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Ionic currents in morphogenesis

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Summary. Morphogenetic fields must be generated by mechanisms based on known physical forces which include gravitational forces, mechanical forces, electrical forces, or some combination of these. While it is unrealistic to expect a single force, such as a voltage gradient, to be the sole cause of a morphogenetic event, spatial and temporal information about the electrical fields and ion concentration gradients in and around a cell or embryo undergoing morphogenesis can take us one step further toward understanding the entire morphogenetic mechanism. This is especially true because one of the handful of identified morphogens is Ca^{2+}, an ion that will not only generate a current as it moves, but which is known to directly influence the plasma membrane's permeability to other ions, leading to other transcellular currents. It would be expected that movements of this morphogen across the plasma membrane might generate ionic currents and gradients of both electrical potential and intracellular concentration. Such ionic currents have been found to be integral components of the morphogenetic mechanism in some cases and only secondary components in other cases. My goal in this review is to discuss examples of both of these levels of involvement that have resulted from investigations conducted during the past several years, and to point to areas that are ripe for future investigation. This will include the history and theory of ionic current measurements, and a discussion of examples in both plant and animal systems in which ionic currents and intracellular concentration gradients are integral components of morphogenesis as well as cases in which they play only a secondary role. By far the strongest cases for a direct role of ionic currents in morphogenesis is the polarizing fucoid egg where the current is carried in part by Ca^{2+} and generates an intracellular concentration gradient of this ion that orients the outgrowth, and the insect follicle in which an intracellular voltage gradient is responsible for the polarized transport from nurse cell to oocyte. However, in most of the systems studied, the experiments to determine if the observed ionic currents are directly involved in the morphogenetic mechanism are yet to be done. Our experience with the fucoid egg and the fungal hypha of *Achlya* suggest that it is the change in the intracellular ion concentration resulting from the ionic current that is critical for morphogenesis.
Key words. Ionic currents; vibrating probe; membrane potential; fucoid egg polarization; animal-vegetal polarity; polarization; voltage gradients; calcium; vesicle secretion; *Achlya;* oocytes; insect follicle; insect ovariole; polarized transport; egg activation; mouse blastomere; epithelial morphogenesis; limb bud.

Introduction

The mechanisms underlying morphogenesis must have the capability of generating pattern over relatively long distances of 10–100 µm, based on the dimensions of many well-studied examples of morphogenesis, including ooplasmic segregation, segmentation in *Drosophila,* somite formation in vertebrates, and tentacle formation in *Hydra.* Such morphogenetic fields must be generated by mechanisms based on known physical forces which include gravitational forces, mechanical forces, electrical forces, or some combination of these. These fields have been referred to as 'electro-mechanico-chemical fields'[39] because they generally involve interactions of these physical forces and morphogens such as Ca^{2+} with specific cellular targets such as the cytoskeleton or a population of membrane proteins. Because of these complex interactions, it is unrealistic to expect a single force, such as a voltage gradient, to be the sole cause of a morphogenetic event. However, spatial and temporal information about the electrical fields and ion concentration gradients in and around a cell or embryo undergoing morphogenesis can take us one step further toward understanding the entire morphogenetic mechanism. This is especially true because one of the handful of identified morphogens is

Ca^{2+}, an ion that will not only generate a current as it moves, but which is known to directly influence the plasma membrane's permeability to other ions, leading to other transcellular currents. As a morphogen, Ca^{2+} is best known for its effect on *Fucus* rhizoid formation [106] and ascidian egg polarity [68]. It would be expected that movements of this morphogen across the plasma membrane might generate ionic currents as well as gradients of both electrical potential and intracellular ion concentration. Such ionic currents have been found to be integral components of the morphogenetic mechanism in some cases and only secondary components in other cases. My goal in this review is to discuss examples of both of these levels of involvement that have resulted from investigations conducted during the past several years, and to point to areas that are ripe for future investigation.

This is not meant to be a comprehensive review of all of the literature on ionic currents, although I have tried to include in the reference list most research articles in this area that have been published since my last reviews of transcellular ionic currents [87, 89]. Other reviews that might be of interest include those by Jaffe and Nuccitelli (1977), Weisenseel and Kicherer (1981), Borgens (1982), DeLoof (1986), Harold (1986a, chapter 14; 1986b), Jaffe (1981, 1986), Robinson (1985), Nuccitelli (1988), and the proceedings of an international meeting on ionic currents in development [90].

History and theory of ionic current measurements

A fundamental property of all cells is that they possess an outer boundary composed of a lipid bilayer that contains transport proteins which generate ion concentration gradients across that boundary. Most animal cells use the Na^+/K^+-ATPase and Ca^{2+}-ATPase to generate gradients in Na^+, K^+ and Ca^{2+}, and most plants and prokaryotes rely more heavily on the H^+-ATPase [13]. These ions will flow down their electrochemical gradients through membrane channels, and a steady state will be established in which the number of ions pumped in equals the number leaking out per unit time. These steady-state ion fluxes have been measured for decades using radioactive ion tracers, so that the total ionic influx and efflux is well known for many cells. If the influx sites are separated from the efflux sites, a net transcellular ionic current will result. Such a current flows through the cell and returns through the extracellular medium where it can be detected noninvasively by measuring the voltage gradient it generates as it flows through this medium. Typical cellular ion fluxes generate steady currents on the order of 1–10 $\mu A/cm^2$ which would produce an extracellular voltage gradient on the order of 10 nV over a 30-μm displacement, 30 μm from the cell's surface in a medium with a resistivity of 100 Ω-cm. [It has recently been pointed out that if the mobility of the ion species carrying the electrical current is greater than the average ion mobility in the medium (as is the case for H^+), the potential gradient detected by the probe will depend on the spatial relation of the convection loops to the probe [36]. Thus, one must exercise caution when measuring proton currents, determining their dependence upon the viscosity of the medium and the frequency of vibration of the probe]. Such very small voltage differences are difficult to detect, but the vibrating probe technique uses low impedance electrodes and signal averaging to attain the required sensitivity. This technique involves the movement of a metal electrode measuring voltage rapidly back and forth between two points outside a cell to detect the voltage gradient between the two points, and signal averages using a lock-in amplifier to increase the signal-to-noise ratio [31, 60, 83, 91, 110, 111]. This technique has been used in about 30 laboratories around the world to study both plant and animal cells and embryos, and a National Vibrating Probe Facility has been supported by the NIH at the Marine Biological Laboratory at Woods Hole. Because these steady ion fluxes are a fundamental characteristic of all cells, it is not surprising that they have been detected in nearly every cell and organism that has been studied with this technique. [The only published report of a vibrating probe study in which no extracellular ionic currents were detected was a study of gliding filaments of the giant cyanobacterium, *Oscillatoria princeps* and two species of *Anabaena* [65].] Many cells utilize these fluxes as an integral part of a variety of mechanisms. The retinal rod, for example, modulates the steady dark current so that we may 'see' photons absorbed by rhodopsin. All epithelia are known to generate a voltage across the monolayer by having each cell in the monolayer pump an ionic current through itself that then leaks back between the cells to generate the transepithelial potential. Ionic currents are often associated with an axis of polarity such as the apical-basal axis in epithelia or the animal-vegetal axis in oocytes, and this leads us to the topic at hand. Might these currents play a role in the process of morphogenesis? This question is actually what led us to develop the vibrating probe technique in the first place. Lionel Jaffe already had evidence that a steady current was traversing the germinating egg of the brown alga, *Pelvetia,* using a multicellular technique in which the voltage drop along 200 eggs in series was measured [55]. The next step was to measure the current pattern around a single egg, and in order to detect the small extracellular voltage gradients generated by this current we developed the vibrating probe technique [60]. The *Pelvetia* egg was the first cell type to be studied with the vibrating probe, and still remains today the best example of how ionic currents can play a primary role in morphogenesis.

Ionic currents and concentration gradients are integral components of some morphogenetic mechanisms such as the polarization of fucoid eggs

The eggs of the brown algae, *Pelvetia* and *Fucus,* are ideal systems for the study of the development of cell polarity

because these eggs have no predetermined axis of polarity and a rhizoid outgrowth can germinate at any point on their surface. This process involves a localized secretion of wall-softening enzymes and an increase in turgor pressure to five atmospheres, accomplished by pumping K^+ and Cl^- into the cell. The cell then bulges or germinates at the weakest region where wall-softening has occurred, and the axis of polarity is established. The establishment of this axis of secretion can be influenced by a variety of environmental vectors including unilateral light and unilateral chemical influences from nearby eggs or plants, and, since the light receptors are located in the plasma membrane[54], it is not surprising that this membrane plays a key role in the signal transduction. The investigations using the vibrating probe indicated that ionic currents were already present around the egg as early as measurement was possible (30-min post-fertilization) and tended to enter on the side at which germination would occur some 8 h later[85]. The early current pattern is unstable and shifts position, often with more than one inward current region. However, the current enters mainly on the side where germination will occur and is usually largest at the prospective cortical clearing region where the rhizoid forms. The current pattern observed during the 2-h period prior to germination is more stable and the site of inward current always predicts the germination site, even when the axis is reversed by light direction reversal.

In order to determine how this transcellular current might be influencing the polarization process, it is important to determine which ions are carrying the current. By varying the composition of the artificial sea waters in which these measurements were made, we determined that Ca^{2+} influx was responsible for a small fraction of the current with Cl^- efflux as the most likely carrier of the bulk of it. This Ca^{2+} component was elegantly demonstrated by Robinson and Jaffe (1975) when they measured $^{45}Ca^{2+}$ influx and efflux at either end of polarized eggs by tightly fitting them into holes in a nickel screen so that one end at a time could be exposed to $^{45}Ca^{2+}$. They directly measured 2 picoamps of Ca^{2+} current traversing each egg. Therefore, one consequence of the transcellular ionic current in this egg is the generation of a Ca^{2+} flux through the cell which has recently been shown to generate a gradient in the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$)[24]. It appears to be this gradient which plays the primary role in polarizing the egg.

We now know that a central component of the polarization mechanism in the egg of the brown alga involves a cytoplasmic gradient in $[Ca^{2+}]_i$. This hypothesis is supported by the three criteria required for physiological significance: 1. There indeed exists an endogenous $[Ca^{2+}]_i$ gradient in the egg (although measured long after polarization)[24]; 2. Modifying the direction of this gradient during polarization changes the orientation of the axis of polarization[106]; 3. Inhibiting the gradient prevents polarization[66]. The gradient was detected in germinated zygotes by Brownlee and Wood using Ca^{2+}-sensitive microelectrodes, and 10-fold higher Ca^{2+} was detected in the tip region than in the sub-tip region of the cell. Robinson and Cone imposed a gradient of Ca^{2+} on unpolarized eggs by placing them near a fixed source of Ca^{2+} ionophore in the dark. A majority of the eggs then germinated on the hemisphere facing the ionophore where the Ca^{2+} influx is expected to be the greatest. Finally, the magnitude of the gradient can be greatly reduced by injecting into the cell a molecule that can rapidly shuttle Ca^{2+} from regions of high concentration to regions of low concentration. Jaffe, Weisenseel and Speksnijder recently used various BAPTA Ca^{2+} buffers to do that and found that tip growth could be completely blocked for weeks while the cell remained viable and even divided. This is the strongest evidence yet that a gradient in $[Ca^{2+}]_i$ is required for cell polarization.

What cellular processes is this gradient influencing to generate the polarization? In this system vesicle secretion determines where the wall-softening enzymes will be secreted to generate the rhizoid outgrowth as the turgor pressure increases. Therefore, the gradient must influence the process of vesicle localization and secretion. Actin filaments have been implicated in this process because zygotes cannot be polarized when incubated with cytochalasin D[21, 101]. Brawley and Robinson (1985) made a connection between this cytochalasin effect and the transcellular current by demonstrating that the cytochalasin D significantly reduces the inward current at the rhizoid pole after 2.5 h of incubation, and also changes the distribution of actin filaments in the growing tip. Thus, they hypothesized that the inward current, carried in part by Ca^{2+}, stimulates F-actin localization at the rhizoid pole and causes more Golgi vesicle transport to that region. They further speculated that the membrane surrounding these vesicles might contain Ca^{2+} channels so that upon fusion an increase in Ca^{2+} influx and inward current into the rhizoid tip could occur. This would explain why the addition of cytochalasin D reduces the current. This is a very plausible hypothesis that comes closest to integrating the observed transcellular ionic currents with the overall mechanism of polarization in the fucoid egg. It also illustrates the point made in the introduction that morphogenesis generally involves interactions between a number of physical forces and cellular structures.

Ionic currents are often not directly linked to morphogenesis

While this evidence that ionic currents play a primary role in the establishment of polarity in fucoid eggs is quite strong, we are reminded by Waaland and Lucas (1984) that we cannot generalize and expect the same predictive role for the current in all algal systems. They carefully

examined the current pattern around elongating rhizoids and repair shoot cells of the marine red alga, *Griffithsia pacifica,* and concluded that the ionic currents were neither sufficient nor necessary for the maintenance or reinitiation of sites of localized growth and organelle accumulation. While there is indeed a region of inward current at the growing tips of both rhizoids and repair shoot cells, rhizoids which have stopped elongating following prolonged periods of darkness can reestablish a polar distribution of organelles and restart localized growth in the absence of measurable current at their tips. Moreover, the rate of elongation is not proportional to the magnitude of the inward current density as it is in *Pelvetia*[94]. This lack of correlation between localized growth and tip current has also been found in other cell types such as the hyphae of the fungus, *Achlya,* discussed below, so the presence or absence of a current is not always an absolute predictor of localized growth.

Ionic currents in other plant systems

A wide variety of other plant cell types have been found to drive ionic currents through themselves, although in most of these systems it has not been determined if such currents are critical components of the morphogenetic mechanism. Thus, currents are found to precede and predict the axis of polarity in pollen grains[125,127], to precede and predict branching in the fungus, *Achlya bisexualis*[2,42–45,48,49,72–75], to enter the rhizoid of the water mold, *Blastocladiella*[117], to enter the rhizoid of *Acetabularia*[19,20], to be associated with chloroplast aggregation in *Vaucheria*[9], to be associated with the banding pattern on characean internodal cells[32,77,78], to exit the growing tip of fern gametophytes[102], to traverse the unicellular green algal desmid, *Micrasterias*[120], to be associated with the growing ends of carrot[23] and tobacco embryos[99], and to enter the growth zone of roots from at least three plant species[4,81,124]. Most plant systems utilize electrogenic ion pumps to generate very large membrane potentials that are often more negative than − 150 mV. With such a large driving force, it is not surprising that the magnitude and frequency of occurrence of transcellular ion currents are greater in plant systems than in animal systems. However, these currents often reflect transport mechanisms such as amino acid uptake that are not directly linked to morphogenesis. Therefore, each case must be examined independently to determine if the causal link between the current and morphogenesis is present. In this brief review, I will not discuss all of the plant systems listed above, but will use a single example to illustrate this point, that of the fungus, *Achlya bisexualis.*

A series of elegant investigations by Darryl Kropf and Franklin Harold have revealed a great deal of information regarding the transcellular current and intracellular voltage gradient in the growing hyphal tips of this fungus[72–75]. They have demonstrated that a proton current enters the growing tip of *Achlya* along with a required transport of amino acids (particularly methionine). This current generates a large voltage gradient of 20 mV/mm in the cytoplasm at the tip region, similar to the large voltage gradient measured in *Neurospora* years ago by the Slaymans[114]. One could argue that the gradient in *Neurospora* might have been an artifact due to greater impalement damage at the tip than farther back along the hypha. However, in *Achlya* Kropf demonstrated that this voltage gradient could be abolished under conditions that inhibit the proton current, so it can be turned off and on while the electrodes are in the cell, and is clearly no artifact. Surprisingly, the voltage gradient measured is about 10-fold larger than expected based on a simple ohmic voltage drop generated by the transcellular current. Therefore, another voltage-generating mechanism such as a fixed charge gradient which can theoretically produce larger intracellular fields[62] must be at work here. That is quite interesting, because, just as in the polarizing fucoid egg, it may be the intracellular ion concentration gradient resulting from the ionic current rather than the "IR" voltage drop resulting from it that is the critical factor. Another observation that supports this idea is the fact that while the inward current precedes and predicts a new branch, the current at the pre-existing tip often reverses direction for up to 40 min during new branch formation without affecting the rate of tip elongation. Apparently, it is not the polarity of the ionic current at a given region that dictates growth, but perhaps it is the intracellular concentration of H^+ or Ca^{2+}. The reversal of the current direction at the pre-existing tip might simply represent a passive cellular response to a large inward current elsewhere at a region of new branch formation. Perhaps the relative membrane permeability of the tip region is simply higher than that of the lateral region so the outward current is "focused" there and is temporarily larger than the endogenous inward current in that region.

Ionic currents in animal cell systems

A wide variety of animal systems have been investigated with the vibrating probe. Extracellular ionic currents have been found in slime molds[1], associated with feeding in *Noctiluca*[84], associated with the polarity of insect follicles[10–12,29,50,67,76,82,98,121,131,132], in immature and activating eggs[70,92,103], in cleaving eggs[30,71], in developing embryos[35,63,79,97,109,116,128,129], predicting the site of limb formation in amphibians[17,104], exiting regenerating limbs[16,18,33,80], in intact and damaged bones[15], in mature epithelia[37,112,113], exiting wounded epithelia[3,64], in rat lens[100,108] and in skeletal muscle[5–8,25]. I do not intend to discuss all of these cases, but instead will review a few most closely related to the area of morphogenesis.

Currents associated with animal-vegetal polarity: A polarizing role for intracellular voltage gradients

In immature oocytes of both frogs [103] and fish (Nuccitelli, unpublished data) a steady transcellular current of approximately 1 $\mu A/cm^2$ enters the animal hemisphere and exits the vegetal hemisphere. No experiments have yet been done to determine if the animal-vegetal current in the oocytes is required for polarization, although it seems to be correlated with the maintenance of the immature state. Upon maturation, the current in frog oocytes fades away and no extracellular current can be detected until fertilization or activation. Robinson found that several blockers of Ca^{2+} influx rapidly reduced this current and led to maturation as readily as did the application of progesterone. In order to determine if this current in immature oocytes might be involved in their polarization, we have been investigating earlier stages in *Xenopus* oocytes and find that the transcellular current is present prior to the development of the pigment asymmetry. Since the oocyte is rightly encapsulated in a follicle layer during this time, any extracellular currents will be forced to pass within the very narrow space between the oocyte and follicle layer. This might increase the resistance to current flow and thereby increase the extracellular voltage generated by the current. Such an extracellular voltage gradient could act to polarize the distribution of mobile proteins in the plasma membrane by lateral electrophoresis [56], however this hypothesis has not yet been tested in the frog oocyte. Nevertheless, in insect oocytes, transcellular currents do appear to be involved in animal-vegetal polarity.

Woodruff and Telfer (1973) were the first to demonstrate that there is a voltage difference of about 5 mV between the nurse cells and oocyte in the follicle of the silk moth cecropia. The only way that such a voltage gradient can be maintained in the conductive cytoplasm is by a steady current flow. The extracellular current pattern is similar to that found near the other oocytes previously mentioned with current entering the nurse cells at the animal pole of the follicle and leaving the vegetal pole of the oocyte [67]. However, when the epithelium surrounding the seven nurse cells is removed, an apparent outward current is observed at the nurse cell surface, indicating that the epithelium may be contributing to the current pattern described above [132]. Whatever the true extracellular current pattern, in order to generate the observed voltage gradient across the cytoplasmic bridge, current must flow along the bridge from the oocyte into the nurse cells. Jaffe and Woodruff proposed that the bridge current would flow in this direction if both the nurse cells and the oocyte exhibited a polarized distribution of inward and outward current regions so that current would be expected to exit the vegetal end of the nurse cells and enter the animal end of the oocyte. Since these two regions are tightly apposed in the follicle, they could act as a battery that would drive current across the apposed membranes and this current would then leak back into the nurse cells along the pathway of lowest resistance through the cytoplasmic bridge. This bridge current would generate a voltage of the observed polarity across the cytoplasmic bridge. There is little direct experimental evidence to support this hypothesis, but there is strong evidence in support of intercellular electrophoresis as a protein segregation mechanism. It has long been known that the transport of cytoplasmic components across the bridge is polarized, flowing only in the direction of nurse cell to oocyte. The polarity of protein transport across the intercellular bridge connecting these two cell types can be reversed by reversing the endogenous electric field within this bridge [133]. While it is perhaps not too surprising that proteins could be electrophoresed along an intercellular bridge by an imposed field, Woodruff and Telfer (1980) then discovered even more compelling evidence that electrophoresis is involved in the transport of proteins across the bridge. They have shown that the polarity of movement of a given protein across the bridge is dependent only on its net electric charge. Lysozyme is a basic protein with an isoelectric point of 11.5 and, when injected into the follicle, will only move from the oocyte to the nurse cell. However, when the net charge was reversed on this same protein by methylcarboxylation of its ε-amino groups, it reversed its transport direction and was only observed to move from the nurse cell to the oocyte. In further support for the electrophoresis mechanism, neutral proteins with isoelectric points near 7 moved in both directions across the bridge. Thus in the cecropia follicle, the polarity of protein transport, which is a critical component of the oocyte's polarity, is determined by the electrical field across the intercellular bridge. The cecropia follicle is still the best example in which transcellular ionic currents play an active, causal role in polarized transport, and is the only insect polytrophic ovary (one in which each oocyte has its own private set of nurse cells) to be studied. The three other insect ovarioles that have been investigated more recently are all telotrophic, so all of the oocytes are linked to a single, shared group of nurse cells in a tropharium that is much farther from the oocyte than in polytrophic ovaries. Charge-dependent translocation of microinjected proteins has been demonstrated in ovarioles of *Rhodnius prolixus* and *Oncopeltus fasciatus*, but this was restricted to the tropharium [118, 130]. However, in the most recent work using the ovariole from *Dysdercus intermedius*, negatively charged proteins were found to migrate according to the voltage gradient from the tropharium into the oocytes via the trophic cords, while positively charged proteins remained in the tropharium [82]. The effectiveness of this polarized transport is greatest on the tropharium side, since both negatively and positively charged proteins injected into the previtellogenic oocytes moved into the trophic cords. Thus, intercellular electrophoresis is used by a number of insect species as part of the mechanism of polarized transport. Moreover, a recent study of the nurse cell-oocyte com-

plex of a polychaete indicates that steep voltage gradients exist there as well.

A much larger intercellular voltage gradient has been reported between oocyte and nurse cell of the spirally cleaving polychaete, *Ophryotrocha labronica*[34]. In this species, the oocyte is supported during vitellogenesis by a single nurse cell which is attached to the oocyte with a cytoplasmic bridge as in cecropia. An amazing 22–32 mV difference has been reported across the 3-μm wide cytoplasmic bridge with the oocyte more positive than the nurse cell. This system clearly merits further investigation into the role of this voltage gradient in the polarized transport.

Dorsal-ventral axis

No transcellular current has been found to be correlated with the dorsal-ventral axis of frog eggs[71], however, there is a strong correlation with the dorsal-ventral axis in cockroach oocytes[76]. Positive current enters the ventral side of the oocyte and exits the dorsal side. It has not yet been determined if the current is playing a direct role in the establishment or maintenance of this axis.

Egg activation

Four vertebrate eggs have been investigated during activation with either the extracellular vibrating probe or patch clamp techniques during fertilization: the eggs of the frogs *Xenopus, Rana pipiens* and *Discoglossus pictus*[53, 69, 70, 96], and the egg of the medaka fish, *Oryzias latipes*[92]. In all but *D. pictus,* the activation current was found to enter the site of activation and spread across the egg in a ring-shaped wave. However, the egg of *D. pictus* exhibited no such current wave, despite the presence of a wave of increased intracellular calcium concentration. Current enters only at the animal pole in the dimple region and the Cl^- channels appear to be more highly localized than in any of these other three vertebrates. This egg appears to be the only one studied thus far that exhibits such a striking localization of the channels responsible for the fertilization potential. In the only other case in which the fertilization-gated channels appear to be localized, that of the invertebrate egg, *Urechis caupo*[41], poor spatial resolution makes a direct comparison with the egg of *D. pictus* difficult.

What is the physiological significance of this activation current? The two main consequences of the activation current are the accompanying changes in the membrane potential (fertilization potential) and the intracellular concentrations of the ions carrying the current. In many species, the fertilization potential provides a fast block to polyspermy[52], however, the egg of *D. pictus* appears to be polyspermic (Talevi, in preparation), and the egg of the medaka exhibits no electrical block to polyspermy[86]. Therefore, we have here an example of a transcellular ionic current that is the result of a localized permeability increase (probably mediated by an increase in intracellular Ca^{2+}), and has no known function or clear relationship to morphogenesis.

Mouse blastomere polarization: A polarizing role for extracellular voltage gradients

Lynn Wiley and I found that polarized, 8-cell stage mouse blastomeres drive an ionic current through themselves, into their apical ends and out of their basal ends[97]. Such an apical-basal current is found in all polarized epithelial cells but this was the first measurement of such a current in a cell in the process of polarizing. Since this current must complete its path by traversing the extracellular space between the blastomeres, it can generate an electric field in this space that will be proportional to the resistance between the blastomeres. Such fields have indeed been measured across blastocyst walls and are found to be on the order of a few millivolts[26]. This led us to propose an ionic current polarization hypothesis which states that a transcellular current is generated in the outer blastomeres due to a greater Na^+ influx at their outer plasma membrane compared to the influx at those regions of their membrane in contact with other cells. This current therefore enters apically and leaves basolaterally, leaking to the outside of the embryo by passing between the cells. This leakage current is responsible for generating the voltage measured across the epithelial layer, and may act back on the outer blastomeres to further polarize them. We have recently tested this hypothesis by exposing isolated, unpolarized blastomeres to imposed electric fields of a similar, physiological magnitude and find that polarization of the intracellular organelle distribution does occur. After 2 h in a field of only 4 mV/cell diameter, there is a microtubule-dependent elongation of the blastomere along the field direction and a segregation of organelles with many of the mitochondria shifting toward the positive pole. During normal embryogenesis, the mitochondria are observed to shift basally, and that is the end expected to be the positive pole according to the transcellular current direction. Thus, the mitochondria are moving in the same direction that they do in vivo. This is the first time that the polarity of a higher animal cell has been found to be influenced by an imposed electric field, and this result strongly supports our hypothesis that the endogenous electric field plays a role in the development of polarity in the mouse system. This also provides evidence that extracellular voltages might play a role in morphogenesis.

One other example of such a role for extracellular fields comes from Stern and MacKenzie (1983) who demonstrated that applying voltages of reversed polarity across isolated sheets of chick epiblast caused a reversal of some of their morphological polarity markers. This cell layer normally exhibits apical-to-basal, unidirectional sodium transport and generates a transepithelial voltage of about 16 mV. This voltage will drive current out of regions in the epithelium with lower resistance, such as the primitive streak[63], and it has been hypothesized that such transepithelial voltages might influence the polarity of the cells forming the monolayer[57]. Stern and MacKenzie (1983) cultured epiblasts in mini-Ussing chambers and

reversed the normal potential across the layer by applying 15–30 mV, apical side positive. After only 60–90 min under this condition, the location of Alcian blue-positive materials showed a shift from the basal side of the tissue to the apical side in every case studied. Alcian blue stains components of the basement membrane which are normally only deposited on the basal side of the cell. Furthermore, the electrical resistance across the sheet of tissue fell to about 1–10% of its normal value by 30 min of reversed field application, suggesting that intercellular junctions had broken down. This very intriguing observation deserves much more attention and investigation.

Ionic currents associated with epithelial morphogenesis

Because most morphogenetic events in the developing embryo involve shape changes in an epithelial sheet and all epithelia are electrically polarized, it is not at all surprising that these shape changes are often correlated with an extraembryonic ionic current. The voltage across the epithelial layer will drive current out between all of the cells forming the layer, but this current will be larger where cells are less tightly apposed, leading to a lower resistance to current flow between them. Thus, rather large currents are measured leaving the primitive streak in chick[63] and mouse embryos[129] where intercellular junctions are breaking down. These currents will generate an internal voltage gradient in the embryo that could be used to provide long-range spatial information to cells in need of guidance, but thus far there is no direct evidence that the internal fields are being used in this way. However, there is some indirect evidence: many embryonic motile cells have been found to be exquisitely sensitive to weak voltage gradients, usually migrating toward the negative pole in imposed electric fields[93, 105]. Since some differentiated motile cell types (such as BHK cells) do not exhibit this galvanotaxis, it is not a universal response of all motile cells, so perhaps these embryonic cells have developed the ability to sense these internal fields as one of the many cues that guide them to their destination. Also, neurons exhibit galvanotropism with a similar low threshold and may also utilize embryonic fields for guidance.

One of the most intriguing observations correlating ionic currents and epithelial morphogenesis is the predictive nature of the currents preceding limb bud formation in amphibians. In both axolotls[17] and frogs[104], outward current can be detected at the site of future limb bud formation long before any anatomical change. For more than a week prior to the emergence of the hind limb in axolotls, a steady ionic current is driven out of the ventrolateral flank and returns through the integument in adjacent regions of the body. A peak in the density of the outward current occurs over the exact area of hind limb formation 4 to 6 days prior to its appearance. In *Xenopus* embryos, the region of largest outward current predicted the site of bud formation at least one day prior to the first thickening of ectoderm over the hind limb bud area. These outward currents indicate that the outer epithelium in the prospective limb bud region is becoming leaky long before bud formation. Whether this current influences the development of this bud region is not known. However, Robinson has pointed out that supernumerary limbs can be induced in amphibians simply by making an incision in the skin which will allow current to leak out[119]. Therefore, it is possible that the outward current might act to organize or stimulate limb development. This is a very promising and exciting area for further research.

Conclusion

Transcellular and transembryonic ionic currents are found in a wide variety of both plant and animal systems. They are associated with many cellular mechanisms, including nutrient uptake, signal transduction and cell polarization. Their involvement in morphogenesis is very clear in some systems and not so clear in many systems. By far the strongest case for a direct role of ionic currents in morphogenesis is the polarizing fucoid egg where the current is carried in part by Ca^{2+} and generates an intracellular concentration gradient of this ion that orients the outgrowth. Another good case is the insect follicle in which an intracellular voltage gradient is responsible for the polarized transport from nurse cell to oocyte. However, in most of the systems studied, the experiments to determine if the observed ionic currents are directly involved in the morphogenetic mechanism are yet to be done. Our experience with the fucoid egg and the fungal hypha of *Achlya* suggest that it is the change in the intracellular ion concentration resulting from the ionic current that is critical for morphogenesis. This has led many researchers including myself to begin using indicators of intracellular free $[Ca^{2+}]$ such as aequorin and fura-2 to visualize the distribution of free Ca^{2+} in cells and embryos. Experimentation on the insect follicle and the polarizing mouse blastomere suggests that voltage gradients generated by ionic currents can also influence morphogenesis in some cases. The challenge that faces us is to design the critical experiments that will uncover all of the steps in a given morphogenetic mechanism. Some of these will no doubt involve ionic currents and gradients, but learning about the interactions between these currents, subsequent intracellular ion concentration gradients and other morphogenetic elements such as the cytoskeleton is the next step needed to improve our understanding of morphogenesis.

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Prospects for NMR imaging in the study of biological morphogenesis

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Summary. Small objects can be visualised with a spatial resolution that approaches microscopic dimensions using the technique of high resolution nuclear magnetic resonance (NMR) imaging. Some important features of the method are described and the prospects for using the technique to study morphogenesis are discussed. It is concluded that NMR imaging, in conjunction with the related method of localised spectroscopy, is capable of producing novel structural information.
Key words. NMR; imaging techniques; morphogenesis; structural information; high resolution imaging.

Introduction

Many isotopes, some of them naturally abundant (e.g. ^1H, ^{31}P) and others not (e.g. ^{13}C, ^{15}N), have nuclear magnetic moments, and it is possible to detect such isotopes using the techniques of nuclear magnetic resonance (NMR). The phenomenon was first observed more than 40 years ago, and since that time NMR has developed into a versatile technique with many applications in physics, chemistry, biology and medicine. NMR detects transitions between the energy levels associated with the nuclear magnetic moments, and much of the success of the technique can be attributed to the fact that the measurable properties of these transitions depend on the molecular environment of the nucleus in a predictable way. Thus, NMR spectroscopy is arguably the most versatile technique for structural analysis in the chemistry laboratory[20], and it also finds many applications in biochemistry[40], for example in the characterisation of the structural and dynamic properties of macro-molecules in solution[58].

NMR techniques can be applied to living systems and the methods of particular interest are high resolution NMR spectroscopy and NMR imaging. In the spectroscopic approach, signals are detected from metabolites in the intracellular fluids and the metabolites are monitored under different physiological conditions[26]. Although the insensitivity of the technique limits it to metabolites present at relatively high concentrations, NMR spectroscopy has found many applications in vivo, including applications to mircroorganisms[6], plant tissues[48] and animal tissues[4,56]. The very small fraction of this work that has been concerned with developing systems (table) has concentrated on two areas: (i) the analytical problem of correlating changes in metabolite levels with the onset of developmental changes; and (ii) the role of intracellular