DEVELOPMENTAL BIOLOGY 49, 518-531 (1976)

The Ionic Components of the Current Pulses Generated by Developing Fucoid Eggs[1]

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Accepted November 19, 1975

Using a newly developed, extracellular vibrating electrode, we studied the ionic composition of the current pulses which traverse the developing *Pelvetia* embryo. External Na^+, Mg^{2+}, or SO_4^{2-}, are not needed for the first 20 min of pulsing. In fact, lowering external Na^+ or Mg^{2+} (or K^+) actually stimulates pulsing. Since tracer studies show that Ca^{2+} entry is speeded by Na^+, Mg^{2+}, or K^+ reduction, these findings suggest that Ca^{2+} entry triggers pulsing. A sevenfold reduction in external Cl^- raises pulse amplitudes by 60%. Moreover, Cl^- is the only major ion with an equilibrium potential near the pulse reversal potential. These facts suggest that Cl^- efflux carries much of the "inward" current. We propose a model for pulsing in which increased Ca^{2+} within the growing tip opens Cl^- channels. The resulting Cl^- efflux slightly depolarizes the membrane and thus drives a balancing amount of K^+ out. Thus, the pulses release KCl and serve to relieve excess turgor pressure. By letting Ca^{2+} into the growing tip, they should also strengthen the transcytoplasmic electrical field which is postulated to pull growth components toward this tip.

INTRODUCTION

The developing fucoid egg has long been considered to be a prototype of the central developmental phenomenon of localization (Rosenvinge, 1889). In the course of a day, the essentially apolar zygote becomes differentiated into two grossly different regions and then cells: the tip-growing rhizoid cell and the initially quiescent thallus cell. During this period we presume that, as in other tip-growing cells such as *Chara* rhizoids (Sievers, 1967), wall precursor vesicles are continually being localized beneath the growth region membrane to fuse with it. The forces responsible for this localization therefore determine the direction of growth and create the cytoplasmic asymmetry leading to differentiation into the two cell types.

These embryos begin to drive an electrical current through themselves along the prospective growth axis some hours before growth begins (Jaffe, 1966; Nuccitelli and Jaffe, 1974), and we think that this current generates a localizing electrical force (Jaffe *et al.*, 1974a). During the two-cell stage, 25% of this transcellular current is carried in large current pulses which have been detected with an ultrasensitive extracellular vibrating electrode system (Jaffe and Nuccitelli, 1974). A study of their spatial current pattern indicated that the largest internal current density lies beneath the growing tip as shown in Fig. 1. In order to better estimate the magnitude of the electrical field that this current density could generate, as well as to learn more about pulse function, it was desirable to identify the ions carrying the current. Here we report on the ionic components of the pulses as determined by varying the composition of the sea water during pulsing.

Since both positive and negative ions can carry the current, we will try to avoid confusion by referring to electrical currents as positive "charge current." This charge current can then be carried by positive ions moving in the same direction or negative ions moving in the opposite direction. For example, an inward charge current could be carried by Cl^- efflux.

[1] From Richard Nuccitelli's Ph.D. thesis.

MATERIALS AND METHODS

Zygotes of the seaweed, *Pelvetia fastigiata,* were obtained as previously described (Jaffe and Neuscheler, 1969), and cultured at 15°C in dishes containing 3 ml of artificial sea water at pH 8.2. We applied 250 ft-candles of unilateral white light to orient growth in the horizontal plane needed for the current measurements.

The sensor of our extracellular current-measuring system is a vibrating probe with a spherical, 25-μm platinum-black electrode at its tip which measures voltages with respect to a reference electrode 3 mm behind the tip (Jaffe and Nuccitelli, 1974). The probe is vibrated at 200 cps in a horizontal plane between two extracellular points 30 μm apart. Vibration between these points converts any steady voltage difference between them into a sinusoidal output measurable with a lock-in amplifier tuned to the vibration frequency. Since the electric field will be nearly constant over this small distance, it is approximately equal to the voltage difference divided by this distance. The current density in the direction of vibration and at the center of vibration is then given by this field multiplied by the medium's conductivity.

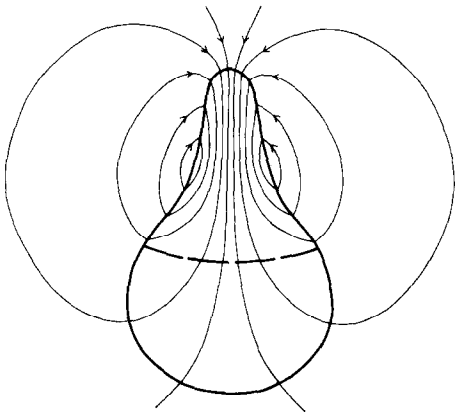

FIG. 1. The spatial pulse current pattern inferred from current density measurements around a two-cell *Pelvetia* embryo (Nuccitelli and Jaffe, 1975).

We have found that the current pulses can be stimulated during the two-cell stage by slight acidification of the sea water with HCl from the natural pH of 8.2 to 6.2 By using this acid-stimulation technique we could systematically study the changes in the stimulated pulses when various ions were omitted from the medium.

The composition of each medium used is listed in Table I. All solutions were made with glass-distilled water. The choline chloride used was purified by recrystallization in hot ethanol and stored at −25°C in a vacuum desiccator. Both Tris (tris(hydroxymethyl)aminomethane) and choline

TABLE I

ARTIFICIAL SEA WATERS

Component	Normal medium (m*M*)	1/250 Na^+ (m*M*)	Mg^{2+}-free (m*M*)	Ca^{2+}-free (m*M*)	3× Ca^{2+} (m*M*)	1/10 × K^+ (m*M*)	10 × K^+ (m*M*)	1/7 Cl^- (m*M*)
NaCl	425	–	425	425	425	434	335	–
Choline Cl	–	425	–	–	–	–	–	–
$NaCH_2SO_3$	–	–	–	–	–	–	–	483
Na_2SO_4	28	–	28	28	28	28	28	–
$NaHCO_3$	2.5	2.5	2.5	2.5	2.5	2.5	2.5	–
$KHCO_3$	–	–	–	–	–	–	–	2.5
KCl	10	10	10	10	10	1	100	7.5
$CaCl_2$	10	10	10	–	30	10	10	–
$CaSO_4$	–	–	–	–	–	–	–	10
$MgSO_4$	–	28	–	–	–	–	–	–
$MgCl_2$	55	27	–	65	55	55	55	37
Tris + Tris-HCl	10	–	83	–	10	–	–	–
EGTA	–	–	–	1	–	–	–	–

were used as sodium substitutes. Methane sulfonate (Eastman Kodak Co.) was substituted for chloride. It has been shown to be a nearly impermeable and nontoxic chloride substitute in frog muscle (Milligan, 1965). Before each experiment the pH, osmotic pressure (Hi Precision Osmometer 3R, Advanced Instruments) and resistivity (Industrial Instruments Conductivity Bridge RC16B2) of the medium to be used was measured. The Ca^{2+}-free sea water was made by replacing Ca^{2+} with Mg^{2+} and adding 1 m*M* EGTA (ethyleneglycol-bis-(β-amino-ethyl ether)N,N'-tetra-acetic acid) to assure that the free Ca^{2+} concentration was less than 10^{-9} *M* even with 0.01 m*M* Ca^{2+} contamination (Caldwell, 1970).

As discussed in the results, Tris and choline did not give the same response when used as Na^+ substitutes. In an effort to determine why they elicited different responses, we examined their influence on the external calcium activity, using 0.1 m*M* of the calcium indicator, murexide, in artificial sea water at pH 8.0. Specific amounts of NaCl were replaced by Tris-HCl or choline-Cl and the ionic strengths were adjusted with NaCl since one-third of the Tris was unprotonated. The free Ca^{2+} concentration was then determined by measuring the absorption difference at the two wavelengths, 470 and 540 nm (Ohnishi and Ebashi, 1963). All measurements were made at room temperature within 2 hr after mixing the solutions. The murexide absorbance difference did not change when the Tris concentration was varied with no Ca^{2+} present. Choline (400 m*M*) had no effect on the Ca^{2+} concentration. However, 400 m*M* Tris reduced the free Ca^{2+} by 30%. The relative Ca^{2+} activity at various Tris concentrations is plotted in Fig. 2. Low Tris concentrations have no effect on Ca^{2+} activity; however, when Tris is used to replace a large fraction of the Na^+ in sea water, the reduction in Ca^{2+} activity is significant.

The procedure for all of the current-measuring experiments reported here was the same. After growing the embryos in unilateral light for 24 hr, the culture dish was moved to an inverted microscope (also with unilateral light) and the vibrating probe was positioned near the tip. A 20-μm gap was maintained between the plasma membrane at the tip and the closest approach of the probe. The embryo's growth rate was determined by measuring its length before and after each experiment with a Vickers image-splitting eyepiece. The medium was exchanged over a 1-min period using a coupled, two-syringe system so that the amount of new medium added was exactly equal to the amount of liquid withdrawn. Nine milliliters of medium were exchanged each time to assure complete medium replacement in the 3-ml chambers. Each embryo was studied for about 2 hr after the medium change.

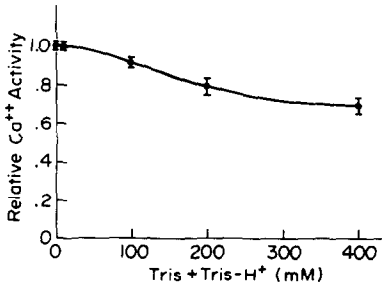

FIG. 2. The reduction of Ca^{2+} activity by Tris in sea water at pH 8.0. Tris was replacing Na^+.

RESULTS

Ion Changes

Hydrogen. Increasing the H^+ concentration 100-fold by a pH change from 8.2 to 6.2 stimulates pulsing immediately, and often about an hour later as described in an earlier paper (Nuccitelli and Jaffe, 1975). Consequently, we have often used this method in these experiments to stimulate pulses while changing other ion concentrations. However, there is no change in pulse amplitudes after this large H^+ increase, and only a 2-mV change in membrane potential after lowering the pH from 9 to 6 and thus increasing H^+ 1000-fold

(Weisenseel and Jaffe, 1972). Furthermore, since the extracellular H^+ concentration is 10^4 to 10^5 times lower than the other common cations, the cell membrane would have to favor H^+ entry by this large factor for H^+ to carry the inward pulse current. Therefore, we feel that it is very unlikely that H^+ is a major current carrier.

Sodium. Na^+ is not required in the external medium for pulsing during the first 20 min after a medium change to low Na^+. In fact, the abrupt reduction of external Na^+ actually *stimulates* pulsing. We studied 32 different embryos using a variety of Na^+ changes from a 50 to a 99.5% reduction in Na^+. Both Tris and choline were used as Na^+ substitutes and Fig. 3 illustrates the pulsing response to two choline substitutions. The nature of the response depends on the magnitude of the Na^+ reduction. While a 75% Na^+ decrease stimulates an increase in the steady current along with a few average amplitude pulses, the more drastic replacement of 99.5% of the Na^+ by choline triggers only two extraordinarily large pulses which slowly fall off over 10 min to a twofold increase in the steady current which lasts about 90 min. There is no further pulsing after 20 min in any choline-substituted medium with less than 10% of the normal Na^+ concentration.

The pulsing response observed when Tris replaces Na^+ is generally milder than for choline. When all of the Na^+ is replaced by Tris, a few small pulses occur and again there is no further pulsing after 20 min. However, in Tris-substituted media this delayed pulse suppression only occurs if less than 2% of the normal Na^+ concentration is present. The difference in the responses to these two Na^+ substitutes may be explained in part by a reduction in Ca^{2+}

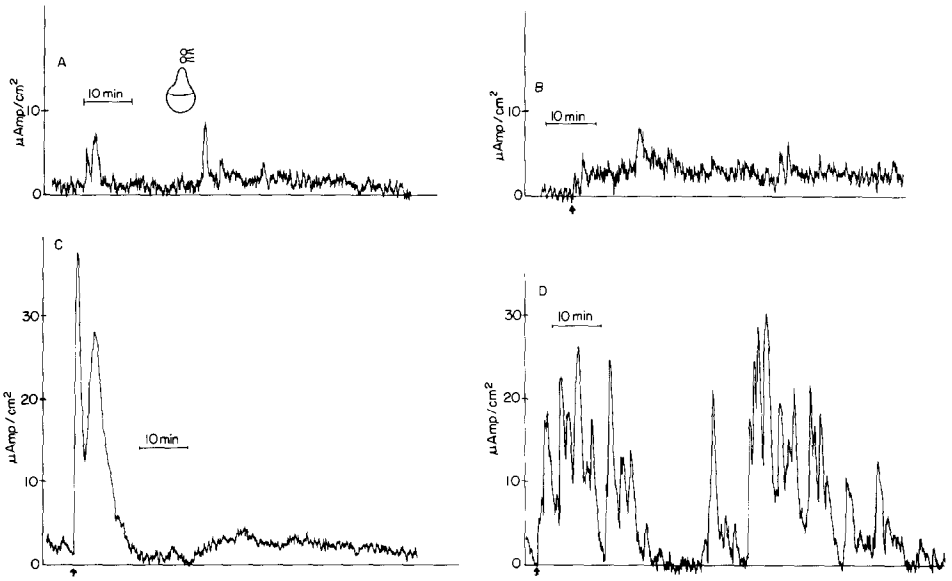

FIG. 3. The pulsing responses to low sodium or magnesium. The probe position indicated in A is the same for all parts of this figure. Ordinates, current densities entering the growth tip; abscissas, time (scale shown in figure). (A) Some spontaneous pulses which occurred in natural sea water at pH 8 in a 27-hr-old embryo. (B) The response to a 75% Na^+ reduction using choline-Cl substitution. The medium was changed at the arrow from artificial sea water at pH 8.2 to low Na^+ sea water at pH 8.2. (C) The response to a 99.5% reduction in Na^+ using choline-Cl substitution. The medium was changed at the arrow from artificial sea water at pH 8.2 to low Na^+ sea water at pH 8.2. (D) The response to Mg^{2+}-free sea water using Tris-HCl substitution. The medium was changed at the arrow from artificial sea water at pH 8.2 to Mg^{2+}-free sea water at pH 8.2.

activity by the high Tris concentration. It will be shown later that the Ca^{2+} activity in the external medium could change the pulsing response. Studies with the calcium-indicator, murexide, show that 450 m*M* Tris lowers the Ca^{2+} activity by 30% as discussed in the Methods section and shown in Fig. 2. Hence, the smaller response observed when Tris is used to replace Na^+ may be due to a reduced Ca^{2+} activity.

Despite these differences, the qualitative results are the same for both Na^+ substitutes. Pulses definitely occur in very low Na^+ at least during the first 20 min. If Na^+ influx were a large component of the current, the pulses should be much smaller in low Na^{2+}. Instead, we often see pulses with a peak surface current density seven times *larger* than average. Consequently, we conclude that Na^+ itself is not a component of the inward pulse current, although its removal somehow stimulates pulsing.

To study the effect of raising external Na^+, we grew four embryos in $^1/_{10}$ Na^+ sea water and subsequently increased the Na^+ by changing to normal sea water at the same pH and osmotic pressure. Since the embryos grow more slowly in low Na^+, two were studied after 36 hr when they had clearly formed cross walls, as well as studying two after the usual 24 hr. In both cases, increasing Na^+ led to a *fall* in pulsing from the average frequency of five per hour to two per hour. Only four pulses occurred during a total observation time of 7.5 hr. For two embryos, one pulse occurred immediately after the change but the other two pulses occurred well after the Na^+ increase. These embryos exhibited normal pulsing when the routine acid-stimulation method was used in low Na^+ medium. Therefore, although they were capable of pulsing normally, increasing Na^+ from 10% to normal partially suppresses spontaneous pulsing.

However, we observed in the earlier experiment that increasing Na^+ from 0.5% to normal usually stimulated a burst of pulses. The main difference between these two cases is the initial Na^+ level. At 0.5% Na^+, *no* pulses occur after the large one or two pulses stimulated by this reduction. When the Na^+ is raised above this level, pulses are stimulated. This suggests that while Na^+ may not be part of the actual pulse current, it is needed in the external medium or the cytoplasm for the proper function of the pulse channels.

Magnesium. External Mg^{2+} is not needed for pulsing and, as with Na^+, lowering the Mg^{2+} concentration (at constant pH and osmotic pressure) stimulates pulsing. Figure 3d shows a typical pulsing response to Mg^{2+}-free medium. This response is very striking because the average pulse amplitude is about four times larger than in normal pulses. There is also an increase in pulse frequency in this medium and this increase was further studied by comparing the pulse frequency in Mg^{2+}-free sea water with that in normal sea water. This was possible because the pulses can be regularly stimulated in normal sea water by lowering the pH from 8.2 to 6.2. In this way a control pulse amplitude distribution was generated and could be compared with the amplitude distributions found when other ion concentrations were modified at pH 6.2. The Mg^{2+}-free pulse amplitude distribution is shown in Fig. 4. The number of pulses in each amplitude interval is indicated in the figure and a second-order fit was made to the intervals in which more than three pulses occurred. The data were plotted on a log-log scale so that the Mg^{2+}-free curve could be easily compared with the control pulse distribution. On this scale, if the Mg^{2+}-free pulses are changed in either amplitude or frequency by a constant factor with respect to the control, it will appear as a shift of the pulse distribution by a constant distance in the respective direction. Figure 4 shows such a shift along the frequency axis indicating a 2.5-fold increase in pulse frequency in Mg^{2+}-free see water. There is no indication of the pulse amplitude increase mentioned above. This is because the amplitude in-

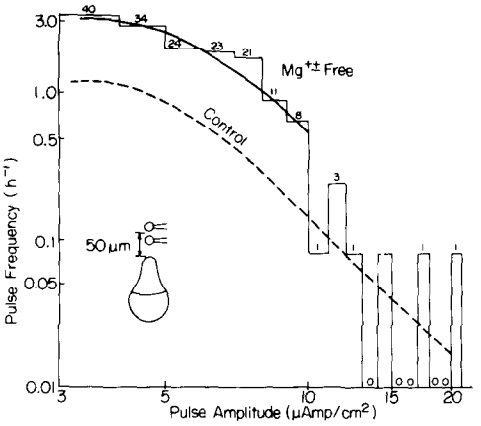

FIG. 4. The frequencies of different pulse amplitudes in Mg^{2+}-free sea water compared with control sea water. The test data (shown by the solid curve and the histogram) represent 169 pulses which entered four embryos during the 2- to 4-hr periods after changing to Mg^{2+}-free, pH 6.2 sea water from artificial sea water at pH 8.2; the control data (shown only by the dotted curve) represents 246 pulses which entered 18 embryos growing for a total of 43 hr in pH 6.2 sea water. Both curves are second-order least-square fits to histograms. The numbers above the histogram indicate the actual number of pulses observed in each test interval. The number of pulses in each control interval was usually about 20% larger. Note that the data is plotted on a log-log scale. The pulse amplitudes represent the peak current density at the rhizoid tip during a pulse.

crease does not occur when both the Mg^{2+} *and* the pH are lowered, but only when the Mg^{2+} is lowered at a constant pH 8.2. Nevertheless, the total current in the response is about the same for the two pH values because at pH 6.2 there is a larger increase in the steady current. The average-amplitude pulses occur on top of this high steady current level of pH 6.2, whereas at pH 8.2 much larger pulses occur on top of a very small steady current level. Thus, the area under these responses is about the same in both cases as indicated in Table 2.

When the medium is changed to one which is both Mg^{2+}-free and has only 0.5% normal Na^+, one pulse five times larger than average occurs and slowly falls off over a 10-min period. Curiously, this response is only about 75% as large as the response for low Na^+ alone.

Calcium. Pulsing is suppressed in Ca^{2+}-free ($<10^{-9}$ *M* free Ca^{2+}) sea water. Four embryos were observed for a total of 6 hr in this medium. Although two were pulsing heavily in normal sea water before changing medium, only one pulse was observed during this time when 30 would have been expected to occur under normal conditions. As mentioned in the Na^+ section, pulsing is also partially suppressed by increasing external Na^+. Since Na^+ and Ca^{2+} are probably competing for entry (Blaustein, 1974; Robinson and Jaffe, in preparation), increasing Na^+ will decrease Ca^{2+} influx. So we have a second case indicating that a decrease in Ca^{2+} influx lowers pulse frequency.

On the other hand, a threefold increase in the external Ca^{2+} immediately stimulates a few pulses. This suggests that Ca^{2+} entry may serve as a trigger for pulse occurrence, since in low Ca^{2+} pulses are suppressed and in high Ca^{2+} they are stimulated.

Increasing external Ca^{2+} is one way to increase Ca^{2+} influx, but an even more effective method is to remove those ions competing with Ca^{2+} for membrane entry sites—specifically, Na^+ and Mg^{2+}. We have tracer evidence which indicates that a 99% decrease in external Na^+ causes a 2.5-fold increase in $^{45}Ca^{2+}$ influx (Robinson and Jaffe, in preparation). As noted earlier, this same decrease stimulated very large pulses. Similarly, the removal of external Mg^{2+} gives a 30% increase in $^{45}Ca^{2+}$ influx (Chen and Jaffe, in preparation) and, as we have seen, this change stimulates large groups of pulses. Finally, it will be indicated below that a 30-mV hyperpolarization in one-tenth K^+ increases pulse frequency by 50% and also enhances Ca^{2+} influx by 40%. Therefore, we find that four different methods for increasing Ca^{2+} influx all stimulate pulsing, and that decreased Ca^{2+} influx radically suppresses pulsing. This leaves little doubt that Ca^{2+} entry is a trigger for the current pulses.

Chloride. When 96% of the external Cl^- is replaced by methane sulfonate, the acid-stimulated pulses are larger than normal.

TABLE 2

PULSE RESPONSE OF 24-HR PELVETIA EMBRYO TO CHANGES IN ION CONCENTRATIONS

Ion	Changes[a]	Stimulates pulsing?	Stimulated response[b] (C × 10^{8})	Nature of pulses
Na^+	1/4×	Yes	6	Average amplitude (av amp)
	1/10×	Yes	18	1 pulse, 7× av amp, 10 min fall-off
	1/250×	Yes	20	1-2 pulses, 7× av amp, 10 min fall-off
	1/250×, Mg^{2+}-free	Yes	16	1 pulse, 5× av amp, 10 min fall-off
	10×	No	–	Suppresses pulsing
Mg^{2+}	Mg^{2+}-free	Yes	13	Groups of pulses, 4× av amp
	Mg^{2+}-free (pH 6.2)	Yes	11	Av amp
Ca^{2+}	Ca^{2+}-free	No	–	Suppresses pulsing
	3×	Yes	6	Av amp
	3×, 1/250× Na^+, 1/2× Mg^{2+}	Yes	48	1 pulse, 9× av amp, 30 min fall-off
H^+	pH 8.2 - 6.2	Yes	12	Av amp
K^+	1/10× (pH 6.2)	Yes	6	Av amp
	5× (pH 6.2)	No	–	Suppresses pulsing
	10×	No	–	A few reversed pulses
	10× (pH 6.2)	Yes	−2	Reversed pulses, 0.7× av amp
	20×	No	–	Suppresses pulsing[c]
	20× (pH 6.2)	Yes	−12	Reversed pulses
Cl^-	1/7×	No	–	1.4× av amp
SO_4^{2-}	SO_4^{2-}-free	No	–	Av amp

[a] All media at pH 8.2 unless specified otherwise.

[b] Coulombs net ion flux per embryo calculated from area under stimulated current pulses during 15 min after medium change. The corresponding value for an unstimulated 15 min would be 1×10^{-8} C. This assumes the current crossed a surface area of 1.3×10^{-5} cm². This surface area was calculated based on the inward current distribution shown in Fig. 1. The current density at the hemispherical tip was exactly as measured but the density along the 25-μm strip below the tip was 25% of the tip density. To adjust for this current density variation, a weighted surface area was used = $7 \times 10^{-6} + 0.25\ (2.5 \times 10^{-5})$ cm² = 1.3×10^{-5} cm².

[c] Pulsing is irreversibly suppressed and will not resume in normal sea water.

This pulse amplitude distribution is shown in Fig. 5. Larger pulses clearly occur more frequently than in the control, while the frequency for smaller pulses is actually lower than normal. It appears that the augmented current flow resulting from the increased frequency of larger pulses is balanced by a decrease in the number of small pulses. Therefore, we do not find a simple shift of the curve to the right along the amplitude axis as would be expected for an amplitude increase in all frequency ranges. Nevertheless, most of the distribution is shifted towards higher amplitudes and the constant shift in this region indicates a 60% increase in pulse amplitude in one-seventh Cl^-.

The substantial average amplitude increase observed in a low chloride medium indicates that some significant part of the pulse current consists of chloride ions. Moreover, our best estimate is that the sevenfold chloride reduction used should raise the electrochemical "force" driving chloride out by 2.3-fold. Comparison of this theoretical expectation with the estimated 1.6-fold increase in pulse amplitude suggests that chloride efflux actually does make up much of the pulse current.

The electrochemical "force" driving chloride out is given by $(E_{Cl} - V_m) = (RT/F)\ln(Cl_i/Cl_o) - V_m$ (Hodgkin, 1958). Estimating an internal chloride activity, Cl_i, of 110 mM (see below) and taking membrane po-

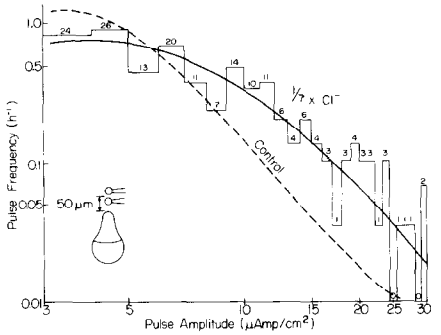

FIG. 5. The frequencies of different pulse amplitudes in $^1/_7\times$ Cl^- sea water compared with control sea water. The test data (shown by the solid curve and the histogram) represent 182 pulses which entered 14 embryos during the 2- to 3-hr periods after changing to $^1/_7\times$ Cl^-, pH 6.2 sea water from artificial sea water at pH 8.2; the control data shown by the dotted curve is the same as described in Fig. 4. Both curves are second-order least-square fits to histograms. The numbers above the histogram indicate the actual number of pulses in each test interval. The pulse amplitudes represent the peak current density at the rhizoid tip during a pulse. Note that the data are plotted on a log-log scale.

tentials, V_m, of -72 and -65 mV in standard and $^1/_7$ chloride sea water (from Weisenseel and Jaffe, 1972), we calculate driving forces of 31 and 72 mV in these two media and hence a 2.3-fold increase. It may be objected that the net efflux depends upon the chloride channels' conductance as well as the driving force. This is true. But a recent analysis of voltage-concentration relations indicates that channel conductances are practically independent of moderate changes in the external concentration of the ions they carry (Jaffe, 1974). (In particular, they certainly do *not* exhibit the conductance changes predicted by constant field theory.) Furthermore, if these channels' conductance *were* significantly effected by a reduction in Cl_o, the conductance should be somewhat reduced; hence, the expected flux increase would only be brought a bit below 2.3-fold and closer to the observed 1.6-fold increase in average pulse amplitude.

Sulfate. Normal pulses occur in sulfate-free sea water. This indicates that sulfate influx is not a major component of the pulse current. Furthermore, if sulfate efflux were involved, one would expect to see an increase in pulse amplitude in sulfate-free medium. This was not found, so we conclude that sulfate is not a component of the current.

The results of the various ion changes are summarized in Table 2.

Membrane Potential Changes

The *Pelvetia* membrane at the two-cell stage is essentially a K^+ electrode with a membrane potential of -72 mV (Weisenseel and Jaffe, 1972). This value can be varied easily by raising or lowering the external K^+ concentration with sodium substitution. (1) When the membrane potential is reduced from -72 to -22 mV by a tenfold increase in K_o^+, the pulses actually *reverse* direction. Six different embryos were studied during this potential change, and the pulses occurring in $10\times$ K^+ had the usual shape but carried current outward at both pH 8.2 and 6.2. This remarkable reversal has never been observed in normal sea water nor during any other ion changes. Figure 6 illustrates this effect and indicates that the pulse amplitudes are roughly equal and opposite at the membrane potentials -72 and -22 mV.

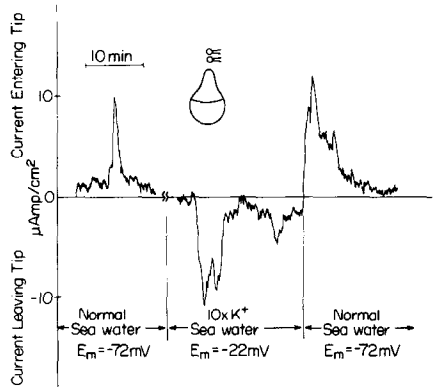

FIG. 6. The pulsing response in $10\times$ K^+ sea water. Ordinate, current density at rhizoid tip; Abscissa, time. While measuring the current density at the rhizoid tip of one embryo, the medium was changed twice at the indicated points from artificial sea water at pH 8.2 to $10\times$ K^+ sea water at pH 6.2 and back again to the artificial sea water.

This approximate equality of amplitudes suggests that the reversal potential lies roughly midway between these two potential values. The steady current level exhibited a small reversal in four of these embryos which usually lasted about 20 min. In one embryo this reversal persisted for an hour accompanied by a large number of outward pulses. (2) The milder reduction of the membrane potential to −40 mV by a fivefold increase in K^+ completely suppresses pulsing. Six embryos were observed for a total of 16 hr in this medium without pulsing, although normal pulsing was observed when these embryos were in normal sea water before and after the period in high K^+. During 16 hr in normal sea water about 80 pulses would be expected to occur. This reversible pulse suppression implies that the reversal potential for these pulses is around −40 mV. This is roughly the same value suggested by the pulse reversal in tenfold K^+. (3) To better study the current reversal we further reduced the membrane potential to −10 mV by a 20-fold increase in K^+. This resulted in two types of responses. Three embryos responded with larger reversed pulses, and 20-40 min after a large group of reversed pulses these embryos burst at their tips. In three other embryos pulsing was irreversibly suppressed and these did not burst. This implies that a large reversed pulse current leads to bursting. (4) In the other direction, an increase in the membrane potential to −106 mV by a 90% reduction in K^+ results in an increase in pulse frequency. Figure 7 shows the amplitude distribution for the 158 pulses measured in this medium. The curve indicates a fairly consistent 50% frequency increase. This suggests that hyperpolarization stimulates pulsing. We now have tracer evidence that this same hyperpolarization enhances Ca^{2+} influx by about 40% (Chen and Jaffe, in preparation). Again we find that increased Ca^{2+} influx results in an increased pulse frequency.

It would be appropriate to consider here which ions could have equilibrium potentials near the −40-mV reversal potential observed. Table 3 lists the major ions involved along with their concentrations. The internal free Ca^{2+} concentration is not known, but in all cells in which it has been studied, it is less than 1 μM (Baker, 1972; Ebashi and Endo, 1968). The internal Mg^{2+} activity is also unknown but is roughly 1 mM in many nerve cells (Baker and Crawford, 1972). The total internal Cl^- is 320 mM (Allen *et al.*, 1972), however our general knowledge of Cl^- compart-

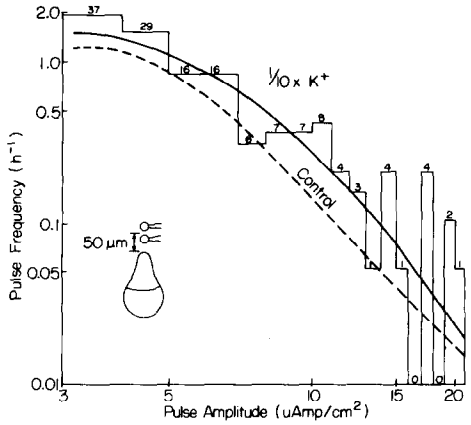

FIG. 7. The frequencies of different pulse amplitudes in $^1/_{10}\times$ K^+ sea water compared with control sea water. The test data (shown by the solid curve and the histogram) represent 146 pulses which entered eight embryos during 2- to 3-hr periods after changing to $^1/_{10}\times$ K^+, pH 6.2 sea water from artificial sea water at pH 8.2; the control data shown by the dotted curve is the same as described in Fig. 4. Both curves are second-order least-square fits to histograms. The numbers above the histogram indicate the actual number of pulses in each test interval. The pulse amplitudes represent the peak current density at the rhizoid tip during a pulse. Note that the data are plotted on a log-log scale.

TABLE 3

EQUILIBRIUM POTENTIALS FOR THE MAJOR IONS

Ion	Internal concentration (mM)		External concentration (mM)	Equilibrium potential (mV)
	Total	Free		
Na^+	20	10?	480	+93
K^+	400	400	10	−91
Mg^{2+}	25	1?	55	+48
Ca^{2+}	4	10^{-3}?	10	+114
Cl^-	320	15 to 150	565	−90 to −30

mentation in algal cells (MacRobbie, 1970) indicates that the cytoplasmic Cl^- is always much less than the average cell Cl^-. Here we assume that $^1/_{20}$ to $^1/_2$ of the cell Cl^- is free. Probably less than half of the cell Na^+ is free (Lev and Armstrong, 1975). From these ion concentrations we calculate the equilibrium potentials predicted by the Nernst equation. It is clear that the only ion with an equilibrium potential even close to −40 mV is Cl^- If it were exactly −40 mV, the internal free Cl^- concentration would be 110 m*M*, the value used earlier to calculate the increase in Cl^- efflux expected by a decrease in external Cl^-

DISCUSSION

The Ionic Components of the Pulses

These ion substitution studies present a rather clear picture of the ions involved in the pulse current pattern shown in Fig. 1. The inward charge current at the growing tip is largely carried by Cl^- efflux. Two results support chloride's involvement. First, the observed pulse current reversal potential is at or near the Cl^- equilibrium potential. Second, lowering the external Cl^- raises pulse amplitudes by roughly the amount expected if Cl^- efflux were responsible for most of the tip current. Na^+, Mg^{2+}, and SO_4^{2-} are not carrying this inward current since pulsing can occur in both Na^+- and Mg^{2+}-free sea water.

The outward charge current is carried by K^+. The Cl^- efflux must be accompanied by an equal efflux of some cation to restore electrical neutrality. Since K^+ is by far the most abundant internal cation and has a permeability 50 times greater than Na^+, K^+ is no doubt the outward current carrier (Allen *et al.*, 1972; Robinson and Jaffe, 1973).

Calcium plays a definite role in pulsing. Although the current reversal potential rules out Ca^{2+} as a major current carrier at both pH 6.2 and 8.2, there is strong evidence to suggest that Ca^{2+} entry acts as a trigger for pulsing. All those ion changes which increase Ca^{2+} influx (low Na^+, low Mg^{2+}, low K^+, high Ca^{2+}) stimulate pulsing; and in low-Ca^{2+} medium, pulsing is greatly suppressed. Furthermore, several general cellular properties of Ca^{2+} make this role as a trigger likely. First, intracellular free Ca^{2+} concentrations are generally extremely low, so that even a small amount of Ca^{2+} influx could result in a large (though perhaps transient) increase in the local Ca^{2+} activity. It could be relatively transient because of the powerful Ca^{2+} pumps generally found in cells. Second, mild increases in internal Ca^{2+} are known to increase K^+ and perhaps Cl^- permeabilities. In *Pelvetia* we have two independent observations which support a Ca^{2+}-mediated increase in K^+ permeability. First, we find that during polarization of the eggs by unilateral light, five times as much Ca^{2+} enters the dark end (and future rhizoid end) as its antipode (Robinson and Jaffe, 1975). This Ca^{2+} asymmetry is accompanied by a K^+ asymmetry which is almost as great (Jaffe *et al.*, 1974a). We think it is likely that the high Ca^{2+} at one end is responsible for the increased K^+ permeability there. Second, microelectrode impalement of closely related *Fucus* eggs results in a large increase in K^+ permeability (Weisenseel and Jaffe, 1974). It was concluded that this K^+ permeability increase was probably due to an increase in the concentration of cytoplasmic Ca^{2+} during the impalement process. In a number of other cell types including red blood cells (Romero and Whittam, 1971) and neurones (Meech, 1974), small increases in internal Ca^{2+} increase K^+ permeability.

One case in which Ca^{2+} entry is thought to increase Cl^- permeability during a voltage spike is in the green alga, *Chara* (Findlay and Hope, 1963). They find that the transient Cl^- permeability increase during an action potential depends on the concentration of Ca^{2+} in the medium. Since Sr^{2+} was the only divalent cation

capable of partially replacing Ca^{2+} in this function, and has the most similar hydrated radius, it is likely that Ca^{2+} entry through a selective channel is involved in this transient Cl^- permeability increase. Similarly, in *Pelvetia,* Ca^{2+} entry at the tip could trigger pulsing by opening Cl^- channels there. These observed effects of Ca^{2+} on K^+ and Cl^- permeabilities further support our inference that Ca^{2+} entry triggers pulsing.

There is also a hint that the transport of Ca^{2+} away from this tip area is involved in *closing* the channels. We observed that the large pulses stimulated by a 99% reduction in external Na^+ take 10 min or more to fall off instead of the normal 1 min. One well-known mechanism for exporting Ca^{2+} is by exchange for external Na^+ (Blaustein, 1974). Therefore, if there is too little external Na^+ available, Ca^{2+} efflux might be slowed and channel closure delayed. Furthermore, we noted earlier that a minimum of 10% of the normal external Na^+ is needed for the proper function of the pulse channels since increasing Na^+ from 0.5% to normal stimulated pulses, while the increase from 10% to normal did not. Since Na^+ does not carry the pulse current, this low Na^+ requirement may well indicate that Na^+ is needed for a Ca^{2+} exchange mechanism which maintains a low internal Ca^{2+} concentration somehow needed for pulsing.

The combination of these results suggests a model for ion movements in pulsing. First, a small amount of Ca^{2+} enters at the growing tip and opens Cl^- channels. Second, Cl^- leaks out through these channels, slightly depolarizing the cell. Third, K^+ is driven out by this depolarization, mainly at the tip since local Ca^{2+} entry has increased its permeability there. Since much of the Cl^- efflux at the tip is therefore accompanied by K^+, the net current measured should underestimate the Cl^- efflux. In fact, preliminary results of $^{36}Cl^-$ tracer experiments indicate that 10 times more Cl^- leaves than indicated by the current pulses. This implies that 90% of the Cl^- efflux at the tip is accompanied by K^+ while the remainder of the K^+ efflux carries the charge current leaving the base of the rhizoid cell and the thallus cell.

We noted in an earlier paper (Nuccitelli and Jaffe, 1974) that "while the time course of a fucoid pulse seems to be under relatively localized control, its amplitude seems to be under some quite separate, more slowly changing, and less localized control." This model suggests that the time course of a pulse is controlled by local Ca^{2+} movements, while its amplitude is determined mainly by the slowly changing internal Cl^- concentration as well as the amount of Ca^{2+} entry. Pulses are therefore larger in low external Cl^- because Cl^- efflux is made larger by this change. They are stimulated by Ca^{2+} entry, and the amplitude of the pulses depends on the amount of Ca^{2+} influx. Thus, in low-Mg^{2+} sea water, pulses are larger because Ca^{2+} has less competition for membrane entry sites and more can enter to raise the Cl^- permeability further by opening more Cl^- channels. There is also a hint that pulse fall-off depends on Ca^{2+} diffusion away from the channels.

Pulse Function

The main purpose of determining the ions carrying the pulse current is to gain some insight into the function of these current pulses. We feel that two possible functions are suggested by these results: turgor pressure regulation and intracellular electrical field generation.

Pulsing may well be involved in a turgor pressure control mechanism since it represents K^+ and Cl^- efflux (both of these ions are constantly pumped into the cell). Since the turgor pressure depends on the osmotic pressure difference between the inside and outside of the cell, changes in the external osmotic pressure must be balanced by corresponding adjustments in the internal osmotic pressure if the turgor pressure is to remain constant. K^+ and Cl^- efflux dur-

ing pulsing will lower the internal osmotic pressure and act as a pressure regulator. Three facts support this role. First, during natural development, pulses do not occur until after germination when the internal osmotic pressure has reached its peak and may need adjustment (Nuccitelli and Jaffe, 1974; Allen *et al.*, 1972). Second, we have found that pulsing is stimulated by decreases in the external salt concentration as small as 3%, while it is suppressed by salt increases. Furthermore, preliminary results indicate that the $^{36}Cl^-$ efflux stimulated by the external salt decrease is of the right magnitude to accomplish the necessary osmotic pressure adjustment. We are currently studying this osmotic response. Third, a large reversed pulse current leads to bursting. Since pulsing represents the opening of Cl^- channels, these reversed pulses probably correspond to Cl^- entry which must be accompanied by K^+. This KCl entry will certainly increase the internal osmotic pressure, and the subsequent turgor pressure increase is the most likely cause of bursting.[2] The large groups of reversed pulses observed before bursting represented an influx of about 8 pmole of ions. This would only increase the total osmotic pressure by about 5%. However, the K^+ and Cl^- ions which entered near each other would not be measured as a net charge current and probably represent a significant amount of the ion influx. Therefore, the 5% increase calculated from the net charge flow is probably a gross underestimate. We feel that this observed bursting after reversed pulsing suggests that normal pulses would relieve pressure increases and thus supports their suggested role as osmotic pressure regulators.

These current pulses will also generate a transcellular electrical field. However, the magnitude of this field depends on the ion involved. The peak field generated by an average Cl^- current of 7 $\mu A/cm^2$ in a cytoplasmic resistivity of about 200 Ω-cm would be on the order of 1 mV/cm. Since cytoplasmic components typically have mobilities around 1 μ/sec per V/cm, this small transient force would probably have little effect. However, the field generated by Ca^{2+} entry could be much larger. This is because the free Ca^{2+} concentration is very low and Ca^{2+} is normally bound very soon after entering the cell. Ca^{2+} entry will therefore generate a very sharp fixed-charge gradient which in theory could create large fields on the order of 100 mV/cm (Jaffe *et al.*, 1974a). A field of this magnitude might well pull negatively charged vesicles towards the growing tip where Ca^{2+} enters. While we have no direct evidence that these vesicles are negatively charged, the literature indicates that most cell organelles do have a net negative charge. The most analogous organelles which have been studied are the secretory vesicles in mouse nerve terminals and in bovine chromaffin cells. Both have been shown to have an appreciable net negative surface charge in physiological media (Vos *et al.*, 1968; Matthew *et al.*, 1972).

Similar Pulses in Other Systems

Strikingly similar pulses are observed along regenerating anucleate posterior stalk segments of the marine green alga, *Acetabularia* (Novak and Bentrup, 1972). Extracellular voltage measurements always indicated current pulses with the same duration, shape, and frequency as these fucoid pulses. Furthermore, they enter the prospective regenerating end long before any regeneration is evident. In in-

[2] We have discussed this bursting in high K^+ in an earlier paper (Jaffe *et al.*, 1974b). At that time tracer studies showed no detectable change in the internal K^+ concentration in 10× K^+ medium. We therefore concluded that high K^+ burst eggs by creating a membrane depolarization which in turn somehow weakened the wall at the growth tip. However, these pulse reversal observations suggest the simpler interpretation that the lowered membrane potential allows Cl^- to leak in and burst the cells by increasing the osmotic pressure. This interpretation also explains why 30× K^+ burst eggs much faster than 10× K^+ and suggests that the larger K^+ increase of 20× should result in a detectable increase in the internal K^+.

tact cells of *Acetabularia,* Gradmann *et al.* (1973) have shown that Cl^- efflux carries the depolarizing inward current during light-triggered action potentials. However, 10,000 times more Cl^- leaves than that needed to discharge the membrane's capacitance. Furthermore, this Cl^- efflux increases when the active Cl^- influx increases. Both these observations support the idea that Cl^- pulses may be part of an osmotic pressure regulator in *Acetabularia* also.

Two freshwater algae, *Nitella* and its close relative, *Chara,* also exhibit action potentials. They occur along internodal cells, and again Cl^- and K^+ efflux carry most of the pulse current (Gaffey and Mullins, 1958; Mullins, 1962). One thousand times more Cl^- leaves than is required to discharge the membrane's capacitance during a pulse. Furthermore, there is some evidence that these spikes are triggered by local Ca^{2+} entry (Findlay, 1962; Findlay and Hope, 1963). Their function has not been determined. However, since so much more Cl^- leaves than needed for the potential change itself, perhaps their function is also osmotic regulation as we propose for the fucoid pulses.

Finally, there have been more recent reports of Cl^- spikes in tissue-cultured muscle cells from the chick (Fukuda, 1975). They are also preceded by Ca^{2+} entry and last up to 2 min. Since they only occur when the cell is hyperpolarized slightly by injecting a Cl^- current, it seems quite possible that these pulses of Cl^- efflux are also acting to relieve excess turgor pressure.

We thank Eric Henss of our machine shop for his excellent skill in the fabrication of the small probe parts and the medium exchange system. We also thank Dr. Yasuko Nakajima for the frequent use of her osmometer.

This investigation was financially supported by a U.S. Public Health Service biophysics predoctoral training grant to Richard Nuccitelli and a National Science Foundation research grant to Lionel Jaffe.

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