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The Interrelationships between Sodium Ion, Calcium Transport and Oxygen Utilization in the Isolated Chick Chorioallantoic Membrane

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Summary. The interrelationships between sodium ion, calcium transport and oxygen utilization have been investigated in the chick chorioallantoic membrane. The oxygen uptakes of the two surface layers of the tissue, the ectoderm and the endoderm, were separated into their basal, Na^+ dependent and Ca^{++} dependent components. The endoderm has a basal rate of respiration of 3.6 μliters $O_2/cm^2/hr$ and a Na^+ dependent component of 1.4 μliters $O_2/cm^2/hr$. The ectoderm has a basal rate of respiration of about 3.5 μliters $O_2/cm^2/hr$, and Na^+ and Ca^{++} dependent components of 1.1 and 3.6 μliters $O_2/cm^2/hr$, respectively. The rate of ectodermal calcium transport and calcium-stimulated oxygen uptake is strictly dependent on the presence of sodium in the bathing medium, and complex kinetics are observed as a function of sodium concentration. On the other hand, in 140 mM Na^+ the rate of calcium transport exhibits simple saturation kinetics as a function of calcium concentration. Ca^{++}/O_2 ratios determined for many different rates of transport give a ratio of about 0.5, a value much lower than similar ratios determined for other transport mechanisms. The calcium transport mechanism in the ectoderm responds to changes in transport rate very sluggishly, taking 30 to 50 min to give a maximum response. The differences between the calcium transport mechanism in this membrane and other known transport systems are discussed and it is suggested that these differences may represent the adaptations necessary for transcellular calcium transport.

The preceding paper described the effect of Ca^{++} on the respiration of the chick chorioallantoic (CA) membrane, an embryonic tissue under study as a model system of active transcellular calcium transport [9]. Ca^{++} markedly stimulated the oxygen uptake of this membrane. Through a combination of techniques, it was determined that the extra oxygen uptake occurred only in the calcium transporting ectodermal layer of cells and only when the transport mechanism was operative. These results suggested that the calcium-stimulated respiration reflected the energy expenditure due to active calcium transport.

During these studies it was noted that ouabain, a specific inhibitor of sodium transport [10], prevented both calcium-stimulated oxygen consumption and membrane calcium uptake. Previous studies with the CA membrane [21, 31], and with intestine [1, 19], have also indicated that either ouabain treatment, or Na^+ withdrawal, effectively inhibit active calcium transport. These results suggested a more detailed examination of the effects of Na^+, *per se*, on the calcium transport mechanism in the CA membrane.

Morphologically, the CA membrane is essentially a bilayer of simple epithelia, each layer performing a different active transport function. The ectoderm is primarily concerned with calcium transport, while the endoderm is responsible for the resorption of Na^+, chloride and water from the allantoic fluid [30, 31]. Using the "stat" apparatus as described previously [8, 9], the effects of both ouabain treatment and sodium withdrawal on the respiration of the ectoderm and the endoderm were examined separately. The data from these experiments, combined with the known effects of Ca^{++} on the oxygen consumption of these cell layers [9], allowed a complete "dissection" of the membrane's respiration into its basic components.

This report presents the results of these investigations as well as more detailed information on the kinetics of calcium transport and its associated oxygen uptake. The calcium transport mechanism is shown to be sodium dependent, and Ca^{++}/O_2 ratios are calculated for many experimentally altered rates of calcium transport. The discussion compares the characteristics of the calcium transport mechanism in the CA membrane with those of other transport systems.

Materials and Methods

A detailed description of the experimental procedures utilized in these studies is given in the preceding paper [9]. CA membrane preparations from 17 to 18 day old embryos were used and their oxygen uptake was measured by standard manometric techniques in a Gilson Model G-20 differential respirometer (Gilson Medical Electronics, Madison, Wisconsin) or in the oxygen "stat" apparatus described previously [8].

Measurements were made in a medium containing 140 mM Na^+, 25 mM Tris, 6.5 mM K^+, 0.5 mM Mg^{++}, 170 mM Cl^-, 0.5 mM SO_4, 1.5 mM PO_4, and adjusted to pH 7.4. $CaCl_2$ was added to this medium at the concentrations described in Results. In Na^+ replacement experiments, the Na^+ removed was replaced by an equimolar amount of choline chloride.

The rates of calcium transport presented in these studies were determined in two ways. In one, the amount of calcium transport was measured as the disappearance of Ca^{45} from the solution bathing the ectodermal layer while the membrane was mounted in a Ussing-type transport chamber. In these experiments, originally performed by Terepka *et al.* [31], the amount of calcium transport from the ectodermal solution was

measured at hourly intervals over a 6-hr period. The published rates of calcium transport determined from these experiments are the arithmetic mean values, expressed as μEquiv/cm²/hr, calculated over the entire experimental period [31]. To perform a meaningful kinetic analysis on these data, the initial rates of calcium transport were determined by plotting the original hourly values of calcium uptake as a function of time, and fitting the best straight line to the points over the first 3 hr of the experiments. Rates of calcium transport were calculated as a function of calcium concentration and membrane age from the slopes of such plots.

The second method for determining the rate of calcium transport in the CA membrane was used for the sodium replacement experiments. This procedure was based on the amount of Ca^{++} accumulated by the membrane during a 1- to 2-hr incubation period. Since calcium transport by this tissue occurs only across the ectodermal cell layer, and since virtually all the Ca^{++} taken up during the first 1 to 2 hr is retained within the membrane [31], this method is a valid measure of the rate of calcium transport. Membrane Ca^{45} uptake was determined in a Dubnoff Metabolic Shaking Incubator (Precision Scientific Co., Chicago, Illinois) in air, at 37 °C and a shaking rate of 150 cpm. One circular outer shell membrane preparation, 2 cm² in area, was placed in a 30-ml beaker containing 4 ml of buffer and approximately 2 μC of Ca^{45}. After 60 to 90 min of incubation the membranes were removed, washed 3 times (5 min each) in 4 ml of calcium-free Tris buffer and the CA membrane was carefully separated from the shell membranes. The CA membranes were then placed in liquid scintillation counting vials with 0.5 ml of NCS® (Amersham/Searle, Des Plaines, Illinois), and solubilized by slowly shaking for 2 hr at 37 °C in the Dubnoff apparatus. Ten ml of scintillating fluid were added and the solutions cooled before counting by standard scintillation techniques.

The rate of calcium uptake is expressed as μmoles/cm²/hr and the rate of oxygen uptake is expressed as μliters O_2/cm²/hr or μmoles O_2/cm²/hr at 37 °C and a barometric pressure of 760 mm Hg. Oxygen uptakes can be converted to standard temperature and pressure by multiplying by 0.88. All data shown are expressed as the mean ± SEM. The number of experiments for each point is shown in parentheses near the data.

Results

Respiratory Components of the CA Membrane

The effect of sodium and calcium withdrawal on the oxygen consumption of the whole CA membrane was measured by manometry, and their individual effects on the ectodermal and endodermal cell layers were determined in the oxygen "stat" apparatus. These results are presented in the first two figures. Fig. 1 shows the marked inhibitory action of ouabain on the total oxygen consumption of the membrane as noted in the preceding report [9]. Compared to the control rate of 13.0 ± 0.2 μliters O_2/cm²/hr, ouabain reduced the oxygen uptake about 50%, to 7.2 ± 0.2 μliters O_2/cm²/hr. In the absence of ouabain, the complete replacement of the NaCl in the medium with choline chloride produced identical results. Note that this inhibition of oxygen uptake occurred in the presence or absence of 1 mM Ca^{++}, and is significantly greater than that due to Ca^{++} removal alone.

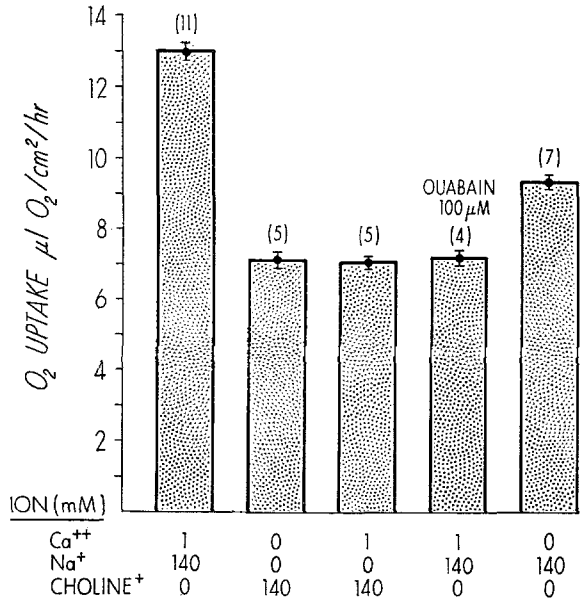

Fig. 1. Comparison of the effects of ouabain treatment and Na^+ withdrawal on the oxygen uptake of the CA membrane as determined by manometry. Na^+ withdrawal experiments were done in the presence and absence of 1 mM Ca^{++}. The abcissa shows the changes in the ionic composition of the medium in mM. The left- and right-hand bars in the figure are 1 and 0 mM Ca^{++} controls, respectively

When similar experiments were performed in the stat apparatus, the results shown in Fig. 2 were obtained. The endodermal respiration could be separated into two components, a "basal" respiration of 3.6 ± 0.1 µliters $O_2/cm^2/hr$, and a sodium dependent respiration of 1.4 ± 0.3 µliters $O_2/cm^2/hr$. The term "basal" respiration refers to oxygen uptake not dependent on Na^+ or Ca^{++}. As shown earlier, the oxygen uptake of the endoderm is not affected by Ca^{++} [9]. On the other hand, ectodermal oxygen consumption has three components, a basal respiration of 3.5 ± 0.1 µliters $O_2/cm^2/hr$, a sodium dependent respiration of 1.1 ± 0.2 µliters $O_2/cm^2/hr$, and a calcium dependent component of 3.6 ± 0.3 µliters $O_2/cm^2/hr$. In the presence of 140 mM Na^+, the effects of 100 µM ouabain on the oxygen uptake of the ectoderm and the endoderm were equivalent to those shown for sodium withdrawal. The two bars on the right in Fig. 2 represent the magnitudes of the basal, sodium and calcium dependent respiration of the whole membrane as measured independently by the stat apparatus and by manometry. The sum of the individual ectodermal and endodermal values for the basal, sodium dependent, and calcium dependent components are 7.1 ± 0.1,

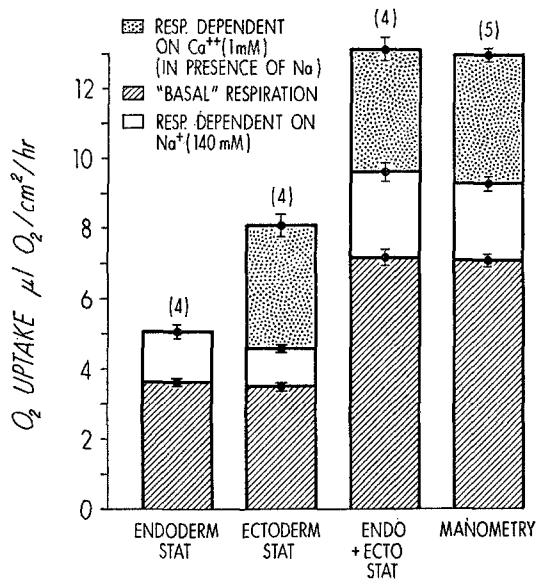

Fig. 2. Summary of the effects of Na^+ and Ca^{++} withdrawal on the oxygen uptake of the CA membrane. The two bars on the left show the effects of Na^+ and Ca^{++} withdrawal on the oxygen uptake of the endoderm and the ectoderm as determined by the stat apparatus. The hatched areas represent basal uptake, the open areas represent sodium-stimulated uptake (in the absence of Ca^{++}) and the dotted areas represent calcium-stimulated uptake (in the presence of Na^+). The Ca^{++} data are taken from the preceding report [9]. The bars on the right compare the sum of the ectodermal and endodermal basal, sodium-, and calcium-dependent respiratory components with the values determined for the whole membrane by manometry

2.4 ± 0.5, and 3.6 ± 0.4 µliters $O_2/cm^2/hr$, respectively, and are in excellent agreement with those determined by manometry.

Sodium Ion, Calcium Transport and Oxygen Consumption

The manometric results presented in Fig. 1 indicate that Ca^{++} is unable to stimulate the oxygen uptake of the CA membrane in the absence of Na^+ or in the presence of ouabain. To further investigate the interrelationship of Na^+ to the energy expenditure involved in active calcium transport, the effect of various sodium concentrations on the oxygen uptake of the tissue was determined in the presence and absence of Ca^{++}. These experiments were performed in the manometric apparatus with the results presented in Fig. 3.

In the absence of Na^+, with or without 1.0 mM Ca^{++} in the bathing medium, the basal oxygen uptake of 7.1 ± 0.1 µliters $O_2/cm^2/hr$ is obtained.

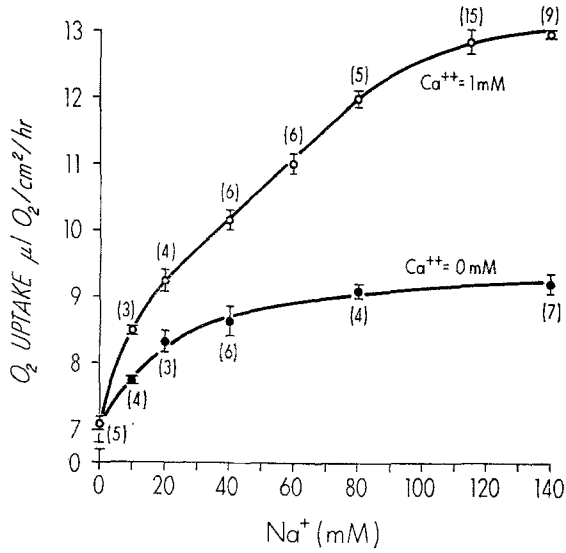

Fig. 3. Effect of Na^+ concentration on the oxygen uptake of the whole membrane in the presence and absence of 1 mM Ca^{++} ion as determined by manometry. Note the broken ordinate. Osmolality of the medium was maintained by replacing sodium chloride with choline chloride

The lower curve shows the effect of increasing sodium concentrations on respiration in the absence of Ca^{++}. Note that oxygen uptake increases with increasing Na^+ and that the effect saturates at sodium concentrations of about 40 mM. On the other hand, in the presence of 1 mM Ca^{++} (upper curve), the respiration of the membrane exhibits more complex behavior. The oxygen uptake of the membrane increases linearly with increasing sodium concentration over the range of 15 to 90 mM, and then plateaus at about 120 mM. A kinetic analysis of these two curves by the method of Lineweaver and Burke [17] indicates that the 0 mM Ca^{++} data fits a straight line on the double reciprocal plot, giving a K_m of 22 mM Na^+ and a V_{max} of 2.7 μliters $O_2/cm^2/hr$. However, the curve obtained in the presence of Ca^{++} does not fit a straight line on such a plot. This line exhibits a definite downward curvature at sodium concentrations above 40 mM.

The direct effect of Na^+ on the rate of calcium-stimulated oxygen uptake can be calculated from Fig. 3 by subtracting the lower curve (0 mM Ca^{++}) from the upper curve (1 mM Ca^{++}). The result of this calculation is shown by the dotted line in Fig. 4. The solid line in Fig. 4 gives the values for membrane calcium uptake in μmoles/cm^2/hr determined as a function of sodium concentration in parallel experiments with Ca^{45} in the Dubnoff apparatus. These experiments were performed at a calcium concentration

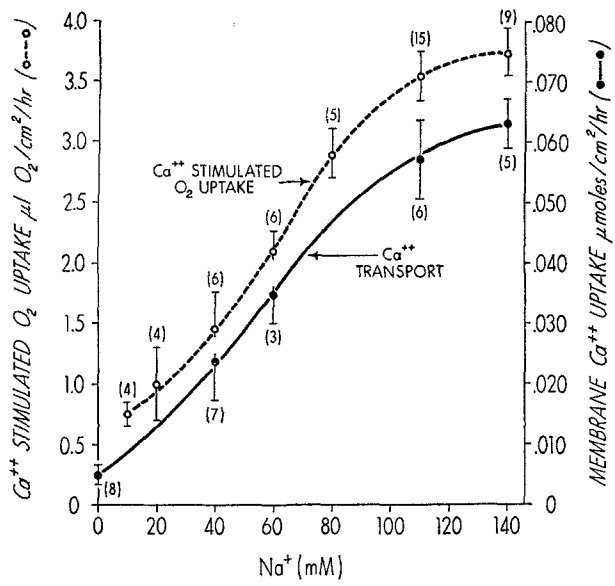

Fig. 4. The effect of Na^+ concentration on the rates of calcium-stimulated oxygen consumption (o— —o) and calcium transport (•——•). The oxygen uptake curve was calculated from Fig. 3 (*see* text) and calcium transport was measured as membrane uptake of Ca^{++} (*see* Materials and Methods)

of 1 mM. It can be seen that the rates of both calcium transport and calcium-stimulated oxygen uptake are dependent on Na^+ and follow a sigmoid relation as a linear function of sodium concentration. It is important to note that the shape of these curves is very different from the rectangular hyperbolas obtained for sodium-dependent transport of sugars or amino acids by intestine and other tissues [28].

The data of Fig. 4 indicate that over a rather wide range of transport rates there is a direct and apparently constant relationship between the rate of calcium transport and the rate of calcium-stimulated oxygen utilization. Since the rate of calcium transport in the CA membrane can also be varied by simply changing the calcium concentration in the bathing medium [31], the relationship between extra oxygen uptake and calcium transport was further investigated as a function of calcium concentration. The effects of various calcium concentrations on the oxygen uptake of the whole membrane, as determined by the manometric apparatus, are plotted in Fig. 5. The figure shows that as the concentration of Ca^{++} is increased, the membrane's rate of oxygen uptake is also increased, and that the effect appears to be maximal at 1 mM Ca^{++}. In these experiments, Na^+ was maintained at 140 mM.

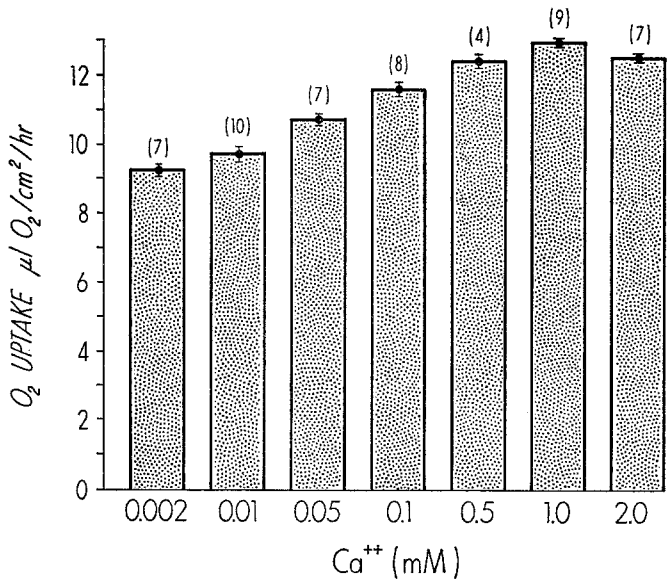

Fig. 5. The effect of increasing the medium Ca^{++} concentration on the oxygen uptake of the whole CA membrane as measured by manometry. The Ca^{++} concentrations shown on the abcissa are those determined in the flask by atomic absorbtion after each experiment

The increments in the calcium-stimulated oxygen uptakes can be calculated as a function of calcium concentration from the data of Fig. 5 and compared directly with calcium transport rates determined at similar calcium concentrations in Ussing transport-chambers [31]. When both parameters are expressed in μmoles/cm^2/hr, the curves shown in Fig. 6*A* are obtained. It can be seen that both transport and extra oxygen uptake exhibit saturation near 1 mM Ca^{++}, but that oxygen uptake rises faster and levels sooner than transport. The Lineweaver-Burke plots [17] of these data are shown in Fig. 6*B* and *C* and indicate that both processes fit simple, carrier-mediated type kinetics [29]. The K_m for calcium transport is 0.28 ± 0.01 mM and the K_m for calcium-stimulated oxygen uptake is somewhat lower at 0.1 ± 0.01 mM. The corresponding V_{max} values are, 0.11 ± 0.003 and 0.16 ± 0.005 μmoles/cm^2/hr, respectively. The two K_m values are statistically different by *t*-test ($p < 0.05$).

The relationship between calcium transport rate and extra oxygen consumption as a function of calcium concentration can be more clearly illustrated by calculating the ratio: μmoles calcium transported/μmoles extra O_2 consumed. Table 1 presents the results of these calculations, as well as Ca^{++}/O_2 ratios calculated for transport rates altered by varying the

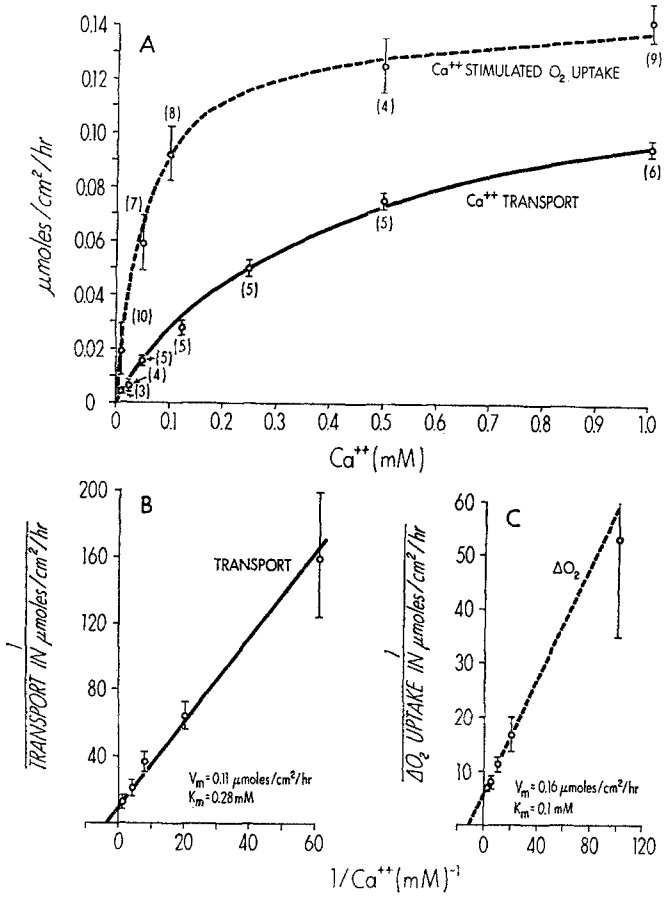

Fig. 6. A kinetic analysis of the rates of calcium transport and calcium-stimulated oxygen uptake. Part *A* shows the rates of calcium and oxygen uptake plotted in μmoles/cm²/hr as a function of Ca^{++} concentration. Rates of transport were determined from the data of Terepka *et al.* [31] (*see* Materials and Methods) and the rates of oxygen uptake were calculated from the increments in O_2 consumption as a function of Ca^{++} concentration presented in Fig. 5. Parts *B* and *C* present double reciprocal plots of the data shown in Part *A*

age of the membrane [31], or the sodium concentration in the bathing medium (Fig. 4). The age and sodium concentration experiments were performed in 1 mM Ca^{++} buffer.

The upper section of Table 1 gives the Ca^{++}/O_2 ratio calculated directly from Fig. 6*A* as a function of calcium concentration. The right-hand column in Table 1 shows that the Ca^{++}/O_2 ratio increases approximately 4 times from 0.16 to 0.67 as the calcium concentration increases from 0.01 mM to 1.0 mM. In the middle section of Table 1, the Ca^{++}/O_2 ratios are calculated

Table 1. Effect of experimentally altered rates of calcium transport on the Ca^{++}/O_2 ratio[a]

Cause of transport rate variation	$[Ca^{++}]$ mM	Calcium transport[b] (μmoles/cm²/hr[d])	ΔO_2[c] (μmoles/cm²/hr[d])	Ca^{++}/O_2 ratio
Changes in $[Ca^{++}]$				
	0.01	3±0.2	19±11	0.16±0.09
	0.05	15±0.7	59±11	0.25±0.05
	0.10	26±1.0	93±12	0.28±0.05
	0.50	75±3.1	125±11	0.60±0.09
	1.0	96±2.2	143±7	0.67±0.05
	2.0	80±6.1	130±7	0.62±0.07
Changes in age				
14 days	1.0	72±9.0	101±3	0.71±0.10
15 days	1.0	75±5.1	101±4	0.74±0.07
16 days	1.0	81±4.2	133±8	0.61±0.06
17 days	1.0	96±2.0	143±7	0.67±0.05
18 days	1.0	90±7.0	137±8	0.66±0.09
Changes in $[Na^+]$				
0 mM	1.0	5±1.4	—	—
40 mM	1.0	24±6.4	56±11	0.43±0.19
60 mM	1.0	35±5.4	82±6	0.43±0.09
115 mM	1.0	57±6.5	137±8	0.42±0.07
140 mM	1.0	63±4.0	143±7	0.44±0.04

[a] Each value represents the mean ± SEM of 3 to 8 experiments.
[b] Transport values for the $[Ca^{++}]$ and age sections were calculated from data of Terepka *et al.* [31] and values for the $[Na^+]$ section were calculated as membrane uptake at 60 min. (*See* Materials and Methods for details.)
[c] ΔO_2 represents the amount of calcium-stimulated O_2 uptake calculated as the difference between the experimental and the calcium-free or sodium-free rate. (*See* Figs. 4 and 5.)
[d] The values presented in these two columns are the experimental values $\times 10^3$.

for membranes aged 14 to 18 days. The transport data were taken from Terepka *et al.* [31] and the oxygen uptake data from the preceding report [9]. Note that as the age of the membrane increases, the rate of transport increases about 35%, but that the Ca^{++}/O_2 ratio remains relatively constant. The average Ca^{++}/O_2 ratio obtained from these calculations, 0.68±0.02, is the same as those calculated for the three highest calcium concentrations shown in the upper section of Table 1.

The lower section of Table 1 shows the effects of various sodium concentrations on the Ca^{++}/O_2 ratio. Note that the Ca^{++}/O_2 ratio does not vary even when the transport rate varies threefold. Although the magnitude of the ratio, about 0.43, agrees well with those presented in the previous

report, 0.45 [9], it is slightly lower than the values shown in the rest of Table 1. This difference can be attributed to the two methods used to estimate calcium transport rates. In the sodium experiments and in the previous report, the rate was determined as the membrane's uptake and retention of Ca^{++} during a 90-min interval in Dubnoff or manometric flasks. On the other hand, the transport rates for the calcium concentration and age experiments were calculated as the disappearance of Ca^{++} from the solution bathing the ectodermal surface over a 3- to 4-hr interval in a transport chamber.

Time Course of Calcium Stimulation of Oxygen Uptake

To gain additional information about the nature of the calcium transport mechanism in the CA membrane, the time course of the calcium-stimulated respiration was examined. These experiments were performed in the stat apparatus with the results shown in Fig. 7. The closed circles represent an experiment begun with a 0 mM Ca^{++} medium bathing a 15-day ectoderm.

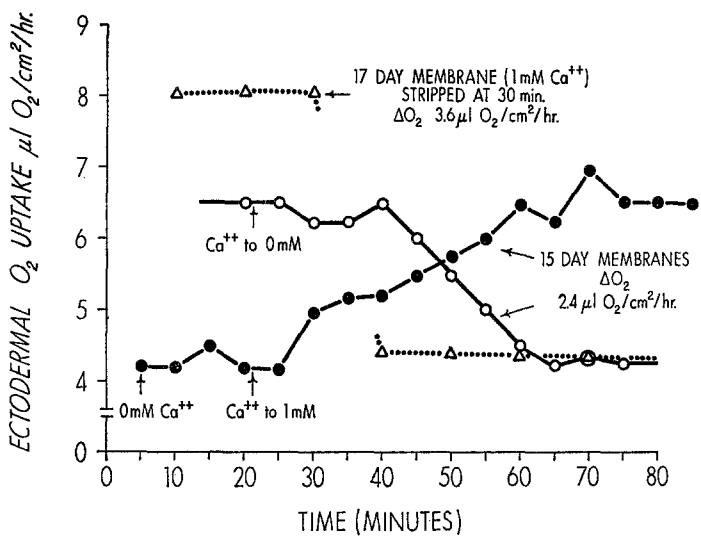

Fig. 7. The time course of the changes in the calcium-stimulated oxygen consumption in the ectodermal layer determined with the stat apparatus. The closed circles (•——•) represent a 15-day membrane initially bathed in 0 mM Ca^{++} and stimulated by the addition of enough calcium to raise the final Ca^{++} concentration to 1 mM. The open circles (○——○) represent a stimulated 15-day membrane that was placed in a calcium-free solution. The open triangles (△ ··· △) represent a stimulated 17-day membrane bathed in 1 mM Ca^{++} that had the inner shell membrane removed from the ectodermal surface (stripped) at 30 min. The differences in the rates of oxygen uptake caused by the alterations in Ca^{++} concentration or by stripping are consistent with those shown previously [9] for these differently aged membranes

At 20 min, the calcium concentration was increased to 1 mM. The calcium-stimulated oxygen uptake responds very slowly, reaching a maximal rate only after 30 to 40 min.

The open circles represent an experiment begun with a calcium-stimulated 15-day ectoderm. At 20 min, the Ca^{++} in the bathing medium was changed to 0 mM. Note that the time required for oxygen uptake to decrease to the 0 mM Ca^{++} rate is also about 30 to 40 min. This gradual change in respiratory rate should be compared with the effects of stripping the inner shell membrane away from the CA membrane, a procedure that abolishes active calcium transport by this tissue [9, 31]. The dotted line represents a 17-day ectoderm run in 1 mM Ca^{++} for 30 min. The chamber was then disassembled and the inner shell membrane was carefully peeled away. The procedure required approximately 10 min, and it can be seen that the first time point obtained (the 40-min point in the figure) shows a rate of respiration equivalent to that obtained in a 0-mM Ca^{++} solution. This suggests that the effect of stripping on calcium-stimulated oxygen consumption is virtually immediate.

Discussion

From Figs. 1 and 2 it can be estimated that almost half of the total respiration of the CA membrane is dependent on the presence of Na^+ and Ca^{++} ions. The remaining "basal" respiration of the tissue is not affected by specific sodium or calcium transport inhibitors such as ouabain (Fig. 1), PCMBS or stripping [9, 31], and is probably not directly related to energy expenditure for active electrolyte transport. The percentage of total respiration involved in the basal functions is 55%, a value similar to that found for: brain, 60% [35]; gallbladder, 55% [20]; toad bladder, 60% [15, 24]; or frog skin, 65% [38].

In considering the interrelationships between sodium, calcium, and oxygen consumption of the CA membrane, the unique morphology of this organ, composed of three distinct tissue layers, must be kept in mind [4]. Ca^{++} is transferred from the exterior egg shell across the ectodermal cells toward the mesoderm. Na^+ is reabsorbed from the internal allantoic fluid across the endodermal cells and into the same space, the mesodermal layer of the membrane. This layer has an elaborate circulatory system so that, *in vivo*, these ions can be readily transferred to the developing embryo. Thus, while active sodium and calcium transport systems are anatomically opposite, they are functionally oriented in the same direction.

The reabsorption of Na^+ from the allantoic fluid, along with chloride and water, has been suggested to involve an endodermal active transport system [23, 30]. The unidirectional Na^+ flux across this cell layer is about 0.4 to 0.6 μmoles/cm²/hr according to the data of Moriarty and Hogben [22]. The data presented in this paper (Fig. 2, first bar) indicate that about 1.4 μliters O_2/cm²/hr of the endodermal respiration is dependent on Na^+ or inhibited by 100 μM ouabain. If one makes the assumption that it is valid to compare these two, separately determined values, and that all of the sodium-dependent respiration in the endoderm is due to transcellular sodium transport, a tentative Na^+/O_2 ratio can be calculated for the CA membrane. The value obtained (8 – 11) is in reasonable agreement with the Na^+/O_2 ratios of 15 to 18 calculated for toad bladder and frog skin following similar assumptions [15, 16, 24]. Support for the suggestion that the sodium-stimulated respiration in the CA endoderm is caused by active sodium transport is derived from the similarity of the K_m value for sodium-stimulated oxygen uptake of the membrane and the K_m for sodium transport in other known sodium transport systems. The K_m value obtained for sodium-stimulated respiration from the lower curve of Fig. 3, 22 mM, compares well with the K_m values found for sodium transport in red blood cells, 18 mM [12, 25], toad bladder, 20 mM [6], or the isolated enzyme, Na^+, K^+-activated ATPase, 24 mM [11].

In the preceeding paper it was suggested that the rate of calcium-dependent oxygen consumption of the CA membrane was proportional to the rate of ectodermal active calcium transport [9], and the data of Figs. 4 and 6 confirm and extend this conclusion. A similar conclusion has been reached concerning the connection between transport and metabolism in many other tissues [2, 15, 26, 35, 38]. Fig. 4 also demonstrates that active calcium transport by the CA ectoderm is a sodium-dependent process, and that the dependency follows a sigmoid shape as a function of medium sodium concentration. The rate of calcium uptake found in sodium-free solutions, 0.005 ± 0.001 μmoles/cm²/hr, is virtually the same as the rates obtained with inhibitors such as PCMBS, ouabain or oligomycin [9], indicating that the sodium dependency is very strict. In the stat apparatus, 100 μM ouabain, added to the ectodermal bathing solution containing 140 mM Na^+, completely inhibited calcium-stimulated respiration. This suggests that a sodium-calcium interaction of some type occurs in or on the ectodermal cell layer of the membrane.

Active accumulation of sugars and amino acids by intestine is also strictly dependent on Na^+, and two convincing models have been proposed to explain these observations, i.e., the co-transport model favored by Crane [5]

and the direct energy input model recently suggested by Kimmich [14]. Although these two models differ fundamentally over the site and mechanism of the energy input for active metabolite transport, both models predict that a quantitatively similar inhibition of metabolite accumulation should occur with ouabain treatment or sodium withdrawal. In addition, the kinetics of sodium-dependent sugar or amino acid uptake derived from both models are identical. Experimental data show the familiar carrier type of saturation curve as a linear function of sodium concentration, and exhibit straight lines on Lineweaver-Burke plots [5, 14, 28].

The active calcium transport mechanism in the CA membrane has features in common with both models for sodium-dependent transport. Both transport and extra oxygen uptake by the ectoderm are inhibited to the same extent by sodium withdrawal or by ouabain treatment. However, the kinetics of the sodium dependency of the calcium transport process are very different than those inherent in the results or models as presented by Crane or Kimmich [5, 14, 28]. The curves of Fig. 4 show a sigmoid, rather than a hyperbolic shape as a function of sodium concentration and do not fit a straight line on a double reciprocal plot. Similar sigmoid-shaped curves have recently been described for the active transport of K^+ in red blood cells as a function of the external potassium concentration [7, 27], and enzyme models exhibiting similar kinetics have been compiled and discussed by Webb [34]. Unfortunately, the information currently available on the calcium transport system in the CA membrane is too limited to allow a critical assessment of these complex models or to suggest a precise mechanism for the sodium-calcium interactions observed. Clear hypotheses have been presented concerning the exchange of Na^+ and Ca^{++} between the cytoplasm and the extracellular fluids in squid axon and cardiac muscle [36]. However, the types of sodium-calcium exchange carrier systems proposed for the cell membranes of nerve and muscle have not yet been demonstrated to be directly applicable to active transcellular calcium transport by epithelial tissues.

When Na^+ is not limiting, both calcium transport and extra oxygen uptake are saturable processes, reaching a maximal rate at a calcium concentration of about 1 mM. A kinetic analysis of these data by the method of Lineweaver and Burke [17] shows that both processes fit a straight line on the double reciprocal plot (Fig. 6). This observation indicates that in 140 mM Na^+, the ectodermal calcium transport mechanism is compatible with the classic kinetic model for membrane transport. This model features: a reversible combination of Ca^{++} with a mobile carrier on the outside of the membrane; transfer of the calcium-carrier complex across the membrane;

and release of Ca^{++} on the inner surface of the membrane [29]. The K_m observed for the CA membrane transport process, 0.28 mM is about 10 times less than the K_m of 1 to 2 mM found for calcium transport in rat intestine [18, 33], and about 100 times lower than the K_m of 24 mM found for calcium transport in chick intestine [1]. On the other hand, the calcium transport system of mitochondria and muscle sarcoplasmic reticulum vesicles have much lower K_m's than the CA membrane, about 45 μM and 3 μM, respectively [3, 37].

Fig. 6 also shows that the K_m for calcium-stimulated oxygen uptake is 0.1 mM, or about three times lower than the K_m for calcium transport. As noted under Results, this value is statistically different from the calcium transport K_m value. However, it must be emphasized that these K_m calculations are based upon data derived from different experiments so that the differences observed could be related to differences in experimental technique. On the other hand, the same result is indicated by the changing Ca^{++}/O_2 ratios shown in Table 1 as a function of increasing calcium concentration. In other systems, such as *E. coli* [13], mitochondria [3], and frog skin [32, 38], the stoichiometry between transport and metabolism does not seem to vary with changing substrate concentration. Also, in the CA membrane Ca^{++}/O_2 stoichiometry does not vary when age or sodium concentration is altered (Table 1). Thus it appears that the changing stoichiometry observed as a function of calcium concentration deserves more thorough investigation. If the changing Ca^{++}/O_2 ratio withstands additional study, it may be that Ca^{++} is activating two oxygen-consuming processes in the ectodermal layer, transport and some other function with a lower affinity for Ca^{++}. Based purely on morphological evidence, it has been suggested that certain cells in the CA ectoderm may be involved in acid and/or carbonic anhydrase secretion [4].

The data presented in Table 1 indicate that whether the Ca^{++}/O_2 ratio is calculated from separate experiments, such as those shown in the upper two sections, or in parallel experiments such as the sodium experiments in the lower section, the Ca^{++}/O_2 ratio obtained is about 0.5. This value is much lower than similar values calculated for other active transport systems. For example, the Ca^{++}/O_2 ratio obtained with isolated mitochondria is 12 [3], the Na^+/O_2 ratio calculated above for the CA membrane is $8-11$, the Na^+/O_2 ratio for toad bladder or frog skin is about $15-18$ [15, 16, 24], and the ratio for sugar accumulation in bacteria is 6 [13]. If Ca^{++} is subsequently found to activate a second energy-dependent function in the ectoderm of CA membrane, the Ca^{++}/O_2 ratio presented will have to be revised. However, even if one-half of the calcium-stimulated oxygen uptake is due

to a process other than transport, the Ca^{++}/O_2 ratio will only increase to 1, a value still much lower than those measured for other transport systems.

Another unique aspect of the calcium transport mechanism in this membrane is its slow response to agents that alter the transport rate. Fig. 7 shows that when the ectodermal transport mechanism is stimulated by the addition of Ca^{++} to a low Ca^{++} medium, the calcium-stimulated oxygen uptake develops slowly, taking 30 to 40 min to reach a maximum rate. Transport studies with chambers indicate a similar slow uptake of Ca^{++} from the solution bathing the ectodermal surface [31]. Interestingly, the transport system also takes about 30 to 40 min to respond to the removal of Ca^{++} from the medium. That this slow response is actually due to transport, and not some other effect, is supported by the experiment with the stripped membrane which shows an immediate drop to the low Ca^{++} rate when the transport mechanism is inhibited by stripping [9, 31]. In striking contrast, mitochondria can transport 100 to 300 μmoles of Ca^{++} within 50 sec [3] and toad bladder can respond to the vasopressin-induced stimulation of sodium transport within 3 to 8 min [6].

From this discussion, it is apparent that there are two major dissimilarities between the calcium transport mechanism in the CA ectoderm and other active transport systems. The transport process in this tissue is not only inefficient but also sluggish when compared with the transport mechanism for Ca^{++} in mitochondria or with the mechanisms for monovalent ions in epithelial tissues. It is possible that these particular properties of the CA membrane reflect the adaptations necessary to enable the transcellular transport of large quantities of Ca^{++} without disturbing the well-regulated concentration of intracellular ionic calcium.

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