J. Membrane Biol. 98, 135–144 (1987)

The Journal of
Membrane Biology

Acetylcholine-Induced Na^+ Influx in the Mouse Lacrimal Gland Acinar Cells: Demonstration of Multiple Na^+ Transport Mechanisms by Intracellular Na^+ Activity Measurements

Yoshitaka Saito, Terutaka Ozawa, and Akinori Nishiyama
Department of Physiology, Tohoku University School of Medicine, Sendai, Miyagi 980, Japan

Summary. In the isolated, superfused mouse lacrimal gland, intracellular Na^+ activities (aNa_i) of the acinar cells were directly measured with double-barreled Na^+-selective microelectrodes. In the nonstimulated condition aNa_i was 6.5 ± 0.5 mM and membrane potential (V_m) was −38.9 ± 0.4 mV. Addition of 1 mM ouabain or superfusion with a K^+-free solution slightly depolarized the membrane and caused a gradual increase in aNa_i. Stimulation with acetylcholine (ACh, 1 μM) caused a membrane hyperpolarization by about 20 mV and an increase in aNa_i by about 9 mM in 5 min. The presence of amiloride (0.1 mM) reduced the ACh-induced increase in aNa_i by approximately 50%, without affecting V_m and input resistance in both nonstimulated and ACh-stimulated conditions. Acid loading the acinar cells by an addition/withdrawal of 20 mM NH_4Cl or by replacement of $Tris^+$-buffer saline solution with HCO_3^-/CO_2-buffered solution increased aNa_i by a few mM. Superfusion with a Cl^--free NO_3^- solution or 1 mM furosemide or 0.5 mM bumetanide-containing solution had little effect on the resting aNa_i levels, however, it reduced the ACh-induced increase in aNa_i by about 30%. Elimination of metabolite anions (glutamate, fumarate and pyruvate) from the superfusate reduced both the resting aNa_i and the ACh-induced increase in aNa_i.

The present results suggest the presence of multiple Na^+ entry mechanisms activated by ACh, namely, Na^+/H^+ exchange, Na-K-Cl cotransport and organic substrate-coupled Na^+ transport mechanisms.

Key Words lacrimal gland · Na^+-selective microelectrode · Na^+/H^+ antiport · Na-K-Cl cotransport · intracellular Na^+ activity

Introduction

In the lacrimal gland acinar cells, a muscarinic receptor stimulation produces a primary isotonic secretion rich in NaCl (Alexander, van Lennep & Young, 1972). The receptor stimulation causes an increase in Na^+ uptake into the acinar cells (Parod, Leslie & Putney, 1980; Parod & Putney, 1980). Three possible mechanisms of the agonist-evoked Na^+ uptake have been proposed in this gland: (1) loop diuretic-sensitive NaCl cotransport (Dartt, Mollar & Poulsen, 1981) or $NaKCl_2$ cotransport (Petersen & Maruyama, 1984; Suzuki & Petersen, 1985), (2) Ca^{2+}-dependent nonselective cation channels (Marty, Tan & Trautman, 1984) and (3) amiloride-sensitive Na^+ influx (Parod & Putney, 1980). Existence of a Na-K-Cl cotransporter in the basolateral membrane has been supported by our previous studies on the intracellular Cl^- activities in the mouse lacrimal acinar cells (Saito et al., 1985*a*; Saito, Ozawa & Nishiyama, 1986*b*). The presence of Ca^{2+}-activated Na^+ channel (nonselective cation channel) has been well documented (Marty et al., 1984). However, physiological significance of this channel in fluid secretion is still equivocal. The amiloride-sensitive Na^+ influx described by Parod and Putney (1980) could be mediated by Na^+/H^+ exchange transport. Recently the existence of Na^+/H^+ exchange in the basolateral membrane has been demonstrated in the acinar cells of exocrine pancreas (Hellmessen et al., 1985; Dufresne et al., 1985; Seow, Lingard & Young, 1986).

The present study was undertaken to characterize the ACh-induced Na^+ influx by measuring the intracellular Na^+ activities with double-barreled Na^+-selective microelectrodes. The results suggest that there are at least three distinct Na^+ entry mechanisms in the isolated mouse lacrimal gland acinar cells; Na^+/H^+ exchange, Na-K-Cl cotransport and metabolic substrate-dependent Na^+ uptake.

Materials and Methods

Preparations and Chemicals

The procedures of tissue preparation and superfusion were previously described in detail (Saito et al., 1985*a*). Briefly, the isolated lacrimal gland tissue was tied on a platform, placed in a chamber and superfused with oxygenated solutions using a peristaltic pump at a rate of 8 ml/min. The volume of perfusate in the bath was 2 ml, and a 90% exchange of the bath solution was accom-

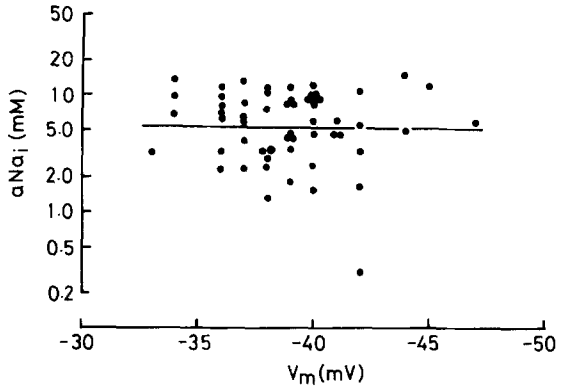

Fig. 1. Relation between basolateral membrane potential (V_m) and intracellular Na^+ activity (aNa_i) of the lacrimal acinar cells (57 cells in 30 preparations) under nonstimulated condition. The least squares regression line shows $\log[aNa_i] = 0.008\ V_m + 1.0$, correlation coefficient $r = 0.069$. The mean V_m and aNa_i are -38.9 ± 0.4 mV and 6.5 ± 0.5 mM, respectively

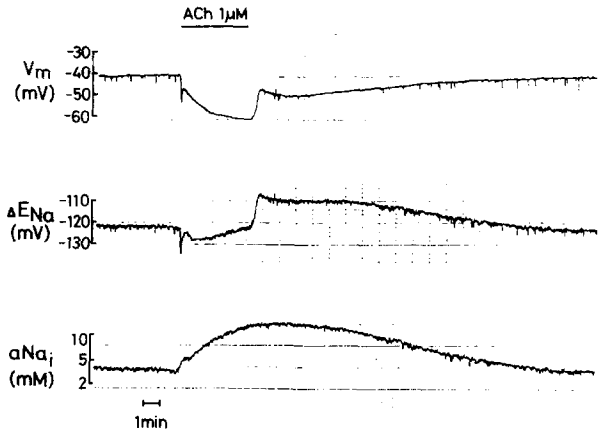

Fig. 2. A typical simultaneous record of membrane potential (V_m), electrochemical Na^+ potential (ΔE_{Na}) and chemical Na^+ potential (ΔE_{Na}-V_m), which is converted to intracellular Na^+ activity (aNa_i) of the acinar cell and the effect of acetylcholine (ACh: 1 μM) obtained by using a double-barreled Na^+-selective microelectrode. Horizontal bar on the top shows the duration of ACh application

plished in 15-20 sec. The bath temperature was kept at 36 ± 1°C. The standard saline solution contained (in mM): Na^+ 139, K^+ 4.7, Cl^- 138, Mg^{2+} 1.1, Ca^{2+} 2.6, $Tris^+$ 5, glutamate 4.9, fumarate 2.7, pyruvate 4.9 and D-glucose 2.8. Chloride-free and K^+-free solutions were prepared by substituting Cl^- with NO_3^- and K^+ with Na^+, on equimolar basis, respectively. Changes in intracellular pH were induced by replacements of the $Tris^+$-buffer solution with a NH_4^+ containing solution (20 mM NH_4^+ substituted for Na^+) or a buffer solution containing 25 mM HCO_3^- and bubbled with a 5% CO_2/95% O_2 gas mixture. The osmolality of the solutions was adjusted to 285 mOsm/kg H_2O with desired amount of D-mannitol. pH was adjusted to 7.4. Drugs tested were amiloride (10^{-6}-10^{-3} M), furosemide (1 mM), bumetanide (0.5 mM), ouabain (1 mM), and acetylcholine (1 μM).

Manufacture of Na^+-Selective Microelectrodes and Measurements of Na^+ Activities

Fibered double-barreled borosilicate glass tubings (o.d. of 1.5 mm and i.d. of 0.9 mm) were soaked in a boiling mixture of conc-HNO_3 and 95% ethanol for about 20 min, rinsed extensively with deionized distilled water and dried in an oven at 110°C (Zeuthen, 1980). Then, using a vertical puller (PA-81, Narishige Sci. Instruments, Tokyo, Japan), the glass capillaries were pulled, applying mild air jet, to a tip diameter of about 0.5 μm and taper and shank length of about 12 mm. Then electrodes were dehydrated by baking on a hot plate at 250°C for 2 hrs. After cooling in the atmosphere of relative humidity of below 50% at 22°C, the inside surface of one barrel of the electrode was silanized by exposing to trimethylchlorosilane vapour for 15-20 min as described by Zeuthen (1980). Silanized electrodes were again baked for 30-60 min. The silanized barrel was back filled with a Na^+-neutral carrier mixture (Steiner et al., 1979) and a 0.1 M NaCl solution. The reference barrel was filled with a 0.5 M KCl solution. Na^+ electrodes were calibrated with solutions of NaCl (100, 50, 20, 10, 5 and 2 mM), Ca^{2+}-free and Ca^{2+}-containing standard saline, and KCl (100 mM). The electrodes showed a slope for Na^+, which was linear between 2-100 mM (least squares regression coefficient $r > 0.99$), of 56.3 ± 0.8 mV, and Na^+ selectivity coefficients against K^+ and Ca^{2+} of 23.9 ± 2.0 and 0.048 ± 0.06, respectively. The intracellular Na^+ activity (aNa_i) was determined according to the Nikolsky formula (Brown & Owen, 1979) with correction for the extracellular Ca^{2+} activity (Zeuthen, 1983) and assuming intracellular K^+ activity to be 90 mM (Saito et al., 1985*b*) and intracellular Ca^{2+} activity to be negligible. The changes in intracellular K^+ activity induced by ACh or ouabain were less than 10 mM in the present experimental conditions (Y. Saito, T. Ozawa, & A. Nishiyama, *unpublished data*) and were neglected in the calculation of aNa_i.

The outputs of the electrodes were fed to a high input impedance electrometer (FD223, W.P. Instruments, New Haven, CT) and recorded on a chart recorder (FBR253A, TOA Electronics, Tokyo, Japan). In order to improve the response time of ion-selective electrodes, most of these were bevelled according to the method of Cassola, Mollenhauer and Fromter (1983) and the outer surfaces were coated with a silver paint (Silvest®, Tokuriki Chem., Osaka, Japan) from the shank and close to the open shaft end and connected to the driven shield of the amplifier (Yoshitomi & Fromter, 1984). The surface of the coating was further covered with insulating varnish.

Statistics

When the effect of a drug was examined, the values for the control and the test conditions were determined in the same cell continuously and normalized. Values were given as the mean ± SEM. Statistical significance of the difference was assessed by paired Student *t*-test. A value of $P < 0.05$ was considered significant.

Results

Intracellular Na^+ Activity and the Effect of Acetylcholine

Figure 1 shows a relationship between the basolateral membrane potential V_m and the intracellular

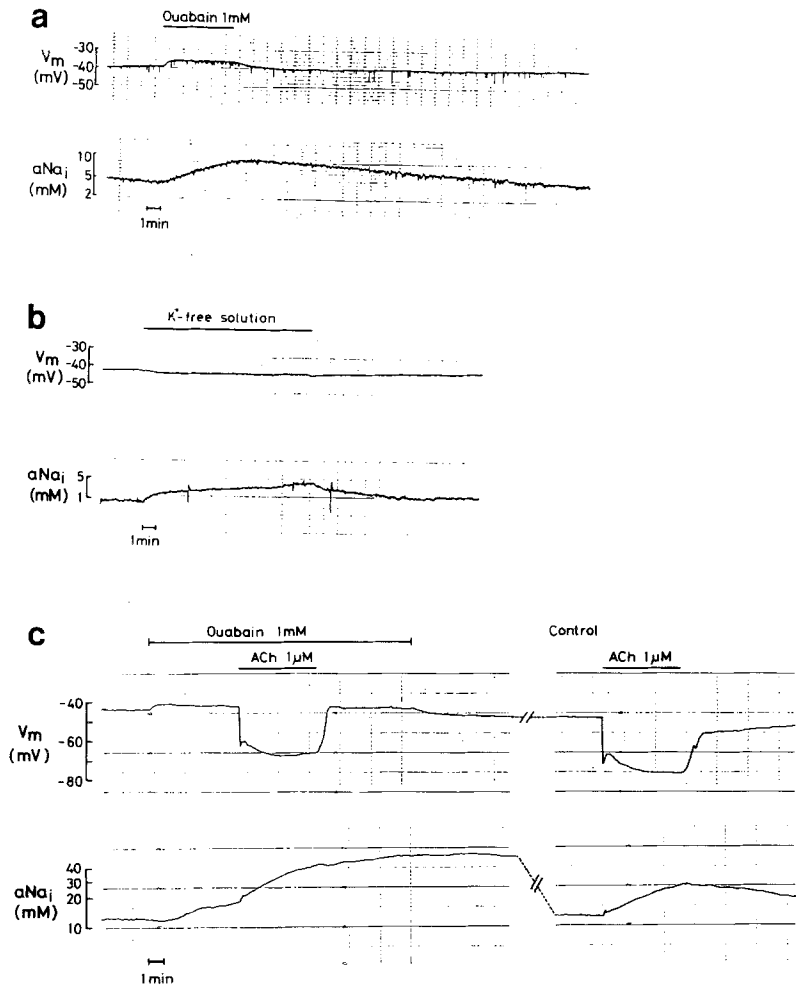

Fig. 3. Effect on membrane potential and intracellular Na^+ activity of ouabain (1 mM, *a*) and superfusion with a K^+-free solution (*b*). *See* Fig. 2 legend for additional explanations. In the presence of ouabain, ACh-induced increase in aNa_i is markedly enhanced and membrane hyperpolarization seen after washout of ACh in the control condition is abolished (*c*)

Table 1. Effect of 1 μM ACh on basolateral membrane potential (V_m) and intracellular sodium ion activity (aNa_i) of the mouse lacrimal gland[a]

	Control		ACh 1 μM		Difference	
	V_m(mV)	aNa_i(mM)	V_m(mV)	aNa_i(mM)	ΔV_m(mV)	ΔaNa_i(mM)
Mean	−38.6	5.3	−58.1[b]	14.5[b]	−19.5[b]	9.2[b]
±SEM	±0.5	±0.7	±0.9	±1.0	±0.8	±0.8

[a] Values in the presence of ACh are those 5 min after the addition of ACh. 26 cells in 18 samples.
[b] Significantly different from the control value $P < 0.001$.

Na^+ activity aNa_i under nonstimulated condition. In 57 cells from 30 preparations the mean V_m was −38.9 ± 0.4 mV and the mean aNa_i was 6.5 ± 0.5 mM (mean ± SEM). There was no correlation between V_m and aNa_i or log[aNa_i], the least squares regression line being $aNa_i = 0.017\ V_m + 7.1$ ($r = 0.013$) or $\log[aNa_i] = 0.008\ V_m + 1.0$ ($r = 0.069$). Figure 2 shows a typical simultaneous record of V_m, electrochemical potential for Na^+ (ΔE_{Na}) and aNa_i and the effects of ACh (1 μM) on these parameters. Addition of ACh caused an immediate membrane hyperpolarization and an increase in aNa_i. In 5 min aNa_i reached a quasi-steady level. On the average in 26 cells of 18 tissue preparations the magnitude of increase in aNa_i (ΔaNa_i) in 5 min was 9.2 ± 0.8 mM (Table 1). The removal of ACh from the perfusate caused an initial rapid depolarization followed by a slow return of the membrane potential to the initial nonstimulated level in about 20 min. The delayed hyperpolarization that appeared after the removal of ACh was not observed in the presence of ouabain or in the K^+-free solution (Fig. 3*c*). Sometimes after

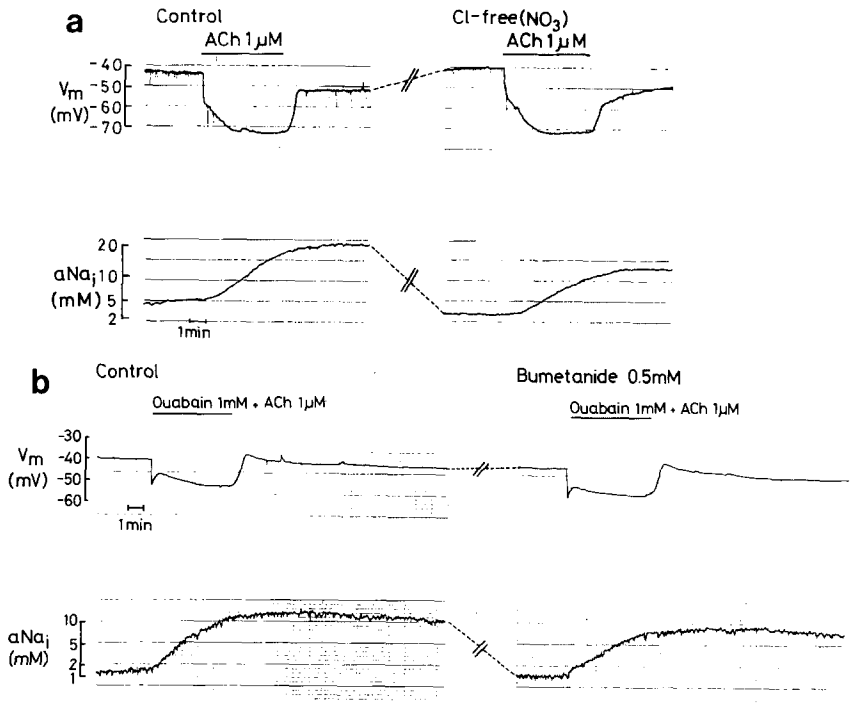

Fig. 4. Effects of removal of external Cl^- (*a*) and loop diuretics (*b*) on ACh-evoked aNa_i change. Upper traces (*a*) show responses in V_m and aNa_i to ACh during superfusion with the control solution and 10 min after switching to a Cl^--free NO_3^- solution. Note that the ACh-induced hyperpolarization is larger and aNa_i increase is smaller in the Cl^--free NO_3^- solution. Lower traces (*b*) show responses in V_m and aNa_i to simultaneous application of ACh and ouabain in the control solution and after shift to a bumetanide-containing solution. Ouabain (1 mM) was applied to eliminate the effect of Na^+/K^+ pump activity

Table 2. Effect of Cl^- substitution with NO_3^- and addition of furosemide (1 mM) and bumetanide (0.5 mM) in the perfusate on ACh (1 μM)-induced increases in the basolateral membrane potential (ΔV_m) and intracellular sodium activity (ΔaNa_i)

		Control		Test			
						% control	
	n	ΔV_m(mV)	ΔaNa_i(mM)	ΔV_m(mV)	ΔaNa_i(mM)	ΔV_m(%)	ΔaNa_i(%)
Cl^--free NO_3^- Ringer	5	−21.0 ± 2.2	9.6 ± 2.0	−22.8 ± 2.2	6.4 ± 0.9	109 ± 3[a]	72 ± 8[a]
Furosemide 1 mM	4	−22.3 ± 2.1	12.0 ± 3.8	−24.8 ± 2.7	7.5 ± 2.2	111 ± 5	65 ± 7[a]
Bumetanide 0.5 mM	4	−16.0 ± 1.6	7.3 ± 1.1	−19.5 ± 1.9	4.3 ± 0.6	122 ± 4[a]	60 ± 7[a]

[a] $P < 0.05$.
n: number of observations.

the removal of ACh, aNa_i continued to increase slightly for a few min and then decreased towards the initial level.

Effect of Ouabain and K^+-Free Solution

Inhibition of the Na^+-K^+-pump by an addition of 1 mM ouabain or removing K^+ from the solution caused an increase in aNa_i (Fig. 3*a* and *b*). When ouabain was applied, membrane potential was depolarized by a few mV before a change in aNa_i indicating the electrogenicity of the pump. In the presence of ouabain the rate of aNa_i increase induced by ACh was augmented (Fig. 3*c*). This proved that ACh-induced increase in aNa_i is the result of enhanced Na^+ influx but not of an inhibition of Na^+ efflux.

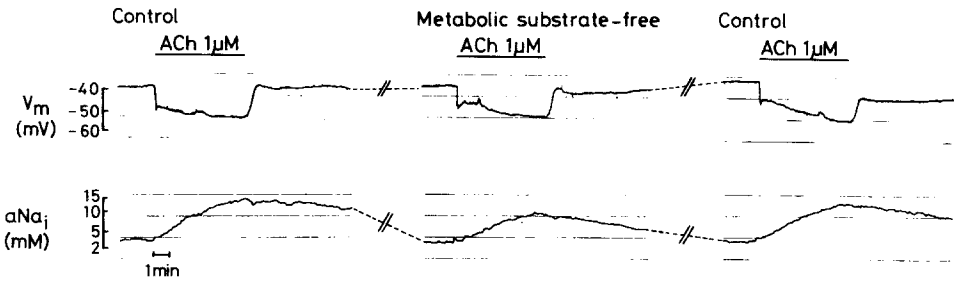

Fig. 5. Effect of metabolite removal from the perfusate on the ACh-induced aNa_i change. During superfusion with a substrate-free solution Na^+ salts of fumarate 2.7 mM, pyruvate 4.9 mM and glutamate 4.9 mM and D-glucose 2.8 mM are replaced with isomolar NaCl and D-mannitol, respectively. In the absence of metabolic substrates the ACh-induced aNa_i increase was decreased as compared with the control conditions

Table 3. Effect of metabolites removal from the perfusate on intracellular sodium activity (aNa_i) in the presence and absence of ACh (1 μM)[a]

	Control			Metabolic substrate removed			
	aNa_i (mM)			aNa_i (mM)			
exp #	ACh(−)	ACh(+)	ΔaNa_i	ACh(−)	ACh(+)	ΔaNa_i	% control
1	3	18	15	2	9	7	47
2	2	9	7	2	8	6	86
3	10	20	11	10	20	10	91
4	9	23	14	8	17	9	64
5	2	12	10	1	6	5	50
6	5	14	9	3	10	7	78
Mean ± SEM	5.2 ± 1.4	16.0 ± 2.1	11.0 ± 1.2	4.3 ± 1.5	11.7 ± 2.3	7.3 ± 0.8[b]	69 ± 8[b]

[a] Effect on basolateral membrane potential of metabolites removal was negligible.
[b] Significantly smaller than the control at $P < 0.05$. aNa_i in the absence of ACh was slightly decreased by substrate removal in four out of six cases. Control values are the average values determined before and after the removal of metabolic substrates.

Effect of Cl^--Free Solution and Loop Diuretics on ACh-Induced aNa_i Increase

In order to test the participation of NaCl cotransport in ACh-induced Na^+ influx, the effect of ACh on aNa_i was observed in a Cl^--free solution or in the presence of loop diuretics (Fig. 4, Table 2). Replacement of Cl^- in the standard solution with NO_3^- caused a slight membrane hyperpolarization and a decrease in aNa_i of the nonstimulated acinar cells. In the Cl^--free NO_3^- solution the magnitude of ACh-induced aNa_i increase (ΔaNa_i) was smaller (ΔaNa_i = 6.0 ± 0.9 mM, 72% control, $P < 0.05$) while the magnitude of membrane hyperpolarization (ΔV_m) was slightly larger (ΔV_m = 22.8 ± 2.2 mV, 109% control, $P < 0.05$). Similar results were obtained in the presence of furosemide (1 mM) or bumetanide (0.5 mM). In all cases ACh-induced membrane hyperpolarization became greater (104-133% control, $P < 0.05$), and ACh-induced aNa_i increase became smaller (50-86% control, $P < 0.05$) than those in the absence of these drugs (Table 2).

Effect of Removal of Metabolic Substrates

Since the presence of Na^+-dependent amino acid transport has been demonstrated in the membrane vesicle preparations of rat exorbital gland (Mircheff, Lu & Conteas, 1983), the effect of replacement of the metabolic substrates in the superfusate (Na^+ salts of glutamate, fumarate and pyruvate and D-glucose) with equimolar NaCl and D-mannitol was examined (Fig. 5). Removal of the substrates caused little change in V_m (±2 mV) but decreased aNa_i slightly in 10 out of 12 cells (6 preps., Table 3). ACh-induced change in V_m was slightly reduced (20.6 ± 1.5 mV to 17.8 ± 1.4 mV) and ACh-induced

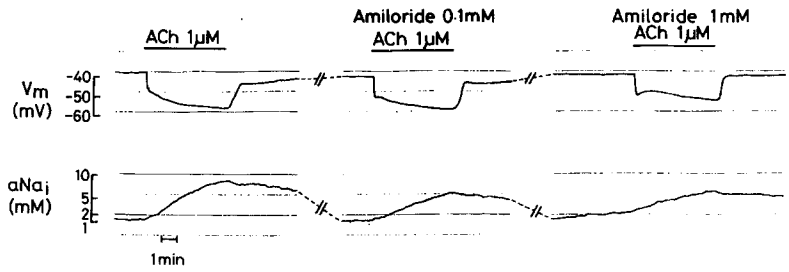

Fig. 6. Effects of amiloride on aNa_i. Addition of 0.1 mM amiloride alone to the superfusate had any effect on neither V_m nor aNa_i, however, it reduced the ACh-induced aNa_i increase by about 50% but not the membrane potential change. At 1 mM, amiloride itself caused a slight membrane depolarization and a gradual increase in aNa_i. The ACh-induced aNa_i increase was also reduced markedly

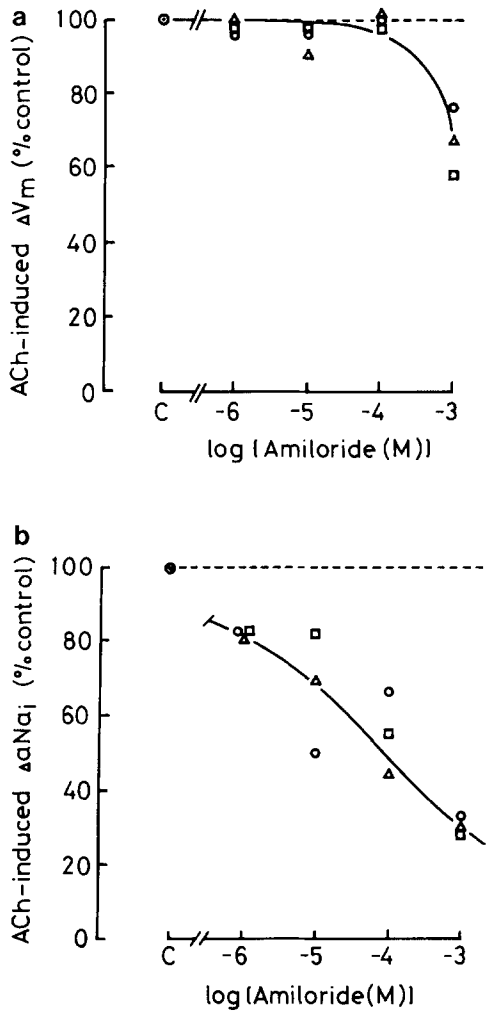

Fig. 7. Effects of increasing doses of amiloride on the ACh-induced membrane hyperpolarization (*a*) and aNa_i increase (*b*). Values are normalized by the control values determined before and after the addition of amiloride. Continuous lines are fitted by eye. Each type of symbol represents individual preparation

change in aNa_i was significantly ($P < 0.05$) inhibited by substrate removal (11.0 ± 1.2 mM to 7.3 ± 0.8 mM). Addition of D-glucose (2.8 mM) or amino acids (alanine, valine, leucine or lysine; 15 mM) to the substrate-free solution had little effect on both V_m and aNa_i, however, replacement of 15 mM NaCl with sodium salt of either glutamate (15 mM) pyruvate (15 mM) or fumarate (7.5 mM) showed a tendency to increase the aNa_i of the nonstimulated condition.

Effect of Amiloride

Amiloride at concentrations from 10^{-6} M to 10^{-4} M inhibited the ACh-induced increase in aNa_i (Figs. 6 and 7*b*), however, it had little effect on aNa_i of the nonstimulated condition, V_m (Fig. 7*a*) or input resistance of the stimulated and nonstimulated acinar cells. At a concentration of 10^{-3} M the drug caused a depolarization of V_m and an increment of aNa_i in the nonstimulated cells and markedly inhibited the ACh-induced hyperpolarization of V_m and increase in aNa_i (Figs. 6, 7*a*, *b*).

Effect of Acid Loading on Intracellular Na^+ Activity

Since the effects of amiloride described above are suggestive of the presence of Na^+/H^+ exchange transport, the effect of intracellular pH change on intracellular Na^+ activity was studied. It has been shown that intracellular pH (pH_i) falls when applied NH_4^+ is removed from the extracellular fluid or PCO_2 of the medium is increased (Thomas, 1974), even when pH of the extracellular fluid is maintained constant by increasing the HCO_3^- concentration (Zeuthen & Machen, 1984). Replacement of the standard $Tris^+$-buffered solution with HCO_3^-/CO_2-buffered solution (Fig. 8*a*) caused a slight but significant membrane hyperpolarization (by −4.5 ± 0.6 mV, $n = 6$) and an increase in aNa_i (by 1.6 ± 0.2 mM). Replacement of 20 mM NaCl of the superfusing solution with 20 mM NH_4Cl (Fig. 8*b*) caused a slight membrane depolarization (+1.3 ± 0.6 mV, $n = 7$) and a decrease in aNa_i (−1.0 ± 0.4 mM). Withdrawal of NH_4Cl from the solution caused an immediate increase in aNa_i, which transiently exceeded

the normal level of aNa_i (Fig. 8*b*). The presence of amiloride (0.1 mM) suppressed the transient surge of aNa_i induced by NH_4Cl removal and subsequent wash-out of amiloride restored the transient increase in aNa_i (Fig. 8*c*).

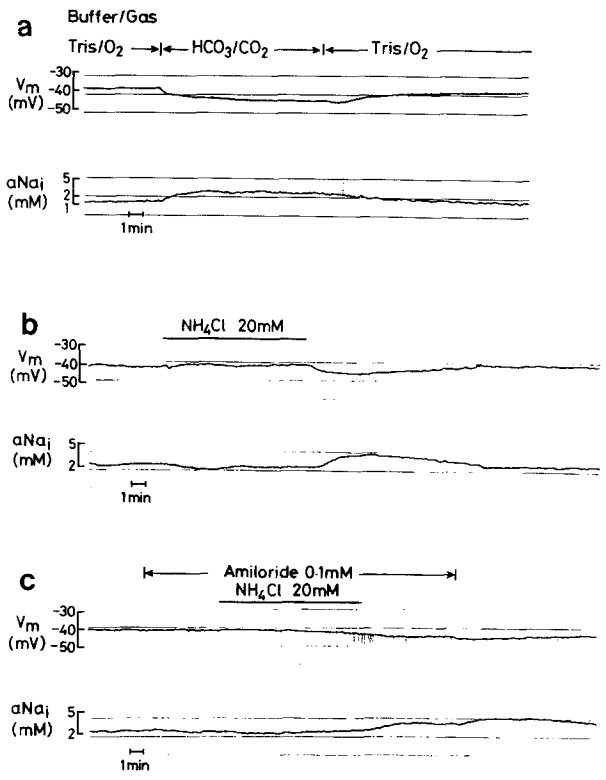

Fig. 8. Effect of acid loading on aNa_i. Tissues were loaded with acid by replacing $Tris^+$-buffered solution with HCO_3^- (25 mM) buffer solution bubbled with 5% CO_2/95% O_2 gas mixture (*a*) or NH_4Cl (20 mM replaced for NaCl) containing solution (*b*). Note that acid loading increased aNa_i (*a*, *b*). Presence of 0.1 mM amiloride suppressed the increase induced by NH_4Cl withdrawal and removal of amiloride produced a transient increase in aNa_i (*c*)

Discussion

Intracellular Na^+ Activity in the Nonstimulated and ACh-Stimulated Acinar Cells

The mean aNa_i determined under nonstimulated condition in the present study was 6.5 ± 0.5 mM (ranging from 0.3 to 24 mM), which was comparable to that determined in the pancreatic acini using Na^+-selective microelectrodes (O'Doherty & Stark, 1983). The least squares analysis showed no correlation between individual aNa_i and corresponding V_m values (Fig. 1). In various tissues of various origin, the aNa_i values so far reported varied, ranging from 3 to 50 mM (*see* Brown & Owen, 1979). The level of aNa_i in a cell could be determined by the balance between Na^+ influx and efflux across the membrane, and either process of Na^+ fluxes could be the rate-limiting step. It is conceivable that, if there is a significant Na^+ influx across the membrane via a Na^+-conductive pathway or a leak pathway due to incomplete sealing of the membrane around the impaled electrode, V_m will be depolarized and aNa_i will show a negative correlation against V_m. The present results, that there is no correlation between aNa_i and V_m, aNa_i is low and V_m (−38.9 ± 0.4 mV) is indifferent from those determined by using conventional single-barreled microelectrodes (Iwatsuki & Petersen, 1978; Suzuki & Petersen, 1985), strongly suggest that leak Na^+ influx around the penetrating electrode is negligible. The presence of continuous Na^+ influx in the nonstimulated condition was demonstrated by the addition of ouabain or superfusion with a K^+-free saline solution (Fig. 3*a* and *b*). In these an inhibition of the Na^+-K^+ pump resulted in an immediate and gradual increase in aNa_i. Thus this slow Na^+ influx seems to be the overall rate limiting step of Na^+ turnover in the nonstimulated condition.

Addition of ACh caused a marked increase in aNa_i due to an increase in Na^+ influx (Fig. 2), being in agreement with the results of ^{22}Na influx measurements in the rat lacrimal gland (Parod et al., 1980; Parod & Putney, 1980). In the lacrimal glands, the cause of the secretagogue-induced aNa_i increase has been attributed to an increase in the Na^+ permeability of the membrane (Parod & Putney, 1980), and patch-clamp experiments in the isolated cells demonstrated the presence of Ca^{2+}-activated non-selective cation channels in the basolateral membrane (Marty et al., 1984). Activation of the NaCl cotransport (Dartt et al., 1981) or $NaKCl_2$ cotransport (Suzuki & Petersen, 1985) has also been suggested for the rabbit and mouse lacrimal glands. On the basis of the present data in the mouse lacrimal gland, we suggest that most of the ACh-induced Na^+ influx consists of Na^+ fluxes mediated by Na^+/H^+ exchange, Na-K-Cl cotransport and metabolic substrate-dependent Na^+ transport, which explains about 50, 30 and 30% of the total Na^+ influx induced by ACh, respectively. At present, however, it is not clear whether these three components of Na^+ influx are independent of each other and additive. Also, our results do not rule out the presence of Ca^{2+}-activated nonselective cation channel in this tissue as one of the mediators of ACh-induced Na^+-influx. Rationale of the presence of three different Na^+ transport mechanisms is discussed below.

Na^+/H^+ Antiport

The findings to support the presence of Na^+/H^+ exchange transport in the mouse lacrimal gland are: (i) the addition of amiloride a potent inhibitor of Na^+/H^+ antiport (Kinsella & Aronson, 1980), significantly inhibited the ACh-induced increase in aNa_i (Figs. 6 and 7*b*) dose-dependently without affecting the membrane potential or the input resistance; (ii) acid loading the tissue by exposing to CO_2 or NH_4^+-containing solution increased aNa_i, while acid depletion decreased aNa_i (Fig. 8*a* and *b*); and (iii) amiloride inhibited the aNa_i increase induced by the withdrawal of NH_4Cl (Fig. 8*c*). In addition, our preliminary experiments (Saito, Ozawa & Nishiyama 1986*a*, and *unpublished data*) of intracellular pH measurements with H^+-selective microelectrode showed that addition of ACh caused an initial transient acidification of the intracellular pH followed by a profound alkalinization beyond the normal pH range, addition of amiloride or the replacement of extracellular Na^+ with N-methyl-D-glucamine inhibited the pH increase induced by ACh. A cholinergic stimulation of the rat lacrimal acinar cells significantly augments the rate of O_2 consumption (Herzog, Sies & Miller, 1976). Therefore, in the present study, an addition of ACh would increase the rate of CO_2 production and via the hydration of CO_2 in the cells the rate of H^+ production would be increased. Therefore, the aNa_i changes induced by ACh and acid loadings as well as the results of intracellular pH measurements are well explained by the presence of Na^+/H^+ antiporter, which is stimulated by an increase in the intracellular H^+ concentration at the modifier site (Aronson, Nee & Suhm, 1982).

Dose-response relations for amiloride (Fig. 7*a* and *b*) need some comments. Amiloride at a concentration of 10^{-6} M, a dose sufficient to inhibit the Na^+ channel of the apical membrane of some tight epithelia, had little effect on V_m and aNa_i of the resting state. It decreased the ACh-induced increase in aNa_i (Fig. 7*b*) only slightly. At concentrations between 10^{-5} and 10^{-4} M, amiloride significantly inhibited the ACh-induced change in aNa_i without affecting V_m and aNa_i of the resting acinar cells. Also the lack of any effect of amiloride on input resistance of both the resting and ACh-stimulated acinar cells suggests that Na^+/H^+ exchange in this tissue is electroneutral. Amiloride at 10^{-3} M, in addition to its inhibitory effect (70%) on ACh-induced aNa_i increase, caused a slight membrane depolarization, a gradual increase in aNa_i and a 30% suppression of ACh-induced hyperpolarization (Figs. 6 and 7). These effects could be attributable to an inhibition of the Na^+-K^+ ATPase of the membrane (Soltoff & Mandel, 1983) and/or Ca^{2+} influx (Parod & Putney, 1980).

Kuijpers et al. (1984) have shown that amiloride acts as a cholinergic muscarinic antagonist in rabbit pancreatic acinar cells with a K_i of about 30 μM. This possibility, however, can be ruled out in the case of the present study since (i) at an amiloride concentration of 0.1 mM, the magnitude of membrane hyperpolarization induced by ACh was unaffected and (ii) the magnitude of ACh-induced hyperpolarization is clearly dose-dependent (Saito et al., 1987) and the hyperpolarization evoked by ACh (1 μM) is completely abolished in the presence of atropine (1.4 μM) (Saito et al., 1985*a*).

Na-K-Cl Cotransport

Evidence for supporting the presence of Na-K-Cl cotransport in the lacrimal glands is manifold: (i) furosemide inhibits fluid secretion in the rabbit lacrimal gland (Dartt et al., 1981), (ii) application of furosemide or piretanide, and Cl^- replacement of the superfusing solution with NO_3^- but not Br^- shift the reversal potential for ACh-induced potential change towards E_K (Suzuki & Petersen, 1985), (iii) intracellular Cl^- activity in the mouse is higher than that predicted for the passive distribution (Saito et al., 1985*a*), (iv) an uphill Cl^- accumulation requires both Na^+ and K^+ in the bathing solution and (v) furosemide inhibits the uphill Cl^- accumulation (Saito et al., 1986*b*). In the present study in the absence of Cl^- or in the presence of loop diuretics in the superfusing solution the ACh-induced increase in aNa_i was reduced to about 70% control (Table 2). This finding, that a part of Na^+ influx induced by ACh requires Cl^- and is susceptible to loop diuretics in the extracellular fluid, offered another support for Na-K-Cl cotransport hypothesis. The mechanism of activation of Na-K-Cl cotransport by ACh stimulation was explained by an increase in the K^+ concentration in the outer surface of the basolateral membrane resulting from an activation of the Ca^+-dependent K^+ channel (Petersen & Maruyama, 1984).

It is noteworthy that furosemide does not interfere with the Na^+/H^+ exchange (Kinsella & Aronson, 1980). However, both furosemide and bumetanide also inhibit the carbonic anhydrase activity (Vogh & Langham, 1981) and the mouse exocrine lacrimal acinar cells contain carbonic anhydrase activity (Henniger, Schulte & Spicer, 1983). In the present experimental condition, where $Tris^+$-buffered solution was used and therefore PCO_2 in the tissue could be very low, the effect of carbonic anhydrase inhibition by loop diuretics on the ACh-

induced Na^+ influx can be neglected. However, under the conditions with ample CO_2 supply, a possibility of indirect effects of loop diuretics resulting from carbonic anhydrase inhibition should be considered.

Metabolic Substrate-Dependent Na^+ Uptake

It was somewhat surprising to find that substitution of metabolizable organic anions with NaCl decreased the aNa_i of the resting state and significantly reduced the ACh-induced Na^+ influx (Fig. 5, Table 3). In these experiments, the Na^+ concentration, pH and osmolality of the substrate-free superfusate were identical with those in the standard solution. Also, it was confirmed that interference of these substrates with Na^+ electrode was negligible. If the supply of the metabolites to the ATP-producing pathways is reduced by removing these anions from the superfusate, aNa_i would increase due to limitation of the Na^+/K^+ ATPase activity. However, the removal of the substrates resulted in a decrease in aNa_i. Therefore we speculate the presence of metabolic substrate-dependent Na^+ transport. Among the substrates present in the perfusate, glucose had no effect on aNa_i. Na^+-dependent amino acid transport has been demonstrated in the plasma membrane vesicles isolated from the rat exorbital lacrimal gland (Mircheff et al., 1983). However, in the present study addition of 15 mM amino acids had no effect on either aNa_i or V_m. Addition of either one of the organic acids, glutamate, fumarate and pyruvate slightly increased the aNa_i. It is possible that these organic anions, the energy sources for TCA cycle, are cotransported with Na^+ like in the kidney proximal tubules (for ref. *see* Wright, 1984) and that activation of the metabolism by ACh addition stimulates the substrate-Na^+ cotransport system. To examine this possibility, Na^+-dependence of the metabolite uptake should be studied.

We thank Merck & Co., Rahway, N.J., and Hoechst Japan, Kawagoe, Japan, for the gifts of amiloride and furosemide, respectively, and Miss Yoko Aizawa for typing the manuscript. This work was supported in part by grants from the Japanese Ministry of Education, Culture and Sciences.

References

Alexander, J.H., Lennep, E.W. van, Young, J.A. 1972. Water and electrolyte secretion by the exorbital lacrimal gland of the rat studied by micropuncture and catheterization techniques. *Pfluegers Arch.* **337:**299–309

Aronson, P.S., Nee, J., Suhm, M.A. 1982. Modifier role of internal H^+ in activating Na^+-H^+ exchanger in renal microvillus membrane vesicles. *Nature (London)* **299:**161–163

Brown, H.M., Owen, J.D. 1979. Micro ion-selective electrodes for intracellular ions. *Ion-Selective Electrode Rev.* 1:145–186

Cassola, A.C., Mollenhauer, M., Fromter, E. 1983. The intracellular chloride activity of rat kidney proximal tubular cells. *Pfluegers Arch.* **399:**259–265

Dartt, D.A., Moller, M., Poulsen, J.H. 1981. Lacrimal gland electrolyte and water secretion in the rabbit: Localization and role of (Na^+ + K^+)-activated ATPase. *J. Physiol. (London)* **321:**557–569

Dufresne, M., Bastie, M.J., Vaysse, N., Creach, Y., Hollande, E., Ribet, A. 1985. The amiloride sensitive Na^+/H^+ antiport in guinea pig pancreatic acini: Characterization and stimulation by celrulein. *FEBS Lett.* **187:**126–130

Hellmessen, W., Christian, A.L., Fasold, H., Schulz, I. 1985. Coupled Na^+-H^+ exchange in isolated acinar cells from rat exocrine pancreas. *Am. J. Physiol.* **249:***G*125–*G*136

Henniger, R.A., Schulte, B.A., Spicer, S.S. 1983. Immunolocalization of carbonic anhydrase isozymes in rat and mouse salivary and exorbital lacrimal glands. *Anat. Rec.* **207:**605–614

Herzog, V., Sies, H., Miller, F. 1976. Exocytosis in secretory cells of rat lacrimal gland. Peroxidase release from lobules and isolated cells upon cholinergic stimulation. *J. Cell Biol.* **70:**692–706

Iwatsuki, N., Petersen, O.H. 1978. Intracellular Ca^{2+} injection causes membrane hyperpolarization and conductance increase in lacrimal acinar cells. *Pfluegers Arch.* **377:**185–187

Kinsella, J.L., Aronson, P.S. 1980. Properties of the Na^+-H^+ exchanger in renal microvillus membrane vesicles. *Am. J. Physiol.* **238:***F*461–*F*469

Kuijpers, G.A.J., Depont, J.J.H.H.M., Nooy, I.G.P. van, Fleuren-Jakobs, A.M.M., Bonting, S.L., Rodrigues De Miranda, J.F. 1984. Amiloride is a cholinergic antagonist in the rabbit pancreas. *Biochim. Biophys. Acta* **804:**237–244

Marty, A., Tan, Y.P., Trautmann, A. 1984. Three types of calcium-dependent channel in rat lacrimal glands. *J. Physiol. (London)* **357:**293–325

Mircheff, A.K., Lu, C., Conteas, C.N. 1983. Resolution of apical and basal-lateral membrane populations from rat exorbital gland. *Am. J. Physiol.* **245:***G*661–*G*667

O'Doherty, J., Stark, R.J. 1983. A transcellular route for Na-coupled Cl transport in secreting pancreatic acinar cells. *Am. J. Physiol.* **245:***G*499–*G*503

Parod, R.J., Leslie, B.A., Putney, J.W., Jr. 1980. Muscarinic and α-adrenergic stimulation of Na and Ca uptake by dispersed lacrimal cells. *Am. J. Physiol.* **239:***G*99–*G*105

Parod, R.J., Putney, J.W., Jr. 1980. Stimulus-permeability coupling in rat lacrimal gland. *Am. J. Physiol.* **239:***G*106–*G*113

Petersen, O.H., Maruyama, Y. 1984. Calcium-activated potassium channels and their role in secretion. *Nature (London)* **307:**693–695

Saito, Y., Ozawa, T., Hayashi, H., Nishiyama, A. 1985*a*. Acetylcholine-induced change in intracellular Cl^- activity of the mouse lacrimal acinar cells. *Pfluegers Arch.* **405:**108–111

Saito, Y., Ozawa, T., Hayashi, H., Nishiyama, A. 1985*b*. Cl and K activities in the mouse lacrimal acinar cells and the effect of secretagogues. *J. Physiol. Soc. Jpn.* **47:**389

Saito, Y., Ozawa, T., Hayashi, H., Nishiyama, A. 1987. The effect of acetylcholine on chloride transport across the mouse lacrimal gland acinar cell membranes. *Pfluegers Arch. (in press)*

Saito, Y., Ozawa, T., Nishiyama, A. 1986*a*. Multiple Na entry mechanisms in the mouse lacrimal gland acinar cells as studied with Na-selective microelectrode method. *In:* Gastroin-

testinal Secretion Symposium. July, 1986, Calgary, Alberta, Canada. The proceedings. (*in press*)

Saito, Y., Ozawa, T., Nishiyama, A. 1986*b*. Transcellular chloride transport by acinar cells of the mouse lacrimal gland. *Proc. Int. Union Physiol. Sci.* **16:***p*480

Seow, K.T.F., Lingard, J.M., Young, J.A. 1986. Anionic basis of fluid secretion by rat pancreatic acini in vitro. *Am. J. Physiol.* **250:***G*140–*G*148

Soltoff, S.P., Mandel, L.J. 1983. Amiloride directly inhibits the Na,K-ATPase activity of rabbit kidney proximal tubules. *Science* **220:**957–959

Steiner, R.A., Oehme, M., Ammann, D., Simon, W. 1979. Neutral carrier sodium ion-selective microelectrode for intracellular studies. *Anal. Chem.* **51:**351–353

Suzuki, K., Petersen, O.H. 1985. The effect of Na and Cl removal and of loop diuretics on acetylcholine-evoked membrane potential changes in mouse lacrimal acinar cells. *Q. J. Exp. Physiol.* **70:**437–445

Thomas, R.C. 1974. Intracellular pH of snail neurones measured with a new pH-sensitive glass micro-electrode. *J. Physiol. (London)* **238:**159–180

Vogh, B.P., Langham, M.R., Jr. 1981. The effect of furosemide and bumetanide on cerebrospinal fluid formation. *Brain Res.* **221:**171–183

Wright, E.M. 1984. Electrophysiology of plasma membrane vesicles. *Am. J. Physiol.* **246:***F*363–*F*372

Yoshitomi, K., Fromter, E. 1984. Cell pH of rat proximal tubule in vivo and the conductive nature of peritubular HCO_3^- (OH^-) exit. *Pfluegers Arch.* **402:**300–305

Zeuthen, T. 1980. How to make and use double-barreled ion-selective microelectrodes. *Curr. Topics Membr. Transp.* **13:**31–47

Zeuthen, T. 1983. Ion activities in the lateral intercellular spaces of gallbladder epithelium transporting at low external osmolarities. *J. Membrane Biol.* **76:**113–122

Zeuthen, T., Machen, T. 1984. HCO_3^-/CO_2 stimulates Na^+/H^+ and Cl^-/HCO_3^- exchange in *Necturus* gallbladder. *In:* Hydrogen Ion Transport in Epithelia. J.G. Forte, D.G. Warnock, and F.C. Rector, Jr., editors. pp. 97–108. Wiley, New York

Received 18 December 1986; revised 9 April 1987