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New Membrane Formation and Intercellular Communication in the Early *Xenopus* Embryo

I. Electrophysiological Analysis

S. W. de Laat, P. W. J. A. Barts, and M. I. Bakker

Hubrecht Laboratory, International Embryological Institute, Utrecht, The Netherlands

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Summary. The ionic permeability of the nonjunctional and newly formed junctional membranes was investigated in embryos of *Xenopus laevis* up to the onset of the fifth cleavage. Continuous measurements were made of the equivalent nonjunctional (R'_0) and junctional resistances (R'_i) in different pairs of adjacent cells separated by one of the four cleavage membranes formed in that period. The specific resistance of the nonjunctional membranes (r_0) and of each cleavage membrane (r_i) as a function of time were derived using a generally applicable computer simulation model. r_0 decreased from about 40 kΩ cm^2 in the uncleaved egg to about 10 kΩ cm^2 at the 16-cell stage, due to the insertion of a small fraction of the relatively permeable newly formed cleavage membranes into the outer surface. Superimposed on this overall decline, a transient decrease of r_0 was observed during each cycle, caused by a temporary partial separation of the peripheral parts of adjacent blastomeres. The changes in R'_0 followed the same pattern. R'_i increased stepwise during each cleavage cycle. At the onset of each cleavage there were no significant differences in R'_i as measured between different pairs of cells. After an initial phase of membrane formation r_i of all cleavage membranes remained constant at about 400 Ω cm^2. In the stages investigated the coupling ratio ranged from 0.8 to 1. It is argued that this close coupling could be the result of the highly impermeable outer surface even in the absence of specialized junctions in the intercellular membranes.

The presence of electrotonic coupling or intercellular communication is established by introducing an electric current into one cell of a multicellular system and measuring its passage to neighboring cells. It has been observed in a large variety of both excitable and nonexcitable adult cells, cultured cells, and embryonic cells (*see*, for reviews, [4, 11, 24]).

Considerable attention has been devoted to the study of electrotonic coupling in embryonic systems, since it has been speculated that the pathways involved provide a means of transferring molecules that play a role in regulating cell division, growth and differentiation [25, 26]. Coupling has been observed in embryos of echinoderms [1, 43, 44], molluscs [32], amphibians [10, 14, 15, 30, 39], birds [38], and teleosts [5, 6].

In many cases the demonstration of electrotonic coupling is associated with the presence of "gap junctions" or, in some cases, "septate junctions" in the intercellular membranes [12, 13, 34, 35, 37, 45]. At present strong evidence is available [2, 12] that gap junctions are mediators for the passage of ions from one cell interior to another.

Not only small ions, but also various dye molecules and other tracer molecules were shown to pass the intercellular membranes of coupled cells [11, 18, 27, 31, 33]. However, fluorescein does not pass between electrically coupled cells of *Asterias* embryos [43] and *Fundulus* embryos [5], and between electrically coupled cells of pre-gastrula embryos of *Xenopus* [41]. In this respect embryonic cells appear to be rather exceptional.

When data on the specific membrane resistance can be deduced, the use of electrophysiological methods provides a quantitative measurement of the ionic permeability properties of the intercellular membrane. For various reasons this appears to be possible in a limited number of systems only. In cases where the geometrical relations between the cells are too complicated to be modelled, or the cells are too small to allow for simultaneous measurement of the intracellular voltage and application of an electric current into a single cell, the coupling ratio has been used as a measure for electronic coupling. Fig. 1 illustrates the principles of this method. A rectangular current pulse is applied to the interior of one cell (I_1) and the resulting deviations of the membrane potential in this cell (V_1) and another cell (V_2) are recorded. The coupling ratio is defined as

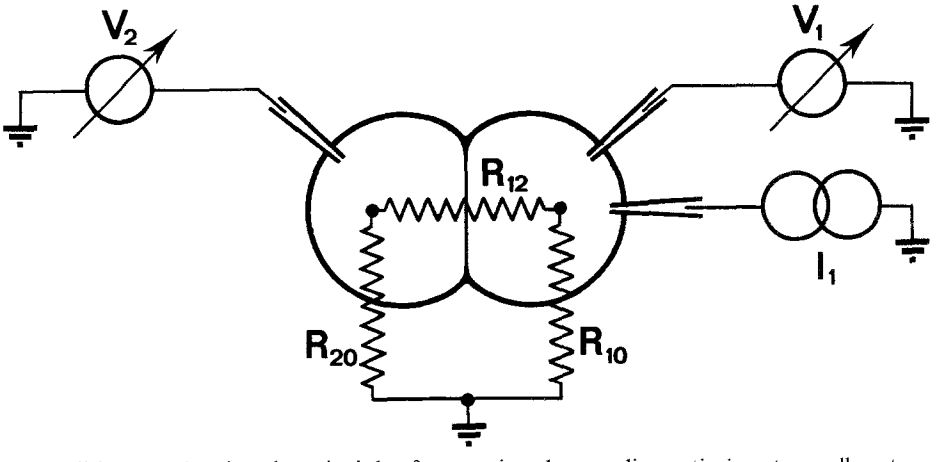

Fig. 1. Diagram showing the principle of measuring the coupling ratio in a two-cell system (*also see text*)

the ratio V_2/V_1 [24]. From Fig. 1 it follows that

$$V_2/V_1 = R_{20}/(R_{20}+R_{12}) = 1/(1+R_{12}/R_{20}). \quad (1)$$

Since V_2/V_1 is dependent on the ratio of the resistances of the junctional and nonjunctional membranes, its value gives no information on the actual membrane resistances R_{12} and R_{20} or on the corresponding specific membrane resistances. Any change of the coupling ratio can be the result of a change of either one of the resistances. Furthermore, a comparison of the permeability properties of the junctional membranes of different systems cannot be made on the basis of their respective coupling ratios.

The actual values of the resistance between two cells (R_{12}) and the resistances from the respective cell interiors to the surrounding medium (R_{10} and R_{20}) can be deduced from the deviations of the membrane potential in the two cells resulting from an applied current on either side of the junctional membrane (Fig. 2). When V_{ij} is the deviation of the membrane potential of cell j caused by a current applied in cell i, R_{pq} is the resistance between compartments p and q, and $I_1 = I_2 = I$, then:

$$R_{10} = (V_{11}\,V_{22} - V_{12}^2)/((V_{22} - V_{12})\,I), \quad (2)$$

$$R_{20} = (V_{11}\,V_{22} - V_{12}^2)/((V_{11} - V_{12})\,I), \quad (3)$$

and

$$R_{12} = (V_{11}\,V_{22} - V_{12}^2)/(V_{12}\,I). \quad (4)$$

It is assumed here that rectification is absent, thus

$$R_{12} = R_{21} \quad \text{and} \quad V_{12} = V_{21} \quad (5)$$

(*see also* [3, 19]).

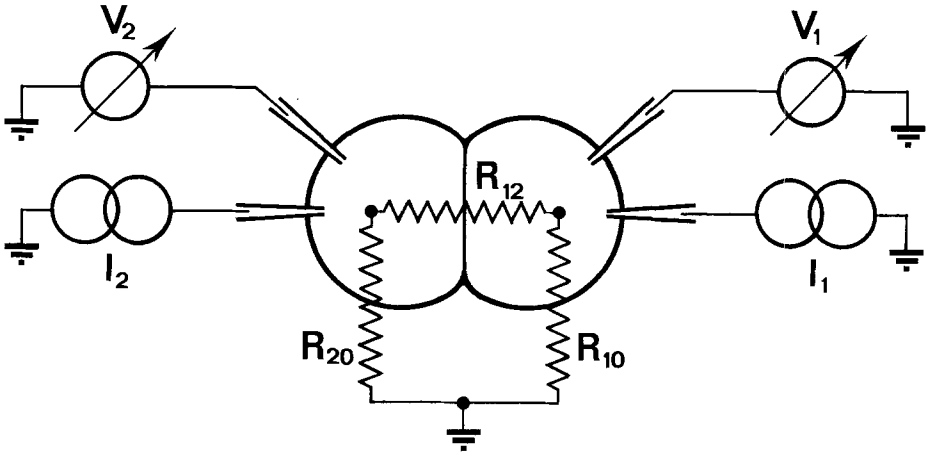

Fig. 2. Diagram showing the principle of measuring the junctional and nonjunctional resistances in a two-cell system (*also see text*)

It should be noted that the "perijunctional insulation" [24] cannot be measured directly with the method illustrated in Fig. 2. In fact the resistances R_{10}, R_{20}, and R_{12} (Eqs. 2–4) are equivalent resistances formed by the junctional, nonjunctional, and perijunctional resistances. In an electrically coupled cell system, the low-resistance part of the intercellular membrane has to be insulated from the external medium, otherwise the coupling could not exist.

In a two-cell system where the surface areas of the respective membranes are known, the specific membrane resistances ($\Omega\,cm^2$) can be directly calculated from the values of R_{10}, R_{20} and R_{12}. When multicellular systems are studied the four-electrode method will yield only equivalent resistances (R'_{10}, R'_{20} and R'_{12}).

In an embryonic system showing rapid cell division without cell growth, such as the early amphibian embryo, we are not dealing with a stationary but with a time-dependent multicellular system. Regional or temporal differences in intercellular communication in such a system can be caused by: (1) differences in specific resistance among the different cleavage membranes, (2) regional differences in specific resistance of any one cleavage membrane, (3) temporal changes of the specific resistance of any one cleavage membrane, (4) temporal changes in the surface areas of the different membranes. We cannot assume *a priori* that the intercellular membranes formed at different cleavages have identical permeability properties, or that the permeability properties of a particular intercellular membrane, once formed, remain constant as development proceeds. On the contrary, such differences or changes in time could play a role in the regulation of differentiation [25, 26]. Up till now early all studies of electrotonic coupling in multicellular embryonic systems have been restricted to the measurement of coupling ratios. Because of the difficulties inherent in making models for such time-dependent systems no data on the specific membrane resistances were obtained. Only very abrupt changes in the coupling ratio could be interpreted as an alteration of the cell membrane properties [44].

In previous papers we showed that removal of the vitelline membrane or treatment with cytochalasin B leads to the exposure of the entire surface area of the first cleavage membrane of the *Xenopus* egg to the medium [7, 8, 22]. Under these conditions we were able to measure the permeability properties of the first cleavage membrane directly. Its mean specific resistance was found to be 1–2 $k\Omega cm^2$, as against 74 $k\Omega cm^2$ for the pre-existing nonjunctional membrane (corrected for surface foldings) [20]. The newly formed membrane has a relatively high permeability for K^+ ions

[21]. Similar results were obtained for the *Ambystoma* egg [23]. It has been suggested by others [42, 46] and by us [20] that the relatively low specific resistance of the cleavage membrane, as compared to that of the non-junctional membrane, in itself could give rise to an appreciable electrotonic coupling in the early cleavage stages of the amphibian embryo, provided that the cleavage furrow is sealed from the external medium. In normally cleaving eggs such a surface barrier is present indeed, as can be concluded from the finding that maximally only 10% of the total area of the first cleavage membrane is in electrical contact with the extraembryonic medium [20]. The terminal junctions reported by Sanders and Zalik [36] could form the morphological basis for the furrow sealing. These authors observed gap junctions from blastula stages on, but not prior to stage 7 [29]. This corresponds to the observed capacity of isolated morula cells of *Triturus* embryos to form low-resistance junctions [15, 16, 17]. However, the presence of gap junctions during the first cleavage has also been suggested [40].

In the present study we have investigated the permeability properties of the newly formed intercellular membranes from the onset of second cleavage till the onset of fifth cleavage in embryos of *Xenopus laevis*. Using the method illustrated in Fig. 2 the equivalent resistances R'_{12}, R'_{10} and R'_{20} were measured continuously in different pairs of adjacent cells separated by one of the four cleavage membranes formed in that period. On the assumption that no differences in permeability properties exist between the animal and vegetative part of a cleavage membrane at any time, the specific resistance of each cleavage membrane as a function of time was determined from the measured equivalent resistances. A generally applicable computer simulation model was developed for that purpose and is described in detail in an accompanying paper [19].

Materials and Methods

Embryos of *Xenopus laevis* were obtained from hormonally stimulated couples, chemically decapsulated, and handled as described earlier [22]. The embryos were kept in Steinberg solution [21]. The experiments were carried out at room temperature (20–24 °C). Developmental stages are according to Nieuwkoop and Faber [29].

Electrical Measurements

Fig. 3 illustrates the principles of the method for a four-cell embryo. Two pairs of 3 M KCl-filled glass microelectrodes (5–20 Ω d-c resistance), connected to Pt-Ag-AgCl electrodes via Steinberg solution bridges, were used to measure intracellular voltages and to pass current into each of two adjacent cells. The medium was grounded via a Pt-Ag-AgCl electrode. The intracellular voltages (V_1 and V_2) were measured with respect to an indifferent Pt-Ag-AgCl

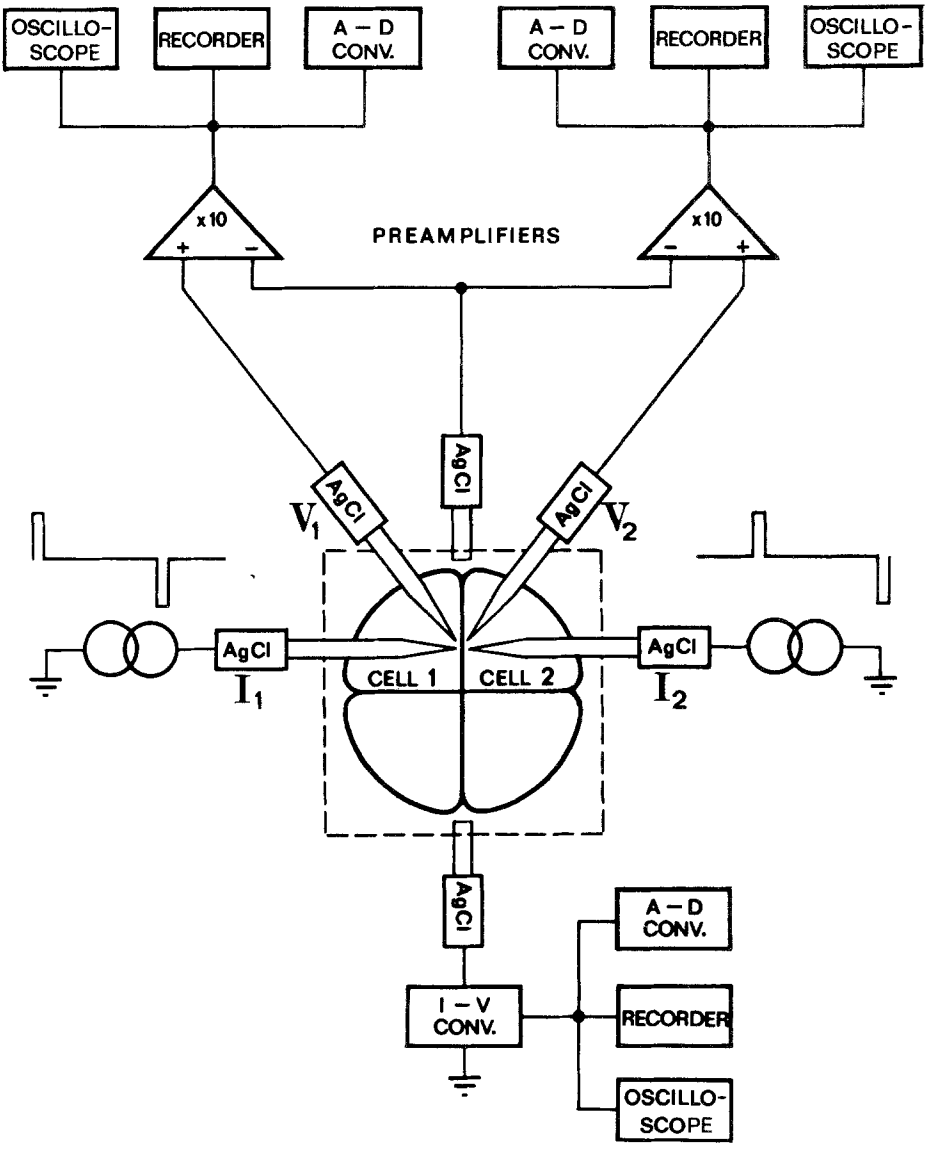

Fig. 3. Diagram of the measuring configuration used for the measurement of the equivalent junctional and nonjunctional resistances (*also see text*)

electrode by means of differential preamplifiers (Transidyne MPA-6, Transidyne general, Ann Arbor, Michigan). Two constant current stimulators (I_1 and I_2) delivered rectangular current pulses of 3×10^{-8} A or 5×10^{-8} A and 1 sec duration. Every minute a sequence of four pulses with 5-sec intervals was passed into the embryo, first two positive pulses into cell 1 and cell 2, respectively, then two negative pulses in the same order. The current was measured by means of a current-to-voltage converter in the ground circuit. The outputs of the preamplifiers and the current-to-voltage converter were recorded on a multichannel pen recorder (Hellige 19, F. Hellige, Freiburg im Breisgau, B.R.D.) and could be monitored on an oscillo-

scope (Tektronix 565, Tektronix, Beaverton, Oregon). Furthermore they were connected via sample and hold amplifiers to A–D converters (Preston X-mod 723 A, Preston Scientific, Anaheim, California). Digital measurements were taken 50 msec before and 950 msec after the onset of each current pulse. The latter time was chosen such that the intracellular voltage had reached a new stable value. The differences between the two measurements yielded the magnitude of the current-induced deviation of the membrane potentials of cell 1 and cell 2 and the magnitude of the current pulse. The timing of the stimulation and the A–D conversion was controlled by two Devices digitimers (Devices Instruments, Welwyn Garden, U.K.). The digital outputs of the A–D converters were connected to a Wang 720C system (Wang International Trade, Tewskbury, Massachusetts) for on-line computation of the equivalent resistances R'_{12}, R'_{10} and R'_{20} (Eqs. 2–5). Since no signs of rectification were observed, the values obtained for positive and negative current pulses were averaged and the mean value of V_{12} and V_{21} was taken for calculation of the equivalent resistances.

In case the coupling ratio approaches one ($V_{12}/V_{11} \to 1$ or $V_{21}/V_{22} \to 1$) it is possible that, due to the errors inherent to the measuring circuit, the measured value for the coupling ratio exceeds the theoretical upper limit of one. This would lead to erroneous results in the on-line calculation of the equivalent resistances. Therefore, an upper limit for these voltage ratios of 0.96 was used during all experiments. If the ratio V_{12}/V_{11} or V_{21}/V_{22} exceeded this value, V_{12} or V_{21} were calculated from

$$V_{12} = 0.96 * V_{11} \quad \text{or} \quad V_{21} = 0.96 * V_{22}.$$

Continuous measurements were made of the equivalent junctional and nonjunctional resistances in different pairs of adjacent cells (Table 1; Fig. 4). Measurements across the third cleavage membrane were made in adjacent animal and vegetative cells. In all other cases animal cells were used only. Only successful measurements with a minimum duration of one cleavage cycle (about 30 min) were taken into account. For each period of development at least four measurements were made per class of adjacent cells.

Table 1. Summary of the different classes of adjacent cells (i, j) used for measuring R'_{ij}, R'_{i0}, and R'_{j0} per cleavage membrane and per developmental stage [a]

Stage	Cleavage Membrane						
	I		II	III		IV	
	d	*v*		*d*	*v*	*d*	*v*
3	1, 2	3, 4	2, 3 or 1, 4				
4	1, 2	3, 4	2, 3 or 1, 4	1, 5 or 2, 6	3, 7 or 4, 8		
5	1, 2	5, 6	3, 4 or 7, 8	1, 9 or 2, 10	5, 13 or 6, 14	1, 8 or 2, 3	4, 5 or 6, 7

[a] The positions of the different cells and cleavage membranes as indicated by the numbers 1 to 16 and I to IV, respectively, are given in Fig. 4. The dorsal and ventral sides are indicated by *d* and *v*, respectively. Adjacent animal and vegetative cells were used for measurements across the third cleavage membrane; in all other cases adjacent animal cells were used.

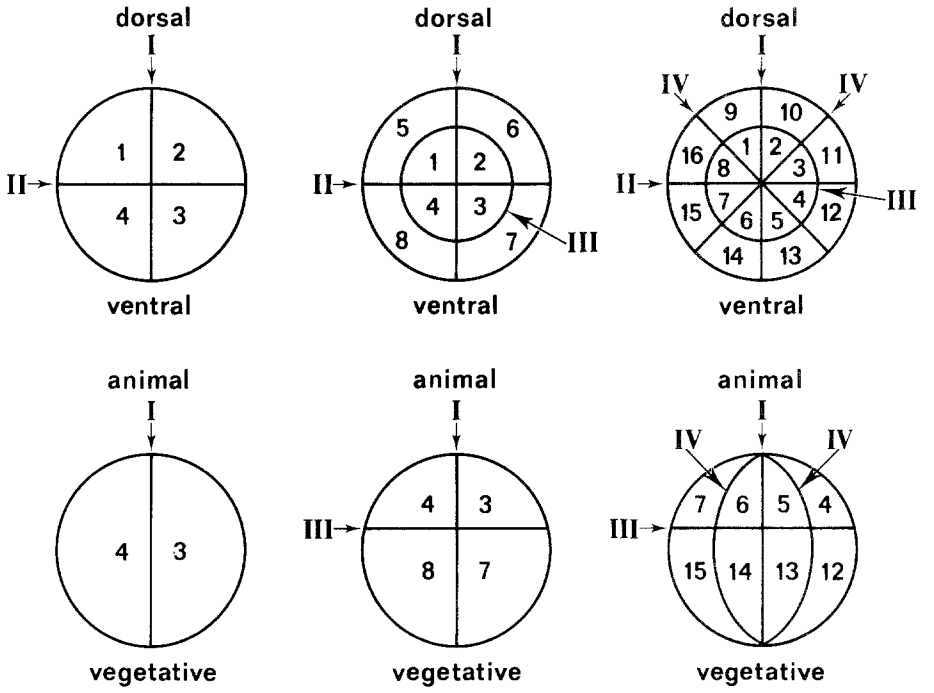

Fig. 4. Diagrammatic representation of the geometry of the 4-, 8-, and 16-cell stage of the *Xenopus* embryo. The successively formed cleavage membranes are indicated by arrows, marked I–IV. The individual cells are numbered separately for each stage. Upper row: animal view. Lower row: ventral view

The egg was oriented on the basis of the position of the first cleavage plane, which as a rule coincides with the dorso-ventral plane, and on the presence of the less darkly pigmented grey crescent on the dorsal side [29].

Derivation of the Specific Membrane Resistances from the Experimentally Determined Equivalent Resistances

The details of the computer simulation model are described in a subsequent article [19].

For a multicellular biological system like the amphibian embryo, we make the following assumptions:

1. The impedance of a cell membrane can be represented as a resistance in parallel with a capacitance.
2. The cell membrane has no rectifying properties.
3. The resistivity of the cytoplasm and extracellular fluids is negligible.

A k-cell embryo consists of $n=k+1$ compartments. It may be represented as a passive linear electrical network with $m=k+2$ nodes: the medium (node 0), the k-cells (node 1 to $n-1$) and the blastocoel (node n). For a network with m nodes, where each node p is connected to each node q via an admittance y_{pq}, and voltages are measured with respect to node 0, we define:

j_{ii}: The current used for excitation of the network at node i.
v_{ij}: The voltage at node j, caused by j_{ii}.

y_{pq}: The admittance between nodes p and q, when current flows from p to q. When rectification is absent $y_{pq}=y_{qp}$.

Furthermore, we define: $y_{ii}=\sum_{\substack{q=0\\q\neq i}}^{n} y_{iq}$.

When a current step function is used for j_{ii}, v_{ij} will be time dependent, due to the capacitive properties of the membranes. If we determine v_{ij} after it has reached a steady state it will be a measure for the conductance. Then $y_{pq}=R_{pq}^{-1}$, where R_{pq} is the resistance between nodes p and q. Hereafter we assume this condition to be fullfilled.

When such a network is excited successively in each node the equilibrium equations can be written in matrix notation:

$$\begin{bmatrix} y_{11} & -y_{12} \cdots -y_{1n} \\ -y_{21} & y_{22} \cdots -y_{2n} \\ \vdots & \vdots \quad \vdots \\ -y_{n1} & -y_{n2} \cdots y_{nn} \end{bmatrix} \cdot \begin{bmatrix} v_{11} & v_{21} \cdots v_{n1} \\ v_{12} & v_{22} \cdots v_{n2} \\ \vdots & \vdots \quad \vdots \\ v_{1n} & v_{2n} \cdots v_{nn} \end{bmatrix} = \begin{bmatrix} j_{11} & & \\ & j_{22} & \\ & & \ddots \\ & & j_{nn} \end{bmatrix} \tag{6}$$

or in short form: $[Y]\cdot[V]=[J]$.

Since $[Y]$ is symmetrical we have $1/2\cdot n\cdot(n+1)$ independent conductances y_{pq}. They can be calculated when the necessary number of v_{ij} and j_{ii} is determined. Knowing y_{pq} (or R_{pq}) and the surface area of the corresponding membrane (A_{pq}), the specific membrane resistance (r_{pq}) can be calculated:

$$r_{pq}=A_{pq}/y_{pq} \quad \text{or} \quad r_{pq}=A_{pq}\cdot R_{pq}. \tag{7}$$

When the surface areas can be measured or estimated, the number of independent parameters will be equal to the number of independent r_{pq}'s.

Experimentally this approach will be nearly impossible in a multicellular biological system. However, an alternative procedure can be followed. The equivalent conductances between pairs of cells (y'_{ij}) and between each of the two cells and the medium (y'_{i0} and y'_{j0}) can be measured by the method illustrated in Figs. 2 and 3. In other words the m-node network is reduced to a 3-node network. For $i=1$ and $j=2$ the equivalent conductances are given by:

$$\begin{bmatrix} y'_{11} & -y'_{12} \\ -y'_{21} & y'_{22} \end{bmatrix} = \begin{bmatrix} y_{11} & -y_{12} \\ -y_{21} & y_{22} \end{bmatrix} - \begin{bmatrix} -y_{13} \cdots -y_{1n} \\ -y_{23} \cdots -y_{2n} \end{bmatrix} \cdot \begin{bmatrix} y_{33} \cdots -y_{3n} \\ \vdots \quad \vdots \\ -y_{n3} \cdots y_{nn} \end{bmatrix}^{-1} \cdot \begin{bmatrix} -y_{13} & -y_{23} \\ \vdots & \vdots \\ -y_{1n} & -y_{2n} \end{bmatrix} \tag{8}$$

and

$$y'_{10}=y'_{11}-y'_{12}; \quad y'_{20}=y'_{22}-y'_{21}. \tag{9}$$

Fortunately the number of independent r_{pq}'s can be reduced drastically on the basis of the geometrical properties of the system. The number of pairs of adjacent cells in which the equivalent resistances R'_{ij}, R'_{i0}, and R'_{j0} were measured, was equal to the number of independent r_{pq}'s. Subsequently, the specific membrane resistances were calculated within preset limits by an approximation procedure. For that purpose, a BASIC program was developed for a Wang 2200-B2 minicomputer. Starting with arbitrary values for r_{pq} and known values for A_{pq}, the resulting equivalent resistances R'_{ij}, R'_{i0}, and R'_{j0} were calculated for the different pairs of cells, using Eqs. (7–9). The calculated values were compared with the experimentally determined mean values. Then the initial r_{pq} values were adjusted until the calculated values of the equivalent resistances corresponded within 1% with the experimentally determined values.

The Geometry of the Early Amphibian Embryo

For simplicity the embryo is considered to be a sphere. The cleavage membranes are taken to be flat planes which section the embryo into different compartments. The blastocoel is considered as a sphere enclosed by the cells. For the stages investigated the surface areas (A_{pq}) of the junctional and nonjunctional membranes of the different compartments can easily be calculated by the proper choice of four parameters: (1) the radius of the embryo (x); (2) the radius of the blastocoel (y); (3) the distance from the third cleavage plane to the center of the embryo (a); (4) the distance from the third cleavage plane to the center of the blastocoel (b) (see Figs. 4 and 5).

No quantitative information is available on the radius of the blastocoel (y) and on the position of the center of the blastocoel (b). It was assumed that y increases by 20 μm during each cleavage cycle between stage 2 and 5. In addition it was assumed that $b = a \cdot x/y$. The implications of these assumptions are analyzed in a subsequent article [19]. Implicit in this geometrical representation is the assumption that the current j_{ii} can only flow directly to the cells adjacent to cell i, to the medium, and to the blastocoel. No direct current flow is possible between diagonally opposed cells or between the blastocoel and the medium (*see also* Introduction). The number of independent r_{pq}'s was reduced further by assuming that at any time:

1. The specific resistance of the animal and vegetative part of a given membrane is identical.

2. The specific resistances of the membranes enclosing the blastocoel are the same and equal to the mean of the specific resistances of the cleavage membranes. The possible influence of the blastocoel is further analyzed in a subsequent article [19].

3. The specific resistances of the nonjunctional membranes are identical. This assumption was confirmed by comparing statistically the equivalent resistances between different cells and the medium.

The seven resulting classes of adjacent cells to be analyzed are given in Table 1. The values of the geometrical parameters used for the calculation of the surface areas (A_{pq}) of the individual junctional and nonjunctional membranes are given in Table 2. The resulting values of these surface areas are given in Table 3.

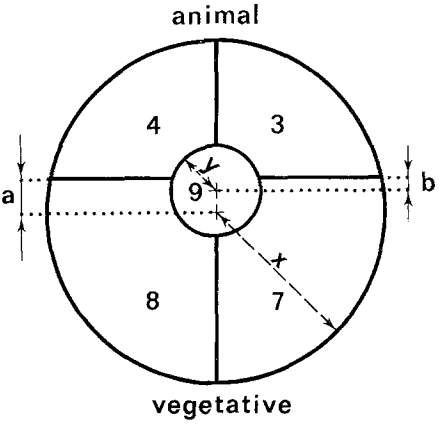

Fig. 5. Diagrammatic representation of a median cross-section through an 8-cell embryo, showing the geometrical parameters that could be varied in the model (*also see text*)

Table 2. Values (in mm) of the geometrical parameters used for the calculation of the surface areas of the individual junctional and nonjunctional membranes[a]

Stage	Number of cells per embryo	x	y	a
2	2	0.65	0.005	
3	4	0.65	0.025	
4	8	0.65	0.045	0.01
5	16	0.65	0.065	0.01

[a] For explanation of the symbols see text and Fig. 5.

Table 3. The surface areas A_{pq} of the individual junctional and nonjunctional membranes at the various stages, calculated on the basis of the geometrical parameters given in Table 2. The areas are given in cm^2

	Number of cells per embryo		
	4	8	16
Nonjunctional membranes	$1.33 * 10^{-2}$		
a. animal cell		$5.62 * 10^{-3}$	$2.81 * 10^{-3}$
b. vegetative cell		$7.66 * 10^{-3}$	$3.83 * 10^{-3}$
Junctional membranes between adjacent cells	$6.63 * 10^{-3}$		
a. animal-animal cells		$2.66 * 10^{-3}$	$2.64 * 10^{-3}$
b. vegetative-vegetative cells		$3.95 * 10^{-3}$	$3.93 * 10^{-3}$
c. animal-vegetative cells		$3.22 * 10^{-3}$	$1.60 * 10^{-3}$
Junctional membranes between cells and blastocoel	$1.96 * 10^{-5}$		
a. animal cell-blastocoel		$2.69 * 10^{-5}$	$2.81 * 10^{-5}$
b. vegetative cell-blastocoel		$3.67 * 10^{-5}$	$3.83 * 10^{-5}$

Results

The Equivalent Junctional and Nonjunctional Resistances

As described above, measurements of the equivalent resistances were made every minute. At room temperature the duration of one cleavage cycle varies from embryo to embryo between 25 to 35 min. Therefore, in order to be able to compare statistically the mean values of the different resistances at a given time, an arbitrary time unit had to be chosen. For each embryo the duration of one cleavage cycle was taken as the basis for this. The appearance of a pigment stripe on the surface of the embryo was used as the criterion for the onset of cleavage. Each cleavage cycle was divided into eight equal time units. The mean value of three successive measurements overlapping with the onset of such a time unit was con-

sidered to be representative for that moment. The values thus obtained for different embryos were averaged. Using this procedure the means $\pm$SEM of the various equivalent resistances were calculated as a function of time.

As a consequence of the geometrical assumptions the equivalent junctional resistance (R'_{ij}) was measured across seven different membranes: the second cleavage membrane and the dorsal and ventral parts of the first, third and fourth cleavage membranes (*see* Table 1). For each of the

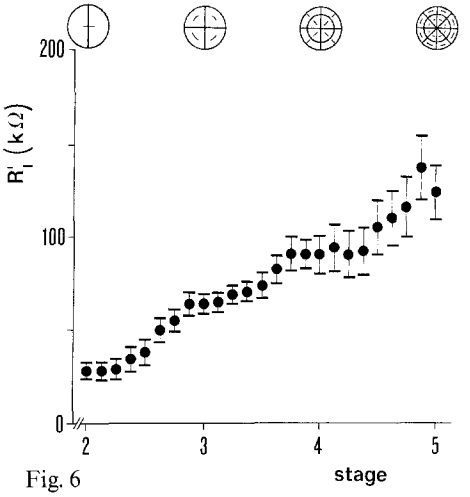

Fig. 6

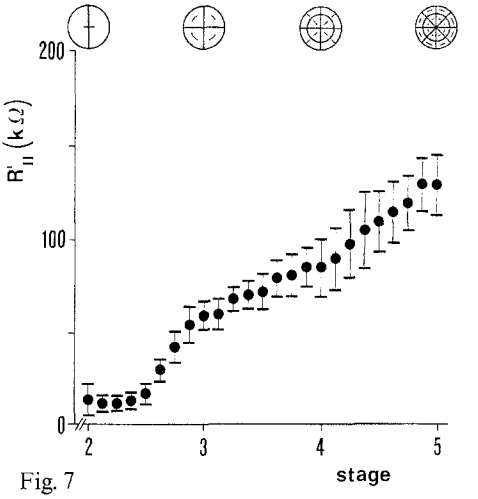

Fig. 7

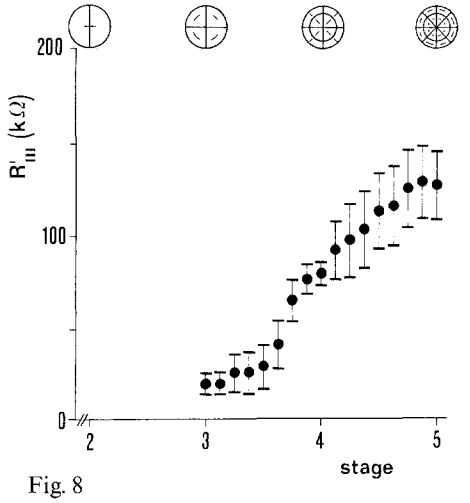

Fig. 8

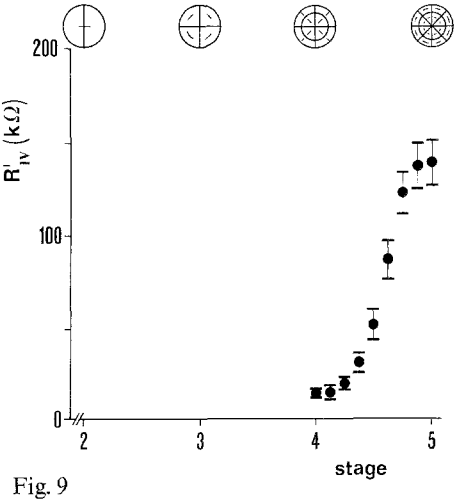

Fig. 9

Figs. 6–9. Equivalent junctional resistance between adjacent animal cells separated by the first (R'_{I}), second (R'_{II}), third (R'_{III}), and fourth (R'_{IV}) cleavage membrane, respectively, as a function of developmental stage. Mean values $\pm$SEM are given. The upper diagrams represent the various stages

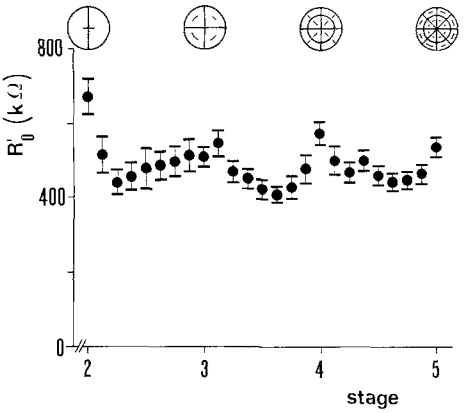

Fig. 10. Equivalent nonjunctional resistance (R'_0) measured in animal cells as a function of developmental stage. Mean values ±SEM are given. The upper diagrams represent the different stages

latter three membranes the dorsal and ventral R'_{ij}'s as measured at the onset of the successive cleavages were compared by an analysis of variance. In all cases differences between the dorsal and ventral R'_{ij} were absent ($p > 0.10$). Therefore, the dorsal and ventral R'_{ij}'s were pooled per cleavage membrane and per time. Figs. 6–9 show the resulting pooled equivalent resistances (R'_i) across the first four cleavage membranes (R'_{I}, R'_{II}, R'_{III}, R'_{IV}, respectively) as a function of time. It should be noted that R'_{I}, R'_{II}, and R'_{IV} were measured in animal cells only. Comparing these figures the following properties of R'_i become apparent:

1. R'_i increases stepwise during each successive cleavage cycle.
2. The initial rate of increase in R'_i is greater for subsequent cleavages.
3. At the end of each cleavage cycle a situation is established in which the equivalent resistance between adjacent cells is independent of which of the cleavage membranes separates them. This conclusion is corroborated by an analysis of variance showing the absence of significant differences between the various R'_i's at the onset of the third, fourth and fifth cleavages ($p > 0.10$ in all cases).

To check the assumption that the specific resistances of the nonjunctional membranes of all cells are identical at any one time, the equivalent resistances at the onset of each cleavage of the animal cells investigated were compared by an analysis of variance. No significant differences were found ($p > 0.05$ in all cases). Therefore their values were pooled per time unit. Fig. 10 shows the resulting equivalent nonjunctional resistance (R'_0) as a function of time. From this Figure the following properties of R'_0 are apparent: (1) During the first half of each cleavage

cycle, R'_0 decreases, followed by an increase during the second half of the cycle. (2) R'_0 shows a maximum at the onset of each cleavage cycle. At the onset of the first cleavage cycle R'_0 would be about 1700 kΩ, as can be calculated from the input resistance measured [20]. Apparently, R'_0 drops drastically during the first two cleavages but almost no overall decrease is seen during the third and fourth cleavage.

The Specific Resistance of the Cleavage and Nonjunctional Membranes

The specific resistances of the cleavage and nonjunctional membranes were derived from the mean values of the respective equivalent resistances, using the simulation method described under Materials and Methods. In this analysis cell division was considered to be a discrete process. This implies that at the onset of each cleavage the number of cells was assumed to double. The surface areas of the individual membranes were assumed to remain constant during each cleavage cycle.

Figs. 11–14 show the resulting specific resistances (r_i) of the first four cleavage membranes (r_{I}, r_{II}, r_{III}, r_{IV}, respectively) as a function of time. After an initial increase the specific resistance of all membranes reaches a level of about 400 Ω cm^2 and shows only minor fluctuations as development proceeds. The initial increase apparently reflects the formation of the new membrane. Comparison of this phase among the four membranes shows that the formation of those membranes that arise later is completed more rapidly. While r_{I} reaches its steady level in 1.5 cleavage cycles, r_{II} and r_{III} do so in one cycle, and r_{IV} even in less than one cycle. Preliminary measurements during the formation of the fifth cleavage membrane indicate that this membrane is completed within the first half of the fifth cleavage cycle, and that r_{V} also reaches a level of 400 Ω cm^2. Therefore, once membrane formation is completed the specific resistance of all cleavage membranes is constant at about 400 Ω cm^2. Thus, the stepwise increase in the equivalent junctional resistance as measured between two cells separated by one of these cleavage membranes (Figs. 6–9) is not caused by a change in time of r_i nor by differences in the specific resistances of the different cleavage membranes. It is only due to the synchronous division of the cells, during which the daughter cells become separated by a cleavage membrane having permeability properties similar to those of the previously formed membranes.

Fig. 15 shows the specific resistance of the nonjunctional membranes (r_0) as a function of time. The pattern of change is similar to that of R'_0

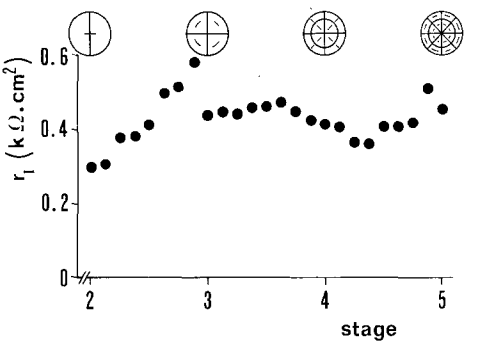

Fig. 11

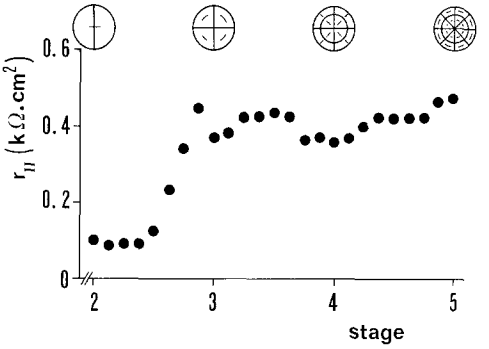

Fig. 12

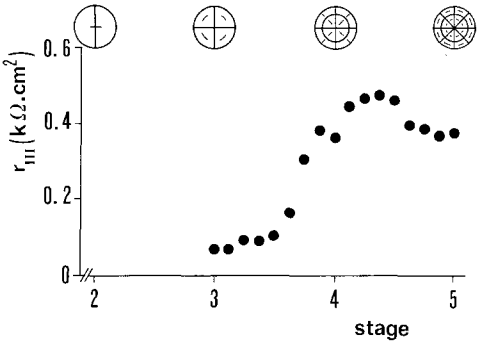

Fig. 13

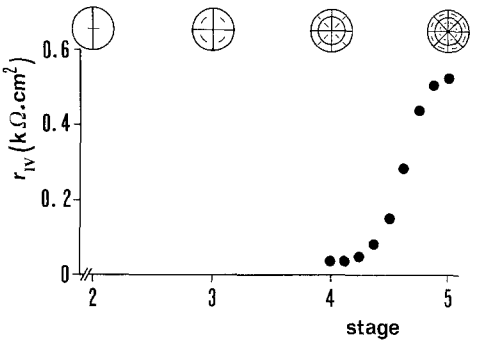

Fig. 14

Figs. 11–14. Specific resistance of the first (r_I), second (r_{II}), third (r_{III}), and fourth (r_{IV}) cleavage membrane, respectively, as a function of developmental stage. The values were derived from the mean values of the equivalent resistances given in Figs. 6–10. The upper diagrams represent the various stages

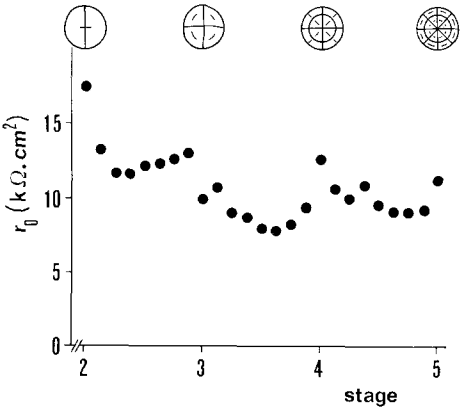

Fig. 15. Specific resistance of the nonjunctional membrane (r_0) as a function of developmental stage. The values were derived from the mean values of the equivalent resistances given in Figs. 6–10. The upper diagrams represent the various stages

(Fig. 10). An overall decrease can be observed, particularly during the first two cleavage cycles (at the onset of the first cleavage $r_0 = 43.5\ \mathrm{k\Omega\,cm^2}$, uncorrected for surface foldings [20]). Superimposed on this decrease r_0 shows a transient decrease during each cleavage cycle. The specific resistance of the nonjunctional membranes is 20 to 100 times greater than that of the cleavage membranes.

Discussion

In the present study we have measured the equivalent junctional and nonjunctional resistances in different pairs of adjacent cells separated by one of the early cleavage membranes. Measurements were made continuously during the cleavage cycles from the onset of the second to the onset of the fifth cleavage. In this way the formation of the first four cleavage membranes could be monitored. In measuring equivalent resistances the multicellular embryo is considered as a three-compartment system composed of the two cells and the surrounding medium. In other words, the equivalent resistances are a measure for the total ability to pass an ionic current between the two cells and between the respective cell interiors and the medium, irrespective of the pathways involved. It was shown that the equivalent junctional resistances increase stepwise during each cleavage. At the onset of each cleavage a situation is established in which no significant differences are found between the equivalent junctional resistances measured between different pairs of adjacent animal cells. The same was found for the equivalent nonjunctional resistance (R'_0). R'_0 shows a decrease during the first two cleavage cycles and superimposed on this, a transient decline during each cycle.

The principles were described of a computer simulation method used to derive the specific membrane resistances from the measured equivalent resistances. The details of this method are described in a subsequent article [19]. The specific resistance is a measure for the ability to pass current per unit membrane area. It was assumed that at any one time differences between the animal and vegetative parts of a given cleavage membrane are absent. From the measurements of the equivalent resistances it was concluded that at any one time differences between the specific resistances of the dorsal and ventral parts of a cleavage membrane, and between the nonjunctional membranes of different cells were also absent. Finally, it was assumed that at any one time the specific resistance of the membranes enclosing the blastocoel is equal to the mean specific resistance of all cleavage membranes at that time. This assumption had to be made

because the blastocoel is hardly accessible to microelectrodes at the stages investigated. This leaves five independent specific resistances to be derived: those of the four cleavage membranes (r_{I}, r_{II}, r_{III} and r_{IV}) and that of the nonjunctional membranes (r_0).

The specific resistance of the nonjunctional membranes is 20 to 100 times greater than that of the cleavage membranes. Apparently the outer surface of the embryo is very impermeable to ions. The changes in r_0 become understandable when we extrapolate our earlier findings on the first cleavage of *Xenopus* eggs [20, 21]. During the early cleavages a small fraction of the newly formed membrane is inserted into the walls of the cleavage furrow and thus forms part of the nonjunctional membrane. Due to the relatively high ionic permeability of this new membrane (especially for K^+ ions) r_0 (and R'_0) will decrease. Moreover, during the first half of each cleavage cycle the peripheral parts of the blastomeres separate partially. They become tightly attached again before the onset of the next cleavage. These movements of the blastomeres can be observed under the dissecting microscope. They lead to a temporary exposure to the medium of a greater fraction of new membrane material in the walls of the cleavage furrow and thus to a temporary decrease of r_0. When the input resistance of the uncleaved egg is 852 kΩ [20] it can be calculated on the basis of $R'_0 = 400\ \mathrm{k\Omega}$ (the minimum value observed) that maximally 0.15 $\mathrm{mm^2}$ of new membrane ($r_i = 400\ \Omega\,\mathrm{cm^2}$) is in parallel with the preexisting membrane. This is about 3% of the total surface area of the nonjunctional membrane. Concomitant with the changes in r_0, we have observed an overall hyperpolarization of the membrane potential (E_m) and superimposed on this, a transient hyperpolarization of E_m during each cleavage cycle. These changes in E_m during the later cleavages are qualitatively similar to those observed during the first cleavage [20, 21]. Most probably the relatively high K^+ permeability of the inserted new membrane is responsible for this hyperpolarization also during later cleavages [21]. The details of the changes of E_m during the early development will be given elsewhere. A possible role of the cyclic changes in E_m in the regulation of the cell cycle has been suggested [21, 28] and is at present the subject of further investigations.

After an initial phase during which the membrane is being formed, the specific resistances of all four cleavage membranes remain constant at about 400 $\Omega\,\mathrm{cm^2}$. The duration of the formation phase decreases during the subsequent cleavages, although the total surface area of membrane to be formed is probably about equal for the first three cleavages and twice as much for the fourth cleavage. Apparently, the division of one large cell

takes more time than the division of k cells having the same total volume. It should be noted that the rate of new membrane growth is extremely high. Within 2 hr the fertilized egg (1.3 mm in diameter) is divided up into 16 cells by the formation of about 6.5 mm^2 of new membrane.

It may seem rather surprising that after the fourth cleavage no large differences are found between R'_{III} on the one hand, and R'_{I}, R'_{II} and R'_{IV} on the other. The fourth cleavage divides the third cleavage membrane into eight equal parts, while each of the other membranes remains divided into four parts (*see* Fig. 4). Therefore, the surface area of the intercellular membrane (A_{pq}) will be much smaller for cells separated by the third membrane than for other cell pairs (*see* Table 3). When all cleavage membranes have equal specific resistances, the junctional resistance R_{pq}, i.e. the resistance of the area of direct contact between cells p and q, will be proportional to A_{pq}. However, the resulting differences in R_{pq} will be obscured when measuring the equivalent junctional resistance, i.e. the actual resistance between the two cells, because of the large number of parallel pathways between cell p and q (*see also* [19]).

In cytochalasin-B treated eggs and in eggs cleaving after the removal of the vitelline membrane the specific resistance of the newly formed membrane, which became exposed to the medium during first cleavage, was found to be 1–2 $k\Omega\,cm^2$ [20]. That is somewhat higher than that found here for the cleavage membranes (0.4 $k\Omega\,cm^2$). This difference may be due to the fact that in the geometrical modelling of the embryo no correction was made for a possible increase in surface area of the cleavage membranes by folding. An alternative explanation would be that a small number of specialized junctions, e.g. gap junctions, are present in the intercellular membranes. In that case 0.1 $\Omega\,cm^2$ junctions occupying about 0.01% of the intercellular membranes could explain the observed difference.

At present there is no clear electron-microscopical evidence for the existence of gap junctions in the developmental stages investigated here [36], although their presence has been suggested during the first cleavage [40]. The absence of cell-to-cell passage of fluorescein in early *Xenopus* embryos [41], as was also found in the early *Fundulus* embryo [5], supports the hypothesis that no gap junctions are involved in the establishment of electrotonic coupling in the early cleavage stages. In conclusion, it is not necessary to assume the presence of specialized low-resistance junctions in the intercellular membranes, but their existence is not excluded by the experimental data.

If the permeability properties of the cleavage and nonjunctional membranes do not change drastically during later division cycles, the

equivalent junctional resistances will increase further as a result of the decrease in surface area of the intercellular membranes and the increase in their number. Preliminary results of measurements during the fifth and sixth division cycle support this prediction. However, a continuation of this process would lead to a rapid loss of coupling as development proceeds. At later stages the involvement of low-resistance junctions is necessary for maintaining a degree of coupling as has been reported [30]. In amphibians, gap junctions have been described in blastula stages [36] and particularly well during neurulation [9]. Furthermore, isolated morula cells are able to form low-resistance junctions [15, 16, 17], and reaggregated and electrically coupled gastrula cells can exchange fluorescein [39]. If indeed gap junctions appear only at later stages, cell-to-cell passage in the early cleavage stages of the amphibian embryo will be mainly restricted to small ions, as was suggested also for the *Fundulus* embryo [5].

The membrane potential is primarily dependent on the K^+ gradient across the cell membrane [21, 23]. Since the membrane potential probably is an important factor in the regulation of the cell cycle [28], the relatively high K^+ permeability of the cleavage membranes [21, 23] may be essential for the synchronous division of the blastomeres during early amphibian development.

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