IONS AND ENERGY METABOLISM IN DUCK SALT-GLAND: POSSIBLE ROLE OF FUROSEMIDE-SENSITIVE CO-TRANSPORT OF SODIUM AND CHLORIDE

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SUMMARY

1. The effects of methacholine on net ionic movements and energy metabolism of the avian salt-gland have been studied, using slices of glands taken from salt-adapted Pekin ducks. The slices were equilibrated with media and drugs for 120 min at 1 °C before the experimental incubation at 38 °C.

2. During incubation at 38 °C the slices accumulated K^+ and lost Na^+ and Cl^-. In the presence of methacholine, they retained more Na^+ and Cl^- and accumulated less K^+, the maximal effects being given by 0·5–1·0 mM-methacholine. Similar results were obtained whether the medium contained 10 mM-Tris (used in most experiments) or 25 mM-HCO_3^- as the major buffer.

3. The higher final levels of cell Na^+ and Cl^- induced by methacholine were not seen when furosemide (1 mM) was also present. Methacholine did not induce a higher level of cell Na^+ when medium Cl^- was replaced by I^-, NO_3^- or SO_4^{2-}, and did not induce a higher Cl^- content when medium Na^+ was replaced by choline or Li^+. The fall of K^+ accumulation caused by methacholine was also prevented by furosemide or by replacing Cl^- in the medium with other anions. The anion-transport inhibitors, SCN^- (up to 10 mM) and 4,4′-diisothiocyano-2,2′-disulphonic acid stilbene (DIDS) (up to 2 mM) did not prevent the effects of methacholine.

4. Methacholine stimulated respiration and lowered the slice ATP contents, and these effects were both prevented by ouabain or furosemide. Ouabain, but not furosemide, also reduced the basal (i.e. in the absence of methacholine) rate of respiration and raised the ATP level. SCN^- and DIDS had no effect on basal or stimulated respiration or on ATP contents.

5. The respiratory stimulation and fall of ATP induced by methacholine were totally prevented if medium Na^+ was replaced by choline. Replacement of Na^+ by Li^+ caused some stimulation of basal respiration; it also permitted some loss of ATP in the presence of methacholine, but the loss was smaller than that seen in the normal Na^+ medium.

6. The respiratory stimulation and fall of ATP induced by methacholine were prevented if medium Cl^- was replaced by SO_4^{2-}. The effects of methacholine were partially blocked when NO_3^- replaced Cl^-.

7. The results are consistent with the stimulation by methacholine of a furosemide-sensitive, coupled entry of Na^+ and Cl^- into the cells, associated with a loss of K^+. This would result in a stimulation of Na^+ extrusion by the ouabain-sensitive transport system for Na^+ and K^+ with increased consumption of ATP.

INTRODUCTION

The secretion of NaCl by the avian salt-gland is stimulated by cholinergic agents *in vivo* (Fänge, Schmidt-Nielsen & Robinson, 1958; Peaker & Linzell, 1975). Precise definition of the underlying ion-transport mechanisms is hindered for want of a tissue preparation in which the polarity of trans-epithelial ion movements can be distinguished *in vitro*, but useful information can be obtained from studies of tissue slices or isolated cells. In particular, observations have been made of the net or unidirectional movements of ions between the cells and their medium (van Rossum, 1964, 1966; Peaker, 1971; Hootman & Ernst, 1981), and of the altered metabolic activity induced by cholinergic agents (Borut & Schmidt-Nielsen, 1963; Hokin, 1966; van Rossum, 1968; Stewart, Sax, Funk & Sen, 1979; Hootman & Ernst, 1980). In the latter case, it is necessary to assume that the cholinergic stimulation of metabolism is closely related to the stimulation of secretion.

The importance of the coupled transport of Na^+ and K^+ as a component of the secretory mechanism is suggested by the finding that the stimulation of respiration and fall of ATP levels induced by cholinergic agents are prevented by ouabain (Borut & Schmidt-Nielsen, 1963; van Rossum, 1964, 1968; Hokin, 1966; Hootman & Ernst, 1980). Further, there is a marked increase in specific activity of the Na^+ and K^+-stimulated adenosine triphosphatase (Na-K-ATPase) in the glands of birds adapting to a high salt intake (Ernst, Goertemiller & Ellis, 1967). However, this enzyme is situated exclusively in the basolateral regions of the epithelial cells (Ernst, 1972; Ernst & Mills, 1977), a distribution which implies that it cannot directly secrete Na^+ across the apical membranes into the lumina of the secretory ducts. Ernst & Mills (1977) and Riddle & Ernst (1979) have proposed that the electrochemical potential maintained by the active extrusion of Na^+ into the intercellular spaces, by the Na-K-ATPase, provides the requisite energy for the uptake into the cells of Cl^- coupled to the downhill movement of Na^+, via a neutral, coupled carrier. In this model, intracellular Cl^- then moves down its electrochemical gradient across the apical interface, so generating a transepithelial potential (lumen negative) favouring paracellular Na^+ entry into the luminal compartment across cation-permeable tight junctions. Cholinergic agents could then act by increasing, directly or indirectly, the entry of NaCl across the basal membranes, thus allowing more ready access of Na^+ from the circulation to the intracellular aspect of the Na-K-ATPase. This would be in accord with experiments on Na^+ fluxes (van Rossum, 1964, 1966). A net increase of intracellular Na^+ in response to cholinergic agents might also be anticipated, but has not yet been observed (van Rossum, 1966; Peaker, 1971). However, the experimental conditions of the earlier work, which included the use of only a single concentration of cholinergic drug, may not have been optimal for detection of net changes of cellular ionic content.

In an alternative hypothesis, Peaker & Stockley (1974) postulated that Cl^- enters

at the basolateral border of the epithelial cells in exchange for HCO_3^-. The Cl^- is then actively secreted across the apical membrane by an anion transporting system which is directly subject to cholinergic stimulation.

In the work described below we have examined some aspects of the hypotheses described above, using slices of salt-gland from ducks. We show that methacholine (acetyl-β-methylcholine) can produce a net increase in the cellular contents of Na^+ and Cl^-, and that the effects of methacholine on the ions, as well as on respiration and ATP content, are sensitive to treatments which are likely to affect a coupled transport of Na^+ and Cl^-. Many of these results are analogous to findings of Hootman & Ernst (1981) who examined the effects of cholinergic stimulation on the binding of [³H]ouabain to isolated epithelial cells of salt-gland.

METHODS

Domestic Pekin ducks (*Anas platyrhynchos*) were obtained as newly hatched ducklings (Ridgeway Hatcheries, La Rue, OH) and were fed *ad libitum* on a chick starting mash. For the first week they were given tap water to drink; for the second week, tap water for approximately 16 h per day and 1 % NaCl (w/v in tap water) for 8 h; for the third week, tap water for 8 h and 1 % NaCl for 16 h. Subsequently they were given only 1 % NaCl *ad libitum* to drink. Ducks were taken for experiments from the end of the fourth week. A regimen of salt stress similar to the above has previously been found to elicit morphological differentiation of the gland (Ernst & Ellis, 1969) and high activity of Na-K-ATPase (Ernst *et al.* 1967; Ernst & Mills, 1977). The ducks were killed by decapitation and slices of the salt glands were prepared as described before (van Rossum & Ernst, 1978). The glands from one or two birds were used for each experiment, the slices being pooled at the start of incubation.

The general procedure involved a pre-incubation period of 120 min at 1 °C followed by experimental incubation at 38 °C. The control incubation medium, referred to as Tris-phosphate Ringer solution, contained (mM): Na^+, 150; K^+, 5·0; Ca^{2+}, 1·2; Mg^{2+}, 1·0; Cl^-, 164; SO_4^{2-}, 1·0; phosphate, 2·0 and Tris, 10·0; the pH was 7·4 and the solution was gassed with O_2. Inulin was present at a concentration of 0·5 % (w/v) as marker for the extracellular water. In some experiments, all the Cl^- anions of the Tris/phosphate Ringer solution were replaced by other anions; NO_3^- or I^- were then present at 164 mM and SO_4^{2-} at 82 mM, the osmotic difference being made up in this last case with mannitol. In analogous experiments, Na^+ was totally replaced with Li^+ or choline. Finally, some experiments were done with a bicarbonate-buffered Ringer solution in which 25 mM-HCO_3^- was used instead of Tris. The Cl^- concentration of this medium and all other components were as in the Tris-phosphate medium; this solution was gassed with 95 % O_2/5 % CO_2 and had a pH of 7·4.

The drugs used, methacholine, ouabain, 4,4'-diisothiocyano-2,2'-disulphonic acid stilbene (DIDS) and furosemide were added to the Ringer solutions without substitution for other components. The first three of these agents were obtained from Sigma Chemical Co., while furosemide was a generous gift from Hoechst-Roussel Inc., Somerville, NJ, U.S.A.

In most experiments, all gland slices were placed together in 20 ml of the Tris-phosphate Ringer solution contained in a beaker standing in ice, for preincubation at 1 °C. They were transferred to a fresh, 20 ml portion of the medium after 10 min, to remove tissue debris and materials leaking from the cells (e.g. K^+ and cytosolic proteins). After 30 min at 1 °C, the slices were distributed in 30–60 mg (wet wt.) lots over fourteen Warburg or Erlenmeyer (25 ml) flasks, each of which contained 3 ml Ringer solution together with the drugs to be tested. Pre-incubation at 1 °C was then continued for a further 90 min. In the ion-substitution experiments this procedure was slightly modified in that the slices were divided at the outset over incubation vessels containing 10 ml each of the anion- or cation-substituted media to be tested; transfer to a fresh portion of the same medium at 10 min, and distribution over Warburg vessels after 30 min, was carried out as above. In this way, the slices were equilibrated in the modified media throughout the pre-incubation, and endogenous contents of Cl^- or Na^+ were greatly reduced by the washing procedure.

After 120 min at 1 °C, the slices from two to three flasks were collected for analysis (see below).

The remaining flasks were transferred to a shaking water bath at 38 °C for the experimental incubation, the period of which is indicated in the text. Readings of O_2 consumption were taken at 10 min intervals, after an initial 10 min equilibration period (Umbreit, Burris & Stauffer, 1949). After incubation, the slices were collected rapidly and treated by one of the two procedures described by van Rossum & Ernst (1978), according to the analyses to be made; i.e. slices to be studied for water and ionic contents were dried and extracted with 0·1 N-HNO_3, while those to be analysed for adenine nucleotides were homogenized in 8 % perchloric acid/40 % ethanol at −20 °C. Analytical procedures were those used by van Rossum (1972).

With the above procedures, the drugs used were present in the incubation media for the last 90 min at 1 °C, to allow equilibration with the tissue, and throughout the experimental incubation at 38 °C. The different treatments were studied in duplicate or triplicate in each experiment, each experiment being performed at least three times. Values given in the text and tables are mean ± S.E. of the mean with number of observations in parentheses, where each observation represents the value obtained from the slices contained in a single incubation vessel. Results are expressed per unit slice dry wt for the slices which were dried, and per unit slice protein in the case of the slices that were homogenized in perchloric acid/ethanol. Tests for statistical difference were done with Student's t test.

RESULTS

Effects of methacholine and ouabain

Ion contents. In previous work, slices of herring-gull salt-gland lost K^+ and gained Na^+ during pre-incubation at 1 °C and then showed no change during subsequent incubation in oxygenated medium at 25 °C, either in the absense or presence of metacholine (van Rossum, 1966). Subsequently, we found that the loss of K^+ and gain of Na^+ could be reversed by metabolism-dependent processes if the incubation in oxygenated medium was conducted at 38 °C (van Rossum & Ernst, 1978), and it seemed possible that an effect of methacholine on the ionic content might also become apparent under these conditions. In order to select a suitable incubation time, we determined the time course of the changes of intracellular K^+ and Na^+ at 38 °C (Fig. 1). A steady state was established in 10 min and was maintained until at least 60 min (the longest time is not shown). Ouabain (0·5 mM) not only prevented the accumulation of K^+ and extrusion of Na^+, but permitted further loss of K^+ and gain of Na^+ beyond that taking place during the pre-incubation at 1 °C, and K^+ did not attain a steady state by 60 min.

We adopted 60 min as the standard period at 38 °C for studying the effects of different concentrations of methacholine. The increase of intracellular K^+ and decrease of Na^+ upon rewarming were partially prevented by methacholine (Fig. 2). A net loss of Cl^- at 38 °C was also reduced. Maximal effects were given by 0·5–1·0 mM-methacholine.

In further experiments we compared the intracellular ionic contents of slices incubated for 120 min at 1 °C and 60 min at 38 °C in Ringer solutions in which either 25 mM-HCO_3^- plus 2 mM-phosphate (Table 1) or 10 mM-phosphate (not shown) replaced the usual Tris and phosphate buffers. The tissue ionic contents were similar in each medium and 1 mM-methacholine increased the intracellular Na^+ and Cl^-, and decreased intracellular K^+, in each case; however, statistical significance was not attained with the effects of methacholine on Cl^- in the bicarbonate Ringer solution. Table 1 also shows that methacholine had no significant effect in the presence of ouabain, all values for treatment with methacholine plus ouabain being similar to those with ouabain alone.

Respiration and adenine nucleotides. The consumption of O_2 was considerably stimulated by methacholine, with maximal effect at 0·2 mM (not shown). Our measurements in the Tris-phosphate Ringer solution show a similar concentration dependence to those of Stewart *et al.* (1979) which were done in a bicarbonate-buffered medium. The time course of respiratory stimulation by methacholine varied from one
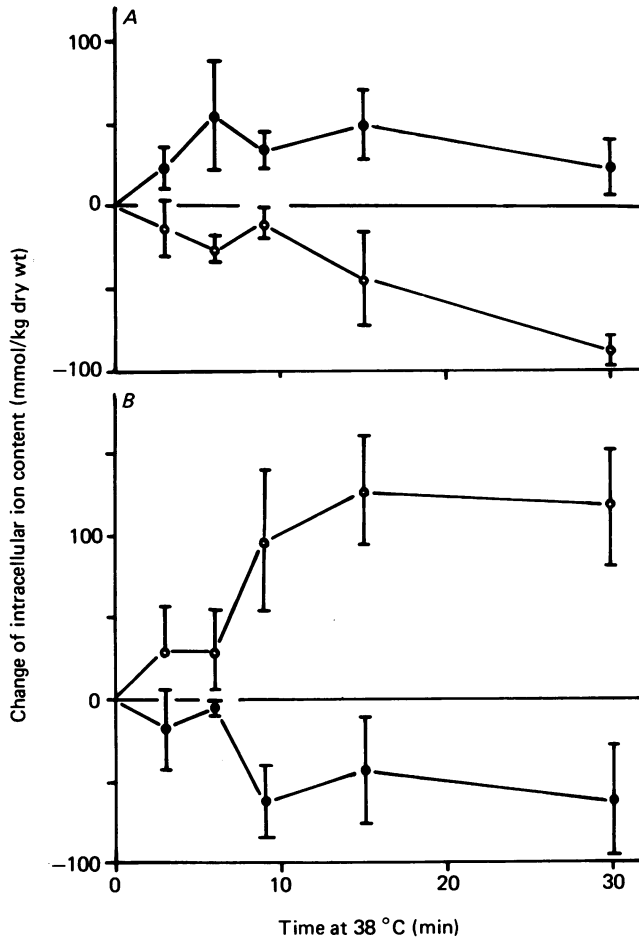

Fig. 1. Time course of the changes of (*A*) intracellular K^+ and (*B*) intracellular Na^+ contents during incubation at 38 °C. Net changes of content were determined as the content of slices analysed after incubation at 38 °C for the indicated times minus the content of slices analysed after the pre-incubation at 1 °C. For each point, $n = 9$, ●, control; ○, with ouabain (0·05 mM).

series of experiments to another. On occasion, there was a tendency for the control rate to decline slightly while the stimulated respiration remained constant for 2–3 observation periods before itself declining; in this case the degree of stimulation showed a maximum after 25–35 min, as in Fig. 3. In other experiments (e.g. Fig. 4), the stimulation by methacholine was already maximal during the first observation

period. Ouabain abolished the methacholine-induced stimulation of respiration and further reduced O_2 consumption to the same depressed rate as that seen in control slices to which ouabain was added in the absence of the agonist (Fig. 3).

The adenine nucleotide contents of incubated slices are shown in Table 2. The ATP content after pre-incubation at 1 °C was higher than after further incubation at 38 °C
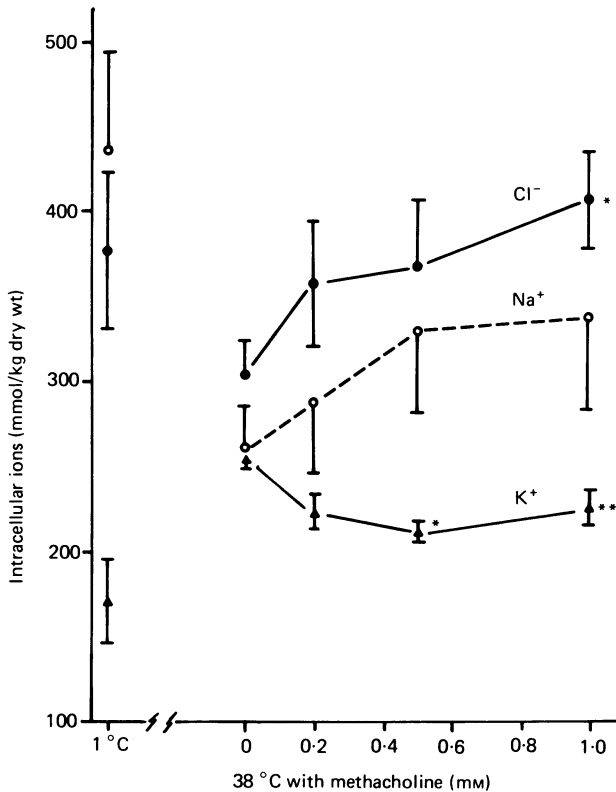

Fig. 2. Effects of different concentrations of methacholine on the intracellular ion contents of salt-gland slices. The three points at the extreme left show the contents of slices analysed after pre-incubation for 120 min at 1 °C; values obtained in the presence of 1 mM-methacholine at 1 °C did not differ significantly from those in its absence, and the results at this temperature have therefore been pooled; $n = 8$. The remaining points represent the contents of slices subsequently incubated for 60 min at 38 °C; $n = 10$. For significance of difference from the value at 38 °C without methacholine, $*P < 0.01$, $**P < 0.05$.

in the absence of drugs, the loss occurring in the first 10 min (van Rossum & Ernst, 1978). However, the ratio ATP:ADP and the adenylate energy charge were unchanged by incubation at 38 °C; the energy charge is defined according to Atkinson (1968) as: $(ATP + 0.5 \times ADP)/(ATP + ADP + AMP)$. The fall of ATP was significantly reduced in the presence of ouabain, suggesting that it resulted from the consumption of ATP by the coupled transport of Na^+ and K^+. Methacholine caused a drastic reduction of ATP content (cf. Hokin, 1966) and of the energy charge, both of which were prevented by ouabain (Table 2).

TABLE 1. Effects of methacholine and ouabain on net movements of intracellular ions in two media. Slices were incubated for the times indicated either in Ringer solution buffered with 10 mM-Tris plus 2 mM-phosphate, or in Ringer solution buffered with 25 mM-HCO_3^- plus 2 mM-phosphate. Other details of medium composition are given in Methods. Each value is the mean ± S.E.M. of six observations in the HCO_3^- and phosphate Ringer solution and of fifteen observations in the Tris and phosphate Ringer solution. Ouabain and methacholine were added after the first 30 min at 1 °C. Neither agent affected the ion contents during pre-incubation at 1 °C, and the results at this temperature have therefore been pooled

Intra-cellular ions	Medium	120 min at 1 °C	Incubation (mmol/kg dry wt)			
			Further 60 min at 38 °C with additions			
			None	Methacholine (1 mM)	Ouabain (0·05 mM)	Methacholine + oubain
Na^+	Tris + phosphate	391 ± 36	250 ± 19	345 ± 38*	535 ± 55***	606 ± 51
	HCO_3^- + phosphate	475 ± 75	283 ± 20	457 ± 42***	662 ± 57***	701 ± 65
Cl^-	Tris + phosphate	379 ± 26	316 ± 15	409 ± 21***	497 ± 59***	509 ± 33
	HCO_3^- + phosphate	406 ± 58	311 ± 21	380 ± 40	530 ± 45***	540 ± 67
K^+	Tris + phosphate	195 ± 8	247 ± 8	214 ± 12**	74 ± 8***	64 ± 8
	HCO_3^- + phosphate	176 ± 14	232 ± 13	169 ± 12***	65 ± 5***	62 ± 6

* $P < 0·05$ for difference from corresponding slices at 38 °C without additions, by Student's t test; **$P < 0·02$; ***$P < 0·01$.
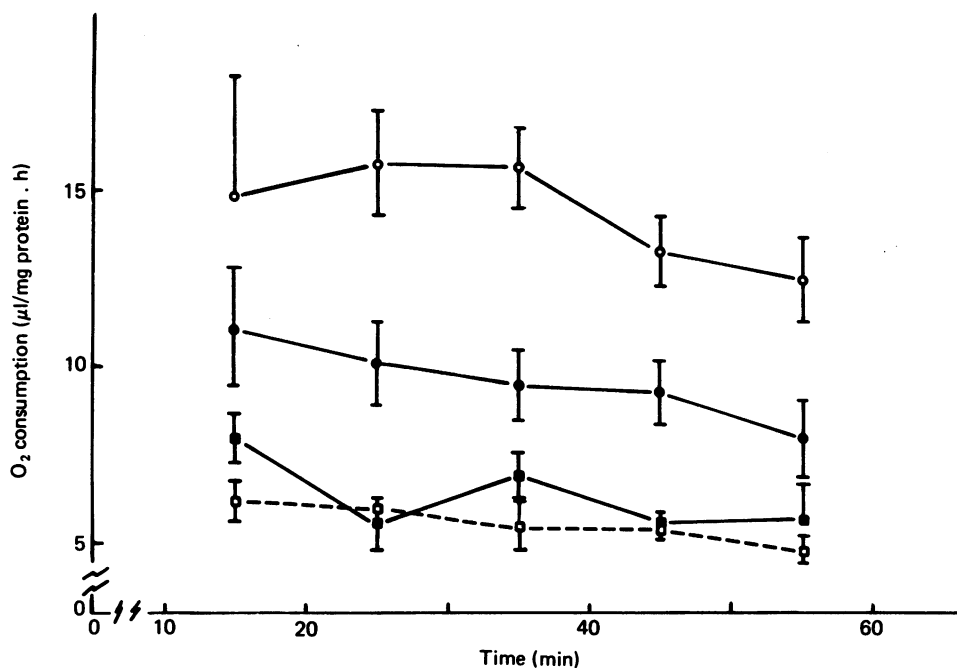

Fig. 3. Effects of methacholine and ouabain on the rate of O_2 consumption by salt-gland slices during the course of incubation. Results are from the same slices as Table 2. ○, methacholine (1 mM); ●, control; □, ouabain (0·05 mM); ■, ouabain plus methacholine. For each point, $n = 6$.

Substitution of medium ions

Experiments were next done to see whether the responses of ions and of energy metabolism to methacholine were affected by the removal of Na^+ or Cl^- from the incubation medium.

Na^+-free medium. When medium Na^+ was replaced by Li^+ or choline, the Na^+ content of the slices after pre-incubation at 1 °C was 85-95 % less than that of slices

TABLE 2. Effects of methacholine and ouabain on adenine nucleotides of salt gland slices. Slices were incubated for the times indicated in Tris plus phosphate-buffered medium. Each value is the mean ± s.e. of mean of six observations. The adenylate 'energy charge' was calculated as in Atkinson (1968). Other details as in Table 1 and Methods

		Incubation			
		Further 60 min at 38 °C with additions			
	120 min at 1 °C	None	Methacholine (1 mM)	Ouabain (0·05 mM)	Methacholine + ouabain
ATP mmol/kg protein	8·1 ± 0·9	4·9 ± 0·7	2·3 ± 0·3	7·0 ± 0·7	6·5 ± 0·5
ADP mmol/kg protein	1·6 ± 0·2	1·1 ± 0·1	1·0 ± 0·1	1·2 ± 0·1	1·1 ± 0·1
AMP mmol/kg protein	1·1 ± 0·1	0·7 ± 0·1	0·8 ± 0·1	0·6 ± 0·1	0·5 ± 0·1
ATP/ADP	4·9 ± 0·1	4·6 ± 0·4	2·3 ± 0·3	5·7 ± 0·5	6·4 ± 0·9
'Energy charge'	0·82 ± 0·01	0·81 ± 0·01	0·68 ± 0·02	0·86 ± 0·02	0·87 ± 0·01

incubated in medium containing Na^+ (Table 3). The Cl^- content was then also somewhat lower, presumably because the substituting cations entered the cells rather less readily than Na^+. In contrast to the results in Na^+-Ringer solution, subsequent incubation of slices in Na^+-substituted media at 38 °C did not lead to a net extrusion of Cl^- and did not result in a higher content of Cl^- in the presence of methacholine (Table 3).

Analogous effects of Na^+-free media were obtained on energy metabolism. Neither choline nor Li^+ permitted significant stimulation of respiration by methacholine, and the substitution of Na^+ by choline completely protected slice ATP against the fall induced by methacholine (Table 4). Replacement of Na^+ by Li^+ also protected ATP, but less completely.

The basal rate of respiration (i.e. in the absence of methacholine) was affected differently by the two cationic substituents. In the choline Ringer solution, respiration was reduced to a rate similar to that seen with ouabain (compare Table 4 with Fig. 3), an effect which may be anticipated from the expectation that removal of Na^+ would inhibit the Na-K-ATPase. By contrast, substitution of Na^+ by Li^+ caused some increase of basal respiratory rate, while not affecting the ATP content (Table 4).

Cl^--free medium. The residual Cl^- in the Tris-phosphate Ringer solution in which NO_3^- or SO_4^{2-} was the principal anion was determined to be 0·04 and 0·06 mM respectively. The Cl^- content of the slices after incubation in these media was 5-10 % of the total Cl^- content of slices incubated in the chloride-containing medium, or about 40 % of the intracellular content (Table 5), indicating some retention of Cl^- in the tissue despite the washing procedure (see Methods). The intracellular Na^+

TABLE 3. Effects of replacing medium Na^+ by other cations on the intracellular ionic content of salt-gland slices incubated with and without methacholine. Sodium in the Tris-phosphate Ringer solution was replaced by Li^+ or choline, as indicated. Methacholine was used at 1 mM. In this series of experiments (in contrast to all others) the ions were assayed in the percholoric acid extract of the wet slices, instead of in a nitric acid extract of dried slices; the ionic contents are therefore expressed in mmol/kg slice protein. For further experimental details, see Methods

Principal cation in medium	Intracellular Na^+ (mmol/kg protein)			Intracellular Cl^- (mmol/kg protein)		
	Na^+	Li^+	Choline	Na^+	Li^+	Choline
Pre-incubated 120 min at 1 °C Then 60 min at 38 °C:	706±100 (4)	118±42 (4)	51±42 (4)	596±80 (3)	241±7 (3)	375±11 (3)
Control	435±40 (7)	63±16 (8)	95±38 (8)	406±27 (6)	307±19 (6)	356±25 (6)
Methacholine	531±50 (8)	55±18 (8)	51±19 (9)	511±23* (6)	293±31 (6)	378±39 (6)
Difference due to methacholine	96±65	-8±24	-44±42	105±36	-14±36	22±45

* Significantly different from value in absence of methacholine, $P < 0.01$.

contents after pre-incubation at 1 °C in the Cl⁻, NO_3^- and SO_4^{2-} Ringer solutions did not differ significantly from each other and the same was true of the K^+ contents. The assay method for Cl⁻ was an electrometric titration with Ag^{2+} which also responds to I⁻, and the values given for slices incubated in the I⁻ Ringer solution thus represent the content of residual Cl⁻ plus I⁻. The Na^+ content of the slices after pre-incubation at 1 °C in the medium containing I⁻ was lower, and the K^+ content higher, than in the other media (Table 5).

TABLE 4. Effects of replacing medium Na^+ by other cations on the respiration and ATP content of slices incubated with and without methacholine. The results are from the same slices as Table 3. Measurements of O_2 consumption were started after 10 min equilibration at 38 °C. The rate of respiration tended to decline during the course of incubation and the initial (10–20 min of incubation at 38 °C) and final (50–60 min) readings are therefore given

Principal cation of medium		Na^+	Li^+	Choline
O_2 consumption (μl/mg protein.h)				
Initial:	Control	13·7 ± 1·0 (8)	15·0 ± 0·9 (8)	6·4 ± 1·0 (8)†
	Methacholine (1 mM)	20·6 ± 1·7 (8)*	15·8 ± 2·0 (8)	9·5 ± 1·3 (8)†
Final:	Control	10·8 ± 0·9 (8)	15·4 ± 1·3 (8)	8·0 ± 0·9 (8)
	Methacholine	16·0 ± 1·4 (8)*	14·5 ± 1·8 (8)	8·5 ± 1·0 (8)†
ATP content at end of incubation (mmol/kg protein)				
	Control	6·4 ± 0·6 (7)	7·1 ± 0·5 (8)	7·7 ± 0·8 (8)
	Methacholine	3·2 ± 0·2 (8)*	4·8 ± 0·3 (8)*†	8·2 ± 0·7 (7)†

* Significantly different from value in the absence of methacholine, $P < 0.01$.
† Significantly different from corresponding value in Na^+ medium, $P < 0.01$.

Upon subsequent incubation at 38 °C, the net accumulation of K^+ which normally occurs in the presence of Cl⁻ was not seen when this anion was replaced by others, while the usual extrusion of Na^+ was reduced. A most marked effect was the failure of methacholine to influence intracellular Na^+ or K^+ contents in any of the Cl⁻-free media, although methacholine caused significant increases of Na^+ and Cl⁻, and decrease of K^+, in the chloride-containing medium (Table 5). Furthermore, as was the case when medium Na^+ was replaced by other cations (Table 4), there was no significant stimulation of respiration by methacholine when I⁻ or SO_4^{2-} were used to replace Cl⁻ (Fig. 4).

The control tissue contents of ATP were not significantly altered when NO_3^- or SO_4^{2-} replaced medium Cl⁻ (Table 5). The fall of ATP content induced by methacholine was prevented if SO_4^{2-} replaced Cl⁻, while replacement with NO_3^- gave a significant, although partial, protection of ATP. Tissue contents of ATP were higher in the few measurements carried out in the I⁻-containing medium, but again methacholine caused no reduction.

The behaviour of the slice respiration when NO_3^- was used to replace Cl⁻ in the medium was rather different from that seen with the other substituting anions in that the basal rate of O_2 consumption in the NO_3^- medium was greater than in the Cl⁻ medium; the difference was statistically significant at 25 and 35 min ($P < 0.05$). There also appeared to be a tendency for methacholine to stimulate respiration in the NO_3^- medium; this failed to reach statistical significance at any of the individual observation periods in Fig. 4, but was significant ($P < 0.01$) in the experiments of

Table 6 which were performed in NO_3^- medium. As noted above, the ATP content of slices in the NO_3^- medium was decreased to a substantial extent in the presence of methacholine (Table 5). Since methacholine did not induce a significant change of slice Na^+ or K^+ content in the NO_3^- medium, we examined the effect of ouabain on

TABLE 5. Effect of medium anions on the intracellular ionic content and ATP content of salt-gland slices incubated with and without methacholine. Chloride in the Tris-phosphate Ringer solution was replaced by the anions indicated and slices were distributed over the appropriate media from the start of pre-incubation at 1 °C. Since methacholine had no effect on the ionic contents at 1 °C, the values shown at this temperature are pooled results from slices incubated with and without methacholine. Methacholine was used at 1 mM. For further experimental details, see Methods. The values given are mean ± s.e. of the mean (number of observations)

Principal anion of medium	Cl^-	NO_3^-	I^-	SO_4^{2-}
		Na^+ content (mmol/kg dry wt.)		
Pre-incubated 120 min at 1 °C	183 ± 17 (10)	217 ± 17 (10)	57 ± 11 (8)	149 ± 15 (8)
Then 60 min at 38 °C:				
Control	123 ± 17 (17)	164 ± 9 (14)	52 ± 17 (10)	72 ± 12 (12)
Methacholine	217 ± 18 (15)*	169 ± 7 (14)	30 ± 12 (10)	103 ± 19 (12)
Difference due to methacholine	92 ± 25	5 ± 11	−22 ± 21	31 ± 23
		Cl^- content (mmol/kg dry wt.)		
Pre-incubated 120 min at 1 °C	110 ± 22 (10)	39 ± 10 (10)	158 ± 29 (8)‡	45 ± 14 (8)
Then 60 min at 39 °C:				
Control	130 ± 9 (17)	20 ± 5 (14)	136 ± 17 (10)‡	18 ± 6 (12)
Methacholine	175 ± 12 (16)*	24 ± 5 (14)	114 ± 20 (10)‡	21 ± 5 (12)
Difference due to methacholine	45 ± 15	4 ± 5	−22 ± 43	3 ± 14
		K^+ content (mmol/kg dry wt.)		
Pre-incubated 120 min at 1 °C	148 ± 8 (10)	161 ± 6 (10)	290 ± 33 (8)	145 ± 9 (8)
Then 60 min at 38 °C:				
Control	193 ± 8 (18)	155 ± 8 (14)	278 ± 26 (10)	155 ± 9 (12)
Methacholine	152 ± 7 (17)*	149 ± 8 (14)	306 ± 34 (10)	157 ± 11 (12)
Difference due to methacholine	−41 ± 11	−6 ± 11	28 ± 43	2 ± 14
		ATP content (mmol/kg protein)		
Pre-incubation 120 min at 1 °C				
Then 60 min at 38 °C:				
Control	5·4 ± 0·5 (13)	5·8 ± 0·5 (10)	9·5 (2)	5·9 ± 0·5 (15)
Methacholine	2·8 ± 0·3 (16)*	4·0 ± 0·3 (12)*†	10·7 (2)	5·1 ± 0·5 (16)†

* Significantly different from value in absence of methacholine in same medium; $P < 0.01$.
† Significantly different from value with methacholine in Cl^- medium; $P < 0.02$.
‡ These values represent residual Cl^- plus I^- (see text).

the slices in this Ringer solution (Table 6). Ouabain inhibited the stimulation of respiration induced by methacholine and also reduced the basal respiration by 50 %. In addition, ouabain led to a large loss of K^+ and gain of Na^+ by the slices during incubation at 38 °C, either with or without methacholine, indicating a considerable activity of the ouabain-sensitive transport of these cations in the NO_3^- medium.
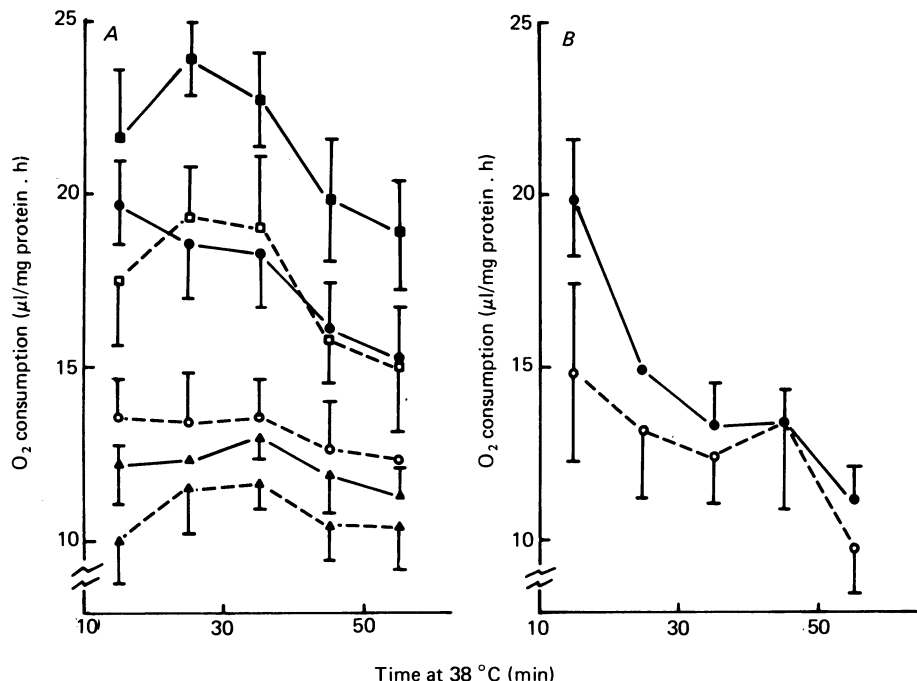
Fig. 4. Effects of methacholine on the rate of O_2 consumption of salt-gland slices incubated in media with different principal anions. Results are from the same experiments as Table 5. Open symbols and dashed lines represent slices incubated without methacholine; filled symbols and continuous lines represent slices incubated with 1 mM-methacholine. Principal anion of the medium: A, $\bigcirc$, $\bullet$, Cl^-; $\square$, $\blacksquare$, NO_3^-; $\triangle$, $\blacktriangle$, SO_4^{2-}; B, I^-.

TABLE 6. Effects of inhibitors and methacholine on respiration and intracellular ion contents in nitrate (chloride-free) Ringer solution. Slices were incubated in the nitrate medium throughout, the composition of which is given in Methods. Ionic contents in slices analysed after pre-incubation at 1 °C are pooled values of slices incubated with no additions and slices incubated with methacholine plus furosemide. In these experiments the rates of respiration were constant throughout the observation period and the mean rate of O_2 consumption is shown. Each value is the mean $\pm$ S.E. of mean of ten observations for Na^+ and K^+ contents and of six observations for O_2 consumption

Incubation	Na^+ content (mmol/kg dry wt.)	K^+ content (mmol/kg dry wt.)	O_2 consumption (μl/mg dry wt.h)
120 min at 1 °C	120 ± 11†	146 ± 6	—
Then for 60 min at 38 °C:			
Control	44 ± 10	155 ± 13	$13 \cdot 3 \pm 0 \cdot 3$
plus ouabain (0·1 mM)	230 ± 19†	41 ± 4†	$6 \cdot 9 \pm 0 \cdot 4$†
plus furosemide (1·0 mM)	42 ± 10	181 ± 11	$12 \cdot 5 \pm 0 \cdot 7$
Methacholine (1 mM)	73 ± 19	169 ± 14	$19 \cdot 8 \pm 1 \cdot 8$†
plus ouabain (0·1 mM)	230 ± 19**	52 ± 7**	$7 \cdot 6 \pm 0 \cdot 4$**
plus furosemide (1·0 mM)	16 ± 12*	164 ± 14	$12 \cdot 9 \pm 0 \cdot 9$**

† Significantly different from value in controls, $P < 0 \cdot 01$.
* Significantly different from value with methacholine alone, $P < 0 \cdot 02$.
** Significantly different from value with methacholine alone, $P < 0 \cdot 01$.

Inhibitors of anion transport

We next studied a number of agents known to affect anion movements in other tissues. Thiocyanate ions inhibit trans-epithelial movements of Cl^- in fish gill and operculum (e.g. Epstein, Maetz & de Renzis, 1973; Karnaky, Degnan & Zadunaisky, 1977) while DIDS inhibits the anion exchange system of red cell membranes (Knauff, Fuhrmann, Rothstein & Rothstein, 1977). We used SCN^- at 0·2, 1·0 and 10·0 mM and
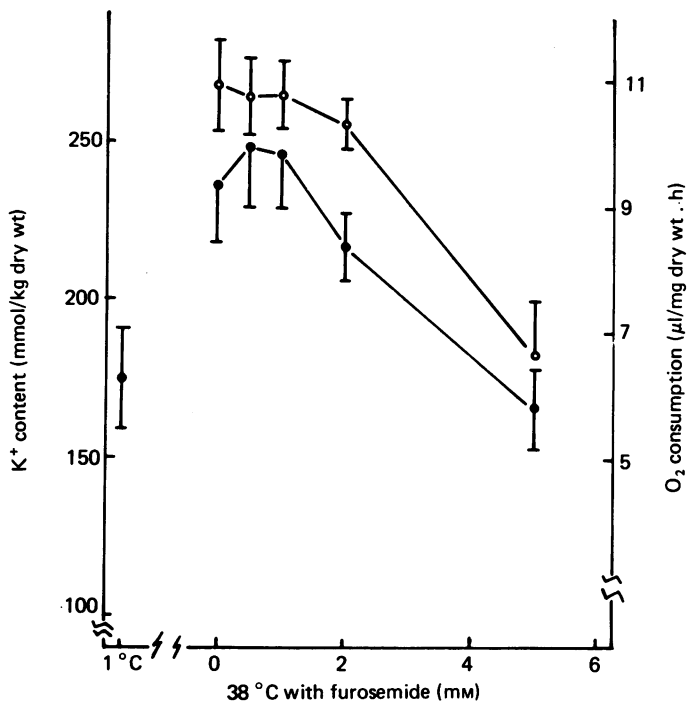

Fig. 5. Effects of different concentrations of furosemide on salt-gland slices. ●, intracellular K^+ content. The point on the extreme left represents the content of slices analysed after pre-incubation for 120 min at 1 °C; furosemide at 5 mM had no significant effect on the K^+ content at 1 °C and the results with and without furosemide at this temperature have been pooled ($n = 8$). The remaining points for K^+ are the contents of slices subsequently incubated for 60 min at 38 °C; $n = 10$. ○, rate of O_2 consumption; $n = 10$.

DIDS at 0·2, 1·0 and 2·0 mM in experiments in which salt-gland slices were incubated in the Tris-phosphate Ringer solution. Neither agent affected the rate of respiration or the ATP contents in the presence or absence of methacholine, nor was any effect observed on the intracellular contents of Na^+, Cl^- or K^+.

The diuretic, furosemide, inhibits coupled movements of Cl^- and Na^+ (reviewed by Frizzell, Field & Schultz, 1979), although it has the complication of also inhibiting mitochondrial O_2 consumption at high concentrations (Manuel & Weiner, 1976). Because of the latter, we first studied the effects of varying concentrations of furosemide on the respiration and K^+ content of salt-gland slices incubated without

methacholine (Fig. 5). Concentrations of up to 1 mM had no effect but 5 mM markedly inhibited O_2 consumption (by 45 %) and totally prevented K^+ accumulation at 38 °C. In further experiments, 1 mM-furosemide alone had no effect on either the ATP or K^+ contents (Table 7). These results, taken together with work on other tissues (van Rossum, Ernst & Russo, 1981), suggest that 5 mM-furosemide had a marked and direct effect on mitochondrial metabolism which was not apparent at 1 mM. In further work, we therefore used the latter concentration.

TABLE 7. Effects of furosemide on intracellular ionic and ATP contents of slices incubated with and without methacholine. The medium was the Tris and phosphate-buffered Ringer solution (see Methods). The values shown at 1 °C are pooled results of slices incubated with methacholine (1·0 mM) or with methacholine plus furosemide (1·0 mM); these treatments showed no significant difference from each other during the pre-incubation

Incubation	Furosemide (mM)	(mmol/kg dry wt)			ATP (mmol/kg protein)
		Na$^+$	Cl$^-$	K$^+$	
120 min at 1 °C	0 and 1·0	239 ± 26 (22)	230 ± 19 (22)	148 ± 8 (22)	—
Then 60 min at 38 °C:					
Control	0	109 ± 10 (30)	188 ± 12 (29)	188 ± 8 (28)	8·6 ± 0·5 (8)
	1·0	82 ± 9 (24)†	149 ± 15 (26)†	199 ± 11 (26)	9·5 ± 0·5 (9)
Methacholine (1·0 mM)	0	176 ± 15 (36)‡	219 ± 13 (36)‖	172 ± 5 (37)†	3·2 ± 0·4 (9)‡
	1·0	102 ± 12 (35)§	162 ± 13 (34)§	196 ± 6 (35)*	9·0 ± 0·3 (9)§

* Significantly different from value with methacholine alone, without furosemide, $P < 0·05$.
† Significantly different from control value $P < 0·05$.
‡ Significantly different from control value $P < 0·01$.
§ Significantly different from value with methacholine alone, without furosemide, $P < 0·01$.
‖ By t test, the probability for difference from the control value was between 0·1 and 0·05. However, by analysis of variance, $P = 0·02$.

While not affecting K^+ content, 1 mM-furosemide caused significant reduction of the intracellular Na^+ and Cl^- contents of the slices incubated at 38 °C without methacholine. This suggests that the rather variable net extrusion of Na^+ and Cl^-, which is seen when slices pre-incubated at 1 °C are incubated at 38 °C under control conditions, is the net result of an active extrusion process and a furosemide-sensitive uptake of these ions.

The effect which methacholine had of increasing the slice content of Na^+ and Cl^- and decreasing that of K^+ during incubation at 38 °C was prevented by 1 mM-furosemide, and the final contents of these ions in the presence of methacholine and furosemide together were not significantly different from the contents with furosemide alone (Table 7). The mean effect of furosemide in the presence of methacholine was to reduce the intracellular Na^+ by 74 ± 19 mmol/kg dry wt. and Cl^- by 57 ± 18 mmol/kg, while increasing K^+ by 24 ± 8 mmol/kg. In this, furosemide differed markedly from ouabain, which induced large net increases of tissue Na^+ and Cl^- and loss of K^+ (see Table 1).

In addition to preventing the changes of ionic content induced by methacholine, 1 mM-furosemide completely abolished the stimulatory effect on respiration (Fig. 6) and prevented the fall of ATP (Table 7). We emphasize that this concentration of

furosemide had no effect on the basal respiration or ATP content and, in this respect also, differed from ouabain.

In the Cl^--free medium containing NO_3^- as the replacing anion, 1 mM-furosemide had no significant effect on the Na^+ content of the cells under basal conditions, but did reduce cellular Na^+ in the presence of methacholine (Table 6). An inhibition by furosemide of methacholine-stimulated respiration was also seen.
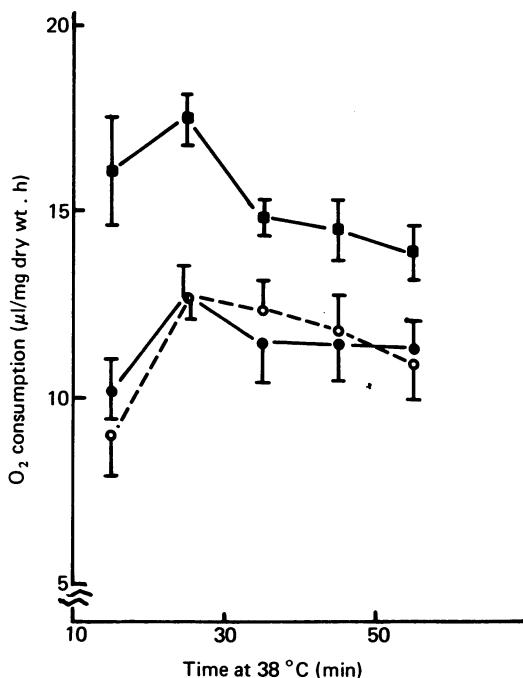

Fig. 6. Effects of methacholine and furosemide on the rate of O_2 consumption of salt-gland slices. ●, control; ■, methacholine (1 mM); ○, methacholine (1 mM) plus furosemide (1 mM). At each point, $n = 14$.

DISCUSSION

Incubation of salt-gland slices with methacholine resulted in raised intracellular contents of Na^+ and Cl^- and lowered K^+ (Tables 1, 3 and 5), changes which were associated with increased O_2 consumption (Table 4, Fig. 3 and 4) and reduced adenine nucleotide energy levels (Tables 2 and 4). There were a number of indications that the effects of methacholine on energy metabolism were closely related to the alterations of ion contents. These include their mutual inhibition by furosemide (Table 7, Fig. 6) and by the replacement of medium Cl^- or Na^+ with other ions (Tables 4–6, Fig. 4). In addition, the effects on energy metabolism appear to be closely related to the Na^+ and K^+ transport which is inhibited by ouabain (Table 2).

The differences of cellular ionic contents induced by methacholine were relatively small compared to the total contents and the estimation of intracellular ionic

contents, especially of Na^+ and Cl^-, is inherently subject to quite large errors. Such errors arise from the persistence of variable quantities of medium adhering to the slices even after a standardized blotting procedure, and to uncertainties of the assumptions involved in the estimation of extracellular fluid and ion contents. Consequently, there were experiments in which the response of one ion or another to methacholine did not attain statistical significance. Such factors, together with the different incubation conditions employed (e.g. lower temperature, lower concentration of drug) probably account for our earlier failure to observe the effect of methacholine on ionic contents (van Rossum, 1966). However, pooling all the results reported in the present work for slices incubated for 60 min at 38 °C in the Tris-phosphate and bicarbonate Ringer solutions, the mean intracellular content of Na^+ in the absence of methacholine was 199 ± 8 (90) mmol/kg dry wt. and in the presence of 1 mM-methacholine was 267 ± 11 (94); the corresponding values for Cl^- were 267 ± 6 (84) and 311 ± 8 (92) mmol/kg, and for K^+ were 215 ± 4 (79) and 177 ± 4 (88) mmol/kg. In each instance, the effect of methacholine was significant at the 0·1 % level of probability.

The changes of ionic content seen with methacholine were qualitatively consistent with those anticipated from the depolarising action of muscarinic cholinergic agents, namely leakage of Na^+ and Cl^- into, and K^+ out of, the cells. However, a simple leakage of ions down their electrochemical gradients would not be expected to require the simultaneous presence of Na^+ and Cl^- (e.g. in the absence of Cl^-, methacholine should still be able to induce an entry of Na^+ in exchange for K^+, but this was not observed) nor to be sensitive to furosemide. The activation by methacholine of a carrier-dependent system for the coupled entry of Na^+ and Cl^- would be more in accord with our observations. The co-transport of these two ions by an electroneutral mechanism that is inhibited by furosemide appears to be a characteristic of a number of absorptive and secretory epithelia (Frizzell et al. 1979). However, the direct stimulation of such a system by a cholinergic agent would, if substantiated, be a novel feature.

The close association of a lower K^+ content with the increased Na^+ and Cl^- in the presence of methacholine, and especially the fact that the depression of cellular K^+ was prevented by furosemide or by omission of medium Cl^-, is not simply explained by a neutral co-transport of Na^+ and Cl^-. One possibility may be that the entry of Na^+ and Cl^- is not electroneutral, and that it produces a depolarization which leads to loss of K^+. Clearly, the more sensitive techniques of isotopic fluxes, together with measurements of membrane potentials, would be needed to study these possibilities more precisely.

While our observations provide evidence for the role of a coupled entry of Na^+ and Cl^- into the cells, we have no evidence consistent with the exchange of Cl^- for HCO_3^- proposed by Peaker & Stockley (1974). Thus, the increased Cl^- content of the cells induced by methacholine was influenced neither by the presence of HCO_3^- in the medium, nor by the anion-exchange inhibitor, DIDS, at high concentrations.

The effects of methacholine on ionic contents of the salt-gland slices show some differences from those observed with isolated cells (Hootman & Ernst, 1981). In the latter, methacholine caused a decrease of Cl^- and no net change of Na^+ content. The cells, like the slices, did lose K^+. The duration of the treatment of the isolated cells

with methacholine was only 10 min, and this may be one factor in the differences of response. Further, movements of Na^+ and Cl^- involved in the secretory activity must involve efflux as well as influx systems, and a different balance between these in the two types of preparation could lead to different intracellular contents in response to stimulation.

The effects of methacholine on aspects of energy metabolism in slices have frequently been used as an indirect indication of cholinergic stimulation of salt-gland secretion (Borut & Schmidt-Nielsen, 1963; Hokin, 1966; van Rossum, 1968; Stewart *et al.* 1979). The underlying assumption is that the increased rate of respiration and fall of ATP content arise from the consumption of ATP by an energy-dependent process, presumably of active transport, which is involved in secretion. The well-established effect of ouabain in preventing the cholinergic effects on energy metabolism suggests an important role for the coupled transport of Na^+ and K^+. Recent studies of the binding of ouabain to isolated cells of the gland have given independent evidence confirming the importance of this transport system in the cholinergic response, for the rate of binding is a measure of the activity of the Na-K-ATPase. Methacholine stimulated the rate of binding of ouabain to isolated cells without changing the total amount of inhibitor bound (Hootman & Ernst, 1981).

Methacholine rapidly increases exchange of ^{24}Na in slices (van Rossum, 1966) and, after more prolonged treatment, raises cellular Na^+ content (Fig. 1) and these findings suggest that the cholinergic activation of the Na-K-ATPase results indirectly from an increased entry of Na^+, probably across the basolateral membranes of the epithelial cells. As discussed above, our experiments on cellular ionic contents suggest that an increased Na^+ entry in response to methacholine occurs by stimulation of a co-transport with Cl^-, and the importance of these ionic movements in determining the eventual consumption of ATP by the Na-K-ATPase during secretory activity is indicated by our measurements of energy metabolism. Thus, replacement of medium Na^+ by choline not only prevented the effect of methacholine on cell Cl^- content (Table 3) but also blocked its ouabain-sensitive effects on respiration and ATP (Table 4), as well as inhibiting the increased rate of ouabain binding to isolated cells (Hootman & Ernst, 1981). Similarly, furosemide blocked the changes of ion content, energy metabolism and ouabain binding normally elicited by methacholine. Our results with Li^+ as replacement for medium Na^+ suggest that this ion may have permitted some activation of the Na-K-ATPase in response to methacholine, since some stimulation of respiration and a partial fall of ATP were seen (Table 4). In this regard it is noteworthy that Li^+ can be secreted by the gland *in vivo* to some extent (Peaker & Stockley, 1973).

Similar considerations hold in the anion-replacement experiments, where the prevention of the effects of methacholine on ion contents and energy metabolism (Table 5 and Fig. 4) showed analogies to the ouabain-binding studies of Hootman & Ernst (1981). These effects were all prevented by the use of SO_4^{2-} as the major anion of the medium and partially prevented by NO_3^-. Our experiments with I^- were also in agreement with the above, but Hootman & Ernst (1981) did not study ouabain-binding in this medium. These findings are consistent with the primary effect of methacholine being closely related to a chloride-dependent system (e.g. that postulated for co-transport of Na^+ and Cl^-) which shows little affinity for SO_4^{2-} or

I^- and a greater affinity for NO_3^-. In other tissues, the relative affinities of transport systems for anions fall in the order: $Cl^- = I^- > NO_3^- > SO_4^{2-}$ (e.g. Soumarmon, Abastado, Bonfils & Lewin, 1980; Warnock & Lyee, 1981). The failure of I^- to support the response of ions and respiration to methacholine indicates that the salt-gland system shows some differences from this order. However, NO_3^- and SO_4^{2-} appear to follow the above sequence as there are indications from our work that NO_3^- shows some affinity for the chloride-dependent system which is stimulated by methacholine. In particular, we noted a cholinergic stimulation of respiration in the NO_3^- medium which was inhibited by both furosemide and ouabain and a significant decrease of cellular Na^+ when furosemide was present together with methacholine (Table 6). At first sight this appears to conflict with the failure of methacholine significantly to increase cell Na^+ in the NO_3^- medium (Tables 5 and 6). This discrepancy may be explained by the lower affinity of NO_3^- for the Cl^- carrier, since the rate of entry of Na^+ into the cells would be limited by the accompanying entry of anions. The rate of entry of Na^+ together with NO_3^-, unlike its entry with Cl^-, may remain within the capacity of the Na–K transporting system to extrude it, so that the intracellular Na^+ content would not increase.

The use of NO_3^- as replacement for medium Cl^- showed some unexpected effects on salt-gland slices under basal conditions (i.e. the absence of methacholine). Thus, NO_3^- induced a stimulation of basal respiration in the slices which was prevented by ouabain (Fig. 4 and Table 6), and also increased the basal rate of ouabain binding in isolated cells (Hootman & Ernst, 1981). In contrast, using SO_4^{2-} or I^- as a replacement for Cl^- had little effect on these activities under basal conditions (although the effect of I^- on ouabain binding was not tested). The results may possibly indicate some stimulation of the Na-K-ATPase by NO_3^-, but there is no evidence for a direct effect of this anion on the isolated enzyme (Opit, Potter & Charnock, 1966) and no indication of increased K^+ accumulation, Na^+ extrusion or ATP consumption in slices incubated with NO_3^- under basal conditions (Table 5). Interestingly, and in contrast to the methacholine stimulation of respiration in nitrate-substituted medium, the increase of basal O_2 consumption was not sensitive to furosemide (Table 6).

Replacement of Na^+ in the medium by Li^+ also gave rather unexpected results which differed in some respects from work in the literature. The stimulation of basal respiration upon replacement of all medium Na^+ with Li^+ is similar to that observed by Borut & Schmidt-Nielsen (1963) with salt-gland slices from herring-gull, but we have been unable to repeat their finding of a stimulation when Li^+ only partially replaced Na^+. We used Li^+ at 10, 20, 50 and 100 mM, and either incubated slices in these media throughout, with Li^+ replacing an equivalent amount of Na^+, or tipped the appropriate quantity of Li^+ from the side-arm of the Warburg vessels after 30 min incubation at 38 °C. In no case were we able to detect a stimulation of respiration by Li^+, or an effect of Li^+ on ATP content or energy charge. We have also been unable to observe the change of ionic content (i.e. increase of intracellular Na^+, Cl^- and K^+) induced by these lower concentrations of Li^+ in the work of Peaker & Stockley (1974) on slices of goose salt gland. We are unable to account for our differences from the earlier work, unless the reason lies in the different species used. The explanation for the observed stimulation of basal respiration upon total replacement of Na^+ by Li^+ is also not readily apparent. While Li^+ can to some extent replace K^+ in stimulation of the Na-K-ATPase and may itself be transported in place of K^+ (Maizels, 1968), it appears to be unable to replace Na^+ in such activities (e.g. Casteels, Droogmans & Hendrickx, 1973; Elshove & van Rossum, 1963; Haugaard, Frazer, Mendels & Haugaard, 1975). A direct effect of Li^+ on a phase of energy metabolism may be responsible for the stimulation of respiration.

In general, our results are consistent with the occurrence in the salt gland epithelial cells of a furosemide-sensitive entry of Na^+ and Cl^- which is stimulated by methacholine to an extent which permits a net increase of cellular Na^+ and Cl^-, these changes being associated with an enhanced leakage of K^+. As a result of the entry

of Na^+ and Cl^-, an ouabain-sensitive consumption of ATP is stimulated. The many similarities between the inhibitor and ion sensitivities of this system and of the binding of ouabain to the cell surface, strengthen the indications of a close relationship between NaCl uptake, stimulation of energy consumption and activity of the Na-K-ATPase, and are consistent with the model of secretion proposed by Ernst & Mills (1977) and Riddle & Ernst (1979). However, studies of unidirectional ion fluxes are required to test more fully the proposed properties of the gland.

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