J. Membrane Biol. 7, 128–145 (1972)

Calcium-Stimulated Respiration and Active Calcium Transport in the Isolated Chick Chorioallantoic Membrane

James C. Garrison and A. Raymond Terepka

Department of Radiation Biology and Biophysics,
University of Rochester, School of Medicine and Dentistry,
Rochester, New York 14642

Received 2 April 1971

Summary. Calcium markedly stimulates the respiration of the isolated chick chorioallantoic membrane. This stimulation of oxygen uptake appears to be closely associated with the membrane's active transcellular calcium transport mechanism. In the presence of 1 mM Ca^{++} the rate of uptake increases from 9.3 ± 0.15 to 13.0 ± 0.2 μliters $O_2/cm^2/hr$, an increase of about 40%. The calcium-stimulated respiration is specific for the ectodermal layer of cells, the known location of the calcium transport mechanism, and only occurs when the calcium transport mechanism is operative. Sr^{++} and Mn^{++} are transported by the tissue at a lower rate than Ca^{++} and cause a smaller stimulation of oxygen consumption. Mg^{++} and La^{3+} have no effect on tissue respiration. In the presence of Ca^{++}, the organic mercurial *p*-chloromercuribenzene sulfonate (PCMBS) inhibits calcium transport and specifically decreases the oxygen uptake of the ectoderm to a rate identical to that obtained in a calcium-free medium. Stripping the inner shell membrane away from the chorioallantoic membrane mimics these effects. The specificity and locus of action of these two inhibitors suggest that a vital component of the active transcellular calcium transport mechanism resides on or near the outer surface of the plasma membrane of the ectodermal cells and that sulfhydryl groups are important to the normal function of this component.

Since excess Ca^{++} is known to adversely affect normal cellular function, it is generally accepted that the concentration of ionic Ca^{++} within the cytoplasm is closely regulated. Ca^{++} ion can inhibit such important enzymes as Na^+, K^+-activated ATPase [10], phosphofructo-kinase [10], pyruvate kinase [3] and pyruvate carboxylase [19]. Ca^{++} can also uncouple mitochondrial energy conservation processes [4]. Consequently, it is not surprising that many, if not all, cells maintain an intracellular Ca^{++} concentration of 10^{-5} to 10^{-8} M by the active extrusion of Ca^{++} [2, 32].

The cells of certain tissues such as intestine, bone, kidney, mammary gland and avian shell gland are specialized for transcellular calcium transport,

and must transfer large quantities of Ca^{++} during the transport process [15, 18, 33, 40]. Since no evidence currently exists to indicate that these cells can tolerate high levels of ionic Ca^{++}, the transfer of Ca^{++} by these tissues is presumably a strictly controlled process, accomplished without significant change in the intracellular ionic Ca^{++} concentration. Although the effects of various vitamins and hormones on the transfer of Ca^{++} across these tissues has been extensively studied, little information is available concerning the transport process itself. It is not known whether transcellular calcium transport occurs via an adaptation of the cells' calcium extrusion mechanism or via a separate, specialized process.

One model system recently described for studying transcellular calcium transport is the chorioallantoic (CA) membrane of the embryonic chick [36]. The CA membrane is an outgrowth of the developing embryo which lines the inside of the shell by the 8th day of incubation. It is composed of three distinct cell layers, an ectoderm, a mesoderm, and an endoderm [34]. The ectoderm is at most two cells thick and lines the fibrous, acellular egg-shell membranes. The mesoderm contains only scattered fibroblasts, collagen fibers and blood vessels, and the innermost layer, the endoderm, contains one cell layer and faces the allantoic fluid. The CA membrane is a convenient tissue for investigating transcellular calcium transport because of its availability, simple morphology, and adaptability to *in vitro* experimentation.

Previous studies with this membrane have shown that the ectodermal layer of cells actively transfers Ca^{++}, liberated from the shell for embryonic bone formation, into the chick's circulatory system [36]. Since the transport process is known to be markedly dependent on oxidative phosphorylation, the energetics of the system have been probed by monitoring the oxygen uptake of the membrane as a function of the Ca^{++} concentration in the bathing medium. This communication presents the effect of Ca^{++}, other divalent ions, and inhibitors of calcium transport on the oxygen uptake of the CA membrane, and discusses the possible location of the transport mechanism within the transporting cells.

Materials and Methods

Measurement of Oxygen Consumption

Two methods were utilized to monitor oxygen consumption. Total tissue oxygen uptake was measured by standard manometric techniques in a Gilson Model G-20 differential respirometer (Gilson Medical Electronics, Madison, Wisconsin). All measurements were made in air, at 37 °C, at ambient barometric pressure, with 4 ml of medium in the experimental flasks, and at a shaking rate of 120 cpm. After a 20-min equilibration period, O_2 uptake in μliters was measured at 15-min intervals for 90 to 120 min. The

data were plotted and the rate of oxygen consumption was determined from the slope of a straight line visually fitted to the points. To be certain that the oxygen uptake of the CA membrane was not limited by the diffusion of oxygen into the cells, some experiments were performed with 100% O_2 as the gas phase. The oxygen uptake of the tissue in these experiments was unchanged.

Oxygen consumption of the ectodermal and endodermal cell layers was determined in a specially designed apparatus that has been described elsewhere [11]. In this apparatus the CA membrane was mounted in a Ussing-type chamber that had an O_2 electrode fitted to one-half of the chamber. An electronic circuit automatically maintained a constant pO_2 in the chamber by infusing 100% oxygen-saturated buffer at a rate which matched the oxygen consumption of the membrane surface facing the O_2 electrode. The actual rates of oxygen uptake were calculated from the amount of oxygen-saturated buffer required to maintain a constant pO_2 in the chamber. The pO_2 of the medium in the other half of the chamber was maintained by bubbling with air. Since this device maintains a constant pO_2 on both sides of the membrane, it is referred to as the "stat" apparatus. Previous studies with this apparatus indicated that it measures only the oxygen uptake due to the layer of cells facing the electrode [11], a suggestion further confirmed by the data in Results. These data show that the sum of the ectodermal and endodermal oxygen uptakes always equaled the total uptake as measured by manometry, and that the respiration measured for one layer was not affected by large variations in the oxygen uptake of the other layer. As discussed earlier [11], these findings may be due to the unique morphology of the CA membrane, but the recent experiments of Nellans and Finn [30] with toad bladder indicate that this experimental approach may prove useful with other tissues. Two minor refinements were added to the original operating procedures. The amount of oxygen-saturated buffer solution delivered by the stepping syringe driver was calibrated on every run by attaching a piece of capillary tubing to the syringe with a three-way stopcock. This allowed calibration of the amount of buffer delivered per step at any time during a run. In addition, temperature and barometric pressure correction were applied for the solubility of O_2 in the medium for each run. The α values (solubility of O_2 in buffer expressed in μliters O_2/ml) used were those determined by Chappell [5]. All stat measurements were at 37 °C and the rates of uptake of both the ectoderm and the endoderm were measured on the same membrane by simply reversing the membrane in the chamber during the run. The reversal procedure did not, in itself, affect the rate of respiration [11].

Preparation of Membranes

Fertile eggs, obtained from Babcock Poultry Farms, Ithaca, N.Y., were incubated for 12 to 19 days at 37 °C, 80% relative humidity, and turned automatically every 2 hr by a Jamesway Model-252 Incubator (Butler Manufacturing Co., Ft. Atkinson, Wisconsin). Eggs of approximately the desired age were selected at random for study, and after the membranes were prepared, the exact embryonic age was determined by the staging techniques of Hamilton [12].

Membranes were obtained by placing the egg with the pointed end down in a 30-ml beaker and cracking away the upper half of the shell with the blunt end of a forceps. Adhering pieces of shell were removed and the outer shell membrane was cut away from the air space, exposing the inner shell membrane and the underlying CA membrane. Three different CA membrane preparations were used: the CA membrane with both the inner and outer shell membranes attached (OSM preparation); the CA membrane with only the inner shell membrane attached (ISM preparation); and the CA membrane after both shell membranes had been removed (stripped preparation).

The OSM preparations are easily obtained from 16 to 19-day eggs and give consistent, linear rates of oxygen uptake for 2 or more hr. A large piece of tissue was cut from the side of the egg, washed 3 times in calcium-free buffer, and opened out on a petri dish containing a layer of dental wax. For manometric experiments, 12 to 15 circular pieces, 2 cm^2 in area, were cut with a device constructed from a brass tube notched to accept the flexible blade from a Gillette Techmatic® razor. The blade was held in place with a rubber band and changed daily. The pieces were washed 3 more times in calcium-free buffer before use. For the stat apparatus, one large piece, 7 cm^2 in area was mounted in the chamber.

ISM preparations were obtained by cracking away the shell and spirally peeling the outer shell membrane down and away from the airspace (counter clockwise). Care is necessary during this procedure to prevent separation of the inner shell membrane from the underlying CA membrane. Enough ISM preparation for 8 to 10 circular pieces, 2 cm^2 in area, could be obtained with this method. Experiments run with ISM preparations in the stat apparatus were performed with fine gauze backing the tissue to give added support. When "stripped" membranes were desired, the inner shell membrane was carefully peeled away from the ISM preparations with fine forceps.

In stat experiments, there was no difference in the control results obtained with the ISM or OSM preparations. However, it was found that OSM preparations gave more consistent results in manometric experiments. Unfortunately, the outer shell membrane contains remnants of the egg shell mammillae in the form of large crystals of $CaCO_3$ [35]. Therefore, in order to maintain a low concentration of calcium, experiments performed in calcium-free media necessitated the use of the ISM preparations.

The ISM preparation is not ideally suited to the *in vitro* measurement of oxygen uptake in the presence of 1 mM calcium in the manometric apparatus. It was found that the control calcium-stimulated rate of oxygen uptake could not be maintained for as long a period of time with the ISM preparation as with the OSM preparation. In some experiments, the rate of oxygen uptake of the ISM preparation in 1 mM calcium would only match the rate found with the OSM preparation for 45 to 60 min. After the first 45 min, the rate would often decline to a rate between the control and the calcium-free rate. When the membranes that showed this decline in respiration were examined, they were found to be rolled up, folded over, and/or partially stripped.

Since this problem did not occur when oxygen uptake was measured with the ISM preparation in the stat apparatus, the vigorous agitation necessary to manometric measurements of oxygen uptake was apparently causing damage to the junction between the CA membrane and the inner shell membrane, a junction vital to the maintenance of active calcium transport [29, 36]. The fragility of this junction was a special problem in membranes aged 13 to 15 days, which were often completely stripped before the end of the experimental period. When we desired to measure the stimulation due to Ca^{++} or another divalent ion with the ISM preparation, the results were selected from those which showed linear oxygen uptakes with time for the first 45 to 60 min of the experiment. In these instances, the rates of oxygen consumption were calculated as the slope of the line drawn through the first 45 min of the experimental period. The rates of oxygen uptake determined in this way with the ISM preparation in 1 mM Ca^{++} were not significantly different from the results obtained with the OSM preparation in 1 mM Ca^{++}.

Solutions

All 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, and 1.5 mM PO_4 with or without 1.0 mM $CaCl_2$, and adjusted to pH 7.4. All salts were of reagent grade. The organic

chemicals utilized in these studies were obtained from Sigma Chemical Co., St. Louis, Missouri, and were added to the above medium at concentrations described in Results.

Although OSM preparations contain approximately 1 mg Ca^{++} per cm^2 in the form of attached calcite crystals [35], the Ca^{++} concentration of control 1 mM Ca^{++} buffer solutions is not materially affected by these preparations. Analyses done after experiments with OSM preparations showed that, at most, they raised the concentration of the medium to 1.1 mM. OSM preparations did, however, raise the Ca^{++} content of the calcium-free buffer to 0.5 mM by the end of the experimental period. ISM preparations, even when washed 6 times before use, also increased the Ca^{++} content of the calcium-free media, but only to 2×10^{-6} M. For these reasons, all experiments requiring a low Ca^{++} concentration were performed with the ISM preparation.

Analyses

Total Ca^{++} was measured on an IL® Model 153 atomic absorption spectrophotometer (Instrumentation Laboratory Inc., Lexington, Mass.). Appropriate standards were run before and after the samples. When the Ca^{++} content of membranes was measured, the tissues were ashed overnight at 600 °C in platinum dishes before analysis. Ca^{++} uptake by the tissue was measured with Ca^{45} as a tracer. Tissues containing Ca^{45} were solubilized in 1 ml of NCS® (Amersham/Searle, Des Plaines, Illinois) at 37 °C for 2 hr and then counted by liquid scintillation techniques. Dry weight was measured on a Cahn Model G Electrobalance (Cahn Instrument Co., Paramount, Calif.) and protein was estimated by the biuret method.

Expression of Results

Oxygen consumption is expressed as μliters $O_2/cm^2/hr$ at 37 °C and a barometric pressure of 760 mm Hg. Uptakes can be converted to standard temperature and pressure by multiplying by 0.88. The results are expressed on an area basis because precisely measured pieces of membrane are simply obtained, give the most consistent results and allow comparison of oxygen uptake rates with calcium transport rates previously expressed on an area basis. The amount of protein and dry weight in mg/cm^2 was determined and is presented in the results in order to facilitate comparison of the respiration of this tissue with others. Averaged results are expressed as the mean $\pm$ SEM, and the number of experiments is shown in parentheses near the data.

Results

Control Experiments

At 17 to 18 days of incubation, the CA membrane is transporting Ca^{++} at a maximal rate. The oxygen uptake of such membranes in 1 mM Ca^{++} is shown in Fig. 1. The oxygen consumption of the whole tissue (dotted bar) was 13.0 ± 0.2 μliters $O_2/cm^2/hr$ in 11 experiments. The dry weight of 17-day membranes (CA membrane only, not including the shell membranes) was 0.825 ± 0.016 mg/cm^2 and protein was 0.79 ± 0.03 mg protein/cm^2. The oxygen uptake of the tissue expressed on a dry weight or protein basis is

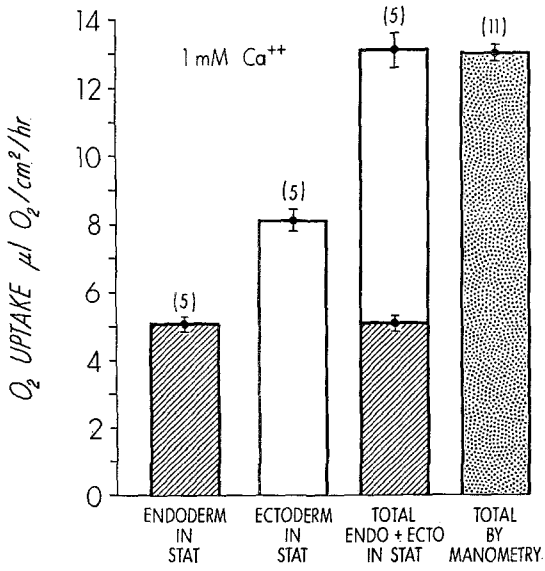

Fig. 1. Oxygen uptake of the CA membrane in 1 mM Ca^{++} ion. Oxygen consumption of the endoderm (hatched bar) and the ectoderm (open bar) was determined in stat apparatus (*see* Methods). The right-hand bars compare the sum of the oxygen uptake of the endoderm and the ectoderm (hatched and open bar) with the oxygen uptake of the whole membrane as measured independently by manometry (dotted bar)

about 16 µliters O_2/mg/hr, a value higher than that found for other epithelial tissues such as frog skin, 1.2 µliter O_2/mg dry wt/hr [23], toad bladder, 1.3 µliter O_2/mg dry wt/hr [22], or gallbladder, 11 µliter O_2/mg dry wt/hr [27].

Since the CA membrane contains no muscle layers and little connective tissue [34], a higher oxygen consumption on a dry weight basis might be expected. However, even when expressed on an area basis, the oxygen uptake of frog skin is about 7 µliters $O_2/cm^2/hr$ [23], still less than half that of the CA membrane.

Fig. 1 also shows the results of using the stat apparatus to monitor the oxygen consumption of the ectoderm and the endoderm of 17–18-day membranes in 1 mM Ca^{++}. The oxygen uptake of the endoderm (hatched bar) was 5.05 ± 0.2 µliters $O_2/cm^2/hr$, while the uptake of the ectoderm (open bar) was 60% higher at 8.07 ± 0.3 µliters $O_2/cm^2/hr$. The sum of the oxygen uptake of the ectoderm and the endoderm shows that the total value, 13.1 ± 0.5 µliters $O_2/cm^2/hr$, compares very well with the value determined for the whole CA membrane in the manometric apparatus, 13.0 ± 0.2 µliters $O_2/cm^2/hr$. The excellent agreement between the two

methods provides confidence in the usefulness of the stat apparatus as a tool for probing the functions of the individual cell layers of the CA membrane.

Effect of Ca^{++} on the Oxygen Consumption of the CA Membrane

Since one of the main functions of the CA membrane is an energy dependent calcium transport process, the oxygen uptake of ISM preparations was determined in the presence and absence of Ca^{++}. Fig. 2 presents the results of these experiments which were performed in the stat apparatus. The oxygen uptake of the endodermal layer of cells was about 5.0 µliters $O_2/cm^2/hr$ and was not affected by changing the Ca^{++} concentration from 0 to 1 mM. In striking contrast, the oxygen consumption of the ectoderm rises from 4.6 ± 0.1 µliters $O_2/cm^2/hr$ in calcium-free medium to 8.1 ± 0.5 µliters $O_2/cm^2/hr$ in 1 mM Ca^{++}, an increase of about 75%.

When the oxygen uptake of the entire membrane is measured in a calcium-free medium, the oxygen consumption is 9.3 ± 0.15 µliters $O_2/cm^2/hr$. Comparing this value with the control rate from Fig. 1 (13.0 µliters $O_2/cm^2/hr$), shows that Ca^{++} stimulates the respiration of the whole tissue about 3.7 µliters $O_2/cm^2/hr$. The data from the stat apparatus indicate that the entire effect is due to the ectodermal layer of cells, and that its magnitude, about 3.6 µliters $O_2/cm^2/hr$, is the same by both methods. The hatched and open bar shows that in 0 mM Ca^{++}, as in 1 mM Ca^{++}, the sum of the oxygen

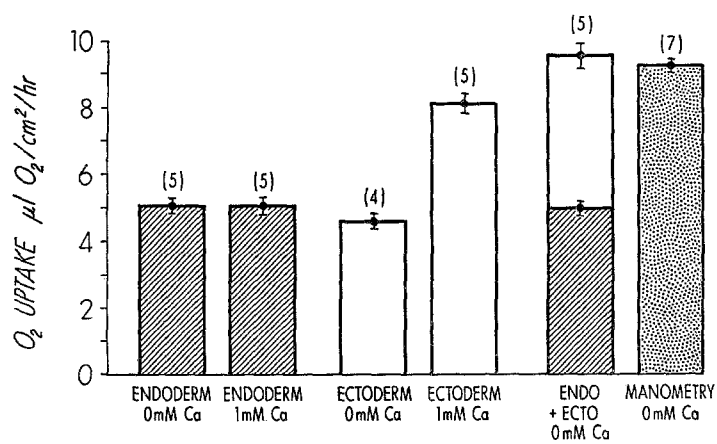

Fig. 2. Oxygen uptake of the endoderm (hatched bars) and the ectoderm (open bars) in the presence and absence of 1 mM Ca^{++} ion. Experiments performed in stat apparatus. The two bars at the right compare the sum of the oxygen uptake of the ectoderm and the endoderm in 0 mM Ca^{++} (hatched and open bar) with that measured for the whole membrane in 0 mM Ca^{++} by manometry (dotted bar)

consumption of the ectoderm and the endoderm measured by the stat apparatus is not significantly different from that for the whole tissue as measured by manometry. Since the calcium transport mechanism is known to be saturated at 1 mM Ca^{++} ion [36], some experiments examined the rate of respiration of the tissue in 2 mM Ca^{++}. The oxygen uptake of the whole membrane in 2 mM Ca^{++} was 12.6 ± 0.2 µliters $O_2/cm^2/hr$, (N = 7), indicating that the Ca^{++} stimulation of oxygen uptake is also maximal at 1 mM Ca^{++}.

Effects of Age

Previous studies have shown that the calcium transport process in the CA membrane is dependent on the age of the embryo, becoming operative on day 14 and increasing in rate from day 14 to day 17 [36]. The effect of 0 and 1 mM Ca^{++} on the oxygen consumption of membranes aged 12 through 18 days is shown in Fig. 3. Note that Ca^{++} has no effect on the rate of respiration of membranes 12 to 13 days old, but stimulates the oxygen uptake of membranes 14 through 18 days old. In addition, the magnitude of the stimulation increases from 2.6 µliters $O_2/cm^2/hr$ at 14 days to 3.7 µliters $O_2/cm^2/hr$ at 17 days. These results closely parallel the known effects of age on the transport mechanism.

The dry weight and Ca^{++} content of the tissue (CA membrane only) were also measured as a function of age with the results shown in Table 1. Although the dry weight of the tissue doubles over the period 14 to 18 days, the oxygen uptake in calcium-free medium increases only slightly. This observation correlates well with morphological evidence showing an increase in connective fibers with age, but little change in the number or type

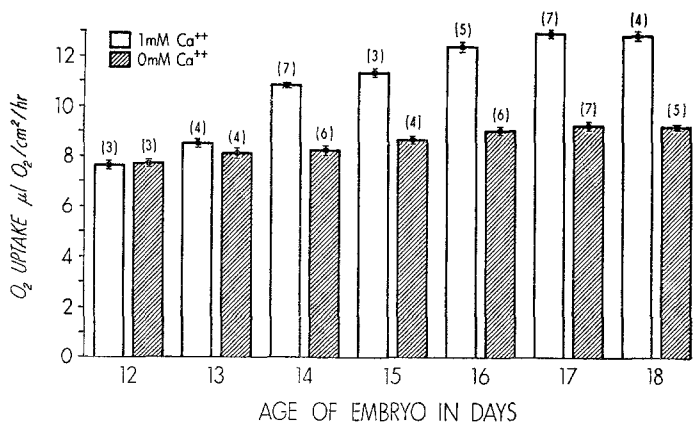

Fig. 3. The effect of embryonic age on the oxygen consumption of the CA membrane in the presence (open bars) and absence (hatched bars) of 1 mM Ca^{++}. Experiments performed by manometry

Table 1. Effect of age on dry weight and calcium content of the CA membrane[a]

	Age of embryo (days)				
	14	15	16	17	18
Dry wt, mg/cm^2	0.620±0.040	0.618±0.022	0.865±0.086	0.825±0.016	1.240±0.085
Ca^{++}, μmoles/cm^2	0.025±0.002	0.021±0.004	0.020±0.003	0.019±0.002	0.024±0.004

[a] All values are the mean ± SEM of 6 determinations.

of cells in the ectoderm or the endoderm [8, 34]. The Ca^{++} content of the tissue does not change with age or transport ability, in agreement with previous results [36].

Effect of Other Ions

The effects of other divalent ions on the oxygen uptake of the membrane was studied using Sr^{++}, Mg^{++} and Mn^{++}. In addition, the effect of La^{+++}, a potent inhibitor of divalent ion transport in isolated mitochondria [28], was tested. The results, presented in Fig. 4, show that Mg^{++}, at concentrations between 0 and 1 mM, had no effect on oxygen consumption in the presence or absence of Ca^{++}. Similarly, La^{+++}, at a concentration of

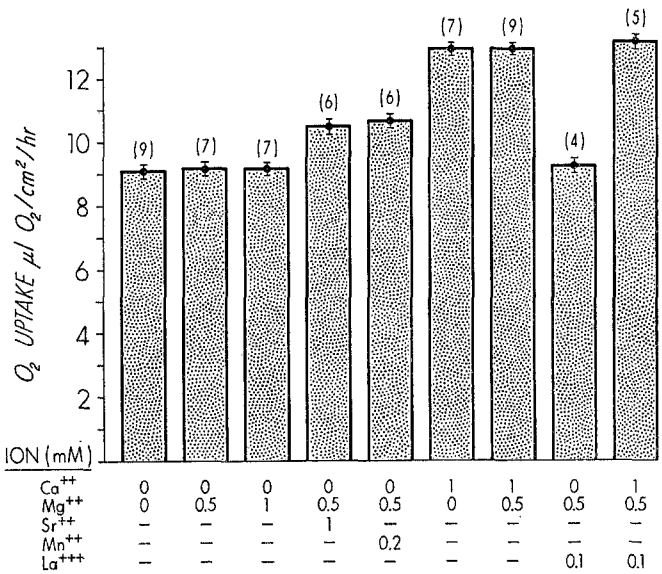

Fig. 4. The effect of various ions on the oxygen uptake of mature (17–18 day) CA membranes as determined by manometry. The concentrations of the ions in mM are shown along on the abcissa. A concentration listed as 0 mM indicates that the ion in question was withdrawn from the control medium which normally contained 1 mM Ca^{++} and 0.5 mM Mg^{++}. A dash (–) indicates that the ion was not normally present in the medium. The third bar from the right is the 1 mM Ca^{++} control and the second bar from the left is the 0 mM Ca^{++} control

0.1 mM, was without effect in the presence or absence of Ca^{++}. On the other hand, Ca^{++}, Sr^{++}, and Mn^{++}, increased oxygen consumption by 3.7, 1.3 and 1.5 μliters $O_2/cm^2/hr$, respectively. Interestingly, Sr^{++} and Mn^{++}, which are actively transported by the membrane, but at a lower rate than Ca^{++}, elicit a smaller stimulation of oxygen uptake.

Effects of Transport Inhibitors

The agents 2,4-dinitrophenol (DNP), ouabain, oligomycin, and *p*-chloromercuribenzene sulfonate (PCMBS), are known inhibitors of active calcium transport in the CA membrane [36]. The oxygen uptake of the whole membrane was measured in the presence of these inhibitors and 1 mM Ca^{++}. DNP, oligomycin and ouabain were present at the concentrations indicated over the entire experimental period. Membranes were pre-treated in 200 μM PCMBS for 15 min, washed, and their oxygen uptake measured in medium free of PCMBS. Since mechanically removing the acellular innershell membrane from the ectodermal layer of cells has been shown to abolish active calcium transport [29, 36], the oxygen uptake of such "stripped" membranes was also studied in 1 mM Ca^{++}.

Fig. 5 shows that DNP stimulated oxygen uptake about 45% above the control while oligomycin and ouabain inhibited respiration about 60% and 50%, respectively. Stat experiments showed that these three inhibitors affected both the ectodermal and the endodermal layer of cells. The last three bars of the figure indicate that PCMBS and stripping produce a rate of oxygen uptake in the *presence* of Ca^{++} that is virtually identical with the rate obtained in a calcium-free medium. The oxygen uptakes of stripped or PCMBS-treated membranes in 0 mM Ca^{++} were not significantly different from those in 1 mM Ca^{++}.

As seen in Fig. 6, PCMBS and stripping reduce the oxygen uptake of the ectoderm about 3.6 μliters $O_2/cm^2/hr$ and have no effect on the endoderm. Data from Fig. 2 are included to stress the similarity of the effects of PCMBS, stripping and Ca^{++} withdrawal on the oxygen uptake of the two layers of the membrane. These experiments were performed on both layers of the same membrane in the stat apparatus by reversing the membrane in the chamber.

Effects of Substrates on Oxygen Consumption

All the experiments described thus far were performed in a simple salt media lacking metabolic substrates. In some experiments, various metabolic substrates were tested for their effect on oxygen uptake. Succinate, pyruvate,

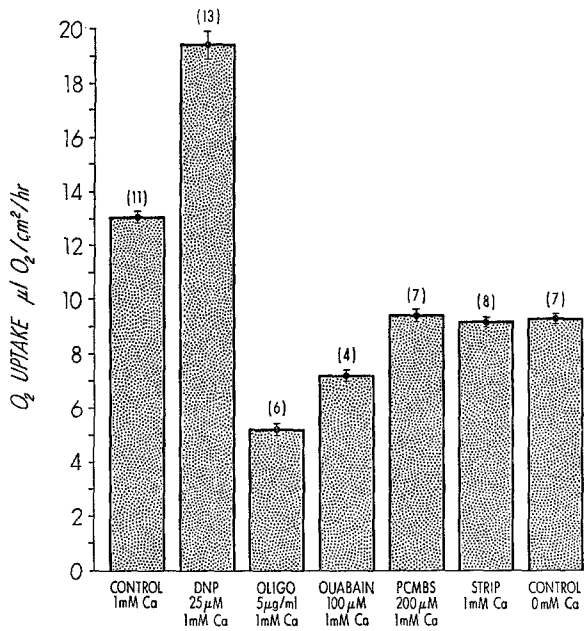

Fig. 5. The effect of various inhibitors of calcium transport on the oxygen uptake of the CA membrane in 1 mM Ca^{++} ion. The concentration of inhibitor is shown below each bar. Similar effects could be obtained with PCMBS at concentrations between 100–400 μM. The last bar on the right is the 0 mM Ca^{++} control value. Experiments performed by manometry

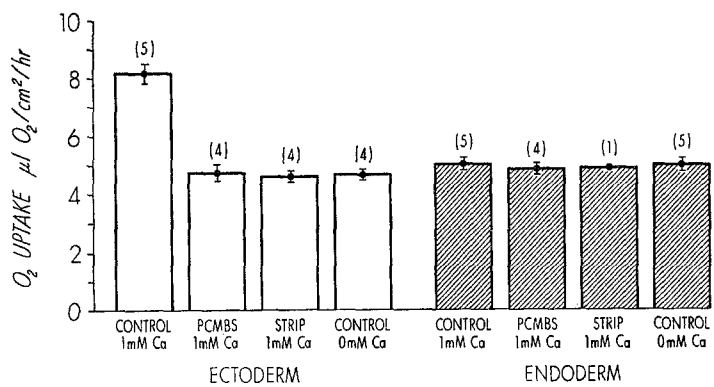

Fig. 6. The effects of the inhibitors PCMBS and stripping on the oxygen uptake of the ectoderm (open bars) and the endoderm (hatched bars) in 1 mM Ca^{++} ion. Conditions for PCMBS treatment are same as for Fig. 5 (*see* Results for full details). Experiments performed in the stat apparatus by reversing the membrane in the chamber during the run. The first and last bar of each set shows the control oxygen uptake in 1 mM and 0 mM Ca^{++}, respectively

Table 2. Effect of inhibitors on calcium and oxygen uptake by CA membrane in 1 mM Ca^{++}

Experiment	O_2 uptake (μliters $O_2/cm^2/hr$)	ΔO_2[a] (μliters $O_2/cm^2/hr$)	Ca^{++} uptake (μmoles/cm^2/hr)	N
Calcium-free medium	9.3 ± 0.2	–	–	(7)
OSM control	12.8 ± 0.3	$+3.6 \pm 0.5$	0.063 ± 0.004	(5)
PCMBS (0.2 mM)	9.5 ± 0.2	n.s.[b]	0.005 ± 0.001	(5)
Stripping	9.2 ± 0.2	n.s.[b]	0.009 ± 0.001	(3)
Ouabain (0.1 mM)	7.2 ± 0.3	-2.0 ± 0.5	0.004 ± 0.001	(4)
Oligomycin (5 μg/ml)	5.2 ± 0.2	-4.0 ± 0.4	0.005 ± 0.001	(6)
ISM preparation	11.3 ± 0.2	$+2.1 \pm 0.4$	0.034 ± 0.003	(3)

[a] ΔO_2 represents the difference in rate of oxygen uptake from that obtained in calcium free medium.
[b] Not stastically significant.

alanine, malate, glutamate and glucose were without effect at concentrations of 1 mM, suggesting that the tissue has a supply of endogenous substrate sufficient to meet its metabolic demands.

Simultaneous Measurement of Calcium and Oxygen Uptake

Some experiments were performed that measured the simultaneous uptake of both Ca^{++} and O_2. These studies were performed in the manometric apparatus with 1 mM Ca^{++} and tracer quantities of Ca^{45} with the results presented in Table 2. Because most of the active calcium transport by the CA membrane over the first hr is due to membrane accumulation of Ca^{++} [36], it is possible to use tissue uptake of Ca^{++} as a reliable indication of transport rate.

The results show that PCMBS and stripping dramatically reduce calcium uptake by the tissue as compared to the control and abolish the calcium-stimulated oxygen consumption. Ouabain and oligomycin also effectively reduce calcium transport, but have distinctly different effects on oxygen uptake than PCMBS or stripping. Note that the effects of PCMBS and stripping on oxygen uptake mimic the effects of a calcium-free medium, whereas the effects of oligomycin and ouabain do not. The ISM preparations are a group of membranes that did not reach a fully stimulated rate (*see* Methods). This group shows a lower calcium uptake and a correspondingly lower Ca^{++}-stimulated oxygen uptake.

Discussion

The results presented show that Ca^{++}, when present in the medium bathing the CA membrane, markedly stimulates its rate of oxygen utilization. The calcium-stimulated oxygen uptake appears to be closely associated with the membrane's active transcellular calcium transport mechanism. This idea is strongly supported by the following correlations. First, the calcium stimulation is specific for the ectodermal layer of cells (Fig. 2), the known location of the transport mechanism [36]. Second, both calcium-stimulated respiration and active calcium transport are dependent on the age of the membrane, appearing only when the transport mechanism becomes operative at about 14 days of incubation (Fig. 3); also, both calcium transport and calcium-stimulated oxygen uptake are maximal at 1 mM Ca^{++}. Third, only those divalent ions that are actively transported by the tissue (Ca^{++}, Sr^{++} and Mn^{++}) stimulated oxygen uptake; Sr^{++} and Mn^{++} elicited a smaller stimulation of oxygen consumption than Ca^{++} (Fig. 4), and published [36] and unpublished data indicate that these two ions are transported at a lower rate than Ca^{++}. Fourth, stripping and PCMBS treatment, two procedures that inhibit membrane calcium uptake (Table 2), lower the oxygen uptake in the presence of Ca^{++} to a rate the same as that found in the absence of Ca^{++} and inhibitor (Fig. 5). Finally, when calcium and oxygen uptake are measured on the same piece of membrane, the results show that the rate of calcium uptake and the rate of extra oxygen uptake are directly related (Table 2).

Indirect support for the hypothesis that the calcium-stimulated respiration is associated with active calcium transport is suggested by the observation that the calcium effect on this membrane is relatively unique. In most tissue preparations or cell suspensions, Ca^{++} ion inhibits or has no effect on respiration. For example, Ca^{++} depresses the oxygen uptake of slices of liver [6, 21, 37], kidney [9, 21], brain [21], lung [21], and spleen [21], and has no effect on the metabolism or respiration of acites tumor cells [3], liver cells [37], intestinal cells (G. Kimmich, *unpublished data*), or toad bladder [14].

On the other hand, it might be argued that the effects of Ca^{++} observed on the CA membrane are, in fact, non-specific. One possibility is that in the *presence* of Ca^{++} the enhanced oxygen uptake is due to calcium transport by mitochondria, a process known to stimulate respiration [4]. The second is that in the *absence* of Ca^{++}, the membrane's structural integrity is so altered that it does not respire normally [24, 25]. In either case, these explanations seem most unlikely when one considers the following infor-

mation: the effects of Ca^{++} on oxygen uptake were limited to only one cell layer, the calcium-transporting ectoderm; the calcium concentration of the calcium-"free" medium was not zero, but about 2×10^{-6} M; in this low Ca^{++} medium, the basal oxygen uptake of both cell layers was linear for periods up to 2 hr, long enough for non-specific cellular deterioration to become apparent; and finally, oligomycin which does not inhibit mitochondrial calcium transport or the associated respiratory activity [4, 31], strongly inhibits both processes in the CA membrane [36].

A hypothesis consistent with all the data is that the transcellular calcium transport mechanism requires a significant energy expenditure by the ectodermal layer of cells. Although the data do not directly indicate an energy donor for the transport mechanism, ATP is implicated by the observation that inhibitors of oxidative phosphorylation, such as DNP and oligomycin, strongly inhibit calcium transport [36]. By analogy with the schemes described for many cells, the calcium transport system in the the CA membrane might act as an ATPase, and the ADP produced then increases the rate of regeneration of ATP [16, 17, 20, 41, 42]. In the CA membrane, this ATP regeneration appears to be dependent on oxidative phosphorylation.

The high degree of correlation between calcium transport and oxygen uptake can be expressed in a quantitative way by calculating the ratio: μmoles Ca^{++} transported/μmoles extra O_2 consumed from the data in Table 2. Control and ISM preparations give the same value, $Ca^{++}/O_2 = 0.45$, indicating a constant stoichiometry between calcium and oxygen uptake in membranes exhibiting quite different transport rates. A more extensive study of the Ca^{++}/O_2 stoichiometry has been completed and will be described in the following paper.

The inhibitors, DNP and oligomycin, exerted expected effects on the oxygen uptake of the CA membrane, and the inhibitory effect of these agents on calcium transport is consistent with their well known action on energy conservation reactions [31]. It is of interest that the sodium transport inhibitor, ouabain, was also effective in inhibiting oxygen and calcium uptake. Although investigators working with the chick and rat intestine have recently suggested an interrelationship between sodium and active transcellular calcium transport, the mechanism of this interaction in these particular systems remains obscure [1, 26]. In any case, it should be emphasized that the effects of all three inhibitors occurred on both cell layers in the membrane, and that the rates of oxygen uptake observed in the presence of these agents were in no way similar to those found by manipulation of the medium calcium concentration. In striking contrast, the

inhibitor PCMBS reduced the oxygen uptake of the membrane in the presence of calcium to a rate the same as the control rate measured in calcium-free media. In addition, PCMBS decreased only the oxygen uptake of the ectoderm, having no effect on the respiration of the endoderm.

PCMBS is an organic mercurical that is known to react specifically with sulfhydryl groups. Because of its high charge, the molecule does not readily penetrate cell membranes, making it an ideal reagent for studying the function of cell surface sulfhydryls [38]. In the CA membrane, PCMBS is an effective inhibitor of calcium uptake, suggesting that membrane sulfhydryl groups are important in the calcium transport mechanism. Interestingly, SH^- binding agents also inhibit calcium transport in erythrocyte ghosts [39], muscle sarcoplasmic vesicles [13], and intestine [1]. In ghosts and muscle vesicles, specific calcium activated ATPases have been implicated as the site action of the sulfhydryl reagents. Whether such ATPases are also essential for calcium transport in the CA membrane remains to be established. In any case, the observation that PCMBS is an effective inhibitor at low concentrations and short time periods (15 min) suggests that the reactive SH^- group is located on or near the outer surface of the plasma membrane of the ectodermal layer of cells.

It is more than coincidental that the metabolic effects of stripping on the CA membrane are indistinguishable from those of PCMBS. The morphological effects of stripping have been clearly established and suggest a common site of action for the two treatments. Correlated electron-microscope and electron-microprobe studies [7] have identified a particular type of ectoderm cell as being involved in transcellular calcium transport. These cells have long thin cytoplasmic processes which are firmly attached to the inner shell membrane. Removing the inner shell membrane from the CA membrane selectively damages the outer plasma membrane of these particular cells [8]. Transport chamber studies with stripped membranes have shown that although stripping abolishes active calcium transport, it does not affect the passive permeability of the tissue [36]. These results, combined with the observation that the calcium-free respiration of the membrane is unaffected by stripping, suggest that stripping is a very specific means of inhibiting calcium transport in the CA membrane. Thus, both PCMBS and stripping inhibit calcium transport, decrease the oxygen uptake of the ectodermal cells in the presence of Ca^{++} to a rate identical to that obtained in a calcium-free medium, and affect the same location within the tissue, the outer plasma membrane of the ectodermal cells.

A previous communication postulated that the initial entry of Ca^{++} into the ectodermal cells was an active process [29]. The results obtained

with Ca^{++}, PCMBS and stripping in this study also suggest that a vital component of the active transcellular calcium transport mechanism in this tissue in located on the external plasma membrane of the ectodermal cells.

The authors wish to thank Dr. George A. Kimmich for helpful discussions, Mrs. Kaye Heitmann for technical assistance in some experiments and Mrs. Jane Leadbeter for typing the manuscript.

This work was supported in part by Public Health Service Grants Nos. 1-T1-DE-175 and 5-RO1-AM-08271, and in part by the U.S. Atomic Energy Commission at the University of Rochester Atomic Energy Project and has been assigned report no. UR-49-1438. A.R.T. is a recipient of a USPHS Career Development Award 9K3-AM-7876.

The material presented is taken from a thesis submitted by J.C.G. to the University of Rochester in partial fulfillment of the requirements for the Ph.D. degree.

References

1. Adams, T. H., Norman, A. W. 1970. Studies on the mechanism of action of calciferol. I. Basic parameters of vitamin D mediated calcium transport. *J. Biol. Chem.* **245**:4421.
2. Borle, A. B. 1967. Membrane transfer of calcium. *Clin. Orthop. Related Res.* **52**:267.
3. Bygrave, F. L. 1966. The effect of calcium ions on the glycolytic activity of Ehrlich ascites-tumour cells. *Biochem. J.* **101**:480.
4. Chance, B. 1965. The energy linked reaction of calcium with mitochondria. *J. Biol. Chem.* **240**:2729.
5. Chappell, J. B. 1964. The oxidation of citrate, isocitrate and cis-aconitate by isolated mitochondria. *Biochem. J.* **90**:225.
6. Cohn, D. V., Griffith, F. D., Levy, R. 1965. Parathyroid extract-induced changes in the oxidation of citrate, succinate and other organic acids by liver. *Endocrin.* **77**:683.
7. Coleman, J. R., DeWitt, S. M., Batt, P., Terepka, A. R. 1970. Electron probe analysis of calcium distribution during active transport in chick chorioallantoic membrane. *Exp. Cell Res.* **63**:216.
8. Coleman, J. R., Terepka, A. R. 1972. Fine structural changes associated with the onset of calcium, sodium and water transport by the chick chorioallantoic membrane. *J. Membrane Biol.* **7**:111.
9. Cutting, M., McCance, R. A. 1947. The effect of calcium ions on the respiration of kidney slices of newborn and mature rats. *J. Physiol.* **106**:405.
10. Epstein, F. H., Whittam, R. 1966. The mode of inhibition by calcium of cell-membrane adenosine-triphosphate activity. *Biochem. J.* **99**:232.
11. Garrison, J. C., Ford, G. D. 1970. An improved method for measurement of oxygen consumption at constant oxygen tension. *J. Appl. Physiol.* **28**:685.
12. Hamilton, H. L. 1952. Lillie's Development of the Chick. Holt, Rinehart and Winston, Inc., New York.
13. Hasselbach, W., Seraydarian, K. 1966. The role of sulfhydryl groups in calcium transport through the sarcoplasmic membranes of skeletal muscle. *Biochem. Z.* **345**:159.
14. Hays, R. M., Singer, B., Malamed, S. 1965. The effect of calcium withdrawal on the structure and function of the toad bladder. *J. Cell Biol.* **25**:195 (No. 3, pt. 2).

15. Howard, J. E. 1957. Calcium metabolism, bones and calcium homeostasis. A review of certain current concepts. *J. Clin. Endocrin. Metab.* **17**:1105.
16. Judah, J. D., Ahmed, K. 1964. The biochemistry of sodium transport. *Biol. Rev.* **39**:160.
17. Kepes, A. 1964. The place of permeases in cellular organization. *In:* The Cellular Function of Membrane Transport. J. F. Hoffman, editor. p. 155. Prentice-Hall, Englewood Cliffs, New Jersey.
18. Keynes, R. D. 1969. From frog skin to sheep rumen: A survey of transport of salts and water across multicellular structures. *Quart. Rev. Biophys.* **2**:177.
19. Kimmich, G. A., Rasmussen, H. 1969. Regulation of pyruvate carboxylase activity by calcium in intact rat liver mitochondria. *J. Biol. Chem.* **244**:190.
20. Klingenburg, M. 1965. Control characteristics of the adenine nucleotide system. *In:* Control of Energy Metabolism. B. Chance, R. W. Estabrook, and J. R. Wiliamson, editors. p. 149. Academic Press Inc., New York.
21. Krebs, H. A. 1950. Body size and tissue respiration. *Biochim. Biophys. Acta* **4**:249.
22. Leaf, A., Page, L. B., Anderson, J. 1959. Respiration and active sodium transport of isolated toad bladder. *J. Biol. Chem.* **234**:1625.
23. Leaf, A., Renshaw, A. 1957. Ion transport and respiration of isolated frog skin. *Biochem. J.* **65**:82.
24. Loewenstein, W. R. 1967. Cell surface membranes in close contact. Role of calcium and magnesium ions. *J. Colloid Interface Sci.* **25**:34.
25. Manery, J. F. 1966. Effects of Ca ions on membranes. *Fed. Proc.* **25**:1804.
26. Martin, D. L., DeLuca, H. F. 1969. Influence of sodium on calcium transport by the rat small intestine. *Amer. J. Physiol.* **216**:1351.
27. Martin, D. W., Diamond, J. M. 1966. Energetics of coupled active transport of sodium and chloride. *J. Gen. Physiol.* **50**:295.
28. Mela, L., Chance, B. 1969. Calcium carrier and the "high affinity calcium binding site" in mitochondria. *Biochem. Biophys. Res. Commun.* **35**:556.
29. Moriarty, C. M., Terepka, A. R. 1969. Calcium transport by the isolated chick chorio-allantoic membrane. *Arch. Biochem. Biophys.* **135**:160.
30. Nellans, H., Finn, A. 1970. Energetics of sodium transport in the toad bladder. *Biophys. Soc. (Abstract)* **10**:196*a*.
31. Pressman, B. C. 1970. Energy-linked transport in mitochondria. *In:* Membranes of Mitochondria and Chloroplasts. E. Racker, editor. p. 213. Van Nostrand, Reinhold, New York.
32. Rasmussen, H. 1970. Cell communication, calcium ion, and cyclic adenosine monophosphate. *Science* **170**:404.
33. Schraer, R., Schraer, H. 1970. The avian shell gland: A study in calcium translocation. *In:* Biological Calcification. H. Schraer, editor. p. 347. Appleton-Century-Crofts, New York.
34. Stewart, M. E., Terepka, A. R. 1969. Transport functions of the chick chorio-allantoic membrane. I. Normal histology and evidence for active electrolyte transport from the allantoic fluid, *in vivo*. *Exp. Cell Res.* **58**:93.
35. Terepka, A. R. 1963. Structure and calcification in avian egg shell. *Exp. Cell Res.* **30**:171.
36. Terepka, A. R., Stewart, M. E., Merkel, N. 1969. Transport functions of the chick chorio-allantoic membrane. II. Active calcium transport, *in vitro*. *Exp. Cell Res.* **58**:107.

37. Thimmappayya, B., Reddy, R. R., Bhargava, P. M. 1970. Preparation of kidney cell suspensions and respiration of kidney and liver cell suspensions. *Exp. Cell Res.* **63**:333.

38. Van Steveninck, J., Weed, R. I., Rothstein, A. 1965. Localization of erythrocyte membrane sulfhydryl groups essential for glucose transport. *J. Gen. Physiol.* **48**:617.

39. Vincenzi, F. F., Schatzmann, H. J. 1967. Some properties of Ca-activated ATPase in human red cell membranes. *Helv. Physiol. Acta* **25**:CR 233.

40. Wasserman, R. H., Kallfelz, F. A. 1970. Transport of calcium across biological membranes. *In:* Biological Calcification. H. Schraer, editor. p. 313. Appleton-Century-Crofts, New York.

41. Whittam, R., Ager, M. E. 1965. The connection between active cation transport and metabolism in erythrocytes. *Biochem. J.* **97**:214.

42. Whittam, R., Wheeler, K. P. 1970. Transport across cell membranes. *Annu. Rev. Physiol.* **32**:21.