Biol. Cell, **55**, 265-272 (1985)

Active Ca^{2+} Transport in Plasma Membranes of Branchial Epithelium of the North-American Eel, *Anguilla rostrata* LeSueur

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A branchial epithelial membrane fraction, more than 20-fold enriched in Na^+/K^+-ATPase activity when compared with the crude homogenate of the tissue, was obtained from adult freshwater American eels. In a membrane vesicle preparation that consisted of 33% inside-out, 23% right-side-out and 44% leaky vesicles, the accumulation of $^{45}Ca^{2+}$ was stimulated by ATP, but not by ADP. Accumulation of $^{45}Ca^{2+}$ was prevented when vesicles were pretreated with detergent or the Ca^{2+} ionophore A23187; Ca^{2+} efflux was observed when the ionophore was added to actively $^{45}Ca^{2+}$-loading vesicles. Oxalate did not affect Ca^{2+} accumulation in these vesicles. Kinetic analysis of the Ca^{2+}-transport process by an Eadie-Hofstee plot revealed that the process is homogeneous; its kinetic parameters are a $K_{0.5}$ for Ca^{2+} of 0.053 μM and a V_{max} of 2.25 nmol Ca^{2+}/min.mg protein (at 37 °C). The calmodulin dependency of this Ca^{2+} transporting process was shown by the inhibitory action of calmodulin antagonists and by the stimulatory effect of calmodulin repletion after EGTA treatment of the membranes.

We conclude that an ATP-energized Ca^{2+} pump is present in the plasma membranes of branchial epithelium, that resembles the Ca^{2+} pumps of *e.g.* mammalian intestinal or renal plasma membranes, and propose its involvement in branchial Ca^{2+}-uptake from the water.

Key-words: *Teleost - Gills - Plasma membranes - Calmodulin - Ca^{2+}-transport.*

INTRODUCTION

For their calcium requirements, freshwater fish may fully depend on the calcium in their aqueous environment. Direct uptake of Ca^{2+} from the water takes place in the gills (5). Reportedly, the major Ca^{2+}-influx in a trout isolated head preparation is associated with the chloride cells (or ionocytes) of the branchial epithelium (19). In reports on Ca^{2+}-dependent ATPase activities in eel gill plasma membranes (2, 3) we advanced biochemical support for a dominant role of the chloride cells in branchial Ca^{2+} absorption. Moreover, the hypercalcemic action of prolactin in the eel is associated with a specific stimulation of a plasma membrane-bound, calmodulin-dependent, high-affinity Ca^{2+}-ATPase, that we tentatively proposed to represent the biochemical expression of the Ca^{2+} pump in the branchial epithelium (3). For freshwater tilapia, *Oreochromis mossambicus,* we have recently shown

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Abbreviations

A23187 - Calimycin, a Ca^{2+} ionophore;
C48/80 - Compound 48/80, a condensation product of formaldehyde and N-methyl-p-methoxyphenethylamine;
EDTA - (ethylenedinitrilo)tetraacetic acid;
HEEDTA - N'-(2-hydroxyethyl)ethylenediamine-NNN-triacetic acid;
Hepes - N-2-hydroxy-ethylpiperazine - N'-2-ethanesulphonic acid;
MS-222 - tricaine methanosulphate; p-NPP - para-nitrophenylphosphate; NP - nitrophenol;
R24571 - 1-[bis-(p-chlorophenyl)-methyl]-3-(2,4-dichloro-β-(2,4-dichlorobenzyloxy)-phenylethyl)imidazoliumchloride;
Tris - tris(hydroxymethyl)aminomethane.

that prolactin-induced hypercalcemia is, indeed, associated with enhanced branchial Ca^{2+} influx (6).

However, to date there are no reports on Ca^{2+}-translocating activity in fish gill plasma membranes. Demonstration of active Ca^{2+} transport is necessary, since the transepithelial potential difference of approximately 20 mV, inside negative, reported for *e.g.* freshwater brown trout (13) might account for passive Ca^{2+} uptake. This report, therefore, deals with the isolation and characterization of a membrane fraction of eel gill epithelium and the demonstration and quantitation of active Ca^{2+} transport in resealed vesicles prepared from these membranes.

BIOLOGICAL MODELS

Yellow female eels, *Anguilla rostrata* LeSueur, with an average body weight of 1.3 kg, were obtained in the early summer of 1984 from a commercial fish dealer in Quebec city, Quebec, Canada. In the laboratory, the fish were kept in running dechlorinated Ottawa city tapwater (0.45 mM Ca, 12 °C) under a photoperiod of 16 hr of light alternating with 8 hr of darkness, until November and December 1984, when the experiments were performed. The animals were not fed.

MATERIALS AND METHODS

Isolation of plasma membranes

In MS-222 (6 g/L, pH 7.4, adjusted with Tris) anesthetized eels, the heart was exposed and after cannulation of the ventral aorta the branchial apparatus was perfused with 40 ml ice-cold isotonic, heparin-containing (20 U/ml) saline to clear the gills of blood cells. Dithiothreitol (DTT, 1 mM), EDTA (0.5 mM) and aprotinin (100 U/ml) were included in the perfusion fluid to enhance enzyme recovery. Next, the branchial epithelium was scraped off onto an ice-cooled glass plate. All subsequent steps were performed at 0-4 °C. The scrapings of individual fish were disrupted (Polytron tissue homogenizer, equipped with a PCU Power control; setting 2, 2 min) in 15 ml of a hypotonic buffer containing 25 mM NaCl, 1 mM Hepes/Tris (pH 8), 1 mM DTT and 100 U/ml aprotinin. The volume was brought up to 50 ml and the homogenate (H_0) was centrifuged at 550 *g* for 15 min (Sorval RC-28) to remove nuclei and cellular debris (pellet, P_0). Membranes were collected by ultracentrifugation of the supernatant (40 Krpm, 45 min, Beckman Ti 42.1 rotor). The pellet (P_1) thus obtained consisted of a firm brownish part well fixed to the wall of the tube and a fluffy layer on top of the former part. The brownish part of pellet P_1 typically contained 80% of the total SDH-activity of the tissue. After mild shaking, the fluffy part of pellet P_1 was removed and resuspended (100 strokes) with a Dounce homogenizer, with a loosely fitting pestle, in a total of 60 ml isotonic buffer containing 250 mM sucrose, 5 mM $MgCl_2$, 5 mM Hepes/Tris (pH 7.4) and 1 mM DTT. This membrane suspension was centrifuged differentially: 1 K*g* 10 min, 10 K*g* 10 min (yielding P_2) and 30 K*g* 30 min (Sorval RC-28). The final pellet (P_3) was rinsed twice and subsequently resuspended by 10 passages through a 23-G needle in 0.5 - 1 ml buffer containing 20 mM Hepes/Tris (pH 7.4), 5 mM $MgCl_2$ and 150 mM KCl (transport studies) or 150 mM NaCl (enzyme studies). These membrane preparations contained approximately 4 mg/ml bovine serum albumin (BSA) equivalents and were used on the very day of isolation without being frozen.

Enzyme assays

Protein was determined with a commercial reagent kit (Biorad), using BSA as reference. The marker enzymes used to characterize the membrane preparations were: Na^+/K^+-ATPase for basolateral plasma membranes, succinic acid dehydrogenase (SDH) for mitochondrial fragments (for assay conditions see 2), NADH-dependent cytochrome-c reductase for endoplasmic reticulum (17), NADPH-dependent cytochrome-c reductase for smooth endoplasmic reticulum (20) and thiamine pyrophosphatase (TPPase; 16) for Golgi membranes. Ca^{2+}-ATPase was assayed in the presence of oligomycin B (5 μg/ml) and sodium azide (NaN_3, 5 mM), at 1 μM Ca^{2+}_{free}, using a 1 mM capacity Ca^{2+}-buffer system (0.5 mM EGTA + 0.5 mM HEDTA). Free Ca^{2+}-concentrations were calculated according to van Heeswijk *et al.* (11).

Vesicular space and membrane orientation

Uptake of D-(^{14}C)-mannitol (Amersham international plc) was measured in the Ca^{2+} transport medium (without ^{45}Ca) to which 100 μM mannitol plus 8.33 μCi/ml ^{14}C-mannitol had been added. The minimum amount of protein in this case was 30 μg per filter.

Resealing of membrane vesicles was substantiated by the fact that SDS treatment of the membranes abolished their ability to accumulate mannitol. The vesicular space for eel gill plasma membranes calculated on the basis of vesicle mannitol content at equilibrium was 2.21 μl/mg protein, a value comparable to the one reported for rat ileal plasma membrane vesicles (22). The steady accumulation of mannitol for at least 2 hr further suggested that no deterioration of the vesicle tightness for mannitol occurred for prolonged incubation times in the Ca^{2+}-uptake medium.

Resealing of membrane vesicles was further analyzed by measuring the effects of detergents on enzymic activity of the Na^+/K^+-ATPase complex. The percentage inside-out orientated vesicles was derived from the increase in ouabain-accessibility upon detergent treatment (ouabain site on the exterior of the cell's plasma membrane) of sealed vesicle preparations, according to Van Heeswijk *et al.*, (11). Assuming rapid permeation of K^+ but not of ouabain through the vesicular membranes the detergent induced increase in ouabain-accessibility - determined via the ouabain-sensitive K^+-NPPase activity - reveals the portion inside-out orientated vesicles of the total vesicle population.

Vesicle Ca^{2+} uptake assays

ATP-dependent Ca^{2+} transport was determined by means of a rapid filtration technique as described by Van Heeswijk *et al.* (11). The composition of the assay medium was (final concentrations in mM, at 37 °C): Hepes/Tris (20, pH 7.4), Tris-ATP (10), KCl (150), Mg^{2+}_{free} (5), Ca^{2+}_{free} (10^{-6}-10^{-3}), HEEDTA (0.5), EGTA (0.5), oligomycin B (5 μg/ml) and NaN_3 (5). The ^{45}Ca radioactive concentration was 3-6 μCi/ml medium. The minimum amount of protein per filter was 15 μg. The membrane filters with retained radioactivity were dissolved in 0.7 ml 2-methoxyethanol, 8 ml of aqualyte (Fisher) was added and the radioactivity determined in an LKB rackbeta LSC, equipped with a dpm-program.

When 10 mM oxalate was introduced into the Ca^{2+} transport assay buffer system, the Ca^{2+} buffering properties of oxalate were taken into account in calculating the free Ca^{2+} concentration, which was 1 μM in these experiments. For the evaluation of the effects of oxalate on vesicular Ca^{2+} uptake, a solubility of 0.0071 g CaC_2O_4/l (at 37C; I = 0.15 M) was used (21).

Calmodulin antagonists R24571 and C48/80 were dissolved in ethanol (100%) and brought to the required concentration in the assay medium (not exceeding 0.1% v/v ethanol). Membrane samples were preincubated with inhibitors or solvent for 15 min at 37C. Ethanol-treated samples served as controls. All assays were performed in plastic tubes. The Ca^{2+} ionophore A23187 was tested at 10 μg/ml assay medium.

EGTA treatment consisted in resuspension of the P_3 pellet with a loosely fitting Dounce homogenizer (100 strokes) in 15 ml 20 mM Hepes/Tris (pH 6.8), 100 mM KCl and 5 mM EGTA; membranes were collected by centrifugation and subsequently washed two times with the basic assay medium (without ATP and Ca^{2+} buffer system).

Statistics and calculations

Values are expressed as mean values ± SE, unless otherwise stated. Statistical analysis of the data was carried out applying Student's *t*-test. Apparent $K_{0.5}$ and V_{max} values were calculated by means of Eadie-Hofstee plots. Linear regression analysis was based on the least squares method.

RESULTS

Membrane isolation and vesicle resealing

As shown in Table I, the procedure applied in this study to isolate plasma membranes from eel branchial epithelium yielded a membrane fraction highly enriched in the plasma membrane marker Na^+/K^+-ATPase, with only minor contaminations of endoplasmic reticulum, Golgi membranes or mitochondrial fragments. The Ca^{2+}-ATPase activity in the purified fraction was 6.36 ± 0.62 μmol P_i/hr.mg protein and the ratio to Na^+/K^+-ATPase was 1 to 12.8 (± 4.2). As shown in Table II, maximum stimulation of Na^+/K^+-ATPase activity was obtained with SDS (at an optimum concentration of 0.03% w/v), coinciding with an apparent loss of the ability of the membrane

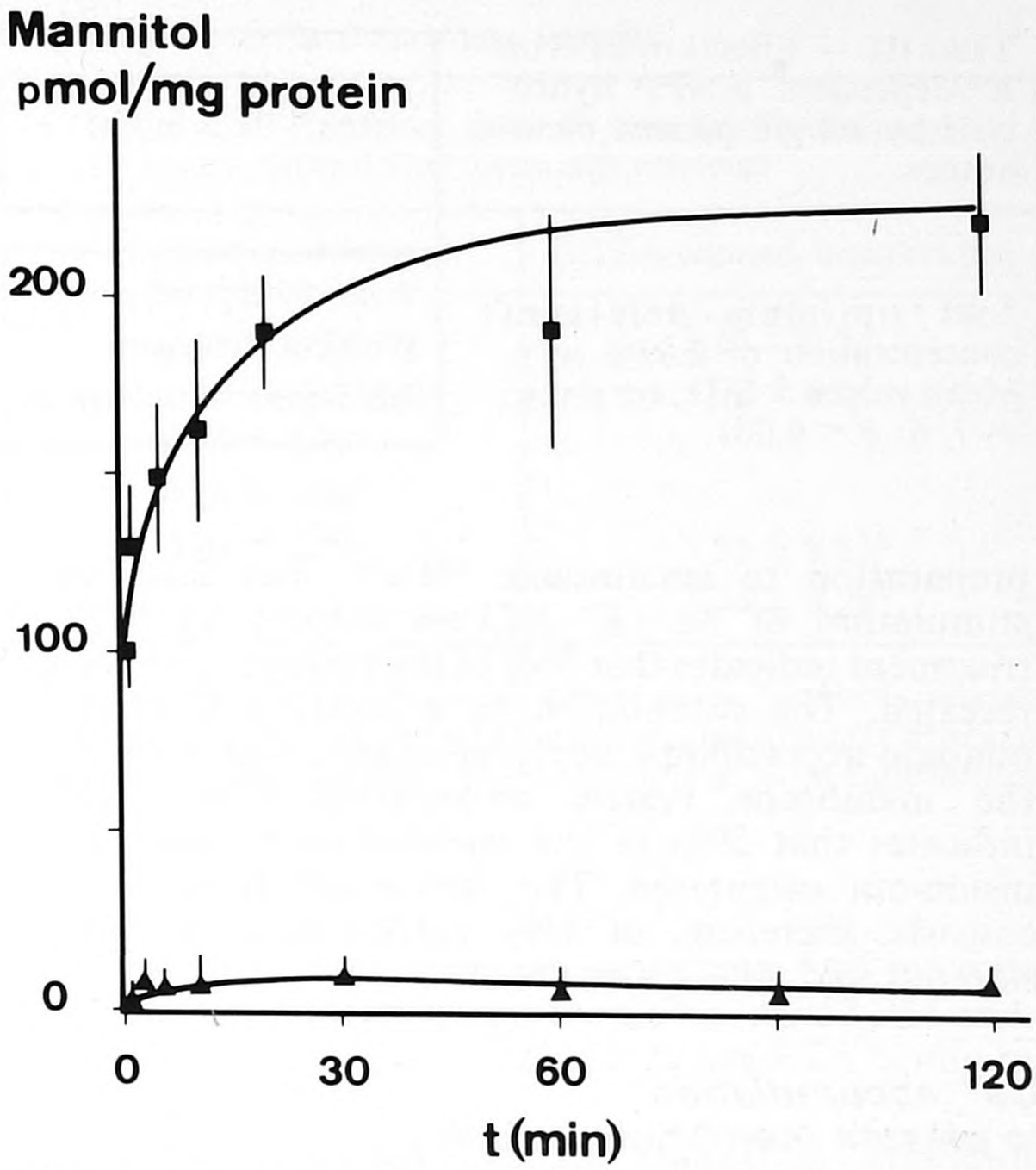

FIGURE 1. — Uptake of D-(^{14}C)-mannitol, in nmol/mg protein, at 37 °C plotted against time. 100 μM mannitol was added to the complete Ca^{2+} uptake medium (1 μM Ca^{2+}_{free}). In membrane preparations pretreated with 0.03% w/v SDS, uptake of mannitol is abolished (▲——▲). The vesicular space of the membrane preparation, as determined on the basis of vesicle mannitol content after 2 hr of incubation, was 2.21 μl/mg protein. Mean values ± S.D. are given; n = 6.

TABLE I. — Relative recoveries and purification of marker enzymes in eel gill plasma membranes.

	H_0 V_{spec}	P_3V_{spec}	% Recovery	Enrichment
Protein	—	—	1.44 ± 0.33	—
Na^+/K^+-ATPase*	3.51 ± 0.56	79.54 ± 16.08	35.31 ± 18.11	23.30
TPPase*	0.37 ± 0.04	0.65 ± 0.08	2.55 ± 0.56	1.78
SDH**	54.86 ± 3.40	12.00 ± 4.28	0.30 ± 0.09	0.22
NADH cyt-c reductase***	4.14 ± 1.10	6.03 ± 2.82	2.46 ± 0.99	1.30
NADPH cyt-c reductase***	10.95 ± 1.13	9.83 ± 4.31	1.40 ± 0.80	0.89
Ca^{2+}-ATPase*	—	6.36 ± 0.62	—	—

* V_{spec} = μmol P_i/hr.mg protein, at 37 °C.
** V_{spec} = ΔA_{490}/min.mg protein, at 25 °C.
*** V_{spec} = ΔA_{555}/min.mg protein, at 25 °C.
Mean values ± S.E. are given for 4 different experiments. Only detergent-treated samples were used.

TABLE II. — Effects of detergents on Na^+/K^+-ATPase and Ca^{2+} uptake by eel gill plasma membranes.

Detergent	Stimulation of Na^+/K^+-ATPase specific activity (n)	% Decrease in ATP-driven Ca^{2+} uptake
SDS (0.03% w/v)*	2.26 × (± 0.35) (7)	100
Tween-80 (0.01% v/v)*	1.96 × (± 0.26) (6)	85
Saponin (0.3 mg/ml)*	1.48 × (± 0.14) (8)	57

* Optimum detergent concentration.
Mean values ± S.D. are given, with the number of observations in parentheses.

TABLE III. — Effects of SDS on K^+-dependent p-NPP hydrolysis by eel gill plasma membranes.

	K^+-induced NP release in the presence of Na^+ and ouabain
	Δ A_{420}/min.mg protein
With detergent*	47 ± 25
Without detergent	114 ± 52
%Increase in oubain accessibility	243 ± 46§

* At optimum detergent concentration of 0.03% w/v. Mean values ± S.D. are given; = 7. §: $P < 0.001$.

preparation to accumulate $^{45}Ca^{2+}$. The 2.26-fold stimulation of Na^+/K^+-ATPase activity by SDS-treatment indicates that 56% of the vesicles have been resealed. The stimulation by a factor 2.43 of the ouabain-accessibility upon detergent treatment of the membrane vesicle preparation (Table III) indicates that 59% of the resealed membranes are inside-out orientated. The membrane preparation consists, therefore, of 33% inside-out, 23% right-side-out and 44% leaky vesicles.

Ca^{2+} accumulation in plasma membrane vesicles

As shown in figure 2, accumulation of Ca^{2+} in membrane vesicle preparations was ATP-, but not ADP-dependent and largely prevented or reversed when the Ca^{2+} ionophore A23187 was added prior to or during Ca^{2+} uptake measurements, respectively. A decrease in the Ca^{2+} buffer capacity from 1 mM to 50 μM, thereby decreasing the total Ca concentration from 4.73×10^{-4} to 2.51×10^{-5} M, did not affect the initial (1 min) Ca^{2+} uptake rates at 1 μM Ca^{2+}_{free}, indicating that the non-ionic Ca concentration did not affect the Ca^{2+} transport system.

As shown in figure 3, addition of 10 mM oxalate did not affect ATP-dependent Ca^{2+} accumulation over a 20 min period. Proceeding from a vesicular space of 2.21 μl/mg protein and 33% inside-out vesicles, the inside-out vesicular space comes to 0.73 μl/mg protein; ATP-driven Ca^{2+} accumulation in the presence of 10 mM oxalate at 1 min amounts to 1.41 ± 0.27 nmol Ca^{2+}/mg protein (n = 5), or 4.27 nmol Ca^{2+}/mg protein when corrected for percentage inside-out vesicles. The intravesi-

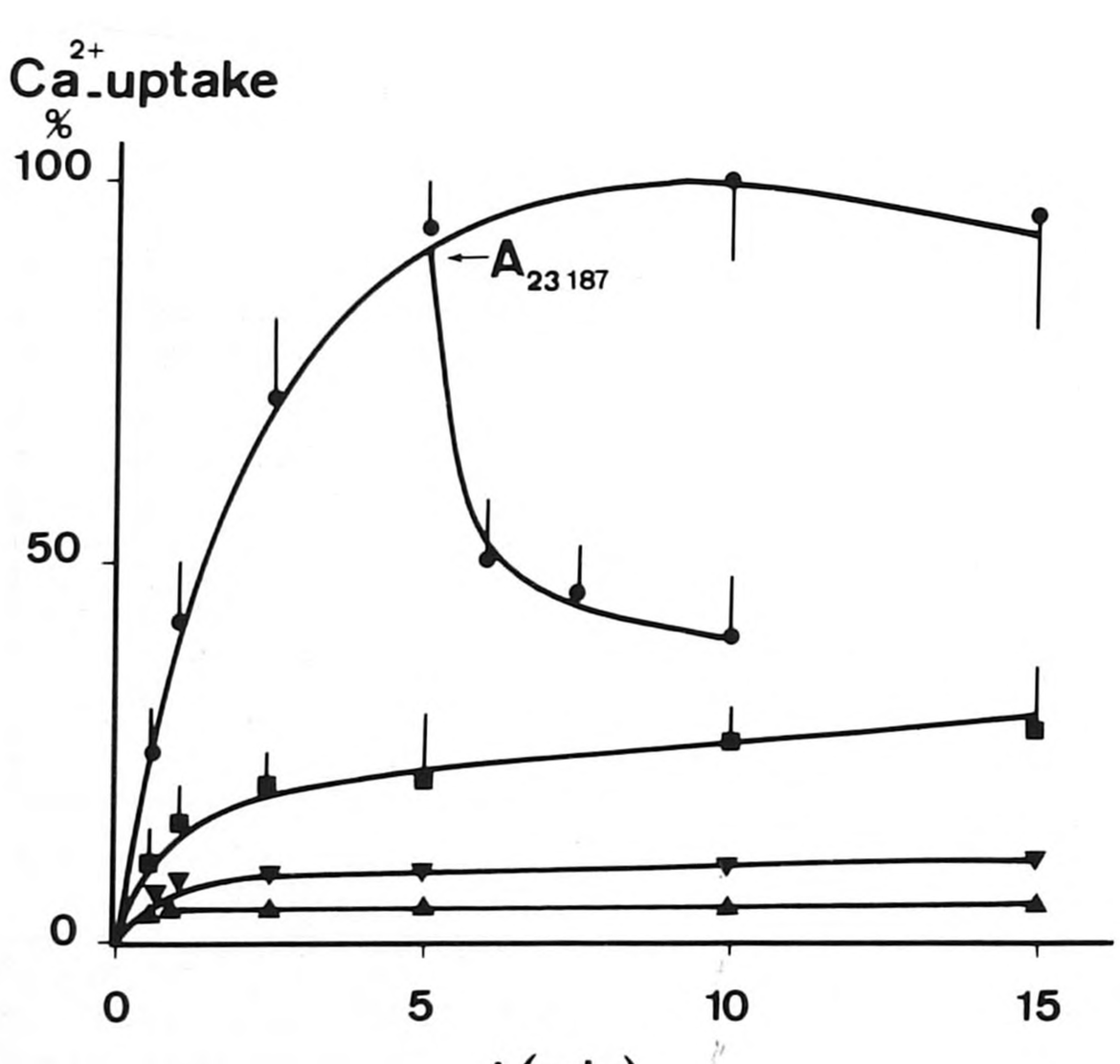

FIGURE 2. — Effect of ATP, ADP and A23187 on Ca^{2+} uptake by eel gill plasma membranes. ▲——▲ uptake in the absence of ATP; ◀——▶ uptake in the presence of 10 mM ADP; ■——■ uptake in the presence of 10 mM ATP and 10 μg/ml A23187; ●——● uptake in the presence of 10 mM ATP. Free Ca^{2+} is 1 μM and free Mg^{2+} is 5 mM. Arrow indicates the addition of 10 μg/ml A23187 to Ca^{2+}-loading vesicles. Ca^{2+}-uptake is expressed as a % of maximum uptake observed in the presence of ATP at t = 10 min. Mean values ± S.E. for 4 to 6 experiments are given.

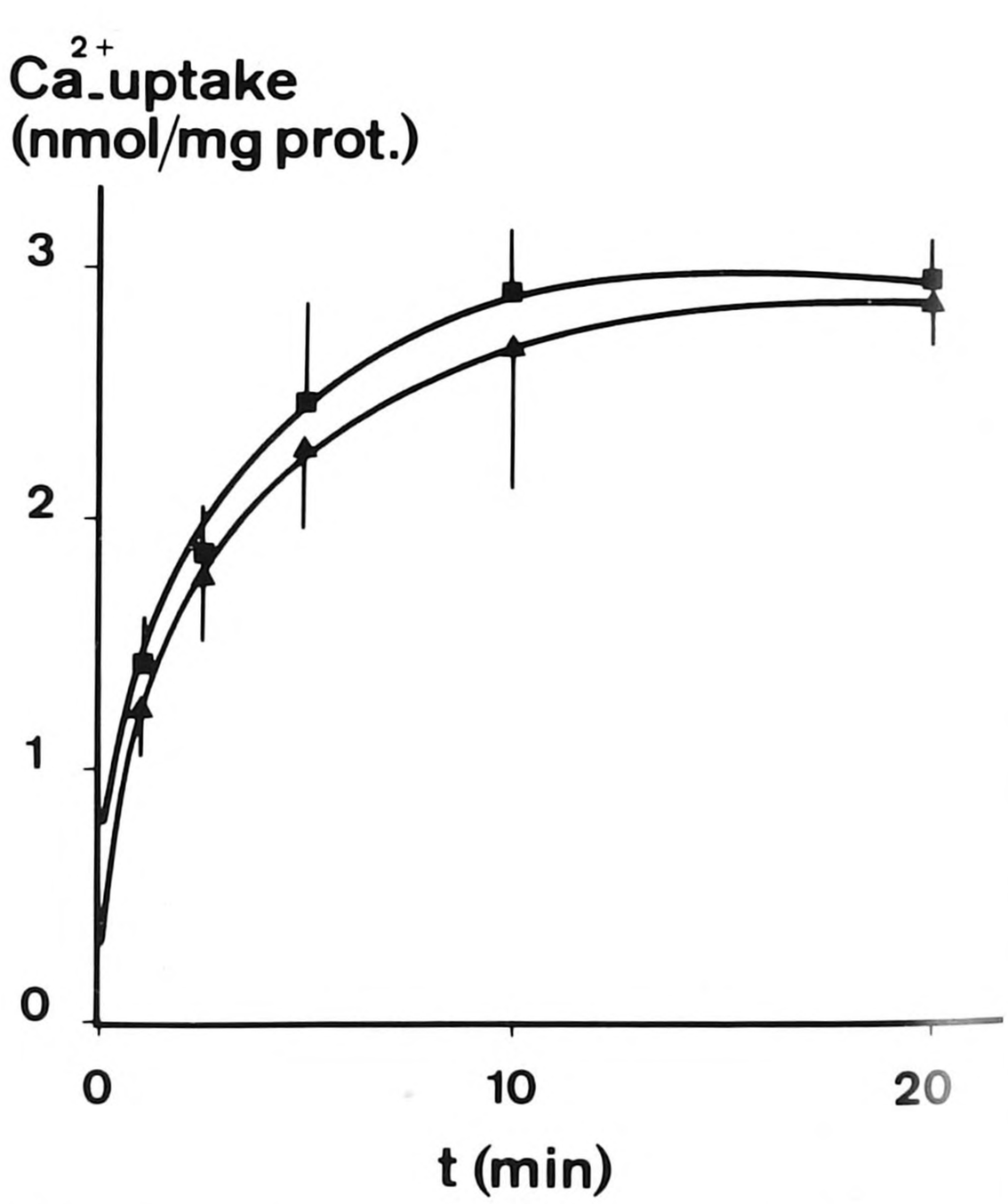

FIGURE 3. — Effects of 10 mM oxalate on Ca^{2+} uptake by eel gill plasma membranes. ▲——▲ uptake in the absence of oxalate; ■——■ uptake in the presence of oxalate. Mean values ± S.D. are given for 4 experiments. Over a 20 min period no statistically significant difference was observed between Ca^{2+} uptake in the presence or in the absence of oxalate ($P < 0.5$). $Ca^{2+}_{free} = 1$ μM.

TABLE IV. — Calmodulin dependency of Ca^{2+} transport rates in eel gill plasma membrane vesicles.

	Initial Ca^{2+} transport rates (1 min determinations) (nmol Ca^{2+}/min.mg protein)	
	Untreated membranes	EGTA-treated membranes
Control (0.1% ethanol)	3.52 ± 0.99	1.20 ± 0.58
R24571 (10^{-7} M)	1.44 ± 0.65[1]	1.08 ± 0.64
R24571 (10^{-6} M)	0.23 ± 0.02[2]	—
C 48/80 (50 μg/ml)	0.26 ± 0.04[2]	—
Calmodulin (10 μg/ml)	3.41 ± 1.08	3.33 ± 0.81[3]
Calmodulin (10 μg/ml) + R24571 (10^{-7} M)	—	1.04 ± 0.28[4]

Mean values ± S.D. are given for 4 to 6 experiments. Values were not corrected for % inside-out vesicles. Symbols indicate statistically significant difference with $P < 0.01$; [1], significantly different from untreated Control; [2], significantly different from R24571 (10^{-7} M); [3], significantly different from EGTA-treated Control and significantly different from calmodulin-repleted EGTA-treatment membranes.

cular Ca^{2+} concentration, then, comes to 4.27/0.73 = 5.85 mM Ca^{2+} 1 min after addition of ATP. This indicates that the solubility of CaC_2O_4 (5.54×10^{-5}) mol/l at this time would have been exceeded more than 100 times. We conclude, therefore, that the Ca^{2+} accumulating membrane vesicles were oxalate-impermeable.

Figure 4 represents an Eadie-Hofstee plot of the Ca^{2+}_{free} concentration dependence of the Ca^{2+} transport process. The kinetic parameters derived from this plot were an apparent half-maximal activation concentration for Ca^{2+} of 0.053 μM and a maximum transport rate of 2.25 nmol Ca^{2+}/min.mg protein at 37 °C.

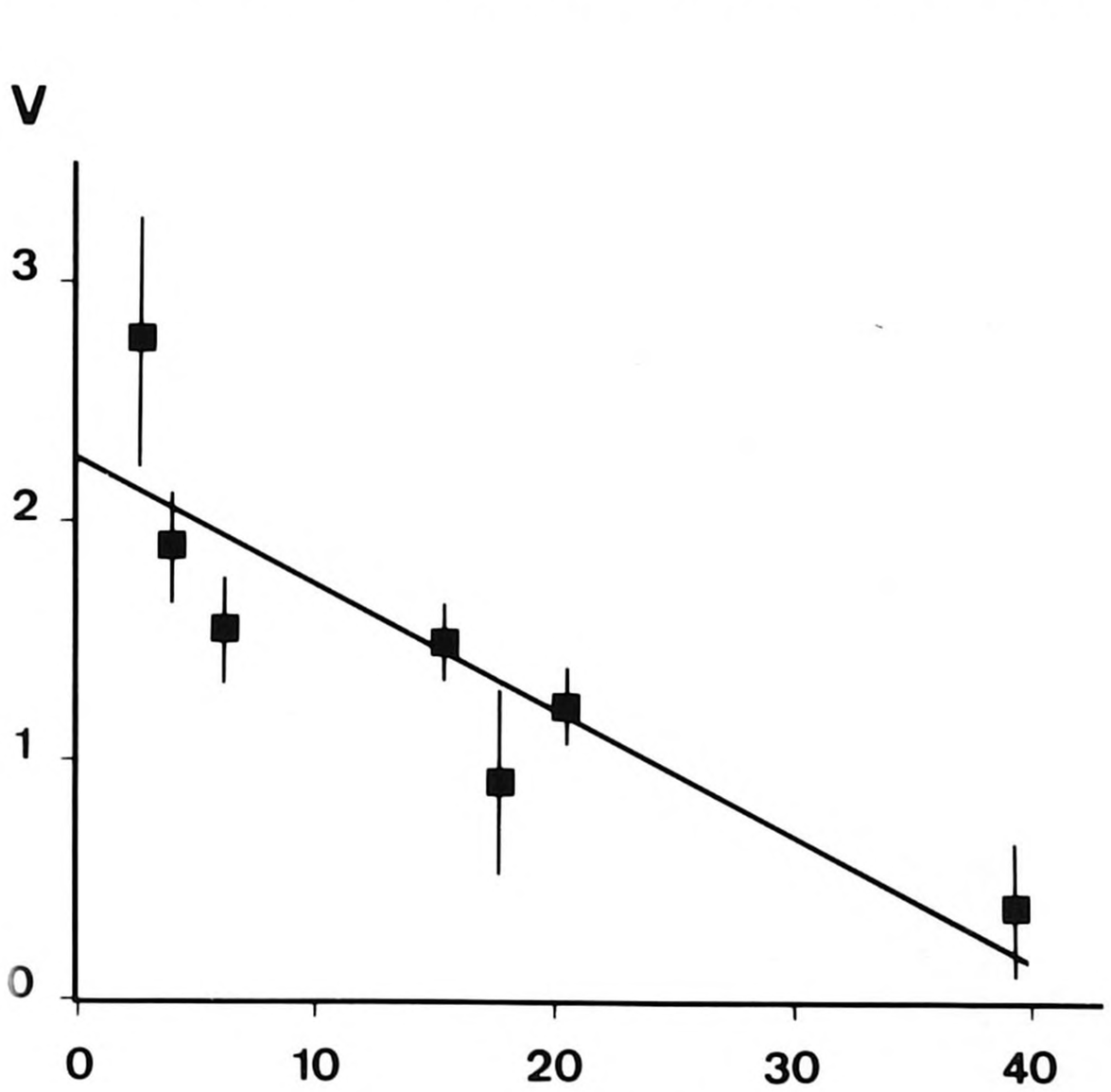

FIGURE 4. — Kinetics of ATP-driven Ca^{2+} uptake in eel gill plasma membranes at 37 °C. Initial rates of ATP-dependent Ca^{2+} uptake (1 min determinations) have been corrected for ATP-independent uptake. Results are presented in an Eadie-Hofstee plot. $K_{0.5}$ for Ca^{2+} is 0.053 μM and V_{max} = 2.25 nmol Ca^{2+}/min.mg protein. Regression: $r_0 = -0.902$; $P < 0.02$. Mean values ± S.D. for 6 to 8 experiments are given. V was expressed as nmol Ca^{2+}/min.mg protein; S indicates the free Ca^{2+} concentrations (10^{-9} — 10^{-6} M).

Table IV summarizes the results on calmodulin dependency of the Ca^{2+} transport process. EGTA treatment of the P_3 membranes significantly decreased the initial Ca^{2+} transport rates at 1 μM Ca^{2+} from 3.52 to 1.20 nmol Ca^{2+}/min.mg protein ($P < 0.001$). The calmodulin antagonists R24571 and C48/80 did not affect EGTA-treatment membranes, but significantly inhibited Ca^{2+} transport rates in "untreated" plasma membranes ($P < 0.001$). The addition of calmodulin to the assay medium (10 μg/ml) did not affect Ca^{2+} transport rates in "untreated" plasma membranes, but restored Ca^{2+} transport rates in EGTA-treated membranes to a level comparable to the transport rate observed in "untreated" membranes. The addition of calmodulin and R24571 did not affect Ca^{2+} transport rates in EGTA-treated vesicles. We conclude from these results that EGTA treatment of the membranes removes endogenous calmodulin and that the Ca^{2+} transport process is calmodulin-dependent.

DISCUSSION

The present data substantiate the presence of an ATP-driven Ca^{2+} pump in plasma membranes of eel branchial epithelium. Studies on rat enterocytes (9) and kidney cortex (14,11) have shown that a similar ATP-energized Ca^{2+} pump is typical for the basolateral plasma membrane fractions of these tissues. In fish gills active transport of ions is concentrated in the chloride cells (3). Most Na^+/K^+-ATPase activity is located in a system of branched membrane tubules that is continuous with the basolateral membranes (12). Since the Ca^{2+} transport system reported in this paper occurs in the Na^+/K^+-ATPase enriched fractions of the tissue, we suggest that it is located in the tubular system and basolateral

plasma membranes of the chloride cells in the gill epithelium. The demonstration of this Ca^{2+} transport mechanism in chloride cells supports our recently presented model for transbranchial Ca^{2+} transport (7).

Membrane isolation

Plasma membranes were isolated using a previously described method with modifications (2). A Polytron tissue homogenizer instead of a Dounce homogenizer for the initial disruption of the tissue was used, EDTA was omitted in the isolation buffers (to improve resealing of the membranes); the bulk of the mitochondria was mechanically separated from the "fluffy" membranes prior to differential centrifugation. These modifications led a to a more than 10-fold decrease in mitochondrial contamination in the final membrane fraction. In order to inhibit any contribution from mitochondrial ATPase activities, NaN_3 and oligomycin were always included in the media used for the study of Ca^{2+}-ATPase activity.

The 23.3-fold enrichment of Na^+/K^+-ATPase activity is roughly 3 times lower than the 61.9-fold enrichment we have reported previously for eel gill plasma membranes (2). This discrepancy, however, apparently derives from the fact that in the present study enrichment factors were calculated exclusively on the basis of enzymic specific activities determined in detergent-treated samples. On the average, detergent treatment of the tissue homogenates (H_0) enhanced Na^+/K^+-ATPase activities 2.5 times; clearly, omission of detergent in determining Na^+/K^+-ATPase in H_0 would lead to a 2.5-fold overestimation of its enrichment in P_3. The Na^+/K^+-ATPase specific activities in the present membrane preparation, however, compare well with those reported previously (3) for eel gill plasma membranes (79.54 and 71.3 μmol P_i/ hr.mg protein; respectively). We conclude, therefore that both membrane fractions were enriched in plasma membranes to a similar extent.

The Na^+/K^+-ATPase activity in our membrane preparation purified 13-, 18- and 26-fold with respect to TPPase, NADH cyt-c reductase and NADPH cyt-c reductase, respectively. Although the contamination with fragments of Golgi apparatus and endoplasmic reticulum (both may contain Ca^{2+} transport ATPase activity; 1, 14, 8) in our membrane preparation is small, such contaminations could result in a overestimation of the capacity of the plasma membrane Ca^{2+} transport system. Moore *et al.* (14, 15) reported for rat kidney and liver significantly higher affinities for Ca^{2+} of the plasma membrane Ca^{2+} pump than for the one of the microsomal Ca^{2+} pump. Should this difference in affinities hold true for the Ca^{2+} pumps in the respective membrane fragments of eel gill tissue as well, one would predict a Ca^{2+} transport process with two affinity sites for Ca^{2+} in significantly contamined membrane preparations. However, kinetic analyses of the Ca^{2+} transport process in our membrane preparations of eel gill tissue show a single class of binding sites with very high affinity for Ca^{2+} (0.053 μM). Moreover, microsomal membranes generally are permeable to oxalate (14). The fact that our membranes proved to be impermeable to oxalate also supports our thesis that the contamination with endoplasmic reticular or Golgi membranes is insignificant with regard to the Ca^{2+} transport process. It seems fair, then to state that this Ca^{2+} transport process reflects plasma membrane Ca^{2+} translocation activity.

Van Heeswijk *et al.* (11) estimated on the basis of ouabain accessibility tests that around 15% of the plasma membrane vesicles of a rat kidney cortex preparation were inside-out orientated. With the same test we found 33% inside-out vesicles in the eel gill membrane preparation. This value compares well with the 30% inside-out vesicles reported for an essentially similar membrane vesicle preparation of tilapia gills (7); the latter value was derived from the effects of detergents on the activities of acetylcholine esterase (exoenzyme) and glyceraldehyde-6-phosphate dehydrogenase (endoenzyme).

Ca^{2+} uptake

The ATP-dependent Ca^{2+} uptake process of eel gill plasma membrane vesicles resembles in many of its attributes the Ca^{2+} transport systems described for plasma membrane of *e.g.* rat kidney cortex (11, 14) or rat gut (9): Ca^{2+} accumulation is promoted by ATP, but not by ADP; the Ca^{2+} transport process is homogeneous, while half-maximal stimulation of the Ca^{2+} transport occurs at intracellular Ca^{2+} concentrations; Ca^{2+} accumulation is prevented by the Ca^{2+} ionophore A23187.

The very high affinity (0.053 μM Ca^{2+}) derived for the Ca^{2+} transport process in eel gill plasma membranes closely resembles the ones reported for high-affinity Ca^{2+}ATPase (0.063 μM) and Ca^{2+} transport (0.13 μM) in tilapia gill plasma membranes (7). Such high affinities are indicative of calmodulin dependency of the Ca^{2+} transport process. For tilapia gill plasma membranes it was shown that radioimmunoassayable calmodulin is present in the Ca^{2+} transporting membranes (7). Calmodulin dependency of the high affinity Ca^{2+}-ATPase activity - the presumed enzymic correlate of the Ca^{2+} pump - in eel gill plasma membranes has been established previously (3).

Calmodulin dependency of the ATP-energized Ca^{2+} pump could be firmly established in the present study. EGTA treatment of membranes and calmodulin antagonists had comparable effects on Ca^{2+} transport rates. Moreover, calmodulin repletion restored Ca^{2+} transport rates in EGTA-treated membranes to levels observed in "untreated" membranes, which indicated that the lipophilic calmodulin antagonists and the Ca^{2+}-chelating EGTA did not affect Ca^{2+} accumulation by interference with vesicle tightness (EGTA treatment had no effect on vesicular space either; results not shown). If we assume that EGTA treatment removes essentially all calmodulin from the membranes, we can state that the Ca^{2+} transport activity observed

after this treatment reflects basal, calmodulin-independent Ca^{2+}-ATPase activity. This contention is further supported by the observation that 10^{-7}M R24571 does not affect Ca^{2+} transport rates in EGTA-treated membranes, but fully antagonizes the stimulatory action of calmodulin on Ca^{2+} transport rates in these membranes. The effects of higher concentrations of R24571 (10^{-6} M) and C48/80 (50 μg/ml) suggest that these inhibitors at these concentrations also affect basal, calmodulin-independent Ca^{2+} transport. Identical results were obtained with R24571 for Ca^{2+}-ATPase activities in eell gill plasma membranes (3).

The observed maximum Ca^{2+} transport rate in the plasma membrane fraction of eell gills (2.25 nmol Ca^{2+}/min.mg protein) is lower than the value for rat kidney cortex preparations reported by Van Heeswijk *et al.* (7.4 nmol Ca^{2+}/min.mg protein; 11) but surpasses the values reported by Gmaj *et al.* for a comparable kidney preparation (1.04 nmol Ca^{2+}/min.mg protein; 10) or the value found for tilapia gill plasma membranes (0.07 nmol Ca^{2+}/min.mg protein; 7). Procedural as well as species-and organ-specific differences may underlie these variations. Also, differences in percentages of inside-out vesicles in the different preparations will contribute to these variations (none of the values given above was corrected for % inside-out vesicles). For tilapia, a comparison made between *in vivo* branchial Ca^{2+} influx rates and the Ca^{2+} transport capacity calculated on the basis of *in vitro* studies on membrane preparations, gave support for a physiological significance of the gill Ca^{2+} pump in the process of uptake of Ca^{2+} from the water. Although no data are available yet on *in vivo* branchial Ca^{2+} influx rates, the capacity of the eel gill plasma membrane Ca^{2+} pump warrants an involvement in the process of branchial Ca^{2+} uptake from the water. We consider passive transport of Ca^{2+} in the gills unlikely: it is bound to follow paracellular routes and this is not compatible with the extremely low permeability to Ca^{2+} that has been demonstrated for the branchial epithelium (18).

Acknowledgements

The authors gratefully acknowledge Dr S.F. Perry and Mrs Suzy Hunt (Dept. of Biology, University of Ottawa, Ottawa), Dr C.H. van Os, Dr M.P.E. van Heeswijk, Mr M.D. de Jong and Dr E. van Corven (Dept. of Physiology, University of Nijmegen, Nijmegen) for their cooperativity and fruitful discussions during these studies.

Experiments were carried out in the Department of Biology, University of Ottawa and were supported by an NSERC of Canada operating grant (A6246) to Dr J.C. Fenwick.

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