capacity of 0·47 nF. *A* (right), membrane current of a single $B_{5\text{-}1}$ blastomere differentiated to a cell of muscle type, input capacity 1·68 nF. *B*, means and S.D. values of the input capacity of four A23187-treated $B_{5\text{-}1}$ blastomeres (left column), seven isolated $B_{5\text{-}1}$ blastomeres which differentiated to cells of muscular type (centre column), and half of mean and S.D. of the input capacity of eighteen bilaterally coupled $B_{5\text{-}1}$ blastomeres which differentiated to cells of muscular type (right column). *C*, percentages of acetylcholinesterase staining in A23187-treated (1/11) and control 16-cell embryos (24/25).

membrane capacity when the blastomere is isolated, or it corresponds to the electrotonic spread among blastomeres when electrical couplings exist. If we assume that the specific membrane capacity is invariable during development, the surface expansion is one of the characteristics of muscular-type blastomeres. An increase in input capacity by electrotonic coupling was always observed in epidermal-type blastomeres.

In addition to being found in blastomeres which differentiated to excitable cells, Ca channels also exist in the membrane of the unfertilized egg. However, the Ca channels in the differentiated epidermal-type blastomeres were activated at more negative potentials than those in the egg membrane and the current decayed more slowly after a voltage step. In the following paper (Hirano & Takahashi, 1984) it is shown that the ion selectivity and inactivation processes of Ca channels in differentiated excitable blastomeres are qualitatively different from those of egg Ca channels. The Na channels in differentiated neural-type blastomeres were similar to egg Na channels with respect to their kinetics, but the threshold potential for activation was 10–20 mV more positive than in the egg.

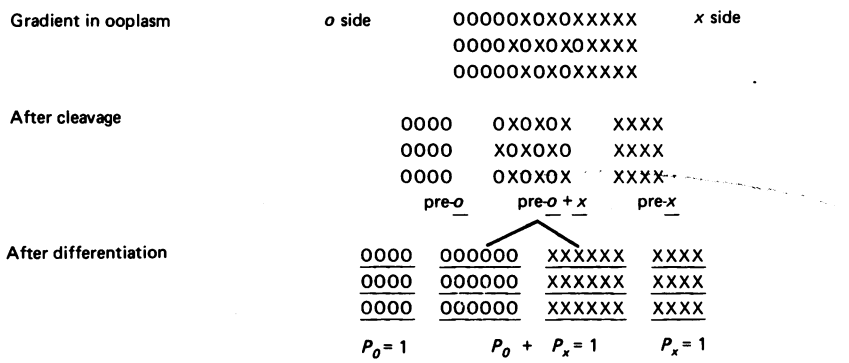


Fig. 13. Model of determination of differentiation. For explanation see text. The symbols ○ and × indicate the cytoplasmic factors for differentiation to *o* and *x* type. P_o and P_x are the probabilities for differentiation to *o* and *x* types respectively.

In the cleavage-arrested embryo the developmental fate of blastomeres might not be determined uniquely, because cytoplasmic segregation is not complete before the 64-cell stage (Conklin, 1905). The potential fates of blastomeres in cleavage-arrested embryos of between 1- and 16-cells are given in Table 4. The cleavage-arrested 1-cell embryo always differentiated into the epidermal type. In the 8-cell embryo, $a_{4\text{-}2}$ blastomeres of the animal hemisphere have the potential to become both brain vesicle and epidermis (Conklin, 1905; Reverberi, 1961). However, the differentiated membrane excitability was not a mosaic of neural and epidermal types, but the blastomere became either a cell of neural type or a cell of epidermal type. Similarly the $a_{5\text{-}3,4}$ blastomeres in the cleavage-arrested 16-cell embryo became cells of either neural or epidermal type. The $B_{4\text{-}1}$ blastomere of the animal hemisphere in the 8-cell embryo is the ancestor of presumptive regions for muscle, mesenchyme and endoderm. This cell differentiated into a muscular, non-excitable or epidermal-type cell. The $B_{5\text{-}1}$ of the cleavage-arrested 16-cell embryo always developed membrane excitability of the muscular type, despite having the potential to form endoderm, mesenchyme and muscle. In conclusion, so far as the membrane excitability is concerned, a single type of differentiation appears in each blastomere after cleavage arrest and no indication of mosaic properties was found.

The probability of a particular type of differentiation seemed to be intrinsic to each blastomere according to the stages of cleavage arrest. The $B_{5\text{-}1}$ blastomere in the cleavage-arrested 16-cell embryo differentiated into a cell of muscular type with a probability of 97%. One $B_{4\text{-}1}$ blastomere of the cleavage-arrested 8-cell embryo differentiated to muscular type in twenty out of fifty-eight embryos (34·5%) and both $B_{4\text{-}1}$ blastomeres in an embryo differentiated simultaneously to cells of muscular

TABLE 3. Identification of cell type

	Egg	Epidermal type	Neural type	Muscular type	Non-excitable type
Channel					
Egg Na channel*	++	+−	−	−	−
Diff. Na channel	−	−	+++	−	−
Egg Ca channel	++	−	−	−	−
Diff. Ca channel†	−	+++	++‡	+++	−
Delayed K channel	?	−	++	+++	+
Anomalous K channel	+	+++	++	++	+
Ca-dependent K channel	−	++	+	+	−
Histochemical					
Acetylcholinesterase	−	−	−	+++	−
Microscopical					
Myofilaments§	−	−	−	+++	−
Tunica	−	++	?	−	−
Surface area					
Increase¶	−	+	++	+++	−

* The shift of potential dependence distinguished differentiated (diff.) Na and Ca channels from egg Na and Ca channels.

† The ion selectivity and inactivation mechanism distinguished differentiated Ca channels from egg Ca channels (Hirano & Takahashi, 1983).

‡ The type of the Ca channel was not determined with respect to selectivity and inactivation.

§ Observation with electron microscope.

¶ Estimated by the measurement of input capacity, assuming the specific capacity to be constant.

type in five cases (8·5%). In the other thirty-three cases (57%), neither of the $B_{4\text{-}1}$ blastomeres differentiated into a cell of muscular type. This distribution of cell types in an embryo can be fitted by the binomial distribution

$$p[x] = [n!/x!(n-x)!]p^x(1-p)^{n-x},$$

where n is the number of the blastomeres in an embryo and p is the probability that a blastomere differentiates to a cell of particular type. For the $B_{4\text{-}1}$ blastomeres, n was 2 and p was estimated by $(20+2\times5)/2\times58 = 0{\cdot}259$. Thus, the equation becomes

$$p[x] = [2!/x!(2-x)!](0{\cdot}259)^x(1-0{\cdot}259)^{2-x}.$$

The binomial distribution predicted $p[2] = 0{\cdot}07$, $p[1] = 0{\cdot}38$ and $p[0] = 0{\cdot}55$ for two, one and no blastomere of muscular type in an embryo respectively. For $a_{4\text{-}2}$ in the 8-cell embryo, two, one and no blastomere of neural type were observed in an embryo in one (2%), thirteen (20%) and fifty-two (78%) cases out of sixty-six embryos

examined. The binomial distribution of ($n = 2, p = 0{\cdot}114$) predicted one, thirteen and fifty-two cases respectively. This suggests that the probability of a blastomere expressing a particular type of differentiation is independent of the states of differentiation of other blastomeres. If similar independence and no difference of potential fates are assumed between $a_{5\text{-}3}$ and $a_{5\text{-}4}$ blastomeres in the cleavage-arrested 16-cell embryo, the probability of differentiation to a cell of neural type can be roughly estimated. Since 41 % of embryos examined did not have any neural-type blastomere, as described in the Results, $p[0; n = 4] = (1-p)^4 = 0{\cdot}41$; thus $p = 0{\cdot}20$.

TABLE 4. Differentiation of blastomeres of cleavage-arrested embryos

Embryo	Blastomere*	Developmental fate	Differentiation†
1-cell	AB_1	All kinds of cell	E
2-cell	AB_2	All kinds of cell	E
4-cell	A_3	Neural plate, epidermis, notochord, endoderm	E
	B_3	Epidermis, endoderm, muscle, mesenchyme	E
8-cell	$a_{4\text{-}2}$ (A)‡	Neural plate (brain), epidermis	N or E
	$b_{4\text{-}2}$ (A)	Epidermis	E
	$A_{4\text{-}1}$ (V)	Neural plate (spinal), notocord, endoderm	nE or E
	$B_{4\text{-}1}$ (V)	Muscle, mesenchyme, endoderm	M or nE or E
16-cell	$a_{5\text{-}3,4}$ (A)	Neural plate (brain), epidermis	N or E
	$b_{5\text{-}3,4}$ (A)	Epidermis	E
	$A_{5\text{-}1,2}$ (V)	Neural plate (spinal), notochord, endoderm	nE
	$B_{5\text{-}1}$ (V)	Muscle, mesenchyme, endoderm	M
	$B_{5\text{-}2}$ (V)	Muscle, mesenchyme	nE

* Names of blastomeres according to Conklin (1905).

† Differentiation observed in the cleavage-arrested embryo. N, neural-type; E, epidermal-type; M, muscular-type; nE, non-excitable-type.

‡ A, animal hemisphere, V, vegetal hemisphere.

For both muscular- and neural-type differentiation the probability increases with advancing stage of the cleavage arrest.

A model for the determination of differentiation which is related to the segregation of cytoplasmic factors by cleavage may be deduced from the results of the present experiments, as shown in Fig. 13. Suppose that before the determination of differentiation the cytoplasmic factors are distributed in a gradient from *o* side to *x* side in a particular region of an ancestor blastomere, corresponding to the fate map for presumptive *o*- and *x*-type cells. After cleavage blastomeres on the *o* side can be purely presumptive *o* type and those on the *x* side can be purely presumptive *x* type, and blastomeres in the intermediate zone may have mixed fates. Such blastomeres in the intermediate zone, however, will differentiate to cells of either *o* type or *x* type with a fixed probability, and will never show the mosaic properties of *o* and *x* types.

Although this model fits the familiar concept of a gradient across the early embryo, we do not suggest that it is the whole story. Our results in 1-cell, 2-cell and 4-cell embryos suggest that there may be some kind of hierarchy among various potential fates, because in these embryos epidermal-type differentiation always resulted despite a wide range of potential fates. In the $B_{5\text{-}1}$ blastomere of the 16-cell embryo muscle-type differentiation is apparently preferentially expressed, whereas in the $B_{5\text{-}2}$ blastomere differentiation into a non-excitable type apparently dominates. For the $B_{4\text{-}1}$ blastomere of the 8-cell embryo epidermal-type differentiation sometimes occurs, despite this cell apparently not possessing the potential to become an epidermal cell. Further, there are some reservations about our all-or-none hypothesis for differentiation because the difference between the voltage dependence of Na channels in differentiated blastomeres and in the egg is apparently greater in the 16-cell than in the 8-cell cleavage-arrested embryo. Therefore, the detailed mechanism of differentiation in the cleavage-arrested embryo still remains to be clarified.

We would like to thank Professor Akira Takeuchi for his helpful comments on the manuscript. We acknowledge the Marine Biology Laboratory of Tohoku University at Asamushi, Miyagi Prefectural Fisheries Experimental Station at Kesennuma, Professor Keiji Suzuki in the Department of Fisheries of Kitazato University at Sanriku, and Wakkanai City Aquarium for collecting materials. This investigation was supported by a grant in aid for scientific research from the Ministry of Education, Science and Culture, Japan, and a grant from the National Center for Nervous, Mental and Muscular Disorders (N.C.N.M.M.D.) of the Ministry of Health and Welfare, Japan.

REFERENCES

Conklin, E. G. (1905). The organization and cell-lineage of the ascidian egg. *J. Acad. nat. Sci. Philad.* **13**, 1–119.

Davidson, E. H. (1976). Localization of morphogenetic determinants in egg cytoplasm. In *Gene Activity in Early Development*, pp. 249–318. New York & London: Academic Press.

Hagiwara, S. & Byerly, L. (1981). Calcium channel. *A. Rev. Neurosci.* **4**, 69–125.

Hirai, E. (1941). An outline of the development of *Cynthia roretzi* Drasche. *Sci. Rep. Tohoku Imp. Univ., Biology* **16**, 217–232.

Hirano, T. & Takahashi, K. (1984). Comparison of properties of calcium channels between the differentiated 1-cell embryo and the egg cell of ascidians. *J. Physiol.* **347**, 327–344.

Karnovsky, M. J. & Roots, L. (1964). A 'direct-coloring' thiocholine method for cholinesterase. *J. Histochem. Cytochem.* **12**, 219–221.

Kozuka, M. & Takahashi, K. (1982). Changes in holding and ion-channel currents during activation of an ascidian egg under voltage clamp. *J. Physiol.* **323**, 267–286.

Miyazaki, S., Takahashi, K. & Tsuda, K. (1972). Calcium and sodium contributions to regenerative responses in the embryonic excitable cell membrane. *Science, N.Y.* **176**, 1441–1443.

Miyazaki, S., Takahashi, K. & Tsuda, K. (1974). Electrical excitability in the egg cell membrane of the tunicate. *J. Physiol.* **238**, 37–54.

Ohmori, H. (1978). Inactivation kinetics and steady-state current noise in the anomalous rectifier of tunicate egg cell membranes. *J. Physiol.* **281**, 77–99.

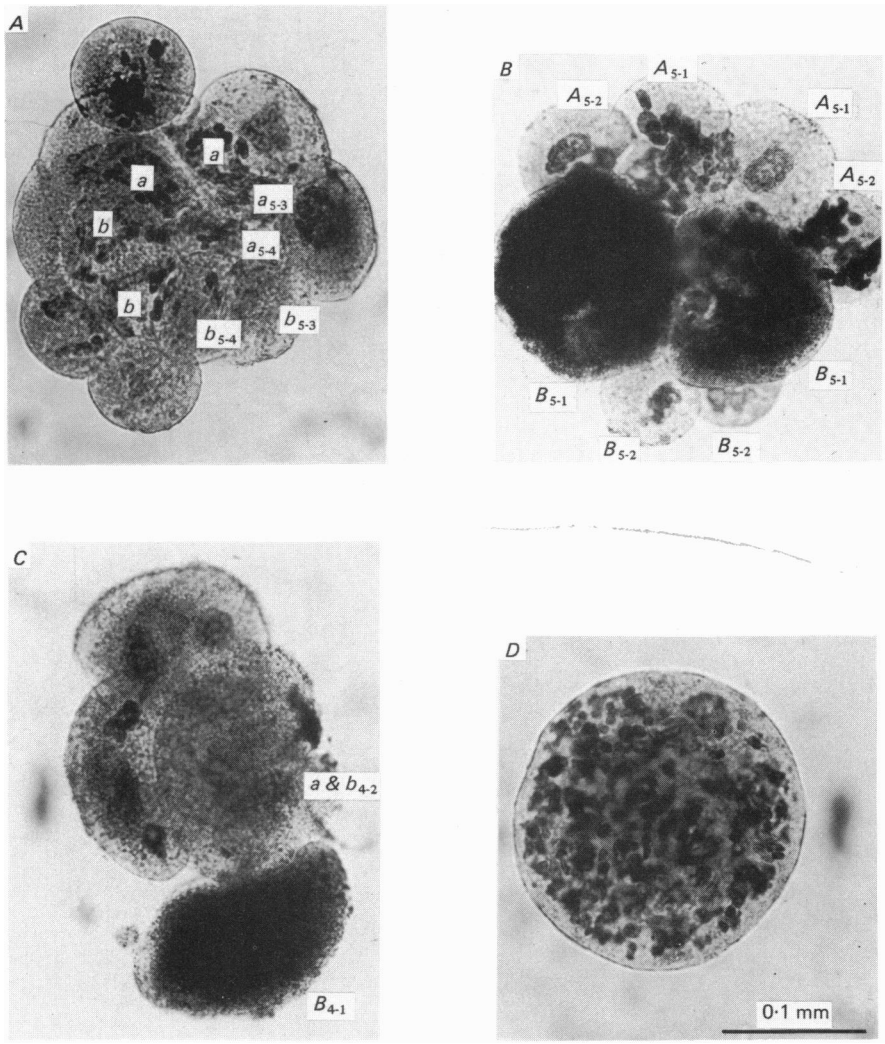
Ohmori, H. & Sasaki, S. (1977). Development of neuromuscular transmission in a larval tunicate. *J. Physiol.* **269**, 221–254.

Okamoto, H., Takahashi, K. & Yoshii, M. (1976*a*). Membrane currents of the tunicate egg under the voltage-clamp condition. *J. Physiol.* **254**, 607–638.

Okamoto, H., Takahashi, K. & Yoshii, M. (1976*b*). Two components of the calcium current in the egg cell membrane of the tunicate. *J. Physiol.* **255**, 527–561.

Reverberi, G. (1961). The embryology of ascidians. *Ad. Morphogen.* **1**, 55–101.

Satoh, N. (1979). On the 'clock' mechanism determining the time of tissue-specific enzyme


A
a
a
a5-3
b
a5-4
b
b5-4
b5-3
B
A5-1
A5-2
A5-1
A5-2
B5-1
B5-1
B5-2
B5-2
C
a & b4-2
B4-1
D
0·1 mm

development during ascidian embryogenesis. I. Acetylcholinesterase development in cleavage-arrested embryos. *J. Embryol. exp. Morph.* **54**, 131–139.

TAKAHASHI, K. & YOSHII, M. (1981). Development of sodium, calcium and potassium channels in the cleavage-arrested embryo of an ascidian. *J. Physiol.* **315**, 515–529.

WHITTAKER, J. R. (1973). Segregation during ascidian embryogenesis of egg cytoplasmic information for tissue-specific enzyme development. *Proc. natn. Acad. Sci. U.S.A.* **70**, 2096–2100.

EXPLANATION OF PLATE

Cleavage-arrested embryo and acetylcholinesterase staining. *A*, animal hemisphere of a cleavage-arrested 16-cell embryo after a developmental time equivalent to hatching of the control larva (75 h at 8 °C). After formalin fixation and dehydration, the embryo was cleared in xylene and embedded in balsam. Naming of blastomeres follows Conklin's (1905) and Satoh's (1979) descriptions. *B*, the vegetal hemisphere of another cleavage-arrested 16-cell embryo at similar developmental age to that in *A*. Acetylcholinesterase activity in both $B_{5\text{-}1}$ blastomeres was stained by the thiocholine method. *C*, a cleavage-arrested 8-cell embryo at the developmental time of hatching of controls (75 h at 8 °C). Acetylcholinesterase activity was demonstrated in a blastomere which developed the muscular-type spike potential in Na–Sr solution. *D*, a cleavage-arrested 1-cell embryo at the developmental time of hatching.