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Bioelectricity and regeneration: Large currents leave the stumps of regenerating newt limbs*

(skin-driven sodium currents/vibrating probe/urodeles/amiloride)

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ABSTRACT **Electrical currents near regenerating newt limbs were measured with a recently developed vibrating probe. Steady currents with local surface densities of 10 to 100 $\mu A/cm^2$ or more leave the end of the stump during the first 5–10 days after amputation and are balanced by currents with densities of only 1–3 $\mu A/cm^2$ that enter the intact skin around the stump. They are immediately dependent upon the entry of sodium ions into this skin and are therefore inferred to be skin-driven. The outward currents are comparable in direction, density, duration, and position to artificially imposed currents previously found sufficient to induce significant regeneration of amputated adult frog limbs. This comparison suggests that the endogenous stump currents play some causal role in initiating regeneration.**

We have recently demonstrated that anuran limb regeneration can be initiated by driving a small steady current through the stump. The effective stimulus is truly electrical (i.e., the effect is not mediated by electrode products). Current must be pulled out of the stump to induce regeneration, whereas inward current actually induces degeneration (1). Many other stimuli are known to induce a measure of regeneration in adult anurans (2, 3). The critical question is whether a comparable electrical component is part of a *natural* control system. As a first step in answering this question, we ask whether normally regenerating amphibian stumps generate comparable currents after amputation.

An old and nearly forgotten paper of Monroy's indicates that endogenous currents do, indeed, leave the regenerating amphibian stump surface (4). Monroy used a relatively low-resistance galvanometer to make these measurements. Hence, as will be discussed below, it is possible to estimate the size of these currents. Two subsequent studies have yielded somewhat contradictory results (5–7). However, both Becker and the Roses used instruments with very high resistance, so the resistance between the points of measurement was essentially the resistance along the skin surface. Because this was not measured, the size of the currents responsible for the measured voltages cannot be inferred.

We have therefore reinvestigated the stump currents in amputated and regenerating newt limbs, using the recently developed ultrasensitive vibrating probe (8). With it, one can directly measure the pattern of current densities entering or leaving a biological source immersed in its natural aqueous medium. Furthermore, the probe is placed at a sufficient distance from the animal as to practically avoid disturbing it. The probe is vibrated between two external points and registers the minute voltage difference generated by any current that may travel between these two points. The only accessory measurement needed to infer the current density is the resistivity of the medium. Use of the vibrating probe allows one to measure extracellular voltage differences reliably (and thereby infer extracellular current densities) at a level $1/100$ to $1/1000$ of that measurable with older techniques.

Using this new method, we find that large steady currents leave the end of the stump for a week or two after amputation. We further show that these regeneration currents are sodium dependent and nerve independent. We infer that they are skin-driven.

MATERIAL AND METHODS

Red-spotted newts, *Notophthalmus viridescens* (formerly *Triturus viridescens*), were purchased from Lee's Newt Farm (Oak Ridge, TN). These animals were adults of mixed sex and 6–11 cm in length. Amiloride (*N*-amidino-3,5-diamino-6-chloropyrazinecarboxamide) was kindly provided by W. B. Gall of Merck Sharp and Dohme Research Laboratories (Rahway, NJ).

Prior to use the animals were kept in well water. All work was done at 19–20°. During electrical measurements and regeneration, the animals were kept in artificial pond water containing 1.5 mM NaCl, 0.2 mM $NaHCO_3$, 0.1 mM KCl, and 0.8 mM $CaCl_2$ (9). This medium had a resistivity of 2300–2600 ohm cm.

Extracellular current densities were measured with a 30-μm diameter, ultrasensitive vibrating probe (8) with an improved vibrator (10). It was operated at a resonant frequency of 310–320 Hz, a peak-to-peak amplitude of 70 μm, and a time constant of 1 sec. The root-mean-square noise was about 0.004 $\mu A/cm^2$.

Prior to amputation and electrical measurements, the animals were anesthetized in 0.35% tricaine methanesulfonate (Sigma), commonly called MS-222. (The standard 0.1% solution did not adequately immobilize the animals for electrical measurements.) The right forelimb was amputated through the elbow joint with surgical scissors. The protruding humerus was then carefully trimmed flush with iridectomy scissors. During regeneration, single animals were kept partially immersed in finger bowls. They were fed freeze-dried *Tubifex* or live "white worms" (*Enchytrae* sp.) twice a week, and their medium was changed at least three times a week. Prior to electrical measurements, each animal was rinsed in anesthetic-free medium and transferred to a shallow, open-topped, transparent measuring chamber (70 × 45 × 5 mm). Enough medium to immerse the limb and to allow convenient measurements (3–5 mm depth) was added. Reference measurements were made at least 1 cm away from the limb. The probe was vibrated horizontally and normal to the nearby surface of the limb to measure the horizontal densities of current leaving or entering this surface. The center of vibration was usually 340 μm away from this

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Abbreviation: meLys, methyl ester of lysine.

* This is paper no. 2 in a series. Paper no. 1 is ref. 1.

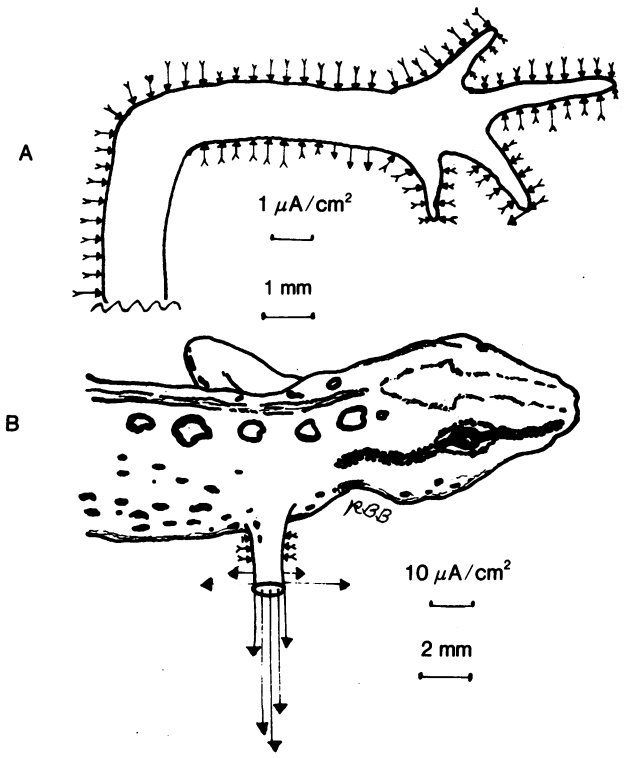

FIG. 1. Current patterns around (*A*) intact and (*B*) amputated newt forelimbs. Each arrow indicates the current density in the mid-horizontal plane, normal to the surface and 340 μm out from this surface. Note that in *A* the current density scale is 10-fold larger than in *B* in order to allow visualization of the relatively low values found near intact limbs. (*A*) Intact limb, Oct. 28, 1976. No measurements were made on the preaxial side of the upper arm because this region was inaccessible to the probe. (*B*) Measurements 20 hr after amputation on March 8, 1976.

surface. At this distance, some decline in current density had occurred (see below). Nevertheless, routine measurements were made at this distance because the optics were better and because this reduced the likelihood that occasional twitches of the lightly anesthetized animal would smash the probe.

RESULTS

We made three preliminary scans of the steady currents leaving or entering the intact forelimbs of adult newts (Fig. 1*A*). Specifically, we made measurements at 340-μm intervals in the midplane around the entire forelimb distal to the elbow as well as in the accessible regions proximal to the elbow. Current was found to enter at almost all locations. It left the base of the first digit and occasionally at the fingertips. Measured entry current densities were generally in the range of 0.01–1 μA/cm², whereas exit currents were between 0.2 and 1.4 μA/cm².

Far larger steady currents, of the order of 50 times larger, left the stumps of recent amputees. Fig. 1*B* shows a typical scan with a peak exit current density of 30 μA/cm². Note that the comparable increases in entry current densities were much smaller. These illustrative values were measured about 340 μm from the animal's surface. Appropriate measurements of the current density variation with distance from the surface indicated that densities at the surface were about 70% higher. All subsequent values were thus corrected.

It was of obvious interest to explore the time course of these large steady stump currents. In 11 cases, the steady surface currents were measured daily from amputation through the medium bud stage to late bud stage of blastema formation, 2–3

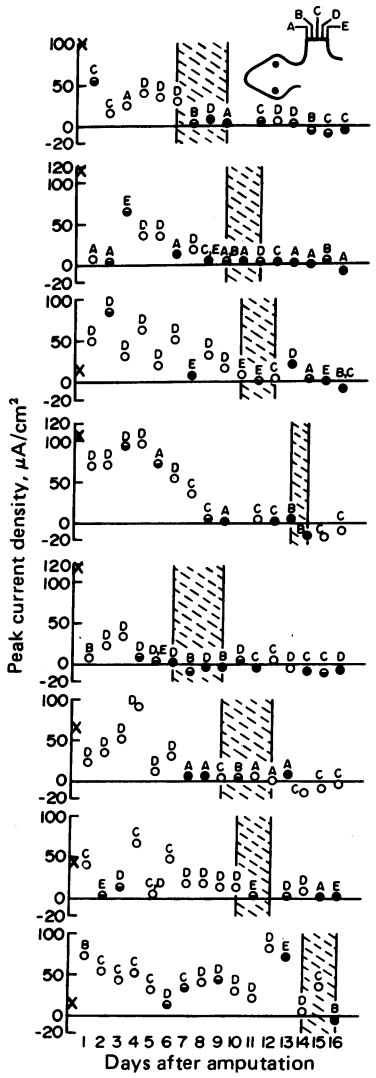

FIG. 2. Peak surface current densities versus time after amputation. Records of eight separate animals amputated Dec. 1, 1976, are shown. Shaded regions indicate approximate time of external blastema appearance. Shading begins at the first hint of this event and ends when it is unmistakable. *Inset* shows standard measuring position at which continuous vertical scans were made. Note that in going from *A* to *E*, one progresses from the preaxial to the postaxial side of the stump. The location of the maximum current density along this direction is indicated above each data point. The symbol used for each data point indicates the character of the integument at this maximum: x, integument may not yet be restored; O, clear epithelium; ●, fully pigmented skin; ⊖, a transitional region. (cf. Fig. 3). The very first measurement was at 5 hr after amputation in every case.

weeks later (11). In all 11 cases, the basic pattern illustrated in Fig. 1*B* persisted for 1–2 weeks after amputation. Relatively intense currents, of the order of 10–100 μA/cm² at the surface, continued to leave the end of the stump, whereas currents of relatively low density, of the order of 1–3 μA/cm² at the surface, continued to enter the rest of the limb.

In eight cases, we extended our exploration to the vertical axis to find the locus of peak current density more exactly. This procedure was repeated daily from the time of amputation through the time of blastema formation. Moreover, camera lucida drawings of the skin pattern at the stump's end were made at the time of each current density scan (Figs. 2 and 3).

These results indicated several substantial regularities. All

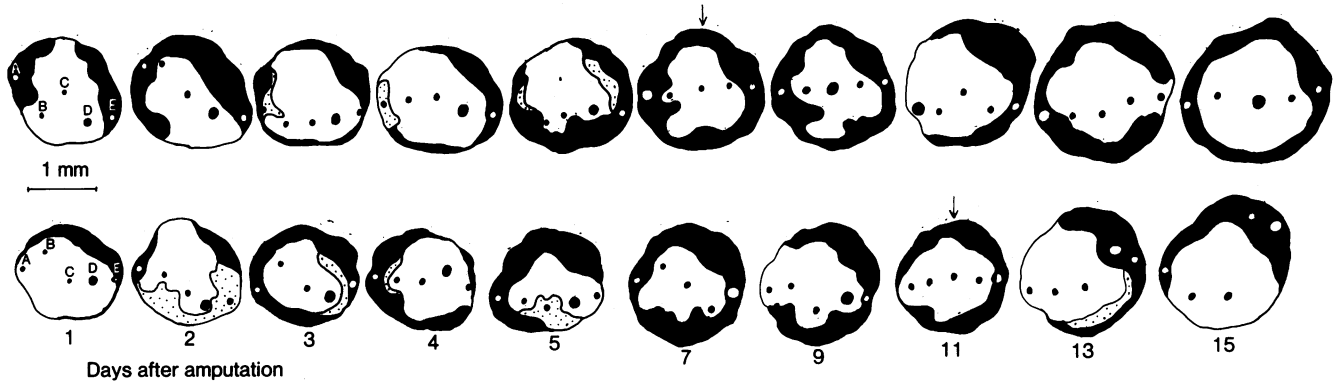

FIG. 3. Two representative series of camera lucida drawings of stump surfaces following amputation. *Upper* series is of animal 6 in Fig. 2; *Lower* series is of animal 3. Black regions, fully pigmented skin; white, unpigmented; stippled, partially pigmented skin. Vertical scans were made at each of the five standard measuring positions *A–E*. Each dot indicates the location of the peak current density along one of these scans. Largest dot indicates the largest of these five. Protuberances associated with development of an externally obvious blastema formed between days 9 and 12 in both cases. Arrows indicate times at which the peak current density fell below 5 $\mu A/cm^2$.

of the peak current densities during the first week were outward and most were very high, in the range of 20–100 $\mu A/cm^2$. They did not necessarily decrease steadily after amputation. Often they reached a maximum several days after amputation, and in most cases this late maximum equaled or exceeded the earliest value, measured 4–5 hr after amputation. They then fell and remained at quite low values or even reversed at about the time of grossly visible blastema formation.

Large outward currents showed a strong tendency to peak over clear, newly formed epithelium and rarely appeared over fully pigmented, mature skin. Of 56 peak outward currents larger than 5 $\mu A/cm^2$, 44 (or 78%) were over clear epithelium, 10 (or 18%) were over borderline or transitional regions, whereas only 2 (or 4%) lay over mature skin. Large outward currents also showed a strong tendency to appear over the postaxial region (positions *D* or *E*, Fig. 3): 41 (or 73%) were postaxial, 11 (or 20%) were central, and only 4 (or 7%) were preaxial.

Source of the Current. The limbs were deprived of their motor and sensory nerves in order to test what contribution, if any, these nerves made to the regeneration currents. The right limbs of three animals were denervated by removing sections at least 1 mm long from all of the trunks of their brachial plexi. A day later, the effectiveness of this process was established by noting that the denervated arms were completely immobile and gave no startle response when pricked. Then, both forelimbs were amputated and the peak currents were measured 30 hr later. In all three cases, the denervated stump generated a larger current than the contralateral control stump. Specifically, the peak control current densities were 28, 39, and 38 $\mu A/cm^2$ whereas the corresponding denervated stump values were 99, 61, and 47 $\mu A/cm^2$.

As a further direct test of the hypothesis that the regeneration currents are skin-driven, we varied the sodium concentration in the medium and observed marked and reversible effects on these currents (Table 1). When the sodium level was raised from the standard 1.7 mM by about 5-fold (to 8 mM), the peak current density leaving the surface of the stump was always found to have risen 15–20 min later. In cases 1–7, in which the starting currents were 16 $\mu A/cm^2$ or less, the increase averaged 4.7-fold. Thus, the relative increase in current tended to equal that in external sodium concentration. In cases 8–11, in which the starting currents were relatively high (29–86 $\mu A/cm^2$), the increase tended to be far smaller, varying from 10 to 45% and averaging only 23%. We lowered the sodium level to nearly zero (i.e., ≤0.001 mM) in two different ways. In cases 12–14, we shifted directly to this medium from the standard medium. The peak current then fell greatly. Within 15–30 min, it fell by 99%, 96%, and 79%, respectively. In cases 4–11, we shifted to near-zero sodium from high sodium and the current fell by 60–98%. Altogether, these data seem consistent with the concept that the regeneration current tends to vary with external sodium concentration over a considerable range of concentrations, which includes the natural level, as well as over a considerable range of current densities between a small sodium-independent base current and a saturating level.

As a confirmatory test of the hypothesis that these currents are generated by the skin, we blocked sodium entry into the skin

Table 1. Effects of changes in external sodium* on peak stump currents 1 day after amputation

Na+	Time†	Animal 1	2	3
	Response to standard, high, then standard sodium			
Std.	0 min	2.8	4.2	16
High	20	17	18	106
Std.	100	12	1.4	14

		Animal 4	5	6	7	8	9	10	11
	Response to standard, high, then low sodium								
Std.	0 min	2.4	3.6	3.6	4.3	29	37	41	86
High	15	2.5	8.2	8.0	46	42	41	50	99
Low	15	1.04	0.2	0.2	0.85	1.0	3.6	3.4	3.7

		Animal 12	13	14
	Response to standard, low, then standard sodium			
Std.	0 min	10.4	20	27
Low	15	8.0	0.89	0.22
	30	2.2		
Std.	2 hr	6.5	1.5	8.1
	3		1.4	9.5
	4	12.2	5.1	26
	5		14	

The values within the body of the table indicate peak current densities in $\mu A/cm^2$.

* Standard sodium was 1.7 mM (artificial pond water); high was 8 mM (artificial pond water plus 6.3 mM NaCl); low contained ≤0.001 mM as determined with a sodium-specific electrode (artificial pond water with sodium salts omitted).

† Time elapsed after the last change of medium.

Table 2. Rapid effects of amiloride and of meLys treatment on peak stump currents (in $\mu A/cm^2$)

Drug	Case	Before drug	15 min after	50 min after
Amiloride	1	3.9	0.48	
	2	6.8	2.5	
	3	22	16	4.4
	4	53	15	4.0
	5	79	24	3.2
	6	120	26	8.8
meLys	7	15	0.4	
	8	21	1.9	
	9	24	8.0	0.2
	10	28	2.3	

Currents were measured 24 (cases 3 and 4) or 30 hr after amputation. In each case, a control measurement was made with standard artificial pond water. Animals were then fully immersed in a solution of either 0.5 mM amiloride or 3 mM meLys in this same medium. Readings were then made 15 min and 50 min later (except for case 9, in which it was made 30 min later).

with amiloride (12, 13) or with the methyl ester of lysine (meLys) (14). Table 2 shows the results. In six cases, including a range of initial values from 4 to 120 $\mu A/cm^2$, 0.5 mM amiloride reduced the peak current by an average of 68% within 15 min. Measurements were continued for 50 min in four cases. In these, the current had fallen by an average of 90%. In three of four cases, 3 mM meLys reduced the peak current by 90% or more within 15 min; in a fourth case, such reduction took 30 min to reach.

We routinely immobilized the animals with tricaine methanesulfonate before measuring currents. However, checks with two other agents [0.5% urethan and *d*-tubocurarine (intraperitoneally)] showed comparable outward stump current densities (see also ref. 15). Because three quite different immobilizing agents yielded comparable values, we conclude that our use of tricaine methanesulfonate did not unduly affect the results.

DISCUSSION

Nature and Source of the Stump Currents. Our main finding is that a current with a peak density of the order of 10–100 $\mu A/cm^2$ is driven out of the end of the newt's forelimb stump for about a week after amputation. Our results agree with those of Monroy and of Becker in showing current leaving the stump end during the first week or two after amputation. Neither Monroy nor Becker determined the size of the currents that produced the surface potentials which they measured. However, it is of at least historical interest to note that Monroy used a 1 KΩ galvanometer to measure these voltages (A. Monroy, personal communication), a meter of such low resistance that much of the current may actually have traversed the meter. Hence, the outward current density (from a 1-mm^2 stump surface) corresponding to his first recorded measurement (of 0.15 mV at 1 day after amputation) can now be estimated as about 15 $\mu A/cm^2$. Thus, in retrospect, it agrees with our new measurements.

Both the skin and the nerves have been suggested as the source of the electrical output of injured or amputated amphibian limbs. Thus, Becker has claimed that denervation largely eliminates the surface potential gradient along intact limbs (16) and has apparently assumed that nerves are the source of limb regeneration currents (5). However, the results of both Monroy (4) and Lassalle (17) argue against a neural source of regeneration currents and argue for a skin source. Both found surface potentials to be unaffected by denervation, whereas Lassalle further reported that ablation of the epidermis eliminates these potentials.

Our own findings clearly point to the skin rather than to the nerves as the pump that drives these stump currents. Transection of the main limb nerves certainly does not reduce these currents; indeed, it seems to actually increase them. Moreover, strong positive evidence for an epidermal origin of these currents is provided by techniques that affect the tendency of external sodium to enter the skin. It is well known that amphibian skin currents consist, in fact, of a sodium influx and therefore require sodium entry (18). When we either removed sodium from the medium or introduced substances (amiloride or meLys) that block sodium entry, the stump current was quickly and greatly reduced. On the other hand, increases in external sodium above the usual pond-water level induced rapid increases in the stump current.

Although the stump surface is covered by a wound epithelium within 5–10 hr after amputation (S. V. Bryant, personal communcation), large outward currents persist for a week or more. Presumably, the new epithelium has a relatively high electrical conductivity, so that the charges pumped into the animal by its intact skin leak out through this region. However, it may be noted that the density of these outward currents does not necessarily decline steadily after amputation. Indeed, it may peak as late as 4 days after amputation. Finally, it should be pointed out that currents driven by injury to individual nerve and muscle cells would be expected to *enter* the stump rather than leave it. To the extent that such cellular injury currents were significant, they would therefore be expected to have reduced the observed outward stump currents and may well explain why the earliest currents are not always the largest.

It may also be asked what specific ions carry the stump current. Most of the current pumped by the skin into the stump undoubtedly consists of sodium ions, whereas the current leaving the cut end of the stump is driven by the electric field in turn produced by this sodium pump. Moreover, until a new epithelium forms, this outward current should also consist largely of sodium ions because these are the dominant interstitial species available for carrying positive charges outward. Inward electrophoresis of chloride ions should contribute relatively little to this current because chloride is so dilute in the external medium, whereas outward diffusion of NaCl, which must also occur, will not of course carry any net charge. After an epithelium covers the stump, the ions that carry current out are unknown.

Relationship of Stump Currents to Regeneration. Our new data suggest that the stump currents are a part of the regeneration mechanism and not just an epiphenomenon. First of all, the natural stump current in newts proves to be comparable to the artificially imposed currents previously used to stimulate a degree of regeneration in frog limbs (1, 19), in direction (Fig. 4), duration (weeks), position (mainly postaxial), and density. The upper limit to the densities imposed in the stimulation experiments can be calculated on the assumption that the total current drawn out of the stump both crossed and affected the region just beneath the stimulating electrode. In Smith's experiment (19), this figure was 0.1 μA divided by 0.1 cm^2 or 1 $\mu A/cm^2$; in our own experiment, it was about 0.2 μA/0.01 cm^2 or 20 $\mu A/cm^2$. These values are clearly comparable to or even smaller than the natural current densities leaving newt stumps (Fig. 2).

Second, a careful reconsideration of some older papers reporting stimulation of frog regeneration by salt solutions reveals that relatively *low* concentrations of sodium salts are quite effective. Among the most effective treatments used by Rose (20)

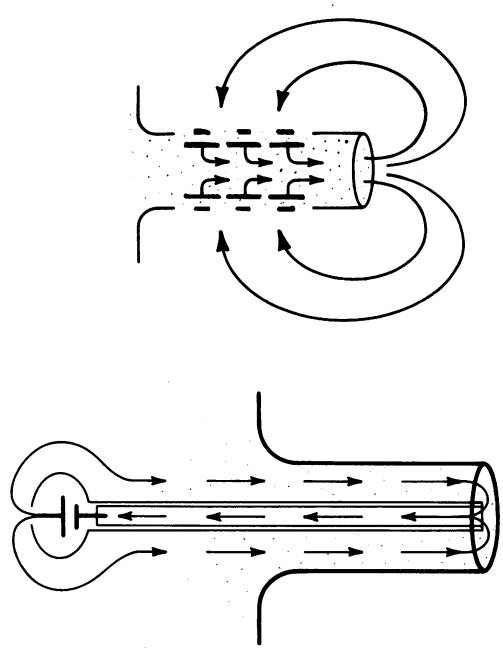

FIG. 4. Comparison of circuits driving endogenous currents through newt limb stumps (*Upper*) to artificially imposed currents through frog limb stumps (*Lower*). The endogenous current was driven by the skin batteries. The artificially imposed one was driven by a mercury cell implanted in the dorsal lymph sac. Current was pulled through the tissue to the mercury cell from the end of the stump through a Ringer's solution-soaked wick that was insulated from the tissue (1).

and Polezhaev (2) were repeated brief applications of 50 mM NaCl and 10 mM $NaHCO_3$, respectively. Comparable increases in external sodium greatly increase the voltage across frog skin (21,22). Hence, these previously inexplicable and largely forgotten effects of dilute sodium solutions can now be interpreted as acting by stimulation of the frog's skin battery.

A third argument that supports a role for the stump currents in newt regeneration is that a plausible general mechanism for possible effects on cell behavior of such currents is now available. We would suggest that skin-driven currents of this magnitude and duration produce sufficient fields in traversing some part of the stump to move and localize certain developmentally critical macromolecules electrophoretically. Both calculation and experiment indicate that steady potential drops as small as 1 mV across a cell can electrophoretically segregate some of the mobile macromolecules floating in its plasma membrane (7, 23, 24). Because the electromotive forces of amphibian skin batteries are known to range from 10 to 100 mV (14), target cells in some sufficiently resistive part of the current's path might well be exposed to a voltage drop of 1 mV or more. Plausible target cells would include epidermal cells within the wound epithelium as well as the neurons that penetrate this tissue (1).

Altogether, these considerations have encouraged us to further explore the hypothesis that there are natural electrical controls of amphibian regeneration.

These experiments also raise a further question. Are there skin-driven electrical currents that promote healing or even regeneration in other vertebrates including man? There is a hint in the clinical literature that artificial application of steady current (which seems to have been of the order of 10–100 $\mu A/cm^2$) may speed healing of skin ulcers in man (25). Moreover, there is an old and largely forgotten literature that has demonstrated what may well be a natural counterpart to these artificially imposed currents: large, steady, endogenous electrical currents invariably leave injured regions of human skin as well as a wide variety of other vertebrate integuments (26, 27). Herlitzka, for example, detected currents as large as 1.4 μA leaving small, superficial wounds made in the skin of his own hand. The area of the damaged regions seems to have been of the order of 1 mm^2, indicating outward current densities of up to 100 $\mu A/cm^2$. Our new results encourage reinvestigation of these old findings.

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