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TTX-Insensitive Sodium Current Develops in Adult Newt Pigment Epithelial Cells in Culture

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ABSTRACT—We cultured retinal pigment epithelial (RPE) cells dissociated from the adult newt eye and examined changes in the voltage-gated ionic currents with time in culture using whole-cell patch-clamp techniques. RPE cells allowed to adhere onto a concanavalin A-coated substrate began to flatten within 1–2 days and some of them started to divide on the 6th day of culturing. Freshly dissociated RPE cells exhibited only voltage-gated outward K^+ currents. Voltage-gated Na^+ currents associated with the initiation of immature action potentials were expressed in more than 50% of sufficiently stretched RPE cells before the first mitosis was observed. This fact may suggest that its expression is not coupled with the cell division. The Na^+ current was insensitive to the Na^+ channel blockers TTX and STX. Trichostatin A (TSA) arrested the cell division of cultured RPE cells reversibly. However, the Na^+ current expression was not blocked by this drug. Since TSA has been shown to act as an inhibitor of the cell cycle at G1 phase, the Na^+ current expression may be regulated by certain factors appearing between G0 and G1 phases before entering S phase. However, this does not mean that the current expression is directly coupled with the cell cycle, because about half of stretched cells do not express the current. We hypothesize that the Na^+ current expression and the presence of immature action potentials may represent a tendency of RPE cells to dedifferentiate towards embryonic neuroepithelial or more neuronal phenotypes in culture.

INTRODUCTION

Many vertebrates can regenerate neural retina after disruption of the original retina sometimes during their development. However, this ability is lost early in embryonic life in most animals. On the other hand, certain species of amphibians, such as newts and salamanders, retain the ability to regenerate a functional retina even in adult life. The process of retinal regeneration in such species has been well studied by light microscopy (Wachs, 1920; Stone, 1950a,b; Hasegawa, 1958). In these investigations there were conflicting theories of the cellular sources of retinal regeneration. Based on electron microscopic analysis coupled with autoradiography in adult newts (Keefe, 1973a,b), the consensus of most investigators now is that the central part of the neural retina is regenerated from the retinal pigment epithelial (RPE) cells, while the peripheral part is regenerated from intrinsic neuroblasts existing at the retinal margin. It is fascinating that after removing the neural retina of the adult newt eye, remaining RPE cells can discard the pigment granules, proliferate, differentiate into various retinal neurons and reform a functional neural network.

One of the most important neuronal phenotypes is the expression of ion channels such as Na^+ and Ca^{2+} , which are responsible for the initiation of action potentials. Recently, using whole-cell patch-clamp techniques, the expression of voltage-gated Na^+ channel in cultured RPE cells has been reported in adult newt (Sakai and Saito, 1994), adult human (Wen *et al.*, 1994) and neonatal rat (Botchkin and Matthews,

1994). Although the voltage-gated Na^+ channels have been observed in cultured RPE cells, the timing of their expression has not yet been specified.

The present work is a continuation of our studies on functional regeneration of newt retina (Negishi *et al.*, 1992; Kaneko and Saito, 1992; Kaneko *et al.*, 1993; Saito *et al.*, 1994; Sakai and Saito, 1994). In the present study we cultured single RPE cells dissociated from adult newt eyes and examined their electrical membrane properties using whole-cell patch-clamp techniques in order to find out when RPE cells start to express voltage-gated inward Na^+ current and whether the Na^+ current expression is associated with the cell cycle or not. We also examined the sensitivity of this current to the specific Na^+ channel blockers tetrodotoxin and saxitoxin. The results suggest that RPE cells acquire TTX-insensitive Na^+ current before the first mitosis and that the Na^+ current expression may not be associated directly with the cell cycle.

MATERIALS AND METHODS

Cell dissociation and culture

Adult newts (*Cynops pyrrhogaster*, 6–10 cm in body length) were anesthetized with 0.1% FA100 (4-allyl-2-methoxyphenol; Tanabe) and decapitated. Eyes were enucleated and sterilized with 70% ethanol. They were hemisected posterior to the *ora serrata* and the neural retinas together with lens were removed from the eye cups with fine forceps in control saline solution (in mM: NaCl 110; KCl 3.7; $MgCl_2$ 1.0; $CaCl_2$ 3.0; Na-pyruvate 1.0; glucose 13; HEPES 5). The eye cups were transferred to Ca^{2+} - and Mg^{2+} -free solution with 10 mM EGTA at pH 7.5 and then incubated at 25°C for about 20 min. The retinal pigment epithelium (RPE) was peeled off from the choroidal layers. Isolated RPEs were collected from several eyes and cut to pieces. Pieces of RPEs were incubated in control solution with 0.1% trypsin for 50 min at 28°C. After rinsing several times with 80%

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Leibovitz's L-15 medium (Flow Laboratories) to dilute the enzyme, RPE cells were dissociated by gentle mechanical trituration. About 500 μ l of cell suspension medium was plated in 35 mm sterile plastic dishes (Falcon, No. 3001). The dish was previously coated with 1% concanavalin A (conA) for 2 hr, washed 5 times with distilled water and air-dried. Freshly dissociated RPE cells were first allowed to adhere for about 20 min, then rinsed a few times. Finally, about 2.5 ml of culture medium with 5% fetal calf serum (HyClone) was added to each dish. A density of cells attached on the substrate was 500–800 cells/cm². All cultures were maintained at 25°C. The culture medium was not changed until cells were examined electrophysiologically.

Solutions and drugs

The dish containing dissociated RPE cells was mounted on the stage of an inverted microscope with phase-contrast optics (Olympus IMT-2). For physiological experiments, the culture medium was replaced with control saline solution described above. In Na⁺-free solution, NaCl was substituted with choline chloride. Test solutions containing tetrodotoxin (TTX), saxitoxin (STX) and tetraethylammonium (TEA) were made by adding an appropriate amount of these drugs directly to the control solution. The solution was fed into the dish by gravity at a rate of about 0.8 ml/min, and withdrawn by suction.

To arrest the cell cycle of RPE cells, we used trichostatin A (TSA) which was originally reported as fungistatic antibiotics (Tsuji *et al.*, 1976) and recently demonstrated as a reversible inhibitor of the proliferation of mammalian cultured cells (Yoshida and Beppu, 1988). This drug at concentrations of 0.1–1.0 μ g/ml was added in the solution at the beginning of cell culture.

Electrical measurements

Electrophysiological recordings were made from freshly dissociated or cultured RPE cells using whole-cell patch-clamp techniques as described (Hamill *et al.*, 1981). The tip diameter of the recording glass pipette was 1–2 μ m, and the resistance was 2–5 M Ω in the bath solution. The recording pipettes were filled with the following solution (mM): KCl 138, HEPES 10, EGTA 5. The pipette was

connected to the patch-clamp amplifier (Nihon Kohden, CEZ-2300). The bath was grounded with an Ag-AgCl wire. Junction potentials at the pipette tip were nulled by an offset current sufficient to make the output current zero. The membrane current was filtered at 2 kHz (a 4-pole Bessel filter). The capacitive current transients were not corrected. Data were sampled and digitized with a 12-bit analog-to-digital converter connected to a microcomputer (NEC, PC-9801VX) and stored on floppy disks for later analysis.

RESULTS

Culture of pigment epithelial cells

Figure 1 illustrates a cultured RPE cell dissociated from adult newt retina under phase contrast microscopy. Freshly dissociated RPE cells are often gourd-shaped, and characterized by the presence of dense pigmentation in the apical portion of the cell and nonpigmented smooth membrane of the basal portion (Fig. 1A). The mean diameter of freshly dissociated RPE cells (long axis) was 39 \pm 5 μ m (SD, n=31). The cells allowed to attach onto a substrate began to flatten within 1–2 days (Fig. 1B). In the following few days, they continued to flatten and then their nuclei (arrow) became visible, because pigment granules spreaded throughout the cytoplasm (Fig. 1C). The shape of stretched cells varied widely from cell to cell. It was spherical or oval in some cases and slender in others. The first mitosis was observed after 6 days in culture (Fig. 1D).

Voltage-gated K⁺ currents in freshly dissociated RPE cells

Figure 2 shows voltage and current records from a freshly dissociated RPE cell. The cell was initially hyperpolarized by a constant current injection to maintain the membrane potential

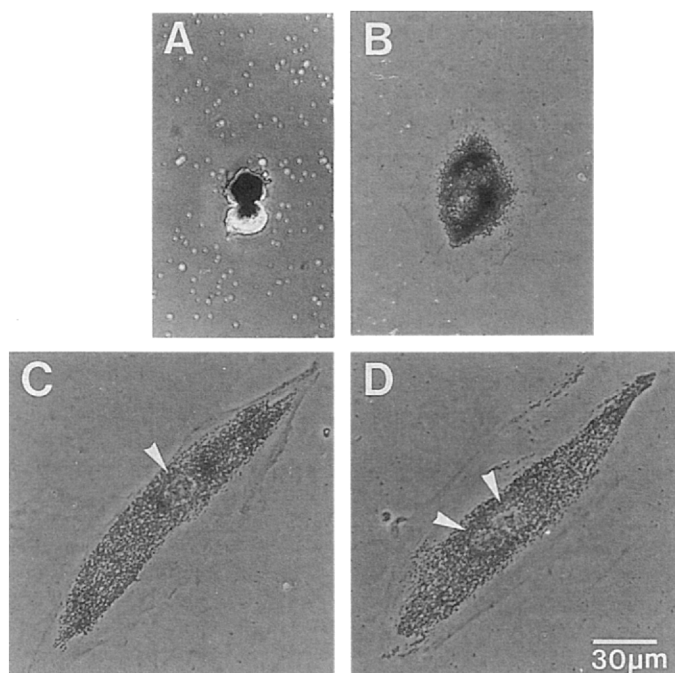


Fig. 1. Phase-contrast photomicrograph of a cultured newt retinal pigment epithelial (RPE) cell. A: RPE cell attached to the plastic dish 5 hours after plating. B: Cell after 2 days in culture. C: Cell after 5 days in culture. D: Cell after 6 days in culture. Arrow head shows a nucleus.

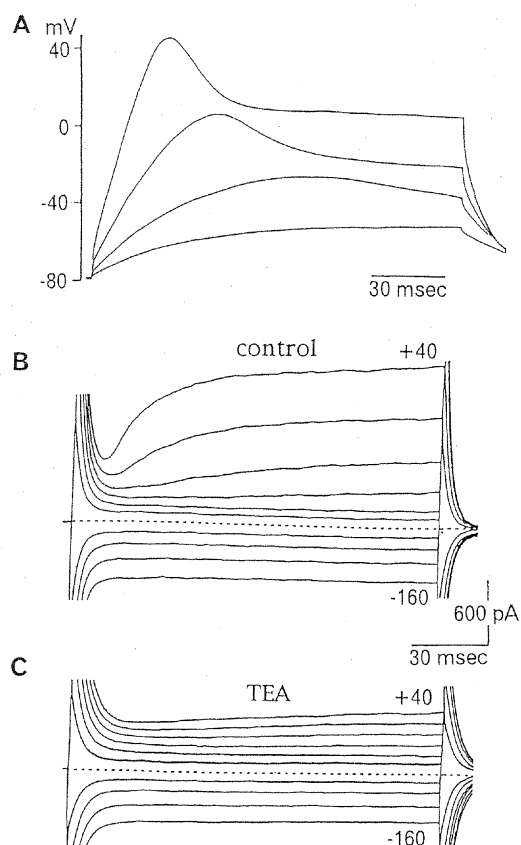


Fig. 2. Voltage and current responses recorded from a freshly dissociated RPE cell. A: Voltage responses under current clamp conditions. The cell was initially hyperpolarized from a resting potential of -45 mV to -80 mV by a constant current injection of about 8 nA and then depolarizing current pulses were applied for 150 msec. Four sweeps were superimposed. A small current pulse produced a response with an exponential time course (passive response). Larger current pulses produced larger passive responses, followed by voltage-dependent decline in amplitude. B: Membrane currents recorded from the cell in control solution under voltage-clamp conditions. Holding potential was -80 mV. The current was evoked by a series of voltage pulses (150 msec) from -160 to $+40$ mV in 20 mV steps. C: Suppression of the voltage-gated outward currents by 50 mM TEA.

around -80 mV. Depolarizing currents under current clamp conditions produced responses with an exponential voltage rise (passive responses) which were followed by a voltage-dependent decline in amplitude (Fig. 2A). Evidence that this voltage decline is due to activation of voltage-gated outward current is shown in Figure 2B and 2C where the same cell was voltage-clamped. When the cell was depolarized by a series of voltage pulses from -160 to $+40$ mV in 20 mV steps, the outward current was activated around -20 mV and exhibited a voltage-dependent increase (Fig. 2B). The same voltage protocol was repeated during superfusion with a medium containing 50 mM TEA (Fig. 2C). TEA suppressed the outward current. Since TEA is known as a blocker of the delayed outward K⁺ current in a variety of excitable cells (Hille,

1984), the result suggests that a major ion channel of freshly dissociated RPE cells is delayed K⁺ channel.

Expression of voltage-gated Na⁺ currents in cultured RPE cells

Within several days in culture, more than 50% of RPE cells examined exhibited immature action potentials and transient inward currents that were never observed in freshly dissociated cells. Figure 3 shows voltage and current records from a cell maintained in culture for 6 days. When depolarizing currents were applied under current clamp conditions, the slow-rising action potentials of long duration was initiated above -40 mV (Fig. 3A). The onset of the response was indicated by an arrow. The same cell was depolarized under voltage clamp conditions from a holding potential of -80 mV to $+40$ mV in 20 mV steps. As the membrane potential was depolarized above -40 mV, a transient inward current was activated (Fig. 3B). This current was suppressed by replacement of NaCl in the external solution with choline-Cl

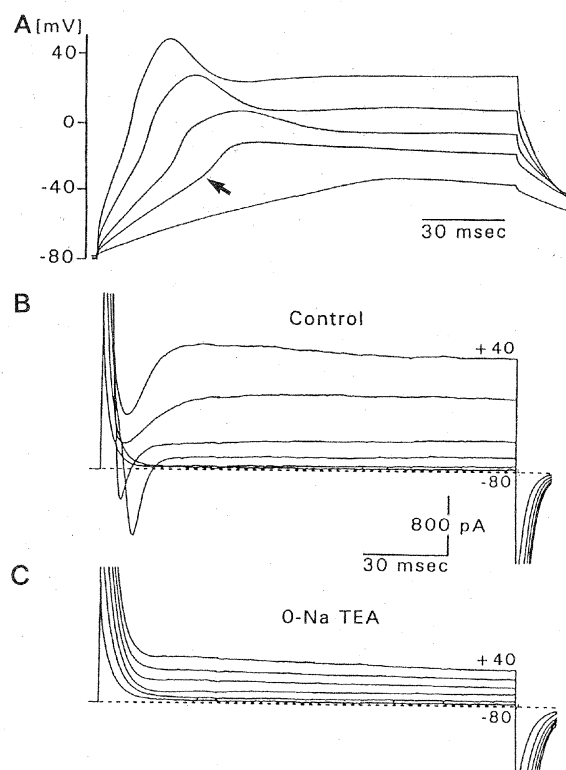


Fig. 3. Voltage and current responses recorded from a RPE cell following 6 days in culture. A: Voltage responses under current clamp conditions. The cell was initially hyperpolarized from a resting potential of -56 mV to -80 mV by a constant current injection and then depolarizing current pulses were applied for 150 msec. Five sweeps were superimposed. Slow-rising action potential of long duration was initiated above -40 mV. An arrow points to the onset of the action potential. B: Membrane currents recorded from the cell in control solution under voltage-clamp conditions. Holding potential was -80 mV. The current was evoked by a series of voltage pulses (150 msec) from -80 mV to $+40$ mV in 20 mV steps. Voltage-gated transient inward current was activated above -40 mV. C: Suppression of the inward and outward currents in Na⁺-free solution containing 20 mM TEA.

(Fig. 3C). The result suggests that the inward current may be carried by Na^+ .

TTX-insensitive Na^+ current

Figure 4 illustrates the effect of TTX on the Na^+ current expressed in a cell maintained in culture for 7 days. When the cell was depolarized from a holding potential of -80 mV to $+20$ mV in 10 mV steps, the Na^+ current was activated with voltage pulses more positive than -40 mV (Fig. 4A). In Figure 4B, the same voltage protocol was repeated during superfusion of a medium containing TTX. The Na^+ current was unaffected by application of 10 μM TTX (Fig. 4B) that would typically be expected to completely block TTX-sensitive Na^+ channels in a variety of excitable cells (for review, see Hille, 1984). Concentrations higher than 50 μM TTX slightly suppressed

the current in some cells (data not shown). Replacement of external Na^+ with choline abolished this current (Fig. 4C). Application of another Na^+ channel blocker STX at concentrations of 10 and 50 μM did not block the Na^+ current (data not shown).

Onset of Na^+ current expression

As stated above, some of RPE cells attached onto a substrate became flat and started the first mitosis within a week. Figure 5 shows the appearance rate of voltage-gated Na^+ current in these cells. Culture period was divided into 3 stages (I, II and III) on the basis of the morphology of RPE cells. RPE cells in the stage I, like a cell in Figure 1B, were flat in shape. Their nuclei were invisible, because pigment granules did not spread throughout the cytoplasm. None of them exhibited the Na^+ current at this stage. RPE cells in the stage II, like a cell in Figure 1C, stretched sufficiently and their nuclei became visible, because the pigment granules spreaded throughout the cytoplasm. Ten out of 18 cells (about 56%) examined expressed the Na^+ current. RPE cells in the stage III included cells during or just after the cell division (Fig. 1D). Six of 12 cells (50%) expressed the Na^+ current at this stage.

Effect of trichostatin on RPE cells

RPE cells were cultured in the presence of TSA (0.1 – 1.0 $\mu\text{g}/\text{ml}$). TSA has been reported to block the cell cycle of rat fibroblasts (Yoshida and Beppu, 1988). When TSA at a concentration of 0.1 $\mu\text{g}/\text{ml}$ was added to the medium at the beginning of cell culture, it arrested the cell division of cultured RPE cells during a 2 week period of observation. After removal of this drug, mitotic cells appeared after 6 days or more. The drug did not inhibit the cell spreading. At higher concentrations, RPE cells tended to exhibit vacuoles in the cytoplasm for a week in culture and some of them detached from the substrate. This fact may imply the deterioration of the cells.

After 7 days of incubation with TSA (0.1 $\mu\text{g}/\text{ml}$), the membrane properties were measured to determine whether the Na^+ current was expressed or not. The result is shown in Figure 6A in comparison with that in the control solution. There was no significant difference in the percentages of the Na^+ current expression in the presence and absence of TSA. That is, in the control solution, 20 of 31 cells (about 64%) expressed the Na^+ current, while in the presence of TSA, 17 of 31 cells (about 53%) expressed the current. The mean value of the maximum Na^+ current in the presence of TSA was 122.9 ± 66 pA (mean \pm SD, $n=17$). This value was less than half of the value (316 ± 109 pA, $n=20$) in the control solution (Fig. 6B).

DISCUSSION

We cultured single RPE cells dissociated from adult newt retina. ConA was used as a substrate, because it caused relatively good adhesion and survival of the retinal cells in comparison with extracellular matrix components, including laminin, fibronectin and collagen (Chiba *et al.*, 1995). RPE cells allowed to adhere onto a substrate began to spread

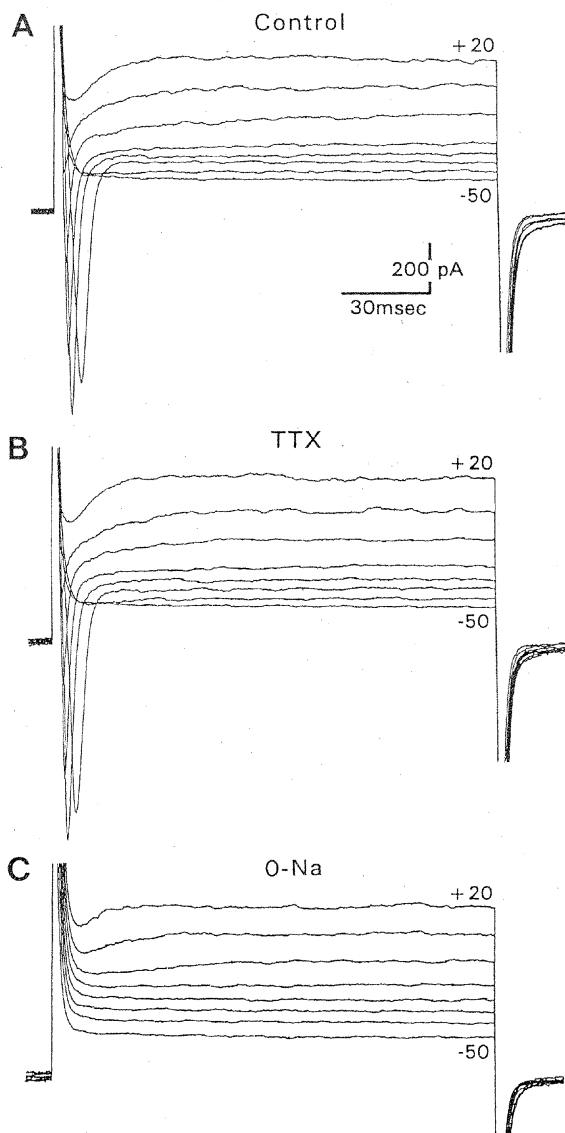


Fig. 4. Effect of TTX on inward Na^+ currents. A: Voltage-gated Na^+ currents in control solution. B: Currents in control solution containing 10 μM TTX. C: Suppression of the inward Na^+ currents in Na^+ -free solution.

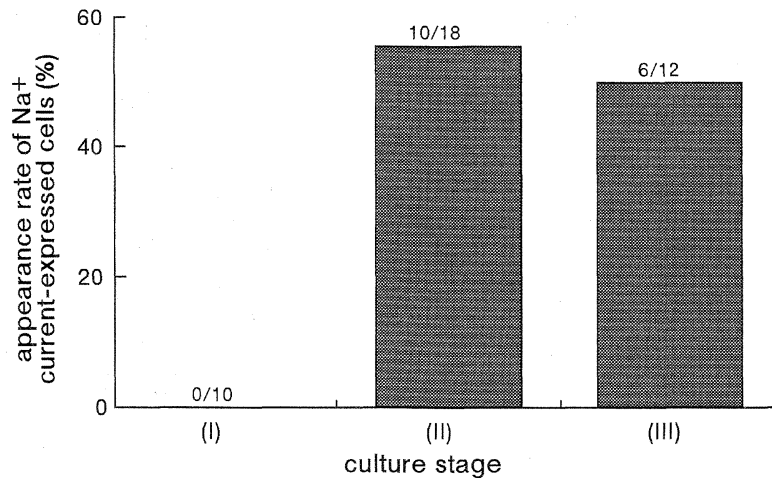


Fig. 5. Appearance rate of Na⁺ current-expressing cells at various times within the first cell cycle. Culture period was divided into three stages (I, II and III). Cells in stages I, II and III showed morphological characteristics similar to those in Figs. 1B, 1C and 1D, respectively. The ratio of the number of current-expressed cells to the total number of cells examined were indicated above each bar.

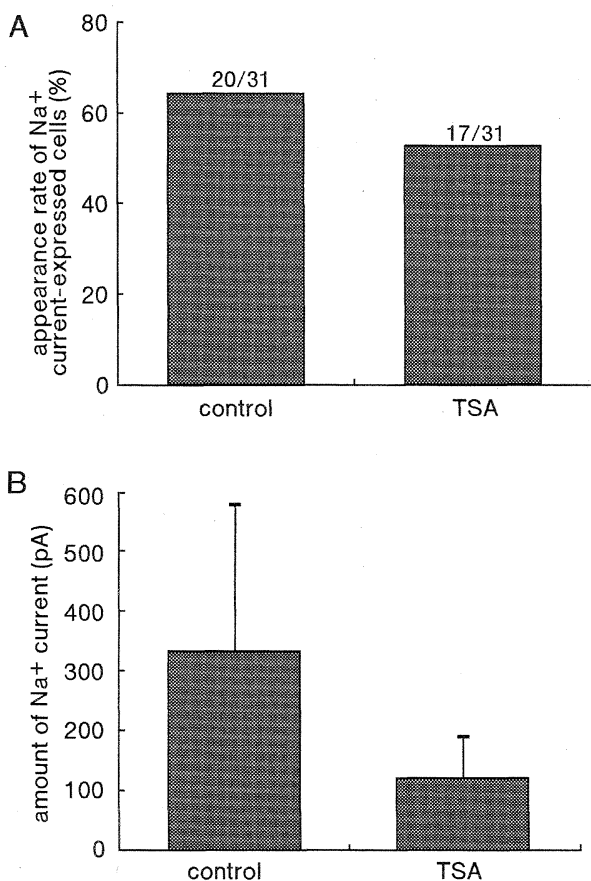


Fig. 6. Effects of TSA on Na⁺ current expression. A: Comparison of the appearance rates of Na⁺ current-expressed cells in control solution and solution containing 0.1 $\mu\text{g/ml}$ TSA. Membrane currents were recorded from sufficiently stretched cells after 7 days in culture. The ratio of the number of current-expressed cells to the total number of cells examined were indicated above each bar. B: Amount of Na⁺ currents in control solution and solution containing 0.1 $\mu\text{g/ml}$ TSA. The error bars show \pm SD.

gradually in Leibovitz's L-15 culture medium with fetal calf serum. Mitosis was first observed in the cultures about 6 days after seeding of cells. In the serum-free culture medium, RPE cells neither spread nor divided, indicating that serum was necessary for cell spreading and/or cell division. The factors included in serum that modulate morphological changes in RPE cells are not yet known.

Adult newt RPE cells in culture acquired voltage-gated Na⁺ currents before the cell division. The appearance of this current corresponded with the initiation of slow-rising action potentials of long duration. The expression of Na⁺ current has also been reported for RPE cells of adult human (Wen *et al.*, 1994) and neonatal rat (Botchkin and Matthews, 1994) during dissociated culture. The Na⁺ current expressed in human RPE cells was very sensitive to the Na⁺ channel blocker TTX, and that of rat RPE cells was also sensitive to TTX in some cells, but insensitive to it in the others. On the other hand, the Na⁺ current expressed in newt RPE cells was consistently TTX-insensitive. One may question whether the TTX-insensitive Na⁺ current would become sensitive to TTX at later stages in culture as in a clonal rat skeletal muscle cell line (Kidokoro, 1975) or embryonic chick cardiac muscle (Sperelakis, 1980). However, we have not yet observed such a change. Furthermore, we have already reported that retinal ganglion cells dissociated from adult newt retina have TTX-insensitive Na⁺ channels (Kaneko and Saito, 1992). This fact may be related to the observation that newt (*Taricha torosa*) has a toxin similar to TTX of puffer fish (Buchwald *et al.*, 1964).

The Na⁺ current expression was observed in the stretched cells as shown in Figure 1C, but not in any of cells showing insufficient cell spreading (Fig. 1B). Since cell morphology has been shown to affect gene expression in many systems (Hay and Svoboda, 1989; Opas, 1994), it might be expected that the stretch of RPE cells in culture influences expression of particular genes which may encode the Na⁺ channels. In the present study, however, the situation was not so simple,

because the Na⁺ current could not be observed in about half of the sufficiently stretched cells.

TSA of a concentration of 0.1 μg/ml was added in the medium at the beginning of the RPE cell culture. It reversibly blocked the cell division of RPE cells. This drug has previously been shown to act as an inhibitor of the cell cycle at the G1 and G2 phases in rat fibroblast cells (Yoshida and Beppu, 1988). Therefore, TSA-arrested RPE cells are expected to remain in either the G0 or G1 phase. More than half of these cells expressed the Na⁺ current under treatment of the drug. This may suggest that the Na⁺ current expression is regulated by certain factors between G0 and G1 phases before entering S phase. However, this does not necessarily mean that the cell cycle contributes directly to the Na⁺ channel expression, because the remaining half of cells did not express the current. A decrease in the Na⁺ current in the presence of TSA may be related to some damage of the cells. In fact, at higher concentration of TSA, some of cultured cells deteriorated and detached from the bottom of the dish. Cellular and molecular mechanisms regulating the Na⁺ channel expression will have to be investigated in the future.

Retinal regeneration in urodale amphibians has been thoroughly described *in vivo* (Wachs, 1920; Stone, 1950a,b; Hasegawa, 1958; Keefe, 1973a,b). In recent years, it has become possible to culture RPE cells and analyse factors affecting their transdifferentiation (an alteration of the state of differentiation of cells that have already differentiated in embryonic development) *in vitro*. Eguchi (1976) preliminary reported that adult newt RPE cells extended fine processes resembling neurons in long-term cell cultures. Reh *et al.* (1987) found that RPE cells of frog tadpoles cultured on laminin-coated substrate expressed neuron specific markers and extended fine processes resembling neurons. In these studies, however, a possibility of the transdifferentiation of RPE cells towards neurons was examined morphologically and immunohistochemically.

One of the functionally important neuronal phenotypes is the voltage-gated Na⁺ channel responsible for generating the action potential. Recently, we described that neuroblasts acutely dissociated from early regenerating newt retina possessed voltage-gated Na⁺ currents and generated immature action potentials (Kaneko and Saito, 1992). We further described that in culture, neuroblasts developed voltage-gated Ca²⁺ and transient K⁺ currents as well as the Na⁺ current resembling those of the retinal ganglion cells (Kaneko *et al.*, 1993). Taking into account the facts that (1) the RPE and the neural retina are derived embryologically from the same neuroepithelial tissue, (2) adult newt RPE retains the ability to regenerate the neural retina *in vivo*, and (3) membrane properties of cultured RPE cells are similar to those of neuroblasts dissociated from early regenerating retina, the expression of voltage-gated Na⁺ channels and the presence of immature action potentials may represent a tendency of RPE cells to dedifferentiate towards embryonic neuro-epithelial or more neuronal phenotypes in dissociated culture.

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