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Cell Wall Is Required for Fixation of the Embryonic Axis in *Fucus* Zygotes

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Establishment of a primary developmental axis generally is thought to involve rearrangements in the plasma membrane or cytoplasm of the egg. In this report the additional requirement for cell wall in polarization of *Fucus* zygotes was investigated. Protoplasts of fertilized eggs were tested for their ability to establish an axis in accordance with an orienting vector of unilateral light. The results demonstrate that cell wall is not required for axis formation. However, the orientation of the axis remains labile until new cell wall is synthesized. The presence of a cell wall is an absolute requirement for axis fixation.

FORMATION OF A PRIMARY EMBRYONIC axis establishes the framework on which all subsequent development depends, yet it is not well understood. The main difficulty in studying axis formation is that in most organisms it takes place early in the differentiation of female gametes. When the egg is mature, the orientation of the preformed axis is clearly visible; in animal eggs it corresponds to the animal-vegetal polarity, whereas in higher plant eggs it is reflected in the asymmetric localization of the vacuole. Thus, this fundamental process transpires within a single, relatively undifferentiated cell buried deep inside somatic tissue and not amenable to experimental manipulation.

Fucus zygotes offer several advantages. Large populations of fertilized eggs develop synchronously and are independent of other cells. During the first hours after fertilization, the orientation of the developmental axis is labile and can be experimentally manipulated by imposing external gradients on the zygotes (*1*). The most convenient polarizing vector is unilateral light; zygotes form an axis parallel to the light gradient. At 11 hours after fertilization, the axis becomes irreversibly fixed in space (*2*). In *Fucus* the axis determines the position of the rhizoid, which emerges from the spherical zygote at germination, 16 hours after fertilization. When unilateral light is applied, the rhizoid grows out from the dark hemisphere.

We recently developed a two-step procedure using cell wall–digesting enzymes for isolating homogenous populations of protoplasts from young *Fucus* zygotes (*3*). At the end of the 4.5-hour digestion period, no cell wall is visible by Calcofluor (a specific probe for β-linked glycosidic polymers) staining, electron microscopy, or birefringence in polarized light. When these protoplasts are placed in regeneration medium [RM; 60% artificial seawater (ASW) containing sucrose], synthesis of new cell wall is detected within 6 hours and is similar to wall assembly in normal zygotes (*4, 5*). Once the sucrose concentration in RM is lowered to less than 0.4*M*, the zygotes germinate, form normal rhizoids, and divide. Cells remain attached to the substratum throughout protoplast isolation and regeneration. We have used this procedure in conjunction with orienting pulses of unilateral light to investigate the role of the cell wall in establishing the polarity of the embryo.

We first investigated the requirement for cell wall in maintaining a fixed, polar axis. Zygotes developing in petri plates in ASW (*6*) were oriented by pulses of unilateral light that terminated at 11 hours, the end of the period of axis fixation (*2*). The cell wall was removed in two steps over 4.5 hours (11 to 15.5 hours), and the resulting protoplasts were allowed to regenerate a wall and germinate in uniform, nonorienting light (15.5 to 48 hours). At 48 hours the embryos were scored for the position of rhizoid outgrowth. When zygotes were polarized by unilateral light, rhizoids grew out parallel to one another on the shaded hemisphere (Fig. 1) (*7*). The percent polarization was calculated as the percentage of embryos bearing rhizoids on the shaded hemisphere. Control cells were treated as above except that no wall-digesting enzymes were added.

Short light pulses did not localize rhizoid outgrowth efficiently in any of the zygotes, probably because they did not receive sufficient energy for orientation. In these cases treated zygotes were not quite as well oriented as controls (the percent polarization was reduced by 10 and 17% in 3- and 5-hour light polarizations, respectively). However, polarization improved when the light pulse was longer; a 7.5-hour light

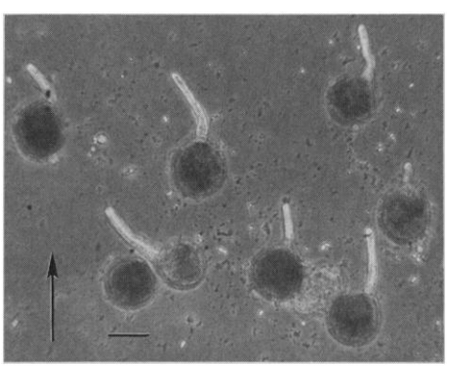

Fig. 1. Photopolarization of zygotes. Zygotes were given a pulse of unilateral light from 2 to 11 hours. Protoplasts were then obtained by treating zygotes for 1.5 hours in medium A [100 m*M* NaCl, 20 m*M* $MgCl_2$, 5 m*M* KCl, 250 μ*M* $CaCl_2$, 0.2% (w/v) bovine serum albumin (BSA), 0.2 m*M* phenylmethylsulfonyl fluoride (PMSF), 10 m*M* tris, and 1*M* sorbitol, buffered to *p*H 7.8 with 2-(*N*-morpholino)ethanesulfonic acid (MES)] containing cellulase (CEL) (2 mg/ml) and alginate-lyase, H45-80 (1 unit/ml), followed by 3 hours in medium P [ASW containing 0.2% (w/v) BSA, 0.2 m*M* PMSF, 0.45*M* NaCl, 10 m*M* MES, buffered to *p*H 5.8 with tris] containing CEL (20 mg/ml) and alginate lyase (10 units of H45-80 and 0.1 unit of AG5-85 per milliliter). [See (*3*) for details of protocol.] Resultant protoplasts were placed in RM containing 0.8*M* sucrose for 12 hours, after which the sucrose concentration was lowered in steps of 0.2*M* every 4 hours. The regenerating protoplasts germinated on the dark side of the light pulse. Embryos were 4 days old when the photograph was taken. Arrow indicates direction of light vector. Scale bar, 50 μm.

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pulse polarized 94% of controls and 88% of protoplasts, and a 9-hour pulse yielded polarizations of 98 and 94%, respectively. (Over 400 embryos were scored for each percentage; replicate dishes varied by <3%.) Thus, cell wall removal from well-polarized zygotes did not disrupt the orientation of the axis. The finding that a high percentage of protoplasts remained oriented to the light confirmed that cells were attached throughout the experiment and did not rotate on the dish.

We next studied whether regenerating protoplasts have the ability to form a polar axis in response to light. Zygotes were grown for 7 hours in uniform light. The walls were then digested from 7 to 11.5 hours (uniform light), and the protoplasts and controls were placed in RM. As soon as RM was added, the dishes were placed in unilateral light for various times. At the end of this orientation period, zygotes were grown in uniform, nonorienting light, and the percent polarization was scored as before at 48 hours. Both regenerating protoplasts and controls were extensively oriented by light vectors lasting 5 hours or longer (Table 1A). Cell wall was first detected with Calcofluor 2 hours after adding RM. Again the percentage of treated zygotes polarized lagged slightly behind controls. Nonetheless, wall removal and regeneration did not affect the zygotes' ability to respond subsequently to a polarizing pulse of unilateral light.

The timing of axis fixation in regenerating protoplasts and controls was investigated by adding a second light pulse (LP2), oriented 180° to the first (LP1) and given immediately after the termination of LP1 (*7*). If the axis was fixed during LP1, rhizoids would emerge on the dark side of this orienting vector. If, however, the axis was still labile at the end of LP1, it would be reoriented by the subsequent LP2 and rhizoids would grow from the dark side of LP2. Axis fixation was measured as the percentage of zygotes oriented by LP1. Three-fourths of control zygotes had fixed axes within 4 hours in RM, whereas regenerating protoplasts reached the same degree of fixation after 8 hours (Table 1B). In both cases the time available to fix an axis in response to unilateral light was prolonged. Three-fourths of the zygotes developing normally in ASW fixed their axes at 11.5 hours (*2*), which in this experiment was the time at which LP1 was initiated. Therefore fixation was delayed 4 hours in controls and 8 hours in regenerating protoplasts. Regenerating protoplasts first stain positively for cell wall with Calcofluor at 1 to 6 hours of regeneration (*3*), suggesting that an intact cell wall may be required for axis fixation.

Recently, we have studied developmental changes in protein synthesis, using two-dimensional gel electrophoresis and fluorography (*8*), and have found that the synthesis of a few, specific proteins changes during early embryogenesis (*9*). These proteins serve as molecular markers for the normal developmental program and allow us to assign a specific pattern of protein synthesis to each developmental stage. During normal development the pattern of protein synthesis shows the following changes in one region of a two-dimensional fluorograph: when zygotes were pulse-labeled from 2 to 6 hours, the lower row of proteins labeled preferentially (Fig. 2A, row 2); whereas, when labeled from 21 to 28 hours, synthesis had shifted to the upper row (Fig. 2C, row 1). Pulsing at an intermediate time, 12 to 16 hours, labeled both rows nearly equally (Fig. 2B). The ratio of relative optical density (OD) (row 1/row 2) calculated after

Table 1. (A) Axis formation during wall regeneration. A single pulse of unilateral light was given in RM for the time indicated. Germination was 50% in treated zygotes and 80% in untreated. (B) Axis fixation during wall regeneration. LP1 and LP2 were given during regeneration in RM for times indicated. Polarization by LP1 was assayed as the percentage of germinated zygotes with rhizoids on the hemisphere shaded during this light pulse. Germination was 70% in treated zygotes and 90% in untreated controls. (C) Axis formation in protoplasts. The procedure for a 2.5-hour light pulse is discussed in the text. Germination averaged 75% in untreated zygotes and 65% in treated ones. (D) Axis fixation in protoplasts. The procedure for 21-hour light pulses is discussed in the text. Because of the long treatment with dilute enzymes during LP1, germination was reduced to an average of 30% of treated zygotes. In all experiments, the first light pulse began at 11.5 hours. Over 250 embryos were scored for every percentage calculated. Polarization in replicate dishes did not differ by more than 6%. NA, not applicable.

Treatment*	Duration (hours)		Polarization (%) by LP1
	LP1	LP2	
(A) Axis formation during regeneration			
+	5	NA	85
−	5	NA	93
+	9.5	NA	94
−	9.5	NA	98
+	12	NA	96
−	12	NA	97
(B) Axis fixation during regeneration			
+	4	4	45
−	4	4	76
+	8	10	75
−	8	10	94
+	18	19	94
−	18	19	94
(C) Axis formation in protoplasts			
+	2.5	NA	76
−	2.5	NA	94
+	6	NA	86
−	6	NA	96
(D) Axis fixation in protoplasts			
+	21	21	5
−	21	21	86
+	45	45	3
−	45	45	99

*(+) indicates treatment with wall-digesting enzymes and (−) indicates controls without enzyme treatment.

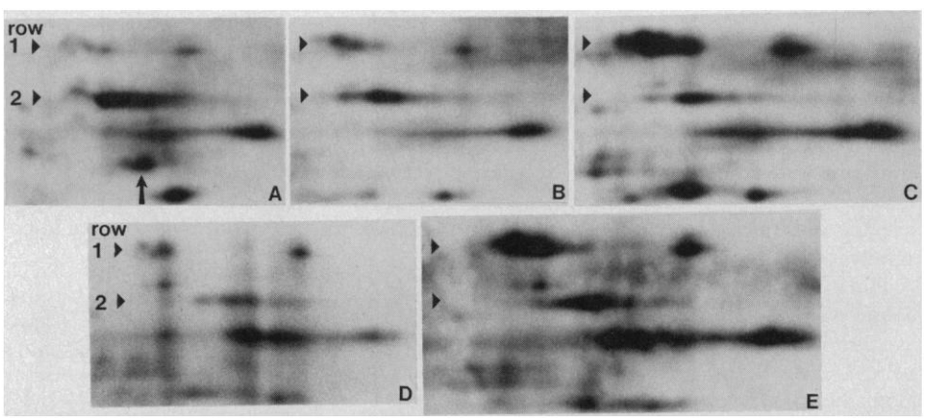

Fig. 2. Comparison of protein synthesis in zygotes developing normally in ASW (A, B, and C), in protoplasts (D), and in regenerating protoplasts (E). The same region of fluorographs of two-dimensional protein gels is shown in each frame. Zygotes were labeled with $Na_2{}^{14}CO_3$ (100 μCi/ml) in ASW for the period indicated, rinsed with nonradioactive medium, and frozen at −20°C in an extraction buffer. At a convenient time proteins were extracted as an acetone powder. Proteins solubilized from the powder were resolved by isoelectric focusing followed by SDS–polyacrylamide gel electrophoresis. (**A**) Zygotes developing normally in ASW labeled from 6 to 12 hours. (**B**) Normal zygotes labeled from 12 to 16 hours of development. (**C**) Normal zygotes labeled from 21 to 28 hours. (**D**) Protoplasts. Wall was removed from 7 to 11.5 hours, and cells were labeled from 12 to 22 hours in medium P containing 15% enzymes to prevent new wall synthesis. (**E**) Regenerating protoplasts labeled in RM from 12 to 22 hours after wall removal (7 to 11.5 hours). All fluorographs were in the linear range for film exposure and were scanned with an LKB Ultrascan XL laser densitometer to calculate the OD ratios given in the text. Any differences in the exposure time of fluorographs was eliminated by calculating ratios. Arrow in (A) indicates the position of protein synthesized exclusively in young zygotes (2 to 6 hours).

fluorographs were scanned was 0.24, 1.24, and 4.36 for the untreated zygotes labeled at 2 to 6 hours, 12 to 16 hours, and 21 to 28 hours, respectively. If wall removal at 7 hours stopped development, one would expect protoplasts to synthesize the 2- to 6-hour pattern. If, however, protoplasts continued along a normal developmental program, we would expect to see a shift to synthesis of proteins in row 1. Protein synthesis in protoplasts pulsed from 12 to 22 hours resembled the normal 12- to 16-hour pattern (Fig. 2D). That is, the intensity of row 1 was modestly greater than that of row 2; the ratio of relative OD was 1.56. The shift to synthesis of proteins in row 1 was more marked in regenerating protoplasts (Fig. 2E). The ratio of relative OD was 2.41 when regenerating protoplasts were labeled from 12 to 22 hours. The protein indicated by the arrow in Fig. 2A was synthesized during normal development in the 2- to 6-hour sample, but not at later times. Protoplasts and regenerating protoplasts did not make this protein, confirming that the normal progression of changes in protein synthesis continued after wall digestion.

Since protoplasts continued along a normal developmental program with respect to protein synthesis (Fig. 2), we were able to study the apparent requirement for cell wall in axis fixation (Table 1B) by investigating the ability of protoplasts to establish an axis. Again axis formation and fixation were studied together. The procedure used for axis formation was similar to that used to generate Table 1A except that the light pulse was given in the presence of wall-digesting enzymes, before wall regeneration. Zygotes were grown for 7 hours in uniform light in ASW, their walls were digested from 7 to 11.5 hours as before, and they were placed in medium P containing 15% of the normal enzyme concentration, which prevented new wall biosynthesis. The light pulse was initiated as soon as the dilute enzyme solution was added to the protoplasts. At the end of the light pulse (at 14 hours for a 2.5-hour light pulse), zygotes were placed in RM and allowed to regenerate a wall and to germinate in uniform, nonorienting light. Orientation was scored at 48 hours. Table 1C shows that three-fourths of protoplasts formed an axis during a 2.5-hour light pulse. Untreated controls with walls were polarized to a greater extent, but this difference diminished as the duration of the light treatment was lengthened to 6 hours.

Axis fixation again was studied by use of a second light pulse oriented 180° from the first. After wall removal at 11.5 hours, protoplasts were given LP1 in a 15% enzyme solution; this lasted from 11.5 to 32.5 hours for the 21-hour light pulse reported in Table 1D. The oriented protoplasts were placed in RM and LP2 was given for 21 hours, from 32.5 to 43.5 hours, during wall regeneration. At the end of LP2, zygotes were placed in uniform, nonorienting light and allowed to germinate. Rhizoid position was scored 24 hours later. Although they formed an axis quite rapidly, protoplasts were unable to fix it irreversibly in place (Table 1D). That is, the orientation of the axis remained labile as long as the cell wall was absent. Even when LP1 lasted 45 hours, only 3% of protoplasts underwent fixation; by comparison, the axis was fixed in >99% of control zygotes by this time.

It has generally been assumed that the plasma membrane and cytoplasm control fixation of the primary embryonic axis (*10*). We have found that the cell wall also plays an obligatory role in this poorly understood process. Enzymatic wall removal inhibited axis fixation almost entirely, yet, when allowed to regenerate a wall, the protoplasts regained fixation competence.

In animal cells the structure most analogous to cell wall is extracellular matrix, and this organelle has also been shown to be involved in establishing cell polarity (*11*). Type IV collagen can orient the apical to basal axis in epithelial cells (*12, 13*), and E-cadherins, components of the Ca^{2+}-dependent cell-cell adhesion system, are involved in polarization of mouse blastomeres (*14*). In light of the results presented here, it is plausible that extracellular matrix also plays a part in establishing the primary developmental axis of the animal embryo, that is, the animal-vegetal axis, which is laid down while the developing oocyte is surrounded by somatic tissue and matrix material.

Our findings extend and refine proposed models for establishing polarity in the *Fucus* zygote. An accumulation of Ca^{2+} channels in the plasma membrane at one end of the fertilized egg (future rhizoid site) is thought to constitute formation of the initial axis. The data presented here confirm that the cell wall does not participate in this process. Quatrano *et al.* (*15*) proposed that fixation entails anchoring these Ca^{2+} channels to microfilaments in the cytoplasm; this proposal was based on the finding that cytochalasins, inhibitors of microfilament polymerization, block axis fixation (*7*). This localized attachment is thought to confer a permanent polarity upon the zygotes. The additional requirement for cell wall during fixation raises the possibility that the wall provides an external matrix to which the microfilaments are connected. From this perspective, fixation would involve the formation of transmembrane bridges between cytoskeletal filaments and wall fibrils at the future rhizoid site. Such transmembrane interactions are well documented in both plant and animal cells and are usually indirect linkages involving many accessory proteins as well as an integral membrane protein (*16*). In *Fucus* the membrane protein may be the localized Ca^{2+} channel involved in axis formation. This arrangement would be reminiscent of the indirect attachment between the anion channel (band 3), spectrin, and actin in erythrocytes (*17*).

It was surprising that the orientation of a fixed axis was not destroyed by wall removal. This finding may indicate that the requirement for cell wall in polarization is transient. Once the axis is fixed, other localized processes (such as metabolic activation) that maintain cell polarity may be initiated rather quickly. Alternatively, once the wall structure required for fixation is in place at the future rhizoid site, it may be insensitive to digestion. This possibility is bolstered by reports that the structure and composition of the wall change around the time of fixation (*18*), and wall removal becomes more difficult in older embryos (*3*).

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Antagonistic Adrenergic-Muscarinic Regulation of M Current in Smooth Muscle Cells

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The β-adrenergic agonist isoproterenol and analogs of adenosine 3′,5′-monophosphate (cAMP) induced a potassium current, M current, in freshly dissociated gastric smooth muscle cells. Muscarinic agonists suppress this current, apparently by acting at a locus downstream from regulation of cAMP levels by adenylate cyclase and phosphodiesterase. Thus, M current can be induced by an agent and regulated in antagonistic fashion by β-adrenergic and muscarinic systems.

THE MODULATION OF IONIC CHANnels by neurotransmitters occurs via a variety of second messengers, including cyclic nucleotides, guanosine triphosphate (GTP)–binding proteins, calcium ions, and metabolites of phosphatidylinositol and arachidonic acid (*1–3*). Dual regulation of the same ionic channel by transmitters exerting antagonistic effects has been documented for Ca^{2+} channels in heart (*4–6*), potassium channels in *Xenopus* ovarian follicles (*7*), and K^+ channels (S channels) in invertebrate neurons (*3, 8*).

The M current is a transmitter-regulated, voltage-sensitive K^+ current suppressed by acetylcholine acting on muscarinic receptors, which was first described in sympathetic neurons (*9*) and since has been observed in a variety of cells, including smooth muscle (*10*). A number of neuropeptides [substance P, luteinizing hormone–releasing hormone (LHRH) (*11, 12*), and bradykinin (*2*)] also suppress M current. But M current has not appeared to be susceptible to dual regulation because only its suppression, and not its induction by transmitters, has been demonstrated. We report here that M current can be induced by the β-adrenergic agent isoproterenol in isolated smooth muscle cells, an induction that can be mimicked by forskolin or phosphodiesterase-resistant analogs of adenosine 3′,5′-monophosphate (cAMP). Hence, this current is regulated in antagonistic fashion by muscarinic and β-adrenergic systems. Some of these results have appeared in preliminary form (*13*).

The effects of isoproterenol on ionic currents were studied in smooth muscle cells freshly dissociated from the stomach of the toad *Bufo marinus* (*14*). Voltage-clamp was accomplished with either conventional microelectrodes (*15*) or with patch electrodes in the whole-cell configuration (*16*). Records from a cell held at a depolarized level and periodically hyperpolarized are shown in Fig. 1. Isoproterenol caused the slow development of outward current at −15 mV, which appears as an upward displacement in the holding current. In response to hyperpolarization of the membrane, the initial rapid current jump (or "ohmic jump"), which is directly proportional to the membrane conductance, increased upon isoproterenol application (compare Fig. 1, a and b). Furthermore, after isoproterenol, the ohmic jump upon hyperpolarization from −15 to −65 mV was followed by a marked slow inward current relaxation, which represents the turning off of the isoproterenol-induced outward current at −65 mV (*10*). This voltage-dependent turnoff of the underlying conductance accounts for the absence of isoproterenol effects at more negative potentials.

The outward current activated by isoproterenol persisted for several minutes but was quickly abolished by acetylcholine (ACh). Suppression of the outward current was accompanied by a decrease in the ohmic jump, indicative of a conductance decrease, and elimination of the inward current relaxations, reflecting the abolition of voltage-dependent current (compare Fig. 1, c and d). The decrease in conductance and absence of current relaxations demonstrate that ACh acts by suppressing outward current and not by activating an opposing inward current. [ACh applied to the cell before any exposure to isoproterenol caused a smaller decrease in conductance, indicating the presence of some M current prior to isoproterenol application, which we designate "endogenous"

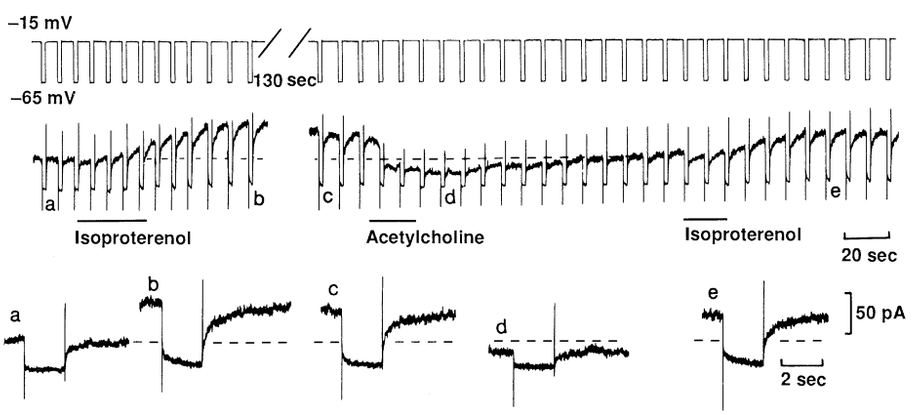

Fig. 1. Isoproterenol activates outward current in smooth muscle cell. (Upper trace) Membrane potential; holding potential, −15 mV, with periodic hyperpolarizations to −65 mV. (Lower trace) Current, with selected responses to hyperpolarizing commands shown on an expanded time scale. Isoproterenol caused slow development of outward current (upwards) above the control dashed line. The induced outward current exhibited voltage sensitivity as indicated by the current relaxations (compare expanded trace a with traces b and c). (Inward current relaxations at the onset of the hyperpolarizing pulses represent K^+ current turning off. Outward current relaxations, at the offset of the pulses, represent the slow activation of K^+ current but with some contamination by voltage-activated Ca^{2+} current.) The magnitude of endogenous M current is indicated by the shift of the current below the control level at −15 mV after ACh application (trace d). Another measure of the endogenous M current is provided by comparing the ohmic current jumps at the onset of the hyperpolarizing pulse in traces a and d; the difference is due to endogenous M current. At −65 mV there is little difference in steady-state current (compare traces a and d), because M current is largely deactivated at this potential. Inward current relaxation due to the endogenous M current is not readily apparent in trace a, because the magnitude of this current is relatively small and its relaxations are faster than those of induced M current. A conventional microelectrode was used in this experiment. The results illustrated are representative of responses from 44 cells.

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