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Wound Currents Following Amputation of Tail Tip in the Japanese Newt, *Cynops pyrrhogaster*

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ABSTRACT—When tail tips of the Japanese newt (*Cynops pyrrhogaster pyrrhogaster*) were amputated, large ionic currents were detected around the stump using a vibrating probe system. The initial wound currents flowed outwardly through the whole stump, and then localized within 24 hr toward the central region of the stump. Current densities reached a peak of about 450 μA · cm^{-2} (about 900 times greater than before amputation) within one half of a day after amputation, and returned to levels similar to the intact tail tip by 4 days, i.e., the wound currents were transient. The swelling of the stump end (regenerate or blastema) occurred about 7 days after amputation. The major ion carrying the currents is thought to be Na^+, because the currents were strongly inhibited by 0.5 mM ouabain added in the external medium or by Na^+-deficient medium. These findings are consistent with those of newt limb stump.

INTRODUCTION

It is very well known that newts and salamanders possess highly regenerative abilities. The loss of an entire limb or tail can be regenerated into a small replica of the missing tissue within one or two months, eventually regaining its original configuration.

This study was undertaken after a series of reports that endogenous ionic currents following injury affect the process of wound healing and/or the regeneration of amphibian limb stump. Borgens and colleagues (Borgens, 1982, 1989) investigated the electrical properties of wound currents and the role in limb regeneration in amphibians, including newts, salamanders and frogs, using a vibrating probe system, which makes possible to detect a minute current. They revealed a number of findings, including: (1) that a strong, steady, endogenous ionic current produces from the limb stump following amputation; (2) that the ion carrying a large part of the current is Na^+ driven by a skin-battery (Borgens *et al*., 1977); and (3) that these electrical properties can be commonly observed in a variety of newts and salamanders (Borgens *et al*., 1984).

It has been now proposed the hypothesis that such wound currents take part in an important role for wound healing in the newt skin (Rhodes, *et al*., 1990) and in the newt digits (Iglesia *et al*., 1996), and regeneration in the newt limbs (Jenkins *et al*., 1996). The purpose of the present study was to confirm whether wound currents are inducible following tail tip amputation in the Japanese newt, and to compare with the electrical properties of wound currents reported in newt limb stumps. Such information is necessary for studying the role of wound currents.

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MATERIALS AND METHODS

Animals

Japanese newts, *Cynops pyrrhogaster pyrrhogaster* Boie, were collected from an agricultural stream in Miyagi Prefecture, Japan, between October and November. Male and female adult newts (8 ~ 11 cm in body length) were stored separately in plastic boxes containing tap water and a small amount of pebbles at 4°C. Healthy newts were maintained without feeding and by exchanging the tap water at regular intervals, for at least one year. Experiments were conducted using only female newts, because their small tail fins were more suitable for electrical measurement. Ten days before use, they were transferred into plastic boxes containing tap water and bait (dried earthworms) at room temperature (23 ~ 26°C).

Tail tip amputation and experimental conditions

Prior to tail tip amputation, newts were anesthetized with 0.02% ethyl *p*-aminobenzoate (stock solution, 2% dissolved in ethanol) dissolved in tap water for 10 ~ 15 min; the anesthetic effect usually continued for about 90 min. The tails of anesthetized newts were cut across 1.5 ~ 2 mm behind the tip of the tail, where the most distal tail bone existed, with a razor blade to give a stump of 0.8 ~ 1.4 mm in width. After amputation of the tail tips, the newts were kept in a moistened chamber for about 45 min until the discharge of blood from the stump completely stopped. The newts were then kept in plastic boxes containing artificial pond water (APW) until electrical measurements were conducted. The APW consisted of 5.8 mM NaCl, 0.7 mM KCl, 0.3 mM $Ca(NO_3)_2 \cdot 4H_2O$ and 0.8mM $MgSO_4$, and was adjusted to pH 7.5 with 1 M Tris-HCl (final concentration, 4.6 mM). The effects of external Na^+ on ionic currents around the stump were examined using 0.5 mM ouabain (a Na^+/K^+-ATPase inhibitor; Mills *et al*., 1977; Helman *et al*., 1979) dissolved in APW or Na^+-deficient APW, in which

NaCl was replaced with choline chloride.

Newts with amputated tail tips were again anesthetized and immobilized, before electrical measurement. Then the whole tail was immersed in an APW filled experimental chamber, and the head and trunk were covered with gauze soaked with APW. The experimental chamber was equipped with a grounded Ag-AgCl wire connection and inlet-outlet tubing to exchange media, and was set on the stage of an inverted light microscope (x100).

Measurement of ionic current around the tail tip stump

The densities and patterns of ionic currents around the stump were measured using a one dimensional vibrating probe system with a modification originally designed by Jaffe and Nuccitelli (1974), and as described elsewhere (Nawata, 1984).

The ionic currents were measured as follows: if an ionic current flows from the stump (current source) and the probe is vibrated in the medium near the stump, a voltage difference between both ends of the vibration was detected with the aid of a lock-in amplifier (LI-75, NF Circuit Design Block, Co. Ltd.). The detected voltage difference (ΔV) can be converted to the current density at the midpoint of vibration by use of Ohm's law and the specific resistance of the medium; the value for both APW and APW containing ouabain was 9.5×10^3 ohm・cm, and that of Na-deficient APW was 10.4×10^3 ohm・cm. In this study, the probe was perpendicularly vibrated against the surface of the stump at a frequency of 170 Hz and with an amplitude of 30 μm. For measurements of ΔV, the probe was placed approximately 50 μm from the surface of the stump at the midpoint of vibration (measuring position) and was sometimes withdrawn to 1 mm away from the stump, where ΔV was null (reference position).

In this paper, the flow of net positive charges leaving the stump was characterized by outward currents and by positive values, and currents in the reverse direction, i.e., those entering the stump as inward currents, were shown by negative values.

RESULTS AND DISCUSSION

Detection of wound currents

First, measurements were performed to confirm the presence of wound ionic currents in the tail tip stump 3 hr after amputation (Fig.1). In the cut face of the stump, a cross-sectional view of the caudal vertebra could be observed, which anatomically consists of the dorsal spine, vertebral foramen, centrum and ventral spine (Ikeda and Inaba, 1971). The vibrating probe was scanned from the dorsal to ventral regions along the median line of the caudal vertebra, at intervals of 190 ~ 230 μm.

Inward currents were detected near both the dorsal fin and spine (measuring positions ① ~ ②) and the ventral fin (⑧), while large outward currents were detected in the large part of the caudal vertebra (③ ~ ⑦). The largest outward currents appeared to flow out of the centrum. When the probe was scanned further along the stump margin (epithelial cell layer), the currents were similar to those at the dorsal and ventral fins (trace not shown).

Changes in the pattern and density of wound currents following amputation

Changes in the wound currents before and after amputation of the tail tip were monitored during a period of 48 hr by a similar manner shown in Fig. 1 (Fig. 2). The currents recorded around the intact tail tip were directed inwardly and were very weak, less than 0.5 μA・cm^{-2} in density (0 hr). In contrast, 1.5 hr after amputation, currents flowed outwardly from the whole stump at a density of about 175 μA・cm^{-2}, whereas currents from the tail surface just behind the edge of the stump were very weak, almost similar to those of intact tails.

The pattern and density of the currents changed according with the times shown at 3, 24, and 48 hr. One marked change in the current pattern was the regional flow of outward currents that localized rapidly toward the centrum, and the

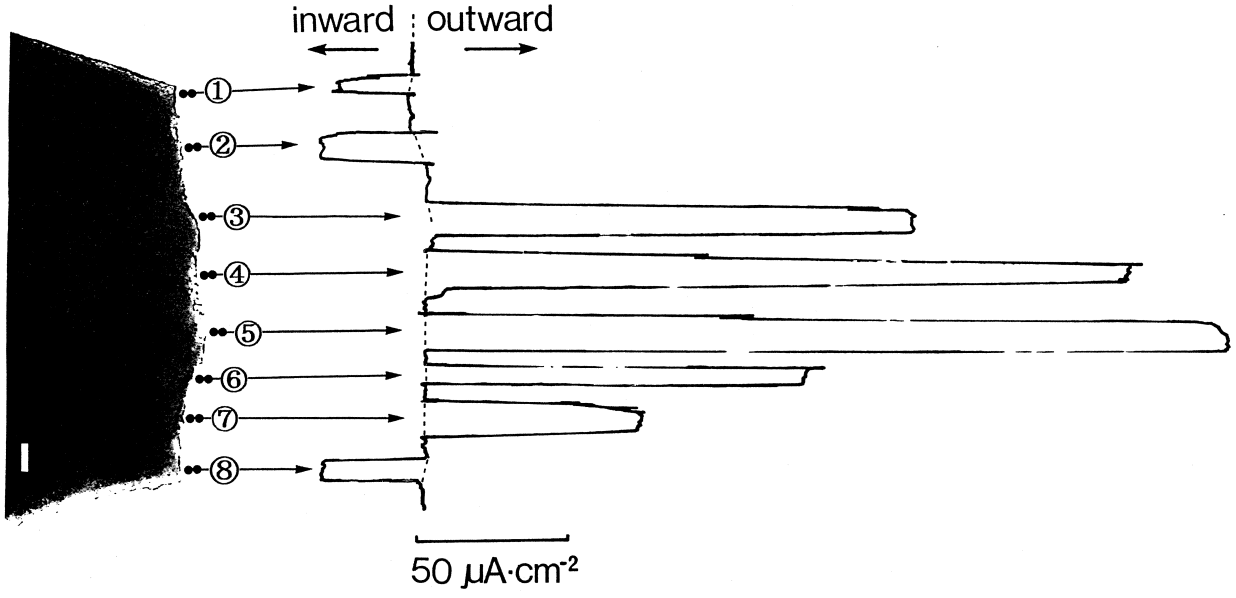

Fig. 1. Representative recording showing density and direction of wound currents. The tail tip was amputated 3 hr before this measurement. The photo (left half) shows a side view of the amputated tail tip (upper: dorsal side) and 8 measuring positions denoted in numerals. Recording trace (right half) at every measuring position shows a clear deflection from the trace measured at the reference positions (dotted line). Measurements at each position were done for 20 ~ 30 sec. Bar in the photo: 100 μm.

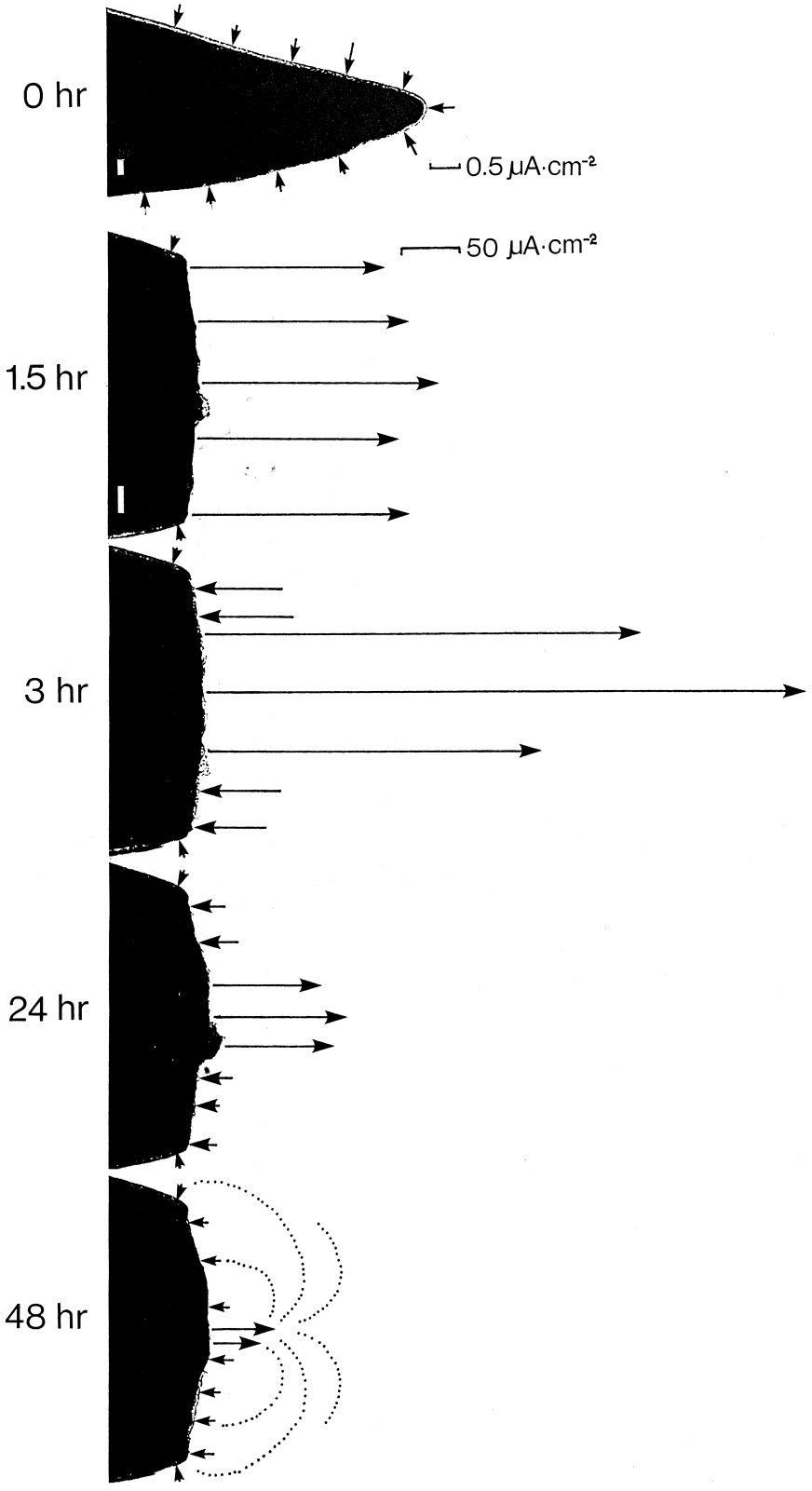

Fig. 2. Changes in density and pattern of wound currents before and after amputation. The currents were recorded along the median line of the stump from the same newt, except for the recording obtained from an intact tail at 0 hr. The length of arrows depicting current direction is proportional to the current density. The dotted six lines depicted with the 48 hr recording show one proposal circuit for the ionic currents. Bars in the photos: 100 μm. Note the photo at 0 hr is magnified 50% compared with the others.

current density also decreased rapidly. McGinnis and Vanable (1986) reported similar changes in the pattern of wound currents in newt limbs. They explained their results with the possibility of wound healing that many intact epithelial cells not only migrate from the stump edge toward the stump center, but also that these migrated epithelial cells connect tightly with each other, resulting in localization of the current path. Their explanation will be also applicable to the present case.

Fig. 3 shows the changes in the current density that were recorded near the centrum of newts 14 days after amputation. The maximum outward currents reached 450 ± 70 μA・cm^{-2} in density within one half of a day, and smoothly decreased to 50 ± 65 μA・cm^{-2} in density within 2 days. The current direction changed from outward to inward in 4 days, and the current density approached that of the intact tail tip before amputation. Thus, the wound currents were transient. In this figure, changes in the lengths of regenerates are also described, which were measured as the distance between the face of the amputation and the pointed end of the swollen stump that was covered with wound epitherial cells. Regenerates began to grow about 7 days after amputation, i.e., after the disappearance of wound currents.

Thus, large wound currents were confirmed in the tail tip stumps of Japanese newts following amputation. The initial currents seem to be formed an electrical/ionic circuit by leaving the stump and by entering largely the undamaged tail skin through the external medium. The current patterns were considerably similar to those of limb stumps from a variety of other urodela (Borgens, *et al.*, 1984).

However, the duration of the outward currents was different, because in the present experiments flowing currents were only measured for the first 3 days after amputation. On the other hand, measurements in the limbs showed that outward currents continued to flow over 14 days after amputation, although flow of especially large currents was only detected during the first several days. The difference may be due to the different stump size. The size of the limb stump was

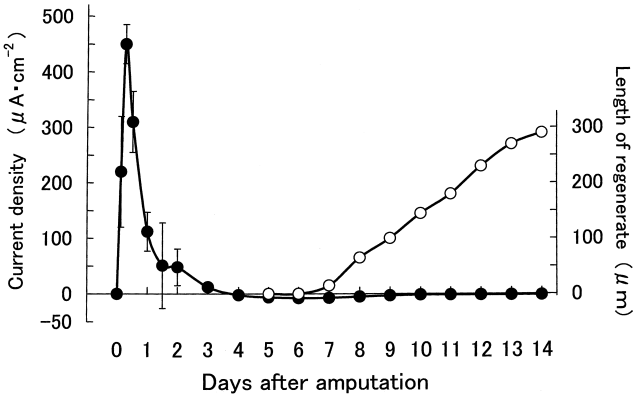

Fig. 3. Changes in density of wound currents monitored near the centrum for 14 days following amputation. Solid circles and bars indicate, respectively, mean values and SE, obtained from 9 newts (left ordinates). Open circles indicate the mean length of the regenerates, separately obtained from 26 different newts (right ordinates).

10 ~ 15 times larger than that of the present tail tip stump. Therefore, it is likely that the quickly full healing of the tail tip stump resulted in a shorter duration of the wound currents. In either case, the large outward currents appeared to be flowing in parallel with the progress of wound healing, because regenerate growth in both limb and tail tip usually occurred to start at almost similar time after amputation. Another difference is that the maximum current density in the present measurements was 5-fold or greater than the maximum values for all other recordings from limbs. This difference is, however, probably because Na^+ concentrations in APW used were several times higher than those of their APW, and wound currents strongly depended on Na^+ concentrations, as described below.

Effect of external Na^+ on wound currents

Wound current dependence on Na^+ was examined using tail tip stumps one day after amputation. When the whole tail was exposed to 0.5 mM ouabain dissolved in APW, the outward currents measured near the centrum decreased within 5 mim and changed to become somewhat inwardly, while the inward currents measured near the dorsal spine also decreased to nearly zero. The reduction in both currents was fully recovered by exchanging the medium for APW. A similar decrease in both currents was also induced by Na^+-deficient APW. Results of the outward currents obtained 10 min after these treatments are summarized in Table 1. The extent of the current reduction seems to be different between ouabain treatment and Na^+-deficient treatment, but the cause was not clear yet.

On the other hand, when the stump region (the range of 1 cm or less in length) was restrictively exposed to these media, neither the outward currents nor the inward currents changed, for at least 30 min after treatment, suggesting that the wide range of the undamaged tail skin is required for the full current flowing. These results support the hypothesis that the currents are driven by a Na^+-pump widely distributed in the undamaged limb skin (Borgens *et al.*, 1977).

In conclusion, as already reported in newt limb stump, the present study also showed that there existed Na^+-dependent wound currents around tail tip stump in the Japanese newt following amputation. Morphological observations (Singer, 1952; Hay, 1959; Iten and Bryant, 1973) have shown that cellular activity in the stump during a few days following amputation is related to wound healing. In preliminary experiments has been observed the fact that the beginning of

Table 1. Effects of ouabain and Na^+-deficient on outward wound currents recorded one day after amputation

treatments	before	after (10 min)
APW + 0.5 mM ouabain	126.4 ± 54.8	–21.7 ± 17.2
Na^+-deficient APW	131.3 ± 64.5	15.1 ± 8.5

Values indicate the current density at peak (mean ± SE) in μA・cm^{-2}. Data was obtained from 6 and 9 newts, respectively, for ouabain and Na^+-deficient treatments.

regerenate growth of tail tip stump is significantly delayed by an artificial reduction of the wound currents. The wound currents, therefore, might play a role for wound healing and the subsequent process of regenerate formation in tail tip stump, as well as in the newt skin, digits and limbs.

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