Comp. Biochem. Physiol. Vol. 79B, No. 1, pp. 9–16, 1984
Printed in Great Britain

0305-0491/84 $3.00 + 0.00

Ca^{2+}-DEPENDENT PHOSPHATASE AND Ca^{2+}-DEPENDENT ATPase ACTIVITIES IN PLASMA MEMBRANES OF EEL GILL EPITHELIUM—II. EVIDENCE FOR TRANSPORT HIGH-AFFINITY Ca^{2+}-ATPase*

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(*Received* 3 *February* 1984)

Abstract—1. Analysis of Ca^{2+}-induced Mg ~ ATP-hydrolysis by eel gill plasma membranes revealed a substrate-specific, high-affinity Ca^{2+}-ATPase activity ($K_{0.5}$: 0.22 μM, V_{max}: 5.41 ± 0.63 μmol P_i/hr/mg protein) and a non-specific, low-affinity phosphatase ($K_{0.5} \geqq 230\ \mu$M, V_{max}: 10.70 ± 1.25 μmol P_i/hr/mg protein).

2. The high-affinity Ca^{2+}-ATPase activity is calmodulin-dependent.

3. The activation energy of the high-affinity Ca^{2+}-ATPase, calculated on the basis of specific activities at 25 and 37°C was 13.84 Kcal/mol.

4. Specific activities of high-affinity Ca^{2+}-ATPase and Na^+/K^+-ATPase were in the ratio of 1:20.4.

5. The calmodulin-dependent, high-affinity Ca^{2+}-ATPase shows characteristics of a transport Ca^{2+}-ATPase; its association with Na^+/K^+-ATPase activity suggests that the branchial Ca^{2+}-transport mechanism is concentrated in the chloride cells of the gills.

INTRODUCTION

In terrestrial vertebrates intestinal absorption of Ca^{2+} performs a pivotal role in calcium metabolism. Studies with mammals and birds showed that intestinal absorption of Ca^{2+} is associated with an active transport process, which is regulated by 1α,25-dihydroxy-vitamin-D_3 (Van Os and Ghijsen, 1982; Wasserman *et al.*, 1982). Martin *et al.* (1969) were the first to report on the presence of a "vitamin-D" stimulated Ca^{2+}-ATPase activity in the brush-border of rat small intestine and comparable activities have subsequently been reported in whole homogenates and brushborder membranes (Kowarski and Schachter, 1973; Lane and Lawson, 1978) and basolateral membranes (Mircheff *et al.*, 1977) of mucosa of the small intestine. However, this Ca^{2+}-ATPase activity was assayed at non-physiological Ca^{2+}-concentrations, had several characteristics which are different from the Ca^{2+}-transporting Ca^{2+}-ATPase of erythrocyte membranes, and as a result it was identified as a plasma membrane (alkaline) phosphatase (Holdsworth, 1970; Haussler *et al.*, 1970) rather than a classical Ca^{2+}-transporting enzyme. On the other hand it is dependent on vitamin-D and its activity is positively correlated with transepithelial Ca^{2+}-transport (Mircheff *et al.*, 1977), so that some involvement, not yet defined, in the process of calcium transport has been presumed.

Recently, Ghijsen and Van Os (1979) and Ghijsen *et al.* (1980) successfully demonstrated the presence of both an alkaline phosphatase and a transport Ca^{2+}-ATPase enzymic activity in the basolateral plasma membranes of rat enterocytes. Differentiation between these two enzyme activities was realized on the basis of inhibitor and substrate specificities. Moreover, studies on phosphorylated intermediates of the phosphatases of these membranes (De Jonge *et al.*, 1981) and on Ca^{2+}-transport in basolateral membrane vesicles (Ghijsen *et al.*, 1982) provided substantial evidence that Ca^{2+}-transport by the enterocyte plasma membrane must be attributed to a high-affinity Ca^{2+}-ATPase activity. This last enzyme, when assayed in the presence of 2 mM free Mg^{2+}, shows high affinity for Ca^{2+} ($K_{Ca^{2+}} < 1\ \mu$M), prefers Mg^{2+}-ATP as a substrate and is stimulated by calmodulin, three characteristics that are also associated with mammalian erythrocyte transport Ca^{2+}-ATPase (Vincenzi and Larsen, 1980). A similar enzyme has been demonstrated in avian oviduct shell gland (Coty and McConkey, 1982). Such Ca^{2+}-transport ATPases are now postulated to be generally present in all animal cells for the purpose of Ca^{2+}-extrusion to maintain low free Ca^{2+}-levels in the cytosol (Schatzmann, 1982). If this is correct it follows that high levels of this enzyme should be found in epithelia specialized for Ca^{2+}-transport such as the teleost gills but to our knowledge no pertinent reports have been published on these animals.

*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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Abbreviations: ADP, adenosinediphosphate; ATP, adenosinetriphosphate; EDTA, (ethylenedinitrilo)tetraacetic acid; EGTA, [ethylene-bis(oxyethylenenitrilo)]tetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; MS222, tricaine methanosulphonate; *p*-NPP, para-nitrophenylphosphate; NTA, nitrilotriacetic acid; PMSF, phenylmethylsulphonylfluoride; Tris, tris(hydroxymethyl)aminomethane.

In this paper results are presented that demonstrate the presence of a high-affinity Ca^{2+}-ATPase in the gills of a teleost fish. Fresh water teleosts take up Ca^{2+} from the water almost exclusively via the gills (Berg, 1968), a process that can be stimulated by the hormone prolactin (Wendelaar Bonga and Flik, 1982). The molecular mechanisms by which prolactin exerts its effect on Ca^{2+}-uptake via the branchial epithelium are unknown. For many teleostean species branchial Ca^{2+}-ATPase activities have been reported (Ma *et al.*, 1974; Fenwick, 1978; Moon, 1978; Ho and Chan, 1980; Shephard, 1981; Doneen, 1981). Moreover, these activities are positively correlated with transepithelial transport rates of $^{45}Ca^{2+}$ in isolated gills (So and Fenwick, 1977). Recently we have shown that the characteristics of the putative branchial Ca^{2+}-ATPase activities, such as low apparent Ca^{2+}-affinity and alkaline pH optima, do not fulfil the requirements for a transport Ca^{2+}-ATPase; rather the characteristics of this branchial Ca^{2+}-ATPase are consistent with those of an alkaline phosphatase (Flik *et al.*, 1983). The observed Ca^{2+}-activated ATP-hydrolysis by these gill membrane phosphatases are most easily explained on the basis of chelation of Ca^{2+} with the substrate and not from stimulatory effects of Ca^{2+} on enzyme activities. We concluded that the assay conditions reported in the literature to measure gill Ca^{2+}-ATPase activities do not allow the resolution of a homogeneous transport Ca^{2+}-ATPase.

We now report studies on the substrate specificity of Ca^{2+}-induced ATP-hydrolysis in gill plasma membranes of the eel. Ca^{2+}-induced ATP-hydrolysis was assayed in the presence of 2 mM free Mg^{2+}, as recommended by Ghijsen and Van Os (1979) and Ghijsen *et al.* (1980, 1982), under conditions that allow predictions of free Ca^{2+}-levels in the micromolar range. By so doing, we demonstrate that the high- rather than the low-affinity Ca^{2+}-ATPase in these membranes has characteristics common to those found in mammalian intestinal and erythrocyte Ca^{2+}-transport systems.

MATERIALS AND METHODS

Materials

All assays were performed in disposable phosphate-free plastic tubes. All reagents used were of the highest purity commercially available. Ultra pure water was used in all assays. Calmidazolium (R24571) was purchased from Janssen Pharmaceutica, Beerse, Belgium. All other chemicals were obtained from Sigma (St. Louis, MO).

Membrane isolation

Female yellow eels, *Anguilla rostrata* LeSueur, with an average body weight of 1.7 Kg were purchased in May 1982 from a commercial fish dealer in Québec City, Québec, Canada. The eels were held in running de-chlorinated Ottawa city tapwater (0.45 mM Ca^{2+}, 12°C) and under a photoperiod of 16 hr of light alternating with 8 hr of darkness until July and August 1982 when the experiments were performed. The animals were not fed.

After quick anesthesia in a Tris-buffered (pH 7.4) MS-222 solution (6 g/l) the heart was exposed. A cannula was inserted into the bulbus arteriosus and the branchial apparatus was perfused with 30 ml of ice-cold isotonic heparin (20 U/ml) containing saline, until the gills were cleared of blood cells. The protease inhibitor PMSF (0.2 mM) was added to the perfusion fluid to enhance enzyme recovery. The branchial epithelium (about 2.5 g wet wt/Kg fish) was scraped off with a glass microscope slide onto an ice-cold glass plate. All subsequent steps were performed at 0–4°C. A highly enriched plasma membrane fraction as judged from high Na^+/K^+-ATPase purification factors, with only minor mitochondrial contamination as judged from low recovery of succinic dehydrogenase activity, was obtained with centrifugation techniques (Flik *et al.*, 1983). Scrapings were disrupted (20 strokes) in large volumes (75 ml) of a hypotonic buffer with a loose-fitting Dounce homogenizer. The homogenate was centrifuged at 550 ***g*** for 15 min (Sorval RC-2B) to remove nuclei and cellular debris (pellet). The membranes and mitochondria were pelleted by centrifugation of the supernatant for 30 min at 27 K rpm (Beckman SW 28 rotor) and the pellet was resuspended with the same Dounce homogenizer (100 strokes) in an isotonic (sucrose) buffer containing EDTA (0.5 mM) to assure a leaky membrane preparation. The resulting membrane suspension was centrifuged differentially: 1 K***g*** · 10 min, 10 K***g*** · 10 min and 30 K***g*** · 30 min (Sorval RC-2B). The final pellet was rinsed twice and subsequently resuspended in a buffer containing 20 mM Hepes/Tris (pH 7.4), 100 mM NaCl and 5 mM $MgCl_2$. Aliquots of this suspension were rapidly frozen in liquid N_2 and stored at −90°C for a maximum of 3 weeks. Recovery in the gill membrane preparation with respect to the crude homogenate was $2.6 \pm 0.57\%$ ($n = 7$) for the total protein and $13.1 \pm 2.33\%$ for the total Na^+/K^+-ATPase. The Na^+/K^+-ATPase was concentrated 60-fold and only $3.4 \pm 0.4\%$ ($n = 7$) of the initial succinic dehydrogenase activity remained in this preparation. To inhibit ATPase activities resulting from mitochondrial contamination, oligomycin B and sodium azide were added routinely to all assays for Ca^{2+}-stimulated ATPase activities. Detergent treatment (Tween-80 or Triton X-100, 0.1% v/v) did not affect Na^+/K^+-ATPase activities of membrane preparations isolated in the presence of EDTA. A maximum increase of 120% in Na^+/K^+-ATPase specific activities was observed after detergent treatment of membranes isolated in the absence of EDTA. These observations were taken as evidence that the use of EDTA during isolation produced the leaky vesicle preparation necessary for optimal substrate and ion accessibility. The Ca^{2+}-concentration of this membrane suspension was 100 nmol Ca^{2+} per mg BSA equivalents membrane protein.

RESULTS

Ca^{2+}-*stimulation and substrate specificity*

In Fig. 1 the results of substrate preference tests are shown for Ca^{2+}-stimulated phosphatase activity in eel gill plasma membranes. At Ca^{2+}-concentrations lower than 100 μM, in the presence of 2 mM free Mg^{2+}, stimulation of ATP-hydrolysis is significantly greater than hydrolysis of ADP ($P < 0.001$). At higher Ca^{2+}-concentrations no significant difference was observed between Ca^{2+}-induced ATP- and Ca^{2+}-induced ADP-hydrolysis ($P > 0.15$). Eadie–Hofstee plots (Fig. 2) of Ca^{2+}-induced ATP- and Ca^{2+}-induced ADP-hydrolysis reveal that two Ca^{2+}-affinity sites are present when either substrate was used. Computed K_m-values for the high-affinity sites amount to 0.22 μM Ca^{2+} for ATP-hydrolysis and 0.32 μM Ca^{2+} for ADP-hydrolysis, whereas the low-affinity sites have K_m values of 0.535 mM Ca^{2+} for ATP-hydrolysis and 0.230 mM Ca^{2+} for ADP-hydrolysis (Table 1). Clearly, these results indicate the simultaneous presence of high-affinity Ca^{2+}-ATPase and low-affinity phosphatase activities in plasma membranes of eel branchial epithelium.

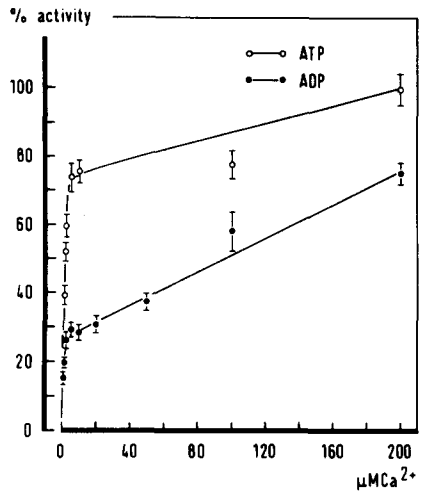

Fig. 1. Effect of Ca^{2+} on ATP- and ADP-hydrolysis by plasma membrane of eel branchial epithelium. Activities are expressed as percentages of maximum ATP-hydrolysis obtained at 200 μM free Ca^{2+} (=100%). Hydrolysis of ATP and ADP in the absence of Ca^{2+} were 41.01 $\pm$ 4.79 and 33.2 $\pm$ 5.48 μmol P_i/hr/mg protein, respectively; 200 μM free Ca^{2+}-induced hydrolysis of ATP and ADP were 8.94 and 6.71 μmol P_i/hr/mg protein, respectively. Mean values $\pm$ S.E.M.; $n = 6$ in all cases. ○——○ ATP, ●——● ADP.

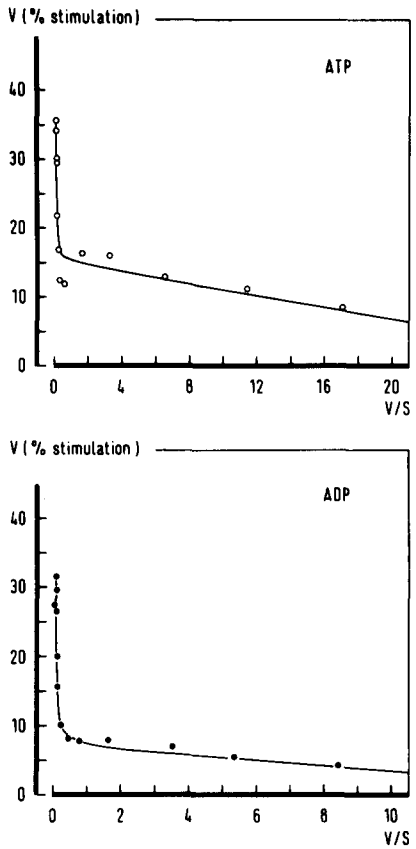

Fig. 2. Eadie–Hofstee plots of Ca^{2+}-induced ATP- and ADP-hydrolytic activities in plasma membranes of eel branchial epithelium at various calcium concentrations. Enzymic activities (V) are expressed as percentage stimulation above background hydrolytic activity. Ca^{2+}-concentrations (S) are expressed in μM. Background activities are 41.01 $\pm$ 4.79 and 33.2 $\pm$ 5.48 μmol P_i/hr/mg protein for ATP and ADP, respectively. Computed $K_{0.5}$ and V_{max} values are presented in Table 1. ○——○ ATP, ●——● ADP. Results are given as mean values of at least 6 determinations.

Effects of phenothiazins and calmidazolium on Ca^{2+}-induced ATP hydrolysis

To further differentiate between either activity we investigated the effects of well-known inhibitors of high-affinity transport Ca^{2+}-ATPases and non-specific phosphatases on Ca^{2+}-induced substrate hydrolysis in the presence of 1 or 200 μM free Ca^{2+} as suggested by Ghijsen *et al.* (1980). As shown in Table 2, chlorpromazine (10^{-5} M), trifluoperazine (10^{-5} M) and calmidazolium (10^{-6} M) inhibited 1 μM Ca^{2+}-induced ATP hydrolysis. Calmidazolium gave complete inhibition without significantly affecting background activities (=zero Ca^{2+}). Trifluoperazine was a more powerful inhibitor than chlorpromazine, but in contrast to the latter inhibitor it also significantly affected background activities. All three inhibitors, at the low concentrations tested, are thought to display their effects as calmodulin antagonists, with calmidazolium being the most potent and specific substance (Van Belle, 1981). We therefore tested the effects of EGTA-treatment and calmidazolium on 1 μM Ca^{2+}-induced ATP-hydrolysis. EGTA-treatment decreased the Ca^{2+}-ATPase activity more than three-fold and led to a 35% decrease in background activities. The Ca^{2+}-ATPase activity resulting after EGTA-treatment could be further specifically inhibited by calmidazolium, without significantly affecting background activities. Table 3 shows the effects of calmidazolium on 200 μM

Table 1. Kinetic parameters of Ca^{2+}-stimulated ATPase and ADPase activities in plasma membranes of eel branchial epithelium

Substrate	High-affinity site		Low-affinity site	
	$K_{0.5}$	V_{max}	$K_{0.5}$	V_{max}
ATP	0.22	5.41 $\pm$ 0.63	535	10.70 $\pm$ 1.25
ADP	0.32	2.32 $\pm$ 0.38	230	8.50 $\pm$ 1.40

K_m-values are expressed in μM Ca^{2+} and V_{max}-values in μmol P_i/hr/mg protein. Enzyme activities were assayed in the presence of up to 5 mM free Ca^{2+} and 2 mM free Mg^{2+} and either 3 mM ATP or 3 mM ADP. V_{max}-values of the low-affinity sites have been corrected in view of the high-affinity sites present. Values were computed from Eadie–Hofstee plots after fitting the data on the basis of a general rate equation for a two-site transport model by means of an iterative procedure (Borst Pauwels, 1973). Mean values $\pm$ S.E.M. are given; $n = 6$.

Table 2. Effects of chlorpromazine, trifluoperazine, calmidazolium and EGTA-treatment on background ATPase and Ca^{2+}-ATPase activities in plasma membranes of eel branchial epithelium

Treatment	Enzyme activities	
	Background ATPase	Ca^{2+}-ATPase
Controls	45.7 ± 3.6 (26)	3.45 ± 0.35 (26)
Chlorpromazine (10^{-5} M)	41.9 ± 3.2 (26)	0.43 ± 0.03 (26)
Trifluoperazine (10^{-5} M)	34.7 ± 2.7 (26)†	0.14 ± 0.10 (26)
Calmidazolium (10^{-6} M)	40.3 ± 3.0 (26)	N.S.* (26)
EGTA + Ethanol 0.1% v/v	29.8 ± 4.7 (8)†	1.05 ± 0.04 (8)
EGTA + Calmidazolium (10^{-6} M)	32.1 ± 4.4 (8)	N.S.* (8)

*No significant stimulation above background ATP hydrolysis in the absence of Ca^{2+}.
†Significantly different from control values $P < 0.001$.
Enzyme activities are expressed as μmol P_i/hr/mg protein and were assayed at 37 C. Inhibitors were dissolved in ethanol. Final ethanol concentrations in the assay media did not exceed 0.1% v/v ethanol (= controls). Membrane suspensions were pre-incubated with inhibitors or solvent for 15 min at 37°C as suggested for calmidazolium (33). EGTA-treatment consisted of resuspension of membranes with a loose-fitting Dounce homogenizer (100 strokes) in 10 vols of 20 mM Hepes/Tris (pH 6.8), 100 mM NaCl and 5 mM EGTA; membranes were collected by centrifugation and subsequently washed two times with the standard basic assay buffer (see Materials and Methods). Mean values ± S E.M. are given with the number of observations in parentheses.

Table 3. Effects of calmidazolium on 200 μM Ca^{2+}-induced ATP- or ADP-hydrolysis

Substrate	200 μM Ca^{2+}-induced substrate hydrolysis (μmol P_i/hr/mg protein)	
	Controls (ethanol 0.1% v/v)	Calmidazolium (10^{-6} M)
ATP	4.06 ± 2.49	3.86 ± 0.97
ADP	4.22 ± 1.29	4.25 ± 0.49

Enzyme activities are expressed as μmol P_i/hr/mg protein and were assayed at 37°C. Membrane suspensions were pre-incubated as described in Table 2. Background ATP- and ADP-hydrolytic activities (zero Ca^{2+}) were not affected by calmidazolium (10^{-6} M). Background activities were 38.5 ± 2.02 and 35.8 ± 1.92 μmol P_i/hr/protein for controls and calmidazolium incubated samples with ATP, respectively; and 25.5 ± 2.56 and 25.33 ± 4.69 μmol P_i/hr/mg protein for controls and calmidazolium incubated samples with ADP, respectively. Values are means ± S.E.M. ($n = 6$).

Ca^{2+}-induced hydrolysis of ATP and ADP. Neither activity was significantly affected. These observations suggest a specific inhibition of the high-affinity Ca^{2+}-ATPase activity by calmidazolium, whereas low-affinity phosphatase activities were not affected.

Effects of theophylline on background and Ca^{2+}-stimulated ATP hydrolysis

As can be seen in Table 4, background ATPase activities at low free Mg^{2+}-concentrations (3 mM ATP + 3 mM Mg^{2+}) are theophylline-sensitive, maximum inhibition amounts to 17.7 ± 2.2% at 1.25 mM theophylline. This observation suggests the occurrence of a non-specific alkaline phosphatase activity (Fawaz and Tejirian, 1972). In the presence of 2 mM free Mg^{2+} background ATPase activities decreased and no theophylline inhibition occurred. When *p*-NPP was substituted for ATP no significant effects of theophylline were observed either at 3 or 5 mM Mg^{2+}. Specific activities were low for *p*-NPP when compared to ATP as substrate; specific activities were significantly lower at 5 mM than at 3 mM Mg^{2+} ($P < 0.05$). Further, theophylline did not inhibit either 1 μM Ca^{2+} or 200 μM Ca^{2+}-induced ATP-hydrolysis at 2 mM free Mg^{2+} (Table 5).

Effects of temperature on Ca^{2+}-induced ATP-hydrolysis

Table 6 gives the specific activities of 1 μM Ca^{2+}-induced ATP-hydrolysis assayed at 25 and 37°C. Assuming linearity of the temperature dependency of this activity in this temperature range, we calculated the activation energy from an Arrhenius plot. A value of 13.84 Kcal/mol was found.

Comparison of ATPase activities in gill plasma membranes

In Table 7 high-affinity Ca^{2+}-ATPase specific activities are compared with Na^+/K^+-ATPase and (Ca^{2+} ~ ATP)phosphatase specific activities. Values for Na^+/K^+-ATPase were about 20 times higher than those for Ca^{2+}-ATPase, whereas (Ca^{2+} ~ ATP)phosphatase activities surpassed Ca^{2+}-ATPase activities more than 30-fold.

Table 4. Effect of theophylline on background hydrolysis of ATP and *p*-NPP in plasma membranes of eel branchial epithelium

Substrate	Background hydrolytic activities		
	Controls	1.25 mM theophylline	Inhibition (%)
3 mM ATP + 3 mM Mg^{2+}	45.2 ± 3.5 (6)	37.2 ± 4.1 (6)	17.7 ± 2.2*
3 mM *p*-NPP + 3 mM Mg^{2+}	4.01 ± 1.17 (4)	3.21 ± 0.79 (4)	N.S.
3 mM ATP + 5 mM Mg^{2+}	39.1 ± 4.5 (6)	39.9 ± 5.2 (6)	N.S.
3 mM *p*-NPP + 5 mM Mg^{2+}	2.53 ± 0.17 (3)	2.48 ± 0.53 (3)	N.S.

*$P < 0.01$.
Enzyme activities are expressed as μmol P_i/hr/mg protein and were assayed at 37 C. Mean values ± S.E.M. are given with the number of experiments in parentheses.

Table 5. Effect of theophylline on Ca^{2+}-induced ATP-hydrolysis in plasma membranes of eel branchial epithelium

	1 μM free Ca^{2+}	200 μM free Ca^{2+}
Control	3.13 ± 0.72 (11)	4.40 ± 0.47 (3)
2.0 mM theophylline	3.18 ± 0.40 (14)	4.40 ± 0.53 (3)

Enzyme activities are expressed as μmol P_i/hr/mg protein and were assayed at 37°C in the presence or absence of 2.0 mM theophylline. Background ATPase activities were not significantly affected by theophylline. Mean values ± S.E.M. are given with the number of experiments in parentheses.

Table 6. Effect of temperature on Ca^{2+}-induced ATP-hydrolysis in plasma membranes of eel branchial epithelium

Temperature (°C)	Ca^{2+}-induced ATP-hydrolysis (μmol P_i/hr/mg protein)
25	1.81 ± 0.24 (6)
37	4.46 ± 0.61 (6)
Activation energy:	13.84 Kcal/mol

Enzyme activities are expressed as μmol P_i/hr/mg protein and were assayed in the presence of 1 μM Ca^{2+}. Association constants of NTA and EGTA for Ca^{2+}, used to calculate 1 μM free Ca^{2+}, are corrected for temperature according to Scharff (1979). Activation energy is calculated from the slope of the ln(activity) vs T^{-1}-curve. Mean values ± S.E.M. are given ($n = 6$).

Table 7. ATP-hydrolytic activities in plasma membranes of eel branchial epithelium

	Specific activity (μmol P_i/hr/mg protein)
Na^+/K^+-ATPase	71.3 ± 6.7
Ca^{2+}-ATPase	3.49 ± 0.35
Ca^{2+} ~ ATP-phosphatase	107.6 ± 5.1

Enzyme activities are expressed as μmol P_i/hr/mg protein and were assayed at 37°C. For Na^+/K^+-ATPase and Ca^{2+} ~ ATP-phosphatase assay conditions are referred to Flik *et al.* (1983) and Fenwick (1978), respectively. High-affinity Ca^{2+}-ATPase is assayed at 1 μM free Ca^{2+} in the presence of 2 mM free Mg^{2+}. Mean values ± S.E.M. are given ($n = 26$).

DISCUSSION

The results presented in this paper show for the first time the presence of a true high-affinity Ca^{2+}-ATPase in the plasma membranes of the branchial epithelium of bony fishes. Further, the characteristics of this Ca^{2+}-ATPase resemble those of the transport Ca^{2+}-ATPases found in the intestine (Ghijsen *et al.*, 1980), kidney (Van Os and Ghijsen, 1981) and erythrocytes (Gietzen *et al.*, 1981b) of mammals and oviduct shell gland of birds (Coty and McConkey, 1982). The following observations lead us to consider the high-affinity Ca^{2+}-ATPase activity described here as the plasma membrane transport Ca^{2+}-ATPase: (1) the high Ca^{2+}-affinity (K_{Ca}: 0.22 μM) observed in the presence of excess free Mg^{2+} indicates that this activity can be specifically stimulated by Ca^{2+} at intracellular concentrations; (2) phosphatase activity with such high affinity for Ca^{2+} preferentially hydrolyzes ATP; (3) the observed high-affinity Ca^{2+}-ATPase activity was inhibited by calmodulin antagonists thus suggesting its calmodulin dependency; (4) the high-affinity Ca^{2+}-ATPase activity was not affected by theophylline or L-phenylalanine which are inhibitors of plasma membrane Ca^{2+}-stimulated alkaline phosphatase activities (Ghijsen *et al.*, 1980); (5) the specific activity of the branchial high-affinity Ca^{2+}-ATPase (V_{max}: 5.41 ± 0.63 μmol P_i/hr/mg protein) is consistent with those reported for transport Ca^{2+}-ATPases of mammalian adipocyte plasma membranes (6.0 μmol P_i/hr/mg protein; Pershadsingh *et al.* 1980) and for erythrocyte plasma membranes (3.6 μmol P_i/hr/mg protein; Waisman *et al.*, 1981); (6) the calculated activation energy for the branchial high-affinity Ca^{2+}-ATPase (13.84 Kcal/mol) is close to the values reported for mammalian erythrocyte Ca^{2+}-ATPase (14–19 Kcal/mol; Larsen *et al.*, 1978); (7) the branchial high-affinity Ca^{2+}-ATPase activity is indeed most likely located in the plasma membranes of the epithelium: it is closely associated with Na^+/K^+-ATPase activity, a basolateral plasma membrane marker (Mircheff and Wright, 1976) and it is insensitive to oligomycin B and sodium azide, which are inhibitors of mitochondrial ATPases (Katz and Doucet, 1980).

Previously we showed that the activity formerly described as Ca^{2+}-ATPase activity in eel gills in fact represents a heterogeneous pool of phosphatases that depend on Ca^{2+}-activation for ATP-hydrolysis (Flik *et al.*, 1983). As was pointed out by Ghijsen *et al.* (1980) for rat intestinal plasma membranes, a transport Ca^{2+}-ATPase enzymic activity should be defined as the ATPase activity that is stimulated by free Ca^{2+} at intracellular concentrations in the presence of a surplus Mg^{2+}. This can be established only under assay conditions with known free Ca^{2+}-levels such as in the presence of a Ca^{2+}-buffer system similar to the one used in this study. Previous studies on Ca^{2+}-ATPase activity in fish gills have not satisfied these requirements (Flik *et al.*, 1983).

The Ca^{2+}-induced ATP-hydrolysis by branchial plasma membrane phosphatase activity could be separated into two distinct kinetic components, one with high affinity and one with low affinity for Ca^{2+}. For the high-affinity component (K_{Ca}: 0.22 μM) ATP was preferred over ADP, the low-affinity component (K_{Ca}: 230–535 μM) showed no preference. We consider the first component as an ATPase involved in Ca^{2+}-transport. Such an enzyme would have an intracellular Ca^{2+}-binding site similar to the comparable enzyme in mammalian intestine and, therefore, a K_{Ca}-value in the range of intracellular Ca^{2+}-concentrations. In general, K_{Ca}-values smaller than 1 μM have now been accepted as an important characteristic of enzymes such as Ca^{2+}-ATPases, adenylate cyclase and cyclic-AMP-phosphodiesterase, that are activated intracellularly (Cheung, 1980). The high-affinity component described here fulfills this criterion. It should be noted that such a K_{Ca}-value is determined by the stability constants of the ligands in the Ca^{2+}-buffer used to calculate the free Ca^{2+}-concentrations (Reed and Bygrave, 1975; Scharff, 1979). In our study a $K_{Ca\text{-}EGTA}$-value of $10^{10.90}$ and a $K_{Ca\text{-}NTA}$-value of $10^{6.40}$, as suggested by Ghijsen *et al.* (1982), were used. These values were taken from the studies of Scharff (1979) who reported closely correlated values for observed and calculated Ca^{2+}-concentrations.

In substrate specificity tests with *p*-NPP, AMP, ADP and ATP, only hydrolysis of the latter two substrates was significantly stimulated by Ca^{2+}. ATP and ADP apparently can serve as substrates for both kinetic components, although the high-affinity component prefers ATP over ADP. Half maximal activation of Ca^{2+}-induced ATP and ADP hydrolysis by

the low-affinity component, however, was calculated to occur at significantly different concentrations (535 and 230 μM Ca^{2+}, respectively). The significant release of ADP that will result from the high background activity when ATP is used as a substrate (Flik *et al.*, 1983) may have led to competitive inhibition of ATP-hydrolysis and therefore the K_{Ca}-value for ATP-hydrolysis may be overestimated. Nevertheless, even the low-affinity site K_{Ca}-value for ADP-hydrolysis (230 μM Ca^{2+}) is too high by at least one order of magnitude to characterize an enzyme functioning at intracellular Ca^{2+}-concentrations. It is unlikely then that this component represents the activity of a Ca^{2+}-transporting enzyme. One striking observation during the substrate specificity tests was that our preparation does not increase the hydrolysis of *p*-NPP or AMP following addition of Ca^{2+}. In this respect it is quite distinct from the preparation of rat enterocyte basolateral plasma membranes used by Ghijsen *et al.* (1980). These authors differentiated between Ca^{2+}-ATPase and Ca^{2+}-stimulated non-specific phosphatase activity in their preparation, i.e. on the basis of substrate specificity: the high-affinity Ca^{2+}-ATPase activity proved to be ATP-dependent, whereas their low-affinity non-specific phosphatase activity showed no distinct preference for either *p*-NPP, AMP, ADP or ATP.

The specific inhibitory action of calmodulin antagonists on the high-affinity Ca^{2+}-ATPase activity suggests the presence of calmodulin in our membrane preparation. Although we did not assay the calmodulin content of these membrane preparations, its presence was indicated by the following observations: (1) Our membrane preparation still contained Ca^{2+}, even though EDTA was used during some steps of the isolation procedure and it may thus be concluded that the endogenous calmodulins were not necessarily extracted from the membranes. Indeed Lynch and Cheung (1979) have shown that the human erythrocyte Ca^{2+}-ATPase-calmodulin complex dissociates only after all Ca^{2+} is extracted. (2) Very low K_{Ca}-values, as found for our high-affinity Ca^{2+}-ATPase activity (0.22 μM), have been shown to be indicative of the presence of calmodulin in erythrocyte Ca^{2+}-ATPase preparations (Schatzmann, 1982). (3) High-affinity Ca^{2+}-ATPase activity in our preparation was decreased by 70% after EGTA-treatment of the membranes. (4) In comparable studies using membranes of tilapia gills we showed the presence of calmodulin by means of radioimmunoassay and the cyclic nucleotide phosphodiesterase assay (unpublished observations).

The effects of the powerful and specific inhibitor (Larsen *et al.*, 1978; Van Belle, 1981) calmidazolium on the Ca^{2+}-induced ATP-hydrolysis are consistent with the assumption that only the 1 μM Ca^{2+}-induced ATP-hydrolysis is caused by intracellularly activated calmodulin-dependent enzyme: this high-affinity component was inhibited, whereas the 200 μM Ca^{2+}-induced ATP-hydrolysis was not affected. Yet, the observed effect of calmidazolium on the high-affinity Ca^{2+}-ATPase activity in EGTA-treated membranes points to the possibility that this inhibitor may also have had some additional non-specific effects at 10^{-6} M. We observed that calmidazolium was at least 10 times more effective than the phenothiazins in its capacity to inhibit the high-affinity Ca^{2+}-ATPase activity. Chlorpromazine and trifluoperazine, at a concentration of 10^{-5} M, both significantly inhibited the high-affinity Ca^{2+}-ATPase activity, but only trifluoperazine markedly affected background activities. This action of trifluoperazine suggested some non-specific action. In a recent paper, Ho *et al.* (1983) showed that trifluoperazine at relatively high concentrations (10^{-5} M) shifted the Ca^{2+}-dependency of sarcoplasmic reticulum Ca^{2+}-ATPase to higher Ca^{2+}-concentrations. They further showed that trifluoperazine's inhibitory action results not from an interaction with the enzyme, but rather from an effect on the membrane resulting in structural perturbations of the ATPase. Keeffe *et al.* (1980) reported for rat liver plasma membranes that chlorpromazine alters membrane fluidity and inhibits ATPase activities non-competitively over a 2–200 μM concentration range of the inhibitor. Calmidazolium on the other hand has been reported to surpass the phenothiazins in potency and to exert its effects at low inhibitor concentrations, and independently of Ca^{2+}, as a purely competitive calmodulin antagonist (Gietzen *et al.*, 1981a). As 10^{-6} M calmidazolium inhibited high-affinity but not low-affinity Ca^{2+}-ATPase activity, we tentatively conclude that the latter activity represents calmodulin-independent phosphatase activity. Thus, this characteristic further differentiates between phosphatase activity and high-affinity Ca^{2+}-ATPase activity.

Ghijsen *et al.* (1980) reported that Ca^{2+}-induced ATP-hydrolysis in rat enterocyte plasma membranes may partly result from alkaline phosphatase activity and that this last activity could be inhibited by L-phenylalanine and theophylline, well-known inhibitors of alkaline phosphatase activity (Fawaz and Tejirian, 1972; Ghosh and Fishman, 1966). We tested both inhibitors but under normal incubation conditions we observed no effect on Ca^{2+}-stimulated ATP-hydrolysis in gill plasma membranes. However, when free Mg^{2+}-levels were low we did observe theophylline inhibition of background ATPase activity, thus corroborating an earlier observation (Flik *et al.*, 1983). At 2 mM free Mg^{2+} (estimated) background activities decreased about 15% and theophylline sensitivity appeared to disappear. Background hydrolysis of *p*-NPP was very low and was not theophylline sensitive at either 3 or 5 mM Mg^{2+}. The weak hydrolysis of *p*-NPP seemed to indicate that the non-specific phosphatase activity of eel gills is distinct from alkaline phosphatases found in teleost intestine (Flik *et al.*, 1982) or mammalian liver and intestine (Jensen, 1979). To date we have not identified an effective inhibitor which can specifically block the ATP-hydrolysis resulting from the Ca^{2+}-stimulated low-affinity component.

In mammals, enzymatic and ion transport studies showed that the transport Ca^{2+}-ATPase and Na^+/K^+-ATPase occur in the same tissue plasma membranes (Mircheff *et al.*, 1979; DeSmedt *et al.*, 1983; Ghijsen *et al.*, 1982). Similarly, both enzymes were found together in the same membranes from eel gills. Transepithelial Na^+-transport in the gills is generally believed to be mediated by the chloride cells of the branchial epithelium (Maetz and Bornancin,

1975). Indeed Hootman and Philpott (1979) have provided ultracytochemical evidence that the bulk of the fish gill Na^+/K^+-ATPase activity is located within the reticular system of the chloride cells. As in the present study high-affinity Ca^{2+}-ATPase and Na^+/K^+-ATPase activities were found in close association in gill plasma membranes we suggest that the Ca^{2+}-transport ATPase is also mainly located in the reticular system of the chloride cells. In freshwater teleosts branchial Ca^{2+} uptake occurs against a transepithelial Ca^{2+}-gradient. The branchial epithelium is a typical tight epithelium and therefore it is likely that transport of Ca^{2+} in the gills follows a transcellular route through the chloride cells. This transport will involve similar events as transcellular Ca^{2+}-transport in, e.g. mammalian enterocytes: (1) entry of Ca^{2+} down its electrochemical gradient at the apical membranes, (2) passage through the cell, mediated by cytosolic Ca^{2+}-binding proteins, and (3) extrusion of Ca^{2+} by a transport Ca^{2+}-ATPase located in the reticular system. The present observations suggest that Ca^{2+}-transport in the gills at least to a large extent is a function of the chloride cells of this epithelium.

The ratio of the specific activities of Na^+/K^+-ATPase and high-affinity Ca^{2+}-ATPase in our membrane preparation (71.3/3.49 = 20.4) is very similar to values reported by De Jonge *et al.* (1981) for such activities in rat enterocyte basolateral plasma membranes (33.9/2.1 = 16.4). This similarity points to an analogous mechanism for the transcellular transport of Ca^{2+}- and Na^+-ions in teleostean gills and mammalian intestinal epithelium.

We are still left with the problem of explaining the function of the branchial alkaline phosphatase (low affinity "Ca^{2+}-ATPase" of the fish literature). The activity of this enzyme appears to reflect the rate of transepithelial calcium transport in eel gills (Fenwick, 1976; So and Fenwick, 1977). Alkaline phosphatase has been implicated in calcium transport in rat intestine (Halloran and DeLuca, 1981). Further time course studies of hormonal effects on this branchial enzyme are under way.

Acknowledgements—The authors gratefully acknowledge Miss Lise Bélanger for her skilful assistance and Dr S. Altosaar (Department of Biochemistry, University of Ottawa) for the use of ultracentrifuge facilities, Miss Jeanne van Rijs, Dr Th. W. Moon and Prof. A. P. van Overbeeke for their critical comments during the preparation of the manuscript and Mrs E. M. Jansen-Hoorweg for typing the manuscript. We are indebted to Dr C. H. Van Os, Dr W. E. J. M. Ghijsen, Mr M. D. De Jongh and Mr M. van Heeswijk for their valuable suggestions and cooperation.

REFERENCES

Berg A. (1968) Studies on the metabolism of calcium and strontium in freshwater fish. I. Relative contribution of direct and intestinal absorption. *Memorie Ist. ital. Idrobiol.* **23,** 161–196.

Borst Pauwels G. W. F. H. (1973) Two site-single carrier transport kinetics. *J. theor. Biol.* **40,** 19–31.

Cheung W. H. (1980) Calmodulin plays a pivotal role in cellular regulation. *Science, Wash.* **207,** 19–27.

Coty W. A. and McConkey C. L. Jr (1982) A high-affinity calcium stimulated ATPase activity in the hen oviduct shell gland. *Archs Biochem. Biophys.* **219,** 444–453.

Doneen B. A. (1981) Effects of adaptation to seawater, 170% seawater, and to freshwater on activities and subcellular distribution of branchial Na^+-K^+-ATPase, low- and high-affinity Ca^{2+}-ATPase and ouabain insensitive ATPase in *Gillichthys mirabilis*. *J. comp. Physiol.* **145,** 51–61.

De Jonge H. R., Ghijsen W. E. J. M. and Van Os C. H. (1981) Phosphorylated intermediates of Ca^{2+}-ATPase and alkaline phosphatase in plasma membranes from rat duodenal epithelium. *Biochim. biophys. Acta* **547,** 140–149.

DeSmedt H., Parys J. B., Borghgraef R. and Wuytack F. (1983) Phosphorylated intermediates of (Ca^{2+} + Mg^{2+})-ATPase and alkaline phosphatase in renal plasma membranes. *Biochim. biophys. Acta* **728,** 409–418.

Fawaz E. N. and Tejirian A. (1972) Inhibition of alkaline phosphatase by theophylline *in vitro*. *Hoppe-Seyler's Z. physiol. Chem.* **353,** 1779–1783.

Fenwick J. C. (1976) Effect of stanniectomy on calcium-activated adenosine triphosphatase activity in the gills of freshwater adapted North American eels, *Anguilla rostrata* LeSueur. *Gen. comp. Endocr.* **29,** 283–287.

Fenwick J. C. (1978) Ca^{2+}-activated adenosine-triphosphatase activity in the gills of freshwater- and seawater-adapted eels (*Anguilla rostrata*). *Comp. Biochem. Physiol.* **62B,** 67–70.

Flik G., Reijntjens F. M. J., Stikkelbroeck J. and Fenwick J. C. (1982) 1,25-Vitamin D_3 and calcium transport in the gut of tilapia (*Sarotherodon mossambicus*). *J. Endocr.* **94,** 40P.

Flik G., Wendelaar Bonga S. E. and Fenwick J. C. (1983) Ca^{2+}-dependent phosphatase and ATPase activities in plasma membranes of the gill epithelium. I. Identification of Ca^{2+}-activated ATPase activity with non-specific alkaline phosphatase activity. *Comp. Biochem. Physiol.* **67B,** 745–754.

Ghijsen W. E. J. M. and Van Os C. H. (1979) Ca-stimulated ATPase in brush border and basolateral membranes of rat duodenum with high-affinity sites for Ca ions. *Nature, Lond.* **279,** 802–803.

Ghijsen W. E. J. M., De Jong H. R. and Van Os C. H. (1980) Dissociation between Ca^{2+}-ATPase and alkaline phosphatase activities in plasma membranes of rat duodenum. *Biochim. biophys. Acta* **599,** 538–551.

Ghijsen W. E. J. M., De Jong M. D. and Van Os C. H. (1982) ATP-dependent calcium transport and its correlation with Ca^{2+}-ATPase activity in basolateral plasma membranes of rat duodenum. *Biochim. biophys. Acta* **689,** 327–336.

Ghosh N. and Fishman W. H. (1966) On the mechanism of inhibition of intestinal alkaline phosphatase by L-phenylalanine. *J. biol. Chem.* **241,** 2516–2522.

Gietzen K., Wüthrich A. and Bader H. (1981a) R24571: a new powerful inhibitor of red blood cell Ca^{2+}-transport ATPase and of calmodulin-regulated functions. *Biochem. biophys. Res. Commun.* **101,** 418–425.

Gietzen K., Konrad R., Tejčka M., Fleischer S. and Wolf H. U. (1981b) Purification, characterization and reconstitution of the Ca^{2+} transport system (high-affinity Ca^{2+}, Mg^{2+}-ATPase) of the human erythrocyte membrane. *Acta biol. med. germ.* **40,** 443–456.

Halloran B. P. and DeLuca H. F. (1981) Intestinal calcium transport: evidence for two distinct mechanisms of action of 1,25-dihydroxy vitamin D_3. *Archs Biochem. Biophys.* **208,** 477–486.

Haussler M., Nagode L. and Rasmussen H. (1970) Induction of intestinal brush border alkaline phosphatase by vitamin D and its identity with Ca-ATPase. *Nature, Lond.* **228,** 119–120.

Ho S. M. and Chan D. K. O. (1980) Branchial ATPases and ionic transport in the eel *Anguilla japonica*. II. Ca^{2+}-ATPase. *Comp. Biochem. Physiol.* **67B,** 639–645.

Ho M. M., Scales D. J. and Inesi G. (1983) The effect of

trifluoperazine on the sarcoplasmic reticulum membrane. *Biochim. biophys. Acta* **730,** 64–70.

Holdsworth E. S. (1970) The effect of vitamin D on enzyme activities in the mucosal cells of the chick small intestine. *J. Membrane Biol.* **3,** 43–53.

Hootman S. R. and Philpott C. W. (1979) Ultracytochemical localization of Na^+,K^+-activated ATPase in chloride cells from the gills of a euryhaline teleost. *Anat. Rec.* **193,** 99–130.

Jensen M. H. (1979) Dephosphorylation of purine mononucleotides by alkaline phosphatases: substrate specificity and inhibition patterns. *Biochim. biophys. Acta* **571,** 55–62.

Katz A. I. and Doucet A. (1980) Calcium activated adenosine triphosphatase along the rabbit nephron. *Int. J. Biochem.* **12,** 125–129.

Keeffe E. B., Blankenship N. M. and Scharschmidt B. G. (1980) Alteration of rat liver plasma membrane fluidity and ATPase activity by chlorpromazine hydrochloride and its metabolites. *Gastroenterology* **79,** 222–231.

Kowarski S. and Schachter D. (1973) Vitamin D and adenosine triphosphatase dependent on divalent cations in rat intestinal mucosa. *J. clin. Invest.* **52,** 2767–2773.

Lane S. M. and Lawson D. E. M. (1978) Differentiation of the changes in alkaline phosphatase from calcium ion-activated adenosine triphosphatase activities associated with increased calcium absorption in chick intestine. *Biochem. J.* **174,** 1067–1070.

Larsen F. L., Hinds T. R. and Vincenzi F. F. (1978) On the red blood cell Ca^{2+} pump: an estimate of stoichiometry. *J. Membrane Biol.* **41,** 361–376.

Lynch T. J. and Cheung W. Y. (1979) Human erythrocyte Ca^{2+}-Mg^{2+}-ATPase: mechanism of stimulation by Ca^{2+}. *Archs Biochem. Biophys.* **194,** 165–170.

Ma S. W. Y., Shami Y., Messer H. H. and Copp D. H. (1974) Properties of Ca^{2+}-ATPase from the gill of rainbow trout (*Salmo gairdneri*). *Biochim. biophys. Acta* **345,** 243–251.

Maetz J. and Bornancin M. (1975) Biochemical and biophysical aspects of salt excretion by chloride cells in teleosts. *Fortschr. Zool.* **23,** 322–362.

Martin D. L., Melancon M. J. Jr and DeLuca H. F. (1969) Vitamin D-stimulated, calcium-dependent adenosine triphosphatase from brush border of rat small intestine. *Biochem. biophys. Res. Commun.* **35,** 819–823.

Mircheff A. K. and Wright E. M. (1976) Analytical isolation of Na,K-ATPase rich membranes and the distribution of enzyme activities. *J. Membrane Biol.* **28,** 309–333.

Mircheff A. I., Walling M. W., Van Os C. H. and Wright E. M. (1977) Distribution of alkaline phosphatase and Ca-ATPase in intestinal epithelial cell plasma membranes: differential response to $1,25(OH)_2D_3$. In *Vitamin D: Biochemical, Chemical and Clinical Aspects related to Calcium Metabolism* (Edited by Norman A. W., Schaefer K., Coburn J. W., DeLuca H. F., Fraser D., Grigoleit H. G. and Herrath D. V.), pp. 281–283. Walter de Gruyter, Berlin.

Mircheff A. K., Sachs G., Hanna S. D., Labiner C. S., Rabon E., Douglas A. P., Walling M. W. and Wright E. M. (1979) Highly purified baso-lateral plasma membranes from rat duodenum. Physical criteria for purity. *J. Membrane Biol.* **50,** 343–363.

Moon T. W. (1978) The characterization of ATPases from the gill of the osteoglossid *Osteoglossu bicirrhosum* (aruma). *Can. J. Zool.* **56,** 795–800.

Pershadsingh H. S., McDonald M. L., Landt M., Bry C. G., Lacy P. E. and McDonald J. M. (1980) Ca^{2+}-activated ATPase and ATP-dependent calmodulin stimulated Ca^{2+}-transport in islet cell plasma membrane. *Nature, London.* **288,** 492–495.

Reed K. C. and Bygrave F. L. (1975) Methodology for *in vitro* studies of Ca^{2+}-transport. *Analyt. Biochem.* **67,** 44–54.

Scharff O. (1979) Comparison between measured and calculated concentrations of calcium ions in buffers. *Analytica chim. Acta* **109,** 291–305.

Schatzmann H. J. (1982) The plasma membrane calcium pump of erythrocytes and other animal cells. In *Membrane Transport of Calcium* (Edited by Carafoli E.), pp. 41–108. Academic Press, London.

Shephard K. L. (1981) The activity and characteristics of the Ca^{2+}-ATPase of fish gills in relation to environmental calcium concentrations. *J. exp. Biol.* **90,** 115–121.

So Y. P. and Fenwick J. C. (1977) Relationship between net ^{45}calcium influx across a perfused isolated eel gill and the development of post-stanniectomy hypercalcemia. *J. exp. Zool.* **200,** 259–264.

Van Belle H. (1981) R24571: a potent inhibitor of calmodulin-activated enzymes. *Cell Calcium* **2,** 483–493.

Van Os C. H. and Ghijsen W. E. J. M. (1981) High-affinity Ca-ATPase in basolateral plasma membranes of rat duodenum and kidney cortex. In *Calcium Transport Across Membranes* (Edited by Bronner F. and Peterlik M.), pp. 159–162. Academic Press, New York.

Van Os C. H. and Ghijsen W. E. J. M. (1982) Calcium transport mechanisms in rat duodenal basolateral plasma membranes: effects of $1,25(OH)_2D_3$. In *Vitamin D: Chemical, Biochemical and Clinical Endocrinology of Calcium Metabolism* (Edited by Norman A. W., Schaefer K., Herrath D. V. and Grigoleit H. G.), pp. 295–297. Walter de Gruyter, New York.

Vincenzi F. F. and Larsen F. L. (1980) The plasma membrane calcium pump: regulation by a soluble Ca^{2+}-binding protein. *Am. J. Physiol.* **233,** 488–494.

Waisman D. M., Gible J. M., Goodman D. B. P. and Rasmussen H. (1981) Studies of the Ca^{2+} transport mechanism of human erythrocyte inside-out plasma membrane vesicles. I. Regulation of the Ca^{2+} pump by calmodulin. *J. biol. Chem.* **256,** 409–414.

Wasserman R. H., Brindak M. E., Meyer S. A. and Fullmer C. S. (1982) Calcium absorption and $1,25(OH)_2D_3$: studies with rachitic and partially vitamin-D-repleted chicks. In *Vitamin D: Chemical, Biochemical and Clinical Endocrinology of Calcium Metabolism* (Edited by Norman A. W., Schaefer K., Herrath D. V. and Grigoleit H. G.), pp. 275–281. Walter de Gruyter, New York.

Wendelaar Bonga S. E. and Flik G. (1982) Prolactin and calcium metabolism in a teleost fish, *Sarotherodon mossambicus*. In *Comparative Endocrinology of Calcium Regulation* (Edited by Oguro C. and Pang P. K. T.), pp. 21–26. Japan Scientific Societies Press, Tokyo.