Contractile Basis of Ameboid Movement VIII. Aequorin Luminescence during Ameboid Movement, Endocytosis, and Capping

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ABSTRACT Aequorin luminescence has been utilized to determine the spatial and temporal fluctuations of the free calcium ion concentration [Ca^{++}] in *Chaos carolinensis* during ameboid movement, pinocytosis, and capping. The [Ca^{++}] increases above ~10^{-7} M during normal ameboid movement. Three types of luminescent signals are detected in cells: continuous luminescence, spontaneous pulses, and stimulated pulses. Continuous luminescence is localized in the tails of actively motile cells, and spontaneous pulses occur primarily over the anterior regions of cells. We are sometimes able to correlate the spontaneous pulses with extending pseudopods, whereas stimulated pulses are induced by mechanical damage, electrical stimulation, concanavalin A-induced capping, and pinocytosis. The localization of both distinct actin structures and sites where [Ca^{++}] increases suggests cellular sites of contractile activity. The independent evidence from localizing actin structures and the distribution of [Ca^{++}] can also be viewed in relation to the solation-contraction coupling hypothesis defined in vitro.

Calcium regulation of cytoplasmic structure and contractility in ameboid cells was first identified in single cell models from *Chaos carolinensis* (66). The calcium regulation of both gelation and contraction was subsequently described in bulk cell-free extracts (21, 68, 69), in partially reconstituted models (31), and in single living cells (70). Recently, a high-affinity calcium-binding protein which regulates gelation and plays a role in regulating contraction (77) (see references 73 and 74 for reviews) has been isolated from *Dictyostelium discoideum*. Evidence for calcium regulation of cytoplasmic structure and/or contractility has also been demonstrated in cell models from other ameboid cells, including *Acanthamoeba* (50), Ehrlich tumor cells (45), and macrophages (78). In addition, a great deal of indirect evidence has also supported the suggestion that calcium regulates at least some of the events involved in ameboid movements (see references 33 and 74 for reviews).

A direct identification of fluctuations in the free calcium ion concentration in living cells is necessary to identify calcium as a physiologically relevant intracellular messenger involved in cell motility. This is especially true because other parameters, including changes in pH, can also affect cytoplasmic structure and contractility in vitro (21, 68, 69).[1] Ultimately, a map of the spatial and temporal distribution of all potential regulatory factors must be constructed and correlated with motile behavior in living cells.

Aequorin luminescence (9–11, 56, 60, 61) has been used as a highly sensitive indicator of intracellular free calcium in several types of cells (see references 9, 10 and 56 for reviews). The high signal to noise ratio, relative specificity for Ca^{++}, lack of toxicity, and ease of signal detection have made this technique attractive for the study of cells containing low free calcium ion concentrations. Aequorin luminescence has already been applied successfully to other motile processes, including the contraction of muscle (3, 4, 8, 23), shuttle streaming in *Physarum polycephalum* (58), fertilization of Medaka eggs (59), and the contraction of *Spirostomum* (26).

This report is aimed at characterizing the relationship between cellular processes, including ameboid movement and the local intracellular [Ca^{++}]. We have also initiated an integration of the available facts on primary messengers, membrane prop-

[1] Heiple, J., and D. L. Taylor. Manuscript submitted for publication.

THE JOURNAL OF CELL BIOLOGY · VOLUME 86 AUGUST 1980 599-607

erties, secondary messengers, and contractile events involved in stimulated motile processes. These studies and the accompanying paper (72) further indicate the value of the giant free-living amebas for studying basic cellular processes. Preliminary reports of this work have appeared in abstract form (67, 71).[2]

MATERIALS AND METHODS

Materials

Materials were obtained as follows: EGTA, PIPES, dimethyl sulfoxide (DMSO), concanavalin A (Con A) (all from Sigma Chemical Co., St. Louis, Mo.), Alcian blue (Eastman Kodak Co., Rochester, N. Y.), and phalloidin (Boehringer Mannheim Biochemicals, Indianapolis, Ind.).

Solutions used in the experiments were prepared as follows: Marshall's medium contained 5.0×10^{-5} M $MgCl_2$, 5.0×10^{-4} M $CaCl_2$, 1.47×10^{-4} M K_2HPO_4, and 1.1×10^{-4} M KH_2PO_4 at pH 7.0 (66). Calcium-free Marshall's was identical, except calcium was absent. Lanthanum Marshall's contained all the components in Marshall's, except that 10^{-4} M $LaCl_3$ replaced all the $CaCl_2$. The calcium buffers contained 5.0 mM PIPES, 5.0 mM EGTA, 75 mM KCl, and various concentrations of $CaCl_2$, pH 7.0. Free calcium ion concentrations in Ca^{++}/EGTA buffers were calculated using an apparent association constant of 2.51×10^6 M^{-1} at pH 7.0, the value determined by aequorin luminescence (11). A phalloidin stock solution was diluted into the Marshall's solution to a final concentration of 100 μg/ml. 0.1% Alcian blue was prepared in distilled H_2O, brought to pH 6.5 with 0.1 N KOH, and filtered through No. 1 filter paper (Whatman, Inc., Clifton, N. J.).

Methods

AMEBAS: Specimens of *C. carolinensis* were cultured in Marshall's medium with mixed ciliates for food. The cells were starved for 2 d, and only vigorously moving specimens were selected for study (70).

AEQUORIN: *Aequorea forskalea* were collected at Friday Harbor, Washington, and the photoprotein aequorin was isolated, purified, and lyophilized as described elsewhere (11). Lyophilized EDTA-free aequorin was dissolved in a buffer containing 2.5 mM PIPES, 75 mM KCl, pH 7.5, to a final concentration of from 1–5 mg/ml.

A calibration curve of aequorin luminescence plotted against the log $[Ca^{++}]$ was determined in vitro (1, 2, 11). The aequorin test solution contained 2.5 mM PIPES, 75 mM KCl, 1.0 mM $MgCl_2$, and either dilutions of $CaCl_2$ or Ca^{++}/EGTA buffers (0.5 mM), pH 7.0. The peak light intensities were measured when constant quantities of aequorin were mixed rapidly with solutions of various calcium ion concentrations (1, 11). All values were expressed in terms of fractional luminescence, that is, as fractions (L/L_{max}) of the maximal signal (L_{max}) obtained in saturating $[Ca^{++}]$. In this way each measurement of light intensity was normalized for the amount of aequorin used in a particular experiment.

To express light measurements from living cells in terms of fractional luminescence, it was necessary to estimate L_{max} under the optical conditions of the experiment. This was done by applying enough pressure to the cover slip to crush the ameba while integrating the light emitted as the aequorin in the cell was discharged by the calcium (5×10^{-4} M) in the bathing medium. L_{max} was determined from the integrated light signal by multiplying the integral by the rate constant for aequorin discharge in saturating $[Ca^{++}]$ at the temperature of the experiment (2).

MICROINJECTIONS: The aequorin solution was loaded into fine-tipped micropipettes (Final o.d., 0.1 μm) drawn from Omega Dot 1.0-mm o.d. (Frederick Haer & Co., Brunswick, Me.) tubing and injected into the cells by applying pressure from a compressed air tank (up to 50 atm). Approximately $^1/_{10}$ of the cell volume (average cell volume was 40 nl) was injected, and we could detect no long lasting influence of the injection on the appearance or behavior of the cells. The aequorin diffused rapidly throughout the cell, independent of the region of the cell injected. The homogeneity of the aequorin distribution was demonstrated in early experiments by gently stimulating the cells with a piezoelectric vibrating probe (57). Stimulation of multiple sites around the perimeter of cells resulted in the same level of luminescence. The aequorin was, therefore, judged to be uniformly distributed. A uniform distribution was also demonstrated for soluble proteins such as FITC-labeled ovalbumin when they were injected into cells and viewed in the fluorescence microscope (72).

LIGHT DETECTION: The aequorin luminescence of cells was analyzed with three different systems. Total luminescence from whole cells was determined with an EMI 9635B photomultiplier with a 1-inch diameter lucite rod serving as a light guide (9, 11). This system allowed the collection of light from the whole cell when the lucite rod was positioned ~0.5 mm above the cell. Light measurements were also made by coupling an EMI 9635B photomultiplier to the microscope containing a 50 × water immersion objective (NA 1.0) to collect light from specific parts of the cells. This setup lost light gathering power but gained spatial resolution. Light signals were recorded in analogue form but were calibrated with a photon counter in terms of counts per second (cps). Image intensification was performed with high gain microscope image intensifier television systems similar to those described elsewhere (54–56). The four-stage image intensifier (EMI 9694) was viewed by means of a TV camera (Philips Plumbicon or RCA SIT). The output was presented in real time on the TV monitor and recorded on magnetic tape for subsequent analysis. The gain of the image tube could be varied from $\sim 10^4$ to 1.5×10^6, depending on the strength of the luminescence. The TV camera was replaced by a Polaroid camera in experiments requiring exposures of more than $^1/_{30}$ s.

MANIPULATIONS OF CELLS: Cells were perturbed by various physical and chemical stimuli and were oriented in an electric field by stimuli of ~10.0–50 V/cm for 50.0 ms (67). The damage response was elicited by inserting an empty micropipette through the plasmalemma into the cell cortex (71). A piezoelectric vibrator was also used to stimulate cells with vibrations conducted through the medium (57). Capping of Con A on the cell surface was induced as described in the accompanying paper (72). Pinocytosis was induced by rinsing the cells with the Alcian blue solution. Pinocytotic channels formed within a few minutes (39). Phalloidin at a final concentration of $\sim 10^{-4}$ M was injected into cells previously injected with aequorin (72). This study represents experiments performed on more than 50 separate cells.

RESULTS

General Characteristics of Luminescence

Microinjection of aequorin or just the carrier buffer into cells in Marshall's medium resulted in an increase in the speed of cell movement away from the site of injection for several minutes. This transient increase in movement (streaming velocity ~1.0–3.0 μm/s) was correlated with a large aequorin luminescence that decreased as the cell slowed to the normal rate of movement (streaming velocity ~0.5–1.0 μm/s) (Fig. 1). This intense luminescence was localized primarily in the tail (Fig. 2).

Cells with normal rates and patterns of movement exhibited fluctuations in aequorin luminescence for at least several hours (Fig. 3). The signals were characterized by a continuous luminescence that usually varied between 5 and 10 times the dark current of the photomultiplier (70–80 cps) and by spontaneous pulses of luminescence of variable amplitude (1,000–3,000 cps) that persisted for ~2–5 s each. Uninjected cells exhibited no signal above the dark current.

The luminescence of motile cells was localized either by

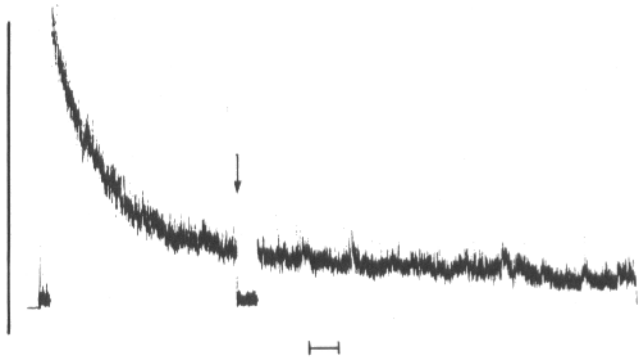

FIGURE 1 Luminescence measured from an entire ameba immediately after microinjecting aequorin into the cell. The cells moved with increased velocity while the luminescence was elevated. There was a decrease in both the rate of movement and luminescence after recovery from the injection. Photomultiplier signal is calibrated in terms of counts per second (cps). Vertical scale bar represents 3,000 cps. Horizontal scale bar represents 10 s. The shutter was closed briefly at the arrow.

[2] P. Cobbold kindly shared his findings on aequorin luminescence in *C. carolinensis* before publication.

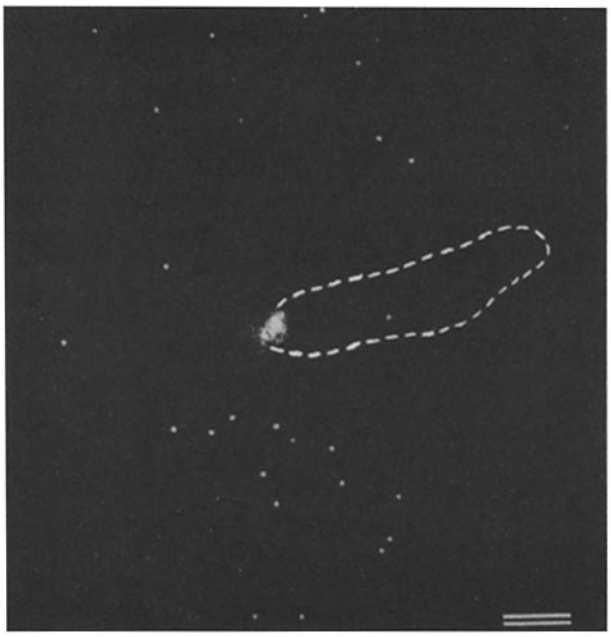

FIGURE 2 Intensified image of the luminescence of the tail of an ameba immediately after microinjection of aequorin as shown in Fig. 1. The level of luminescence corresponds to ~3,000 cps (see Fig. 1). The rest of the cell extended to the right of the luminescence and is outlined by the dashed line. Scale bar, 300 μm. This image was recorded in 1/60 s.

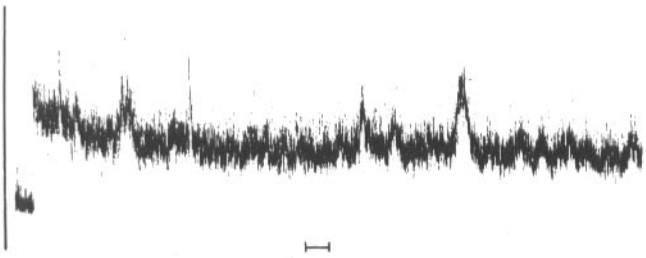

FIGURE 3 Aequorin luminescence measured from an entire cell moving normally (after recovery from injection) and exhibiting both continuous luminescence and spontaneous pulses of luminescence. Vertical scale, 1,000 cps; horizontal scale, 5 s. The shutter was closed at the beginning of the record.

image intensification (Figs. 2 and 4) or by light measurement through a microscope. The form and direction of cell movement was always judged by viewing the cells in the microscope just before and after image intensification or microphotometry and sometimes could be seen simultaneously with luminescence by weak backlighting. Both techniques indicated that the continuous luminescence was localized primarily in the tails, whereas the spontaneous pulses of luminescence occurred primarily over the anterior regions (Figs. 2 and 4). The time required to shut down the intensifier high voltage, to increase the intensity of the light source, and to manually close shutters made it difficult to record the spontaneous pulses and to verify the site of luminescence and the direction of streaming. Therefore, only a qualitative description of spontaneous pulses can be presented at this time.

Luminescence was correlated with cell movements by reversibly inhibiting ameboid movement with lanthanum chloride, which has been shown to inhibit movement of *C. carolinensis* (30) and other cells (13, 41, 42, 76) by blocking the calcium channels in the membrane or by competing for bound calcium at the cell surface. The resting free calcium ion concentration in vivo was calculated by measuring the aequorin luminescence in those cells inhibited with lanthanum chloride (see Materials and Methods). The log of the fractional luminescence of these nonmotile cells was -5.98 ± 0.11, which represents $\sim 1.0 \times 10^{-7}$ M free calcium (Fig. 5). This calculation assumes that the free $[Ca^{++}]$ was uniform throughout the space containing the aequorin. The luminescence from motile cells was two or three times greater than that in La^{+++}-inhibited cells. However, the free calcium ion concentration could not be calculated accurately because the percent volume of the motile cells exhibiting elevated luminescence could not be determined. Cells stopped extending well-defined cylindrical pseudopods (lobopodia) within 30 s after the replacement of calcium, with 10^{-4} M $LaCl_3$, but the endoplasm continued to stream out of the tail, forming broad blebs for ~2 min. Spontaneous pulses of luminescence also stopped within 30 s, and both types of luminescence decayed to a low level as the cells came to a rest in a rounded form (Fig. 6). Most significantly, washing the cells with normal Marshall's solution (containing $\sim 10^{-4}$ M Ca^{++}) induced the cells to resume normal movement slowly. Recovery from $LaCl_3$ inhibition was characterized by the initiation of spontaneous pulses of luminescence and the abortive formation of pseudopods (Fig. 6) followed by larger spontaneous pulses of luminescence and a rise in the continuous luminescence until normal movement returned.

Injection of calcium buffers into cells containing aequorin indicated the possible range of free calcium during movement. The injection of ~1/10 cell volume with the 3.6×10^{-6} M Ca^{++} buffer induced extensive intracellular contractions and an in-

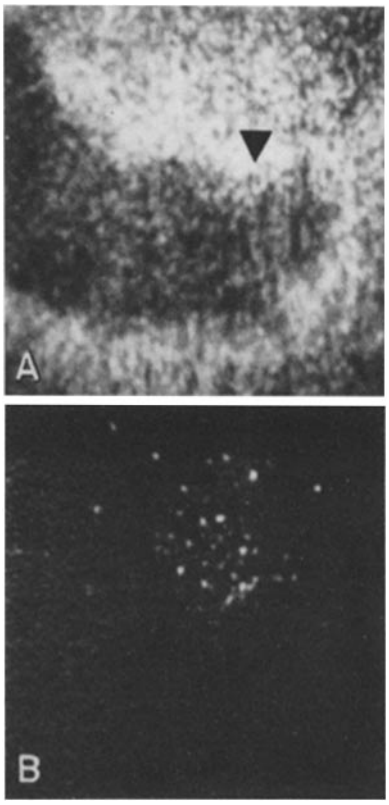

FIGURE 4 Intensified image of aequorin luminescence in a cell moving normally. Background illumination was used to locate the tail of the cell (arrow) (*A*). The continuous luminescence was localized in the tail (*B*) and corresponds to ~500–700 cps. This image was recorded over a 5-s period.

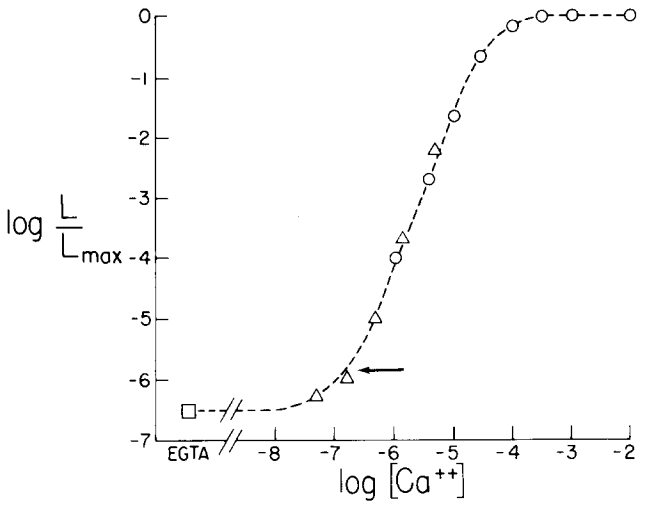

FIGURE 5 Calibration curve relating the intensity of aequorin luminescence to free calcium ion concentration under conditions thought to be appropriate to the cytoplasm of amebas. Curves were determined in vitro in solution containing 75 mM KCl, 1.0 mM $MgCl_2$, 2.5 mM PIPES buffer, pH 7.0 at 22°C. Three rounded, nonmotile cells exhibited an average log fractional luminescence of −5.98 (arrow). (○) $CaCl_2$ dilutions. (△) Ca-EGTA buffers (0.5 mM EGTA). (□) 0.5 mM EGTA with no added Ca^{++}. The arrow indicates the $[Ca^{++}]$ measured in resting (nonmotile) cells.

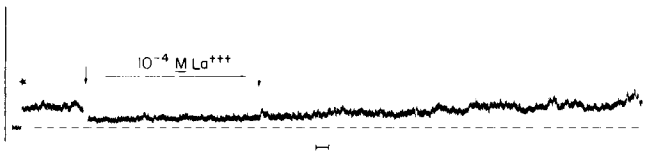

FIGURE 6 Aequorin luminescence recorded from an entire cell moving normally in Marshall's solution. The cell was rinsed with calcium-free Marshall's containing 10^{-4} M EGTA for 10 s and then with calcium-free Marshall's containing 10^{-4} M lanthanum chloride (1st arrow). The continuous luminescence was depressed, and the spontaneous pulses of luminescence were inhibited in the presence of lanthanum. Replacing the medium with normal Marshall's (2nd arrow) led to the reappearance of spontaneous pulses of luminescence and an increase in the continuous luminescence as the cell began moving. Vertical scale, 3,000 cps; horizontal scale, 5 s. The short section at the beginning of the record is the photomultiplier signal with the shutter closed.

crease in luminescence at the site of injection comparable to that observed during the injection response (Figs. 1, 2, and 7). The injection of ~1/10 cell volume with 2.1×10^{-8} M Ca^{++} buffer caused a transient decrease in cell movement and a small decrease in total luminescence. Therefore, normal cell movements probably involve fluctuations in the free calcium ion concentration somewhere between the resting level ($\sim 1.0 \times 10^{-7}$ M) and ($\sim 3.6 \times 10^{-6}$ M) (24, 25).

Continuous Luminescence

The continuous luminescence was the most consistent and easily detected signal. All motile cells exhibited continuous luminescence that was maximal in the tail (Figs. 1, 2, and 4) and was localized in approximately the same region as the wrinkled area (see reference 72, Fig. 3*a*). The geometry and extent of the maximal luminescence varied from cell to cell. The continuous luminescence of unperturbed cells was not immediately dependent on the presence of calcium in the medium because both streaming and continuous luminescence continued for ~1–2 min after being washed with calcium-free stabilization solutions (68) or $LaCl_3$ (Fig. 6). However, external calcium was a requirement for continued movement as well as luminescence.

Spontaneous Pulses of Luminescence

The spontaneous pulses of luminescence were the most difficult signals to localize in the cell. Whereas these pulses were detected easily by measuring the light output from whole cells (Fig. 3), the short duration of the signals and the loss in light-gathering power using microscope optics made their exact localization with the microscope very difficult. The most definitive observation was that they occurred primarily in the anterior half of the motile cells, although not all spontaneous pulses of luminescence could be correlated with the extension of pseudopods (71).[2] Placing cells in calcium-free solutions or in $LaCl_3$ (Fig. 6) caused pseudopods to stop extending almost immediately (66), and the spontaneous pulses of luminescence disappeared. However, cells subsequently washed with calcium-containing Marshall's solution exhibited pulses of luminescence and a rise in the total luminescence before extending pseudopods.

The spontaneous pulses of luminescence were correlated with the extension of pseudopods in cells recovering from cold treatment. Immediately after being rewarmed from 4° to 22°C, the cells extended more pseudopods than the controls (6–10 vs. 1–3 per cell) and exhibited spontaneous pulses of luminescence with higher frequencies (data not shown). These cells eventually resumed normal movement and patterns of luminescence after warming (Fig. 3).

Stimulated Pulses of Luminescence

Pulses of luminescence were induced in cells by a variety of physiological and nonphysiological stimuli. Damaging the plasmalemma and underlying cortex has been shown to induce

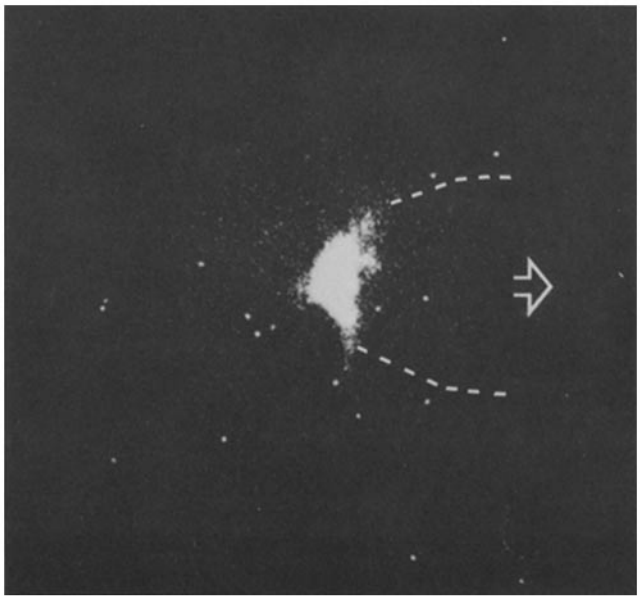

FIGURE 7 Injection of 1/10 cell volume with 3.6×10^{-6} M free calcium into the tail of an ameba caused an extensive cytoplasmic contraction in the tail. The aequorin luminescence was localized at the site of injection. The tail is outlined by the dashed line. Recorded in 1/60 s. The arrow indicates the direction of movement.

a wound-healing contraction of the cortical actin and myosin (35, 72). Damaging the aequorin-injected cells in Marshall's solution by micropuncture resulted in intense localized increases in luminescence at the sites of damage that spread only a few microns from the wounds (Fig. 8). The luminescence persisted for only 3–6 s, and the cells reacted by forming dense intracellular "clots" and by streaming away from the damaged region with an increased rate of movement. There was also a subsequent decrease in luminescence as the cell resumed normal movement, which is similar to the results described for the injection damage response (Fig. 1). In contrast, neither luminescence nor the wound-healing response was elicited when cells were damaged in calcium-free solutions (66, 71).

Electrical stimulation (~5–50 V/cm with a 10–50-ms duration) in Marshall's solution caused the cells to orient in the electrical field toward the cathode (29) and to move with increased velocity, much as they did in response to the injection (Fig. 1). After several stimuli the cells became monopodial, and the luminescence remained elevated primarily in the tail (anodal side). Each stimulus was correlated with a pulse of luminescence in the tail (1–5-s duration) (Fig. 9*A*). However, transient pulses of weaker luminescence could also be observed at the tips of the advancing pseudopods (cathodal side).

Capping of Con A on the surface of *C: carolinensis* (72) was correlated with a transient increase in luminescence (Fig. 9*B*). Therefore, the formation of a distinctly dense and wrinkled tail with an increased amount of actin and most of the Con A (72)

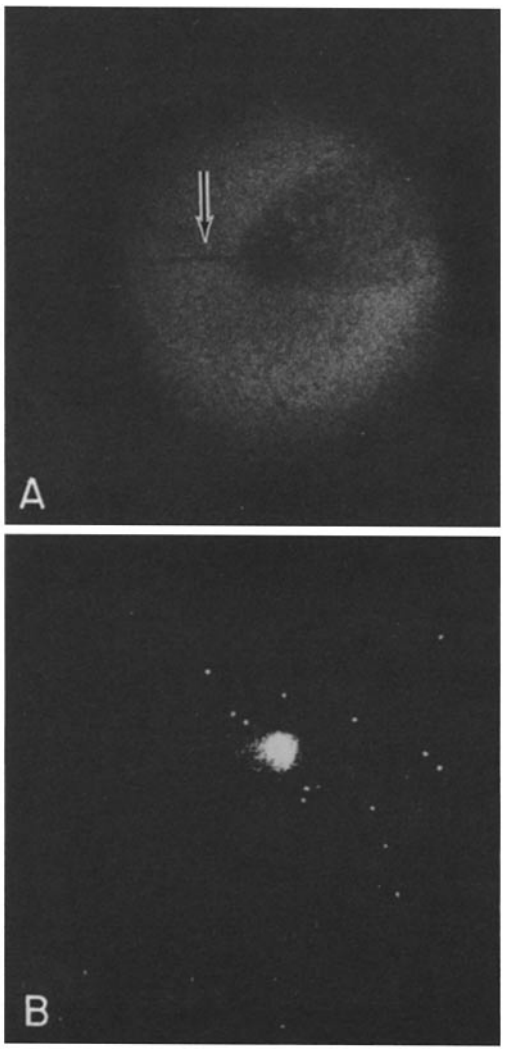

FIGURE 8 Damaging a cell by inserting a microneedle into the cortex initiated the wound-healing response. The micropipette (arrow) and the tail of the cell are shown by raising the background illumination (*A*). The damage response involves an intensive but localized rise in the free calcium ion concentration (*B*), which causes cortical contraction. Recorded in 1/60 s.

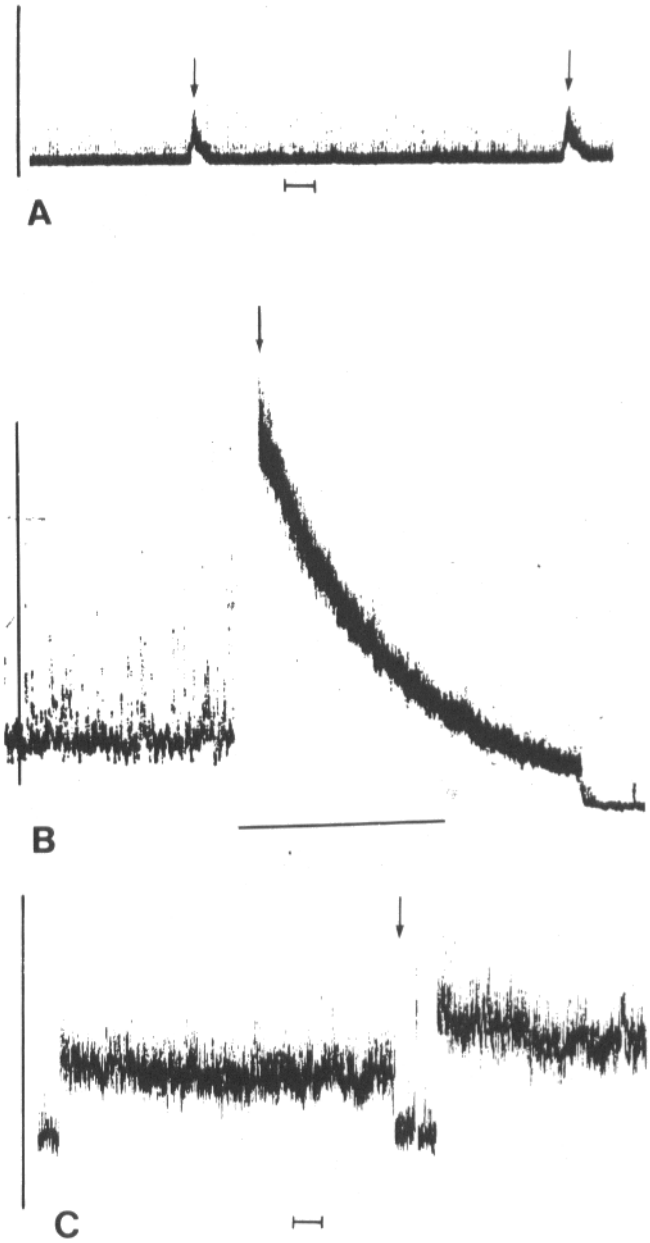

FIGURE 9 (*A*) Single electrical stimulii (arrows) of ~10 V/cm for 50.0 ms caused cells to orient from the anode to the cathode. Luminescence was greatest in the region of the cell closest to the anode, which then became the tail. Vertical scale, 1,000 cps; horizontal scale, 5 s. (*B*) Capping of Con A was preceded by a large pulse of aequorin luminescence. Vertical scale, 3,000 cps; horizontal, 5 s. Shutter closed at end of the record. (*C*) Application of the pinocytosis inducer, 0.1% Alcian blue to an ameba (arrow) resulted in an elevation of the luminescence which preceded the formation of pinocytotic channels as judged by inspection. Vertical scale, 1,000 cps; horizontal scale, 5 s. The shutter was closed during a short initial section of the record and during the section between the control recording and the induction of pinocytosis.

involved a transient rise in the intracellular free calcium ion concentration (16, 20).

Pinocytosis induced with 0.1% Alcian blue was correlated with a small rise in the intracellular free calcium ion concentration. The amplitude of the continuous luminescence increased before the detection of either cortical actin fibrils (72) or fully formed pinocytotic channels (14, 38) (Fig. 9*C*). (Correlations are based on observations made in separate experiments.)

A piezoelectric vibrator (56, 57) induced directed movement and luminescence that depended on the proximity of the vibrator to the cells. Piezoelectric stimulation at a distance (~40–50 μm) induced the formation of pseudopods that extended toward the vibrating probe (40, 47). This positive mechanical taxis (vibrotaxis) was correlated with transient and highly localized pulses of luminescence at the site of pseudopod formation. Immediately after the stimulated pulse of luminescence, a hyalin cap formed that contained a distinct plasma gel sheet (70, 71). When the stimulator was moved closer to the cells (5–10 μm), they did not extend pseudopods toward it but rather exhibited a negative mechanical taxis. The cells always moved away from this stimulus, as they did from electrical stimulation and mechanical damage (Figs. 8 and 9*A*). The formation of a tail at the site of stimulation was correlated with an extensive area of continuous luminescence (data not shown).

Microinjection of phalloidin (final intracellular concentration $\sim 10^{-4}$ M) caused the cortical actin to pull away from the membrane and to contract to one end of the cell over a 1–2-min period (62, 72). The effect of this agent on the intracellular free calcium ion concentration was tested by injecting it into aequorin-loaded cells. Interestingly, the resultant massive contraction was not associated with an increase in luminescence. Control experiments indicated that phalloidin did not interfere with the luminescence of aequorin. Therefore, phalloidin induced contraction without causing a rise in the intracellular free calcium ion concentration. In this respect, the action of phalloidin resembled those of high pH, low temperature, high pressure, and cytochalasin B, which have been shown to produce calcium-independent solation-contractions in cell extracts (21, 63, 69).

DISCUSSION

Correlation between Aequorin Luminescence and Pseudopod Formation and Extension

Our experiments indicate that the cytoplasmic free [Ca^{++}] fluctuated within the range 10^{-7}–10^{-6} M during ameboid movement (Fig. 6). This range of calcium correlates well with the submicromolar threshold identified as regulating both contraction and the sol-gel transformation in model systems of motility (21, 31, 66–69). In addition, we have shown that the velocity of cytoplasmic streaming and cell movement are related to the level of [Ca^{++}] (aequorin luminescence) (Fig. 1). This observation is consistent with the fact that the rate of contraction of cytoplasmic fibrils in single-cell models is related directly to the [Ca^{++}] (66). Therefore, cellular regulation of the [Ca^{++}] below the micromolar level could be an important regulatory mechanism of cell structure and contractility. However, it must be emphasized that other intracellular messengers, e.g. [H^+], could play an important or even preemptory role in regulating cell movement and must be investigated in more detail and compared with the role of calcium (21, 31, 69).[1]

The spatial and temporal distribution, as well as the source of calcium, could explain the ability of this messenger to perform the dual functions of force generation and pseudopod initiation (steering mechanism). The elevated free calcium concentration is sustained over a large area in the tail during movement and is not affected immediately by lowering the extracellular calcium concentration. Therefore, a cellular source of calcium must continue to regulate cytoplasmic solation-contraction in the tail (75). The source of calcium might be the glycocalyx on the cell surface (32, 51) or an intracellular pool of calcium, either bound to the cytoplasmic surface of the plasmalemma (22) or in calcium-sequestering vesicles (19, 53). However, continued movement requires calcium in the medium, suggesting that intracellular or bound calcium must ultimately be replenished from the medium. In contrast, the elevated free calcium ion concentration is transient and localized during the pulses of luminescence. Furthermore, extending pseudopods are much more sensitive to the extracellular free calcium ion concentration, suggesting the requirement of an inward calcium current (47). Therefore, the cellular expression of movement could be regulated in a sensitive manner by the control of the site, rate, and extent of fluctuations in the free calcium ion concentration. These changes, coupled with, or in response to, local variations in other possible messengers, could control very delicate changes in cytoplasmic structure and contractility, resulting in ameboid movement, cell division, endocytosis, and exocytosis. In fact, some of the pulses of luminescence that do not appear to be related to pseudopods could be triggered by endocytosis or exocytosis. A summary of the localization of distinct actin structures and aequorin luminescence is presented in Fig. 10.

Several previous observations have suggested or supported the concept that the free calcium ion concentration increases transiently at the tips of advancing pseudopods and probably originates from the external medium. Pollack (49) injected the Ca^{++} precipitator, alizarin, into amebas and observed the sites of calcium precipitation into purple-red granules during movement. As cells attempted to extend pseudopods, "a shower of purple-red granules" appeared at the site of protrusion. Pollack (49) interpreted these observations as indicating a local increase in free calcium at the site of pseudopod formation. In addition, Taylor et al. (67) demonstrated that *C. carolinensis* emitts continuous luminescence in the tail (anodal side) and pulses of luminescence at the tips of advancing pseudopods (cathodal side) when oriented in an electric field. Stimulation of cells with low frequency vibrations through a microprobe induced the extension of pseudopods toward the microprobe (47) and inward calcium currents, which were measured with the same extracellular vibrating probe (47). This observation is consistent with a positive vibrotaxis observed by Kolle-Kralik and Ruff (40) and the identification of positive spike potentials of the membranes of *C. carolinensis* (65). Finally, Nuccitelli demonstrated that amebas exhibit positive taxis toward a micropipette coated with a calcium ionophore.[3] Therefore, transient influxes of calcium from the medium could be the key step in the formation of new pseudopods.

The mechanism of calcium sequestration in *C. carolinensis* has not been identified. However, vesicles called refractive bodies have been shown to contain high concentrations of calcium (19), and Reinhold and Stockem (53) demonstrated an ATP-sensitive membrane system capable of accumulating calcium in *A. proteus* and *C. carolinensis*. The mechanism(s) for

[3] R. Nuccitelli kindly shared his observations on the effect of calcium ionophores on directed movements.

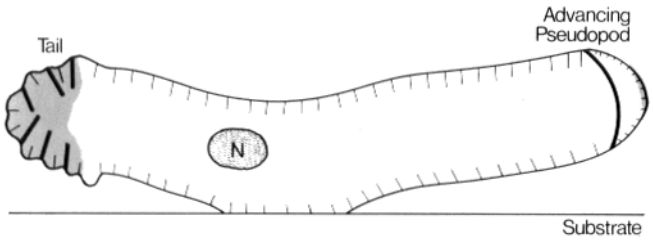

FIGURE 10 Summary diagram showing the comparative distribution of distinct actin structures (heavy and light lines perpendicular to cell surface) in actively moving cells and the localization of free calcium (shaded areas in tail and tip of pseudopod). The heavy lines represent actin structures visible after injection of IAF-actin. Actin appears in distinct structures only in the tail and tips of pseudopods, and is otherwise distributed randomly in motile cells.

reducing the intracellular free calcium ion concentration could involve many separate processes, including binding to, or extrusion across, the plasmalemma (22), incorporation into vesicles (53), and binding to soluble proteins (31).

The Distribution of [Ca^{++}] and Actin Structures in Living Cells: Correlation with the Solation-Contraction Coupling Hypothesis

Fluorescently labeled actin appears to be organized into dense structures close to the plasmalemma, primarily in the tail ectoplasm and in the plasma gel sheets at the tips of advancing pseudopods. Elsewhere, the labeled actin appears to be distributed rather uniformly in motile cells (72). It has been suggested that the control of directed movements and structure in ameboid cells involves specific changes in the local concentration of intracellular messengers (72, 73, 75) that would change both the cytoskeletal and contractile states of an otherwise uniformly distributed system. Calcium could serve both as a messenger for initiating pseudopod extension and for triggering the forces responsible for movement. The continuous elevated calcium concentration in the tails of motile cells could induce solation-contraction coupling (21, 31, 69, 73) of the ectoplasm in the tails (44, 48, 70, 73), resulting in the formation of contracting fibrils that would generate force against the solating ectoplasm (myosin deficient microdomains) (see references 31 and 75 for details). The solated ectoplasm (recruited endoplasm) would then be pushed forward to produce streaming. This suggestion can be correlated with the fact that contracting fibrils and cytoplasmic streaming have been induced in cell extracts (21, 31, 68, 69) and in living cells (70) by raising the free calcium ion concentration to above $\sim 1.0 \times 10^{-7}$ M.

The pulses of elevated free calcium ions observed at the tips of some advancing pseudopods and indicated by numerous other experiments (47, 49, 67),[3] could cause a local and transient solation and contraction. This transient pulse of calcium would be maximal at the membrane-cytoplasm interface and would occur within a small volume, in contrast to the tail luminescence. The extent of solation-contraction would be limited due to the transient nature of the pulse and the small volume of cytoplasm involved. The major effect of the pulse of calcium would be to decrease the gel structure of the ectoplasm locally (31, 75). One consequence of this transient rise in [Ca^{++}] might be the formation of actin fibrils, the plasma gel sheet, caused by solation-contraction coupling, and the separation of these fibrils from the cortex, making this site weaker than the adjacent ectoplasm (72). The force of ectoplasmic contractions centered in the tail could then force endoplasm toward this site of weakened ectoplasm, thus permitting the extension of directed pseudopods (72, 75). Therefore, pseudopod initiation and extension could be the result of a localized weakening of the gel that permits endoplasm to protrude into the forming pseudopod (44, 48). The key to the process is the local activation of the membrane at the site of a new pseudopod followed by the separation of part of the cortical actin from the membrane. The possible role of the plasma gel sheet in force generation cannot be evaluated at this time.

The transformation from endoplasm to ectoplasm at the tips of extending pseudopods would probably require a lowered calcium concentration to maximize gelation (31). Therefore, a balance between localized solation-contraction and gelation must be maintained. It must be emphasized that the above working hypothesis does not explain the formation of all types of pseudopodia in all cells (74). Large cylindrical pseudopods (lobopodia) and broad flat pseudopods (pharopodia or lamellipodia) in some cells could be explained by these processes. However, long slender pseudopods that look like microvilli (filopodia) probably involve other steps.

A major unanswered question is, do the events at the tips of extending pseudopods communicate with the contracting tail, which is sometimes 600 μm away from the tip. In the absence of action potentials (5, 6, 12, 36, 65), various mechanisms of communication are possible, including movement of primary messengers to the tail on the cell surface (analogue of capping) and the formation of a membrane potential gradient (5). However, this question will require more extensive investigations on the electrical and cell surface properties of these cells (64).

The role of calcium as a messenger for inducing wound healing, Con A capping, and pinocytosis has also been indicated by correlating the rise in intracellular free calcium with the formation of actin fibrils during these events (72). For example, the transient rise in the free calcium ion concentration at the site of a wound (Fig. 1) resulted in the formation of fibrils that contracted quickly to form a knot stopping the efflux of cytoplasm. The aequorin luminescence from a single wound lasted only a few seconds, whereas the resultant contracted knot persisted for many minutes. Furthermore, the rise in the [Ca^{++}] during capping and pinocytosis could be the result of transmembrane signaling steps.

Signal Transduction by the Plasmalemma: the Key to Cell Movements

Primary messengers (environmental signals) and the mechanism of signal processing by the plasmalemma must be defined before stimulated events such as pinocytosis, phagocytosis, or chemotaxis can be explained in molecular detail (73). The link connecting the contractile-cytoskeletal system and the intracellular messengers to the environment is the key to understanding complex cell behavior and function.

The induction of pinocytosis represents a model process for correlating the intracellular free calcium ion concentration (Fig. 9*C*), the formation of cortical actin fibrils (72), distinct electrical changes in the plasmalemma (14, 15, 37), and stimulation by primary messengers (pinocytotic inducers) (17, 32). Brandt and Freeman (15) and Gingell and Palmer (27) demonstrated that the initiation of pinocytosis is accompanied by a decrease in electrical resistance of the plasmalemma, implying an increase in the ionic permeability. This might be related to the observation that the binding of pinocytosis inducers causes

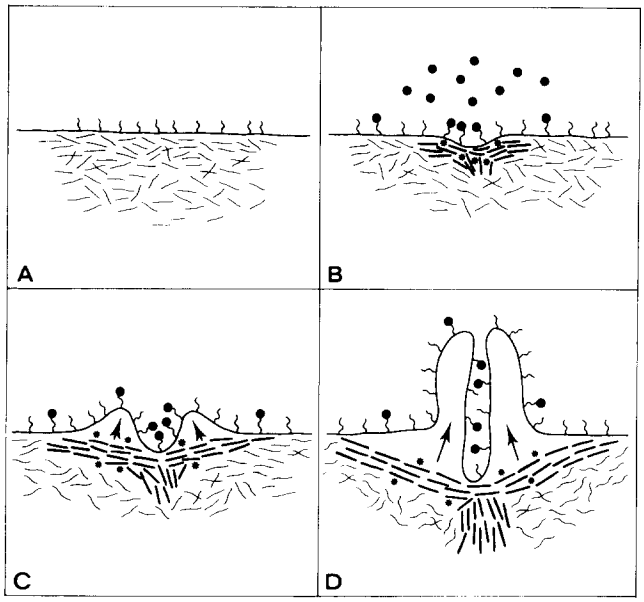

FIGURE 11 Diagramatic summary of the early steps in the induction of pinocytosis. The diagrams represent cross sections of the cell surface, plasmalemma, and cortex. (*A*) Section of cell before activation showing the unperturbed cell surface membrane and cortex in the gelled state. (*B*) Pinocytosis inducers (●) bind to the cell surface (glycocalyx) causing a membrane depolarization and a local rise in the free calcium ion concentration (*). A solation-contraction orients contracting fibrils (heavy lines) that are anchored in the gelled cortex. (*C*) The contracting fibrils separate from the plasmalemma and force the solated cortex outward, forming part of the pinocytotic channel. The membrane remains attached to the contracting fibril at the base of the channel and is pulled down into the cortex during the contraction. (*D*) The pinocytotic channel is formed by a combination of positive pressure due to solation-contraction and tension perpendicular to the cell surface.

membrane depolarization (7, 36, 37) regulated by extracellular calcium and pH (32, 37, 51, 52). Gingell (28) has suggested that the negative surface potential might be decreased when the inducers were bound and that this would directly affect the local membrane potential. These initial changes in the membrane might then permit an inward current of Ca^{++} alone or with other ions (e.g., Na^{+}) that could in turn cause the release of intracellular calcium (43). This rise in free calcium would then initiate solation-contraction coupling, forming fibrils derived from the cortex (72), which would then generate the forces required for channel formation. This proposed mechanism correlates well with many of the original suggestions by Brandt (14), Nachmias (46), Jeon and Bell (34), Josefsson et al. (37), and recently by Klein and Stockem (38) (Fig. 11).

Future studies will be aimed at correlating the distribution of actin structures, possible secondary messengers, including $[Ca^{++}]$ and $[H^{+}]$, and stimulated movements such as chemotaxis (18, 34). Further improvements in our detection and recording techniques should improve our spatial and temporal resolution.

The authors would like to thank V. Fowler, J. Heiple, Skip Virgin and Yu-Li Wang for their helpful suggestions and for critically reading the manuscript. The facilities at the Friday Harbor Laboratories, Friday Harbor, Washington, are gratefully acknowledged.

This work was supported by National Institutes of Health grants AM 18111 to D. L. Taylor and HL 12186 to J. R. Blinks and by Department of Energy contract EY-76-S-02-3120 to G. T. Reynolds.

Received for publication 6 December 1979, and in revised form 26 March 1980.

Note Added in Proof: A recent article by P. Cobbold (*Nature* [*Lond.*]. 285:441–446) also describes aequorin experiments in *C. carolinensis*. His basic data are consistent with our results. However, he reported that La^{+++} treatment did not decrease the aequorin luminescence, and nonmotile cells exhibited more luminescence than motile cells. We found that cells must be pretreated with EGTA to remove the Ca^{++} in the glycocalyx before the La^{+++} treatment would succeed. Furthermore, cells that stop moving randomly, as reported by Cobbold, can actually be stopping because of endocytotic or exocytotic processes that can raise the intracellular $[Ca^{++}]$.

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