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ACTIVE Na^+ AND Cl^- TRANSPORT BY THE ISOLATED CHICK CHORIOALLANTOIC MEMBRANE*

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SUMMARY

Unidirectional Na^+ and Cl^- fluxes across 15–18-day chick chorioallantoic membranes were determined *in vitro* using the short-circuit technique of USSING AND ZERAHN[6]. In the absence of an electrochemical gradient across the membrane, the observation of any net ion movement indicates the existence of an active transport process. In the normal chorioallantoic membrane both Na^+ and Cl^- were found to be actively transported from ectoderm to endoderm. The observed short-circuit current could be fully accounted for by their algebraic sum. Upon removal of the acellular inner shell membrane from the ectodermal side of the chorioallantoic membrane, the direction of net Na^+ transport reversed and net Cl^- transport was abolished. The short-circuit current of these stripped membranes was nearly 3 times greater than the normal membranes and could not be entirely accounted for by the observed active Na^+ transport. It is suggested that the active Cl^- transport site resides in the ectodermal cells and that there exist two opposing active Na^+ mechanisms presumably located in the ectodermal and endodermal layers, respectively.

INTRODUCTION

It has been shown that the isolated chick chorioallantoic membrane is capable of actively transporting Ca^{2+} from ectoderm to endoderm[2]. Upon removal of the acellular inner shell membrane from the ectodermal surface the direction of net Ca^{2+} transport under short-circuit conditions was shown to be reversed. In both cases the magnitude and direction of the observed short-circuit current indicated the existence of at least one additional actively transported ion. In addition, it was found previously that *in vitro* bathing solutions consisting of physiological concentrations of electrolytes supplemented with exogenous glucose were incapable of supporting the tissue, as evidenced by the progressive decline in the transmural potential difference (PD).

The purpose of the present investigation was 2-fold. First, we wanted to determine whether suitable bathing solutions could be found which would enable the membrane to remain in an electrical steady-state as determined by a time-independent PD.

Abbreviation: PD, potential difference.

* Part of this study has previously appeared in abstract form[7].

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Second, we sought to establish the identity of the other actively transported ion(s) and thus characterize the origin of the observed transmural PD in both the normal and stripped chorioallantoic membranes.

MATERIALS AND METHODS

The chorioallantoic membranes used in this study were isolated from fertile hen's eggs of the Babcock Leghorn breed obtained from a local hatchery. All eggs were incubated at 38° and 60 % humidity. Only membranes isolated from eggs of 15–18 days of incubation were used. Periodic candling eliminated infertile or obviously deformed embryos.

After a suitable egg had been selected the shell around the air space (blunt end of the egg) was gently fractured and the fragments carefully removed with forceps. A circular segment of chorioallantoic membrane together with the accompanying inner and outer shell membranes was then removed and quickly transferred to one-half of a modified Ussing-type transport chamber described elsewhere[3]. After the outer shell membrane was spirally peeled away the transport chamber was assembled and 10 ml of test solutions introduced on each side. In those experiments utilizing stripped membranes the inner shell membrane was also carefully removed from the ectodermal surface of the chorioallantoic membrane prior to mounting. The entire isolation procedure required approx. 3 min. All experiments were performed at 37° using thermoelectric heat exchangers (Cambion 7220-2). Aluminum plates mounted on the bottom of the flux chambers provided thermal contact between the heat exchangers and the bathing solutions. In each case 1 cm^2 of membrane was exposed to the bathing media.

After considerable experimentation, bathing solutions were found which would allow the membrane to maintain an electrical steady-state for over 6 h. The solution finally employed was a standard tissue culture medium, Eagle's minimum essential medium[1] with Earle's salts, supplemented with 10 % calf serum (Grand Island Biological Co., Grand Island, N.Y.). The electrolyte composition of this media in mequiv was: Na^+, 144.5; K^+, 5.4; Ca^{2+}, 3.6; Mg^{2+}, 1.6; Cl^-, 125.1; HCO_3^-, 26.2; phosphate, 2.0; and SO_4^{2-}, 1.6. The osmolality of the bathing media was 281 mosM/kg water as determined using an Osmette Precision Osmometer. All solutions were continually gassed with O_2–CO_2 (95:5, by vol.).

Preliminary experiments using paired membranes were discontinued since the delay in mounting the second half often resulted in a substantially lower PD. All data presented here were obtained from randomly selected, unpaired membranes.

Agar bridges containing electrolyte of the same composition as the bathing solutions were used to measure the transmural PD. The small tips were positioned approx. 4 mm away from the ectodermal and endodermal surfaces. The salt bridges were connected *via* calomel half-cells (Radiometer K401) to a self-balancing recording potentiometer having a 100-mV sensitivity and less than 0.5 mV error with a 1-MΩ input resistance. All measurements were corrected for half-cell and bridge-junction asymmetry potentials. Short-circuit current was applied *via* carbon electrodes in agar saline separated from the bathing solutions by a porous disk diffusion barrier. The current was applied continuously and automatically adjusted every 2 min. At hourly intervals the current was interrupted for 1 min and the resultant PD recorded. Membrane resistance was calculated from the PD/short-circuit current ratio.

After mounting, the membrane was permitted to operate in the open-circuited condition for 1 h. The radioisotopes $^{22}Na^+$ and $^{36}Cl^-$ were then added to the appropriate sides and electrical short-circuiting begun. At hourly intervals the originally unlabeled solutions were replaced with fresh, prewarmed solutions of identical composition for the flux measurement of the subsequent period. 6 h after the introduction of isotope the labeled solutions were removed, diluted with identical, isotope-free solution and aliquots removed for counting. All samples were counted in duplicate in both a liquid scintillation counter (Nuclear-Chicago Series 720) and a crystal scintillation counter (Nuclear-Chicago Model 8725). The relative counting efficiency of $^{22}Na^+$ of each counter, determined by counting identical solutions of $^{22}Na^+$ in each machine, was used to calculate the $^{36}Cl^-$ contribution to the activity recorded by the liquid scintillation counter.

All fluxes are expressed in μequiv$\cdot$cm$^{-2}\cdot$h^{-1} with appropriate standard errors. Values were considered to be significant when the difference in means exceeded the 95 % confidence limits calculated from the Student's t test.

RESULTS

The spontaneous potential difference exhibited by the isolated normal chick chorioallantoic membrane when both surfaces are bathed with the above media is shown in Fig. 1. The time-independent character of the PD reflects the ability of the normal chorioallantoic membrane to maintain an electrical steady-state for prolonged periods. The mean PD ranged from 5.0 $\pm$ 0.4 mV at the beginning of the experiment to 6.0 $\pm$ 0.7 mV at the end with the ectodermal side always positive. The short-circuit current, also relatively time-independent as seen in Table I, is designated by a positive sign representing electron flow from ectoderm to endoderm.

With the bathing media employed in these studies there was a marked increase in the transmural PD when the acellular inner shell membrane was removed. The PD of these stripped membranes ranged from 5.9 $\pm$ 1.0 mV initially to 17.7 $\pm$ 1.5 mV at

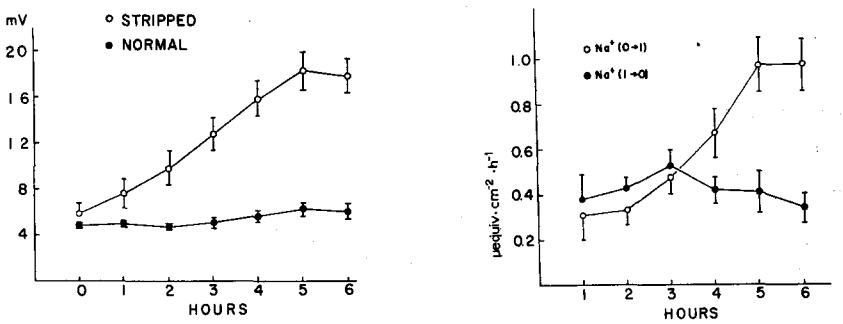

Fig. 1. Time dependence of the transmural potential difference of both normal and stripped chorioallantoic membranes. The ectoderm, or outside, of the membrane is always positive. Each normal and stripped membrane value represents the mean of fourteen experiments with standard errors given by vertical bars.

Fig. 2. Unidirectional Na^+ fluxes in μequiv$\cdot$cm$^{-2}\cdot$h^{-1} across the short-circuited normal chorioallantoic membrane as a function of time. Each ectoderm to endoderm (O $\rightarrow$ I) hourly flux value represents the mean of eight experiments while endoderm to ectoderm (I $\rightarrow$ O) value is the mean of six experiments. Standard errors are represented by vertical bars.

the end of the experiment as indicated in Fig. 1. After the second hour the short-circuit current of the stripped membrane stabilized at a level nearly 3 times greater than that of the normal chorioallantoic membrane (*cf.* Table II). A monotonic increase in the resistance of the stripped chorioallantoic membrane accounted for the majority of the observed rise in the transmural PD. In neither the normal nor the stripped membranes was there any indication of the decay in the electrical parameters reported previously when physiological saline containing only exogenous glucose was used as bathing solutions[2].

The unidirectional Na^+ and Cl^- fluxes across the short-circuited normal chorioallantoic membrane are shown in Figs. 2 and 3, respectively. The Na^+ endodermal to ectodermal (inside (I) to outside (O)) flux remained relatively constant over the 6-h period at approx. 0.4 μequiv·cm^{-2}·h^{-1} whereas the ectodermal to endodermal (outside (O) to inside (I)) flux increased progressively with the suggestion of a plateau of approx. 1.0 μequiv·cm^{-2}·h^{-1} during the fifth and sixth hours. There was no statistically significant net Na^+ flux until after the third hour. In contrast, there was a demonstrable

TABLE I

NET Na^+ AND Cl^- FLUXES IN THE SHORT-CIRCUITED NORMAL CHORIOALLANTOIC MEMBRANE

Direction of fluxes denoted by (O → I) = outside (ectoderm) to inside (endoderm) and (I → O) = inside (endoderm) to outside (ectoderm). Net flux values are expressed as mean ± 95 % confidence level, and were obtained from the corresponding unidirectional fluxes as given in the appropriate figure. Short-circuit current values are expressed as mean ± S.E.

Period (60 min)	*ΔNa^+ (O → I)*	*ΔCl^- (O → I)*	*Short-circuit current*	*Short-circuit current ($\Delta Na^+ + \Delta Cl^-$)*	*Resistance ($\Omega \cdot cm^{-2}$)*
	($\mu equiv \cdot cm^{-2} \cdot h^{-1}$)				
1	−0.08 ± 0.27	0.24 ± 0.32	0.51 ± 0.03	0.19 ± 0.43	366
2	−0.10 ± 0.14	0.38 ± 0.27	0.50 ± 0.03	0.02 ± 0.30	355
3	−0.06 ± 0.19	0.40 ± 0.25	0.52 ± 0.04	0.06 ± 0.31	361
4	0.25 ± 0.23	0.66 ± 0.31	0.53 ± 0.05	0.12 ± 0.39	405
5	0.55 ± 0.27	0.97 ± 0.34	0.49 ± 0.05	0.07 ± 0.44	461
6	0.63 ± 0.26	0.95 ± 0.24	0.42 ± 0.05	0.10 ± 0.37	531

TABLE II

NET Na^+ AND Cl^- FLUXES IN SHORT-CIRCUITED STRIPPED CHORIOALLANTOIC MEMBRANE

Direction of fluxes denoted by (O → I) = outside (ectoderm) to inside (endoderm) and (I → O) = inside (endoderm) to outside (ectoderm). Net flux values are expressed as mean ± 95 % confidence level, and were obtained from the corresponding unidirectional fluxes as given in the appropriate figure. Short-circuit current values are expressed as mean ± S.E.

Period (60 min)	*ΔNa^+ (I → O)*	*ΔCl^- (O → I)*	*Short-circuit current*	*Short-circuit current-ΔNa^+*	*Resistance ($\Omega \cdot cm^{-2}$)*
	($\mu equiv \cdot cm^{-2} \cdot h^{-1}$)				
1	0.45 ± 0.50	0.37 ± 0.70	1.07 ± 0.10	0.62 ± 0.53	269
2	0.69 ± 0.80	0.03 ± 0.77	1.23 ± 0.10	0.54 ± 0.83	298
3	0.68 ± 0.52	0.07 ± 0.58	1.33 ± 0.07	0.65 ± 0.54	355
4	0.90 ± 0.26	−0.11 ± 0.39	1.41 ± 0.07	0.51 ± 0.29	420
5	0.97 ± 0.23	−0.14 ± 0.32	1.44 ± 0.07	0.47 ± 0.26	473
6	0.89 ± 0.33	0.02 ± 0.26	1.33 ± 0.09	0.44 ± 0.37	496

net transport of Cl^- ($O \rightarrow I$) across the short-circuited, normal chorioallantoic membrane after the first hour. The increase in net Cl^- transport with time resulted both from an increase in the unidirectional ($O \rightarrow I$) flux together with a decrease in the ($I \rightarrow O$) movement as seen in Fig. 3. For both Na^+ and Cl^- the demonstration of net transport across the normal choriallantoic membrane in the absence of an electrochemical gradient constitutes proof of active transport. The algebraic sum of net Na^+ and Cl^- transport completely accounts for the observed short-circuit current of the normal membrane as indicated in Table I.

The demonstration of a time-independent unidirectional ($I \rightarrow O$) Na^+ flux in the normal membrane suggested that we were operating in an isotopic steady-state. To verify this, experiments were performed to determine the Na^+ pool of the normal chorioallantoic membrane using an ethanol extraction. With the estimated value of 0.8 μequiv$\cdot$cm^{-2} an equilibration half-time of approx. 30 min was calculated for Na^+ across the entire tissue. Similarly, a maximum value of 30 min was estimated for Cl^-.

When the acellular inner shell membrane was removed the patterns of ionic transport changed appreciably. The unidirectional Na^+ fluxes across the short-circuited stripped chorioallantoic membrane are shown in Fig. 4. The Na^+ flux ($I \rightarrow O$) remained relatively constant at approx. 1.5 μequiv$\cdot$cm$^{-2}\cdot$h^{-1}. The Na^+ flux ($O \rightarrow I$), on the other hand, initially 1.1 μequiv$\cdot$cm$^{-2}\cdot$h^{-1} declined to 0.7 μequiv$\cdot$cm$^{-2}\cdot$h^{-1} at the sixth hour. There is a significant net Na^+ movement ($I \rightarrow O$) after the second hour. The direction of net Na^+ transport in the stripped membrane is, therefore, opposite to that found in the normal chorioallantoic membrane.

In contrast to the normal membrane, net Cl^- transport in the short-circuited stripped chorioallantoic membrane could not be demonstrated. As indicated in Fig. 5, both unidirectional Cl^- fluxes in the stripped membrane followed a parallel decline over the 6-h period.

Table II indicates that there is a significant amount of unidentified active ion transport in the stripped chorioallantoic membrane as seen by the failure of net Na^+

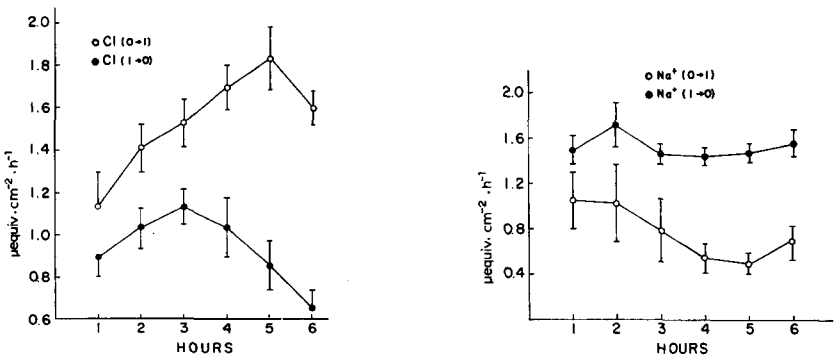

Fig. 3. Unidirectional Cl^- fluxes in μequiv$\cdot$cm$^{-2}\cdot$h^{-1} across the short-circuited normal chorioallantoic membrane as a function of time. Each ($O \rightarrow I$) hourly flux value represents the mean of eight experiments while each ($I \rightarrow O$) value is the mean of six experiments. Standard errors are represented by vertical bars.

Fig. 4. Unidirectional Na^+ fluxes in μequiv$\cdot$cm$^{-2}\cdot$h^{-1} across the short-circuited stripped chorioallantoic membrane as a function of time. Each ($O \rightarrow I$) hourly flux value represents the mean of six experiments while each ($I \rightarrow O$) value is the mean of eight experiments. Standard errors are represented by vertical bars.

transport to fully account for the observed short-circuit current. Electrically, the net effect of these unidentified transport mechanisms must be in the direction of electron transfer from ectoderm to endoderm (O → I). Experiments were performed to examine the possibility of active K^+ and/or H^+ transport contributing to the short-circuit current of the stripped membrane. The results of the K^+ experiments were consistent with the tendency of isolated tissues to leak intracellular K^+ into the bathing solutions.

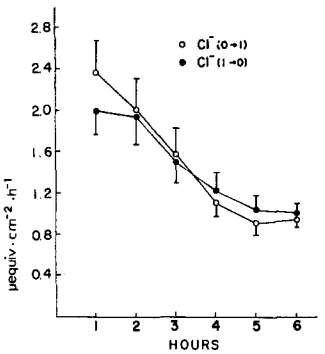

Fig. 5. Unidirectional Cl^- fluxes in μequiv·cm^{-2}·h^{-1} across the short-circuited stripped chorioallantoic membrane as a function of time. Each (O → I) hourly flux value represents the mean of six experiments while each (I → O) value is the mean of eight experiments. Standard errors are represented by vertical bars.

There was no evidence of any net K^+ movement. Similarly, there was no indication of any appreciable net H^+ movement. However, a net H^+ movement on the order of 1 μequiv·cm^{-2}·h^{-1} would be difficult to determine in the presence of approx. 300 μequiv of bicarbonate and phosphate.

DISCUSSION

Previous flux studies on the isolated chick chorioallantoic membrane indicated the existence of an active Ca^{2+} transport from ectoderm to endoderm[2]. The inability of this active Ca^{2+} transport to account for the observed short-circuit current was interpreted as indicating the existence of at least one other actively transported ion.

The experiments reported here indicate that when enriched bathing solutions are employed, enabling a stable transmural PD to be maintained, the normal chorioallantoic membrane actively transports both Na^+ and Cl^- from ectoderm to endoderm. Under these conditions the observed short-circuit current can be entirely accounted for by their algebraic sum. This, of course, requires that the algebraic sum of all other active ion transport processes be zero in order that no net charge transfer results.

When the acellular inner shell membrane is removed from the ectodermal surface, a marked change in the nature of ionic movements results. In particular, under short-circuit conditions, the direction of net Na^+ transport is reversed and net Cl^- transport is abolished. Previously it has been shown that removal of the inner shell membrane reverses the direction of net Ca^{2+} transport as well[2]. The short-circuit current of the stripped membrane is nearly 3 times greater than that of the normal membrane and is not fully accounted for by the observed net Na^+ transport. The

nature of the ion(s) accounting for the remainder of the short-circuit current are as yet undetermined, but the orientation of the required charge transfer is in the direction of electron flow from ectoderm to endoderm. Electrically, this is equivalent to the direction of net Ca^{2+} transport observed when these stripped membranes are bathed with glucose-containing physiological saline. The magnitude of this net Ca^{2+} transport, unless increased due to the present enriched solutions, would not, however, contribute significantly to the short-circuit current observed in the present experiments. As yet we have no measurements of Ca^{2+} fluxes across either the normal or stripped membrane with the enriched bathing solutions. The presence of the serum proteins necessitates a slightly more sophisticated approach in order to obtain accurate specific activities of the transported Ca^{2+}.

The chorioallantoic membrane consists essentially of two epithelia (ectoderm and endoderm) in series separated by a mesodermal layer of connective tissue and blood capillaries. The *in vitro* flux measurements reported here relate to the entire composite membrane of approx. 100 μ thickness. Preliminary electron microscopic evidence suggests that, morphologically, the effect of removing the acellular inner shell membrane appears localized to the outer portion of those ectodermal cells originally in intimate contact with the shell membrane (G. C. MORIARTY, personal communication). Scrutiny of the electrical resistance of the chorioallantoic membrane with and without the inner shell membrane (Tables I and II) suggests that the acellular membrane presents a relatively small barrier to ionic movement. Indeed, direct measurements of the electrical resistance of the inner shell membrane indicates a value which is approximately what one would expect from an equivalent thickness of saline. On the other hand, it is apparent from the flux data that the dynamics of ion transport, specifically Na^+ and Cl^-, are intimately affected by the retention of the acellular inner shell membrane.

The abolishment of net Cl^- transport in the short-circuited stripped chorioallantoic membrane suggests that localization of the active mechanism to the ectodermal cells and perhaps to the outer plasma membrane of such cells. Similarly, we postulate the existence of two active Na^+ transport mechanisms, oriented in opposite directions, operative in the normal membrane. The locus of the active ectoderm to endoderm Na^+ transport would presumably be in the ectodermal epithelia while the endoderm to ectoderm mechanism would probably be localized in the endoderm. The effect on transport of removing the acellular inner shell membrane would then be essentially restricted to those mechanisms in the ectoderm. Preliminary attempts to separate the two epithelia in order to test the above hypothesis directly have to date been unsuccessful. Current microelectrode experiments examining the potential profile of the chorioallantoic membrane will hopefully afford a more precise localization of the transport sites.

At the present time the essential ingredient(s) of the enriched bathing solutions used in these studies is unknown. The ability of the chorioallantoic membrane to maintain a stable PD with these solutions can most probably be attributed to an enhancement of cellular metabolism resulting from the presence of the exogenous metabolites. However, we can not, at this time, rule out the possibility of a more direct relationship between an ingredient of the media and the individual transport processes such as the co-transport of Na^+ with amino acids and sugars found in the small intestine[4,5].

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