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STIMULATION OF Cl^- SECRETION BY EXOGENOUS ATP IN CULTURED MDCK EPITHELIAL MONOLAYERS

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Cultures epithelial monolayers of MDCK cells were grown upon Millipore filter supports and mounted in Ussing chambers for ion-transport studies. Addition of exogenous ATP to the basal bathing solutions resulted in a stimulation of the short-circuit current which was due to both an increased transmonolayer p.d. and an increased conductance. Measurements of tracer Na^+ and Cl^- fluxes demonstrate that the ATP-stimulated short-circuit current, results from basal to apical Cl^- movement (secretion) across the cultured monolayer. ATP-stimulated net Cl^- secretion was inhibited by furosemide ($1 \cdot 10^{-4}$ M) added to the basal bathing solution and by elevating the basal medium K^+ concentration from 5.4 to 54 mM. Both furosemide and elevated basal K^+ exert their inhibitory action upon the ATP-dependent short circuit current primarily by abolishing the electrogenic component without affecting the increased transmonolayer conductance. Hyperpolarization of the transmonolayer potential difference by applied currents also reduces the ATP dependent increase in the short-circuit current. The increased short-circuit current was insensitive to replacement of medium Na^+ by choline$^+$, but was linearly related to Cl^- concentration with isethionate (2-hydroxyethanesulphonate) replacements. NO_3^-, I^-, and the thiocyanate anion were all ineffective substitutes for Cl^- whereas Br^- and acetate were only partially effective. Sodium thiocyanate (10 mM) in the presence of NaCl inhibited the ATP-stimulated short-circuit current.

Introduction

A knowledge of the nature and control of the mechanisms involved in transepithelial Cl^- transport is important in terms of an understanding of the physiology of certain epithelia (e.g. thick ascending limb of the Loop of Henle [1]; cornea [2] fish gills [3]; shark rectal gland [4]; fish intestine [5]) and in the pathophysiology of others (e.g. mammalian small intestine [6–9].

Considerable insight into the mechanisms underlying transepithelial Na^+ transport have been previously derived from studies of convenient model systems such as anuran skin and bladder [10,11]. Significant progress has now been made in the introduction of semi-artifical model systems composed of monolayers of epithelial cells grown in tissue culture upon permeable supports [12–14]. Such cultured systems offer certain technical advantages over traditional in vitro systems such as their simple geometry and their extended viability during experimentation [12–14].

This paper presents evidence for rheogenic Cl^- secretion by cultured epithelial layers of MDCK cells grown upon Millipore® filter supports following stimulation by exogenous application of ATP. The ATP-stimulated Cl^- secretion is examined in respect to (a) its ionic dependence (Na^+, Cl^-), (b) inhibition by furosemide, phloretin and other Cl^- transport inhibitors such as thiocyanate [4], (c) variation in medium K^+ content, and (d) transepithelial p.d.

Abbreviation: SITS, 4-acetamide-4′-isothiocyanatostilbene-2,2′-disulphonic acid.

A preliminary account of some of the present data has been published [15].

Methods

Cell culture. MDCK dog kidney cells were obtained from Flow Laboratories (Irvine, Scotland) at 60 serial passages (Strain I [22]). Culture conditions and preparation of epithelial monolayers upon Millipore filters were identical to those previously used [22] except that epithelial monolayers were grown in Minimum Essential Medium Eagles with 10% v/v foetal bovine serum, 1 unit/cm^3 gentamycin antibiotic, non-essential amino acids and 2 mM glutamine (Flow Laboratories) with 1 I.U./cm^3 insulin (Boots Chemical Co., Nottingham, U.K.) as an additional growth stimulant. Cell monolayers were used for resistance and p.d. measurements after 3–4 days.

Solutions and electrical measurements. Cell monolayers were mounted in Ussing chambers (0.75 cm window radius, 1.76 cm^2 exposed monolayer), thermostatically controlled at 37°C for measurement of p.d. and resistance [7,16]. An automatic voltage clamp [16] was connected to the Ussing chamber via matched calomel half cells (for potential measurement), silver/silver chloride half cells (for current passage) and saturated KCl-agar bridges.

The use of saturated KCl-agar salt bridges minimised error due to liquid-junction potentials and prevented contamination of experimental solutions with AgCl. All p.d. values are expressed with relation to the Millipore filter. Resistance determinations under open circuit conditions were made as previously described [23], also resistance was measured in some voltage-clamp experiments by using small non-zero square-wave excursions of the clamp command voltage.

The experiments were carried out, except where otherwise stated at pH 7.4 in modified Krebs' solution containing 137 mM NaCl, 5.4 mM KCl, 2.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.3 mM NaH_2PO_4, 0.4 mM KH_2PO_4, 12 mM HCl, 14 mM Tris base, 10 mM glucose, 2 mM glutamine, 2 mM sodium pyruvate and 2% (v/v) foetal bovine serum and amino acids for Eagles medium (Flow Laboratories).

A Cl^--free solution was prepared using Na_2SO_4 for NaCl, K_2SO_4 for KCl, $CaSO_4$ for $CaCl_2$ and H_2SO_4 for HCl. Mannitol was added to maintain isosmolarity. Substitutions of Cl^- for Br^-, isethionate, acetate, and thiocyanate were made by replacing NaCl by Na-(anion), Cl^- being substituted by SO_4^{2-} (with mannitol) to achieve zero Cl^- in the other salts.

A Na^+-free solution was obtained by substituting NaCl isosmotically by choline chloride and $NaHPO_4$ by $KHPO_4$. The serum added to all Krebs' solutions in which ionic composition was varied was dialysed overnight against two changes of X50 vol. distilled water to remove salts.

Na^+-flux measurements. Bi-directional Na^+ fluxes were determined simultaneously on the same cell monolayer voltage clamped to zero p.d. using ^{22}Na and ^{24}Na as tracers (Radiochemical Centre, Amersham, U.K.) as previously described [23].

Cl^--flux measurements. Cl fluxes were measured using ^{36}Cl as tracer (Radiochemical Centre, Amersham) [23]. Bidirectional fluxes were determined upon adjacent cell monolayers, or upon the same monolayer using a randomized order to avoid error due to deterioration in monolayer condition. Results were rejected if monolayer resistance fell by 5% over the flux period.

Chemicals. All chemicals were of ANALAR grade. Adenosine triphosphate was the synthetic sodium salt from B.D.H. Chemicals, Poole, U.K. The stilbene, 4-acetamide-4′-isothiocyanatostilbene-2,2′-disulphonic acid was also obtained from B.D.H. 4-Aminopyridine and phloretin were obtained from the Sigma Chemical Co., Fancy Road, Poole, U.K. Furosemide and piretanide were gifts from Dr. Dombey of the Hoescht Chemical Co. Milton Keynes, U.K. Ethacrynic acid was a gift from Merck Sharp and Dohme (Hodderson, Herts, U.K.). Stock solutions of SITS, phloretin, furosemide, piretanide and ethacrynic acid were made in 10^{-2} M Tris base.

Statistical methods. Variation in results is expressed as the standard error of the mean (±S.E.). Tests for significance of difference were made by a two tailed Student's *t*-test (un-paired means solution). One-tailed tests and paired tests were used where appropriate.

Results

(a) The effect of exogenous ATP upon the electrophysiological parameters of MDCK epithelium

MDCK cells of between 60 and 66 passages form

epithelial monolayers of high electrical resistance ($4\ k\Omega \cdot cm^2$) which maintain a small spontaneous p.d. (basal solution electropositive) of 2 mV [18]. Fig. 1 shows that addition of exogenous ATP to the basal solution in a typical experiment, causes a prompt stimulation of the short circuit current from $0.5\ \mu A \cdot cm^{-2}$ to a peak value of $8.3\ \mu A \cdot cm^{-2}$. The increased short circuit current is due both to an increased transmonolayer p.d. (basal solution electropositive) and a decreased monolayer resistance (Table I). The decline in short-circuit current from peak values on continued stimulation with ATP

TABLE I

EFFECTS OF RAISED MEDIUM Ca^{2+} AND Mg^{2+}, ELEVATED K^+ AND VOLTAGE-CLAMPING AT NON-ZERO p.d.'s UPON THE ATP-STIMULATED CHANGES IN TRANSMONOLAYER ELECTRICAL PARAMETERS

ATP ($1 \cdot 10^{-4}$ M) was added to the basal bathing solution in all conditions at $t = 0$, recordings of control parameters were made 1 min prior to ATP addition. Subsequent recordings were made at 1 and 5 min after ATP addition. The high K^+-containing Krebs' was obtained by isosmotic substitution of KCl for NaCl. The Ca^{2+} and Mg^{2+} concentration was raised to 5.3 and 3.7 mM respectively, without adjustment of other salts. Resistance determinations under voltage clamp were made by square wave excursions (+5mV) of the clamp command voltage, alternatively resistance was measured under open-circuit conditions with hyperpolarizing current pulses of $2\ \mu A \cdot cm^{-2}$. All potential differences are expressed positive with respect to the basal solution. Errors are expressed as ± S.E.

Condition	n	Resistance ($k\Omega \cdot cm^{-2}$)			p.d. (mV)		
		−1 min	1 min	5 min	−1 min	1 min	5 min
Control	4	4.2 ± 0.7	1.8 ± 0.1 a3	3.3 ±0.6 a1	2.1 ± 0.3	26.2 ± 5.1 a4	18.1 ± 5.1 a4
Raised $Ca^{2+} + Mg^+$	4	3.8 ± 0.4 b1	2.0 ± 0.4 a3,b1	2.6 ± 0.5 a1,b1	1.4 ± 0.2 b1	28.2 ± 5.3 a4,b1	18.0 ± 9.7 a4,b1
Control	5	3.2 ± 0.6	1.4 ± 0.1 (5) a3	1.6 ± 0.3 a2	1.3 ± 0.1	39.4 ± 3.2 a4	23.8 ± 3.4 a4
Elevated basal K^+ (54 mM)	5	2.4 (5) ± 0.4 b1	0.7 ± 0.0 (5) a3,b3	0.8 ± 0.0 (5) a3,b2	0.1 ± 0.1 b4	8.9 ± 1.4 a4,b4	5.8 ± 1.2 a4,b4
Elevated apical K^+ (54 mM)	5	1.9 ± 0.3 b1	0.7 ± 0.2 a3,b2	1.0 ± 0.1 a3,b1	1.8 (5) ± 0.2 b1	36.8 ± 1.8 a4,b1	25.2 ± 3.1 4a,b1
					ATP-dependent current ($\mu A \cdot cm^{-2}$)		
					−1 min	1 min	5 min
Control	4	1.5 ± 0.3	0.6 ± 0.0 (5) a3	0.8 ± 0.1 (5) a3	–	39.0 ± 5.7	19.0 ± 3.2
40 mV depolarized (basal solution electronegative)	4	0.9 ± 0.1	0.7 ± 0.1 a1,b1	0.7 ± 0.1 a1,b1	–	49.8 ± 8.7 b1	26.7 ± 3.6 b1
40 mV hyperpolarized (basal solution electropositive)	4	1.4 ± 0.2 b1	0.7 (5) ± 0.1 a3,b1	0.9 ± 0.1 a2,b1	–	± 1.5 b4	4.5 ± 0.9 b4

a Significantly different from pre-ATP values at t = −1 min.
a1 Not significant.
a2 $P < 0.05$.
a3 $P < 0.01$.
a4 $P < 0.001$.

b Significantly different from untreated controls ± ATP.
b1 Not significant.
b2 $P < 0.05$.
b3 $P < 0.01$.
b4 $P < 0.001$.

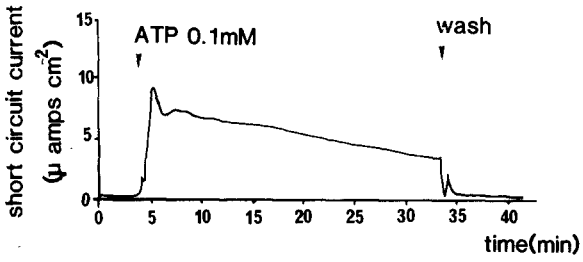

Fig. 1. Effect of addition of 0.1 mM exogenous (ATP) to the basal bathing solution upon the short circuit current maintained by the MDCK epithelial monolayer. The increased short-circuit current is rapidly reversed by washing. Basal short-circuit current is 0.5 $\mu A \cdot cm^{-2}$ and the spontaneous open circuit p.d. is basal bathing solution electropositive.

(Fig. 1) occurs due to both a fall in transmonolayer p.d. and a recovery of resistance values towards pre-stimulation levels (Table I). The peak increase in short-circuit current observed at $1 \cdot 10^{-4}$ M ATP is variable. Table I shows that for three separate batches of epithelial monolayers the short circuit current 1 min after ATP stimulation was 14.5 ± 2.8 ($n = 4$), 28.1 ± 2.3 ($n = 5$) and 39.0 ± 5.7 ($n = 4$) $\mu A \cdot cm^{-2}$. The reason for this variation is unknown, but may involve the use of separate batches of foetal serum for monolayer growth.

It has previously been demonstrated that the action of exogenous ATP upon MDCK epithelium is consistent with an extracellular mode of action [17] most probably by interaction with a surface purine

TABLE II

SUMMARY OF THE BIDIRECTIONAL Na^+ AND Cl^- FLUXES ACROSS CONFLUENT MONOLAYERS OF MDCK CELLS VOLTAGE CLAMPED TO ZERO POTENTIAL DIFFERENCE

J_{A-B} denotes the flux of an ion from the apical to basal surfaces of the cell layer. J_{B-A} denotes the reverse flux. Values of conductance and short-circuit current are mean values recorded continually throughout the flux measurement period at 5-min intervals. In the high K^+-containing Krebs' KCl replaced NaCl. ATP and furosemide were added to the basal bathing solution. Errors are expressed ± S.E. Figures in parentheses are the number of separate adrenaline stimulations.

Condition	n	J_{A-B} (μmol · $cm^{-2} \cdot h^{-1}$)	J_{B-A} (μmol · $cm^{-2} \cdot h^{-1}$)	J_{net} (μmol · $cm^{-2} \cdot h^{-1}$)	SCC (μmol · $cm^{-2} \cdot h^{-1}$)	Conductance (mho · cm^{-2}) × 10^3
A. Na^+ fluxes						
Control	6	0.50 ± 0.13	0.26 ± 0.04	0.26 ± 0.15	0.054 ± 0.001 (6)	1.46 ± 0.21 (6)
$+1 \cdot 10^{-4}$ M ATP	6	0.34 ± 0.08	0.22 ± 0.05	0.13 ± 0.07	0.287 ± 0.034 [b] (6)	1.02 ± 0.18 (6)
B. Cl^- fluxes						
Control	31	0.67 ± 0.15	0.72 ± 0.22	−0.04 ± 0.26	0.038 ± 0.001 (62)	1.02 ± 0.08 (62)
$+1 \cdot 10^4$ ATP	18	1.05 ± 0.25	1.60 ± 0.18 [a]	−0.55 ± 0.21 [a]	0.310 ± 0.047 [b] (36)	1.32 ± 0.21 (36)
54 mM serosal K^+-containing Krebs'	8	0.77 ± 0.15	0.59 ± 0.13	0.18 ± 0.18	0.018 ± 0.003 (16)	0.57 ± 0.07 (16)
54 mM serosal K^+-containing Krebs' $+1 \cdot 10^{-4}$ ATP	8	0.94 ± 0.22	0.75 ± 0.19	0.19 ± 0.26	0.047 ± 0.006 (16)	0.74 ± 0.11 (16)
$1 \cdot 10^{-4}$ M furosemide	6	1.54 ± 0.36	1.29 ± 0.28	0.25 ± 0.38	0.036 ± 0.016 (12)	0.70 ± 0.16 (12)
$1 \cdot 10^{-4}$ M furosemide $+1 \cdot 10^{-4}$ M ATP	6	2.30 ± 0.40	2.02 ± 0.48	0.28 ± 0.50	0.092 ± 0.014 (12)	1.44 ± 0.38 (12)

[a] Significantly different from control values $P < 0.05$.
[b] Significantly different from control values $P < 0.01$.

(P2) receptor [17] as described for other tissues [19, 20]. ATP action was also shown to be dose-dependent, half-maximum stimulation being observed at 1.91 ± 0.06 μM [17].

Medium divalent cation chelation by ATP has been discounted as a likely mechanism of action for ATP stimulation of the short-circuit current on the basis of the half-maximal stimulation [17] and by the observation that a series of purine and pyrimidine, nucleotides though possessing similar divalent cation chelating properties, have markedly different capacities to stimulate the short circuit current [17,21]. Table I also shows that raising the medium Ca^{2+} and Mg^{2+} concentrations to 5.3 and 3.7 mM, respectively, has no significant effect upon the changes in transmonolayer p.d. and resistance observed following ATP stimulation.

(b) The effect of exogenous ATP upon transepithelial Na^+ and Cl^- fluxes

Table II shows that the control net transepithelial fluxes of Na^+ and Cl^- are small (0.26 and –0.04 μmol · cm^{-2} · h^{-1} respectively) and do not differ significantly from zero ($P > 0.2$ and $P > 0.5$, respectively) in agreement with the low values of current-flux equivalent recorded over the Na^+ and Cl^- flux measurement period (0.054 and 0.038 μmol · cm^{-2} · h^{-1}, respectively).

The bi-directional transepithelial fluxes of Na^+ and Cl^- are also small (~0.5 μmol · cm^{-2} · h^{-1}) reflecting the low transepithelial conductance of 1.46 and 1.02 mmho · cm^{-2} (Table II). The values of bi-directional and net fluxes recorded in the present experiments are similar to previous measurements made in this laboratory [18] upon this strain of MDCK cells. Measurements of Na^+ fluxes reported by Cereijido et al. [14] were made upon a different cell strain [22].

Exogenous ATP added to the basal solution results in a significant increase in the current-flux equivalent compared with control values (Table II, Column 6, $P < 0.01$ for values in the presence of ATP versus controls, except where furosemide or high K^+ media was used). The decrease in monolayer resistance noted with ATP-stimulation (Table I) is not consistently observed in grouped conductance data due to between-epithelium variation in control resistance values which may result from edge damage [18,23].

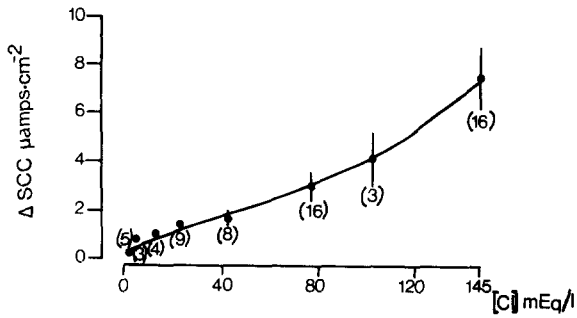

Fig. 2. Effect of substitution of bathing Krebs' NaCl by sodium isethionate (see Methods) upon the ATP-dependent increase in short-circuit current. Figures in parentheses are the number of separate determinations. Error bars are ±S.E. and lie within the point where not shown. Solid line drawn by eye. Epithelial monolayers were preincubated in the appropriate Kreb's solution for 10 min prior to addition of ATP to the basal bathing solution.

In the presence of exogenous ATP net Na^+ flux does not differ significantly from net Na^+ flux in control tissues ($P > 0.5$) (Table II). In contrast net Cl^- flux in the presence of ATP is increased compared with controls ($P < 0.05$ for paired data) by a sufficient magnitude in a secretory direction to account for the increased short circuit current. The ATP-stimulated net Cl^- secretion arises primarily as a result of an increased basal to apical flux of Cl (J^{Cl}_{B-A}) Table II.

(c) The effect of ionic substitutions on the ATP-stimulated short-circuit current

At 139 mM Na^+ the peak ATP-stimulated short-circuit current was 9.59 ± 2.16 ($n = 12$) μA · cm^{-2} and this was reduced to 6.43 ± 1.72 ($n = 8$) μA · cm^{-2} in a Na^+-free Krebs' solution where Na^+ was replaced by choline$^+$. This result is consistent with the ATP-stimulated short-circuit current being due solely to rheogenic Cl^- movement.

With replacement of medium NaCl by isosmotic substitution with sodium isethionate in the range 0 to 145 mM NaCl (see also Methods for details of other Cl^- substitutions) there is a linear dependence of the ATP-stimulated short-circuit current upon Cl^- concentration (Fig. 2). In Cl^- free solutions ATP does not affect either the transepithelial p.d. or transepithelial resistance (data not shown). A series of anion substitutions were tested (Table III) for their ability

TABLE III

EFFECT OF ANION REPLACEMENT UPON THE ATP (10^{-4} M)-STIMULATED PEAK INCREASE IN SHORT-CIRCUIT CURRENT (ΔSCC PEAK)

For SO_4^{2-} the isotonicity was maintained by mannitol addition (see Methods).

Anion	n	ΔSCC (peak) ($\mu A \cdot cm^{-2}$)	P vs. Cl^-
Cl^-	15	8.32 ± 0.18	
$Acetate^-$	8	1.07 ± 0.25	<0.01
Br^-	9	0.25 ± 0.07	<0.01
SCN^-	3	0.10 ± 0.06	<0.01
$Isethionate^-$	10	No response	<0.01
SO_4^{2-}	10	No response	<0.01

to support the ATP-stimulated short-circuit current. Isethionate, SO_4^{2-} and thiocyanate anions were ineffective substitutes for Cl^-, whereas Br^- and the acetate anion were only partially effective. The relative potency of anions in supporting the ATP-dependent short-circuit current is $Cl^- \gg$ acetate, $Br^- \gg SCN^-$, isethionate, SO_4^{2-}.

(d) The action of furosemide

Addition of 0.1 mM furosemide to the basal bathing solution results in a prompt (~1 min) inhibition of the ATP-stimulated short-circuit current (Fig. 3). Addition of an equimolar dose to the apical bathing solution has no effect upon the ATP-stimulated short-circuit current (Fig. 3). To test whether furosemide is inhibiting ATP interaction with its surface receptor, or whether furosemide action is consistent with ion transport inhibition, ATP concentration was increased to 1 mM and the effect of $1 \cdot 10^{-5}$ M furosemide tested. The action of furosemide was no different at 0.1 mM, or 1 mM ATP. Additionally exogenous ATP may be applied to the apical bathing solution to give an identical short-circuit current response [17], in this instance apical furosemide (0.1 mM) has no effect upon the ATP-dependent short-circuit current, inhibition being only observed with basal addition to furosemide. This data is consistent with furosemide inhibition of ATP induced ion transport.

Table IV shows the effect of furosemide upon the ATP-stimulated open-circuit potential and conductance.

ATP stimulation results in the usual decreased

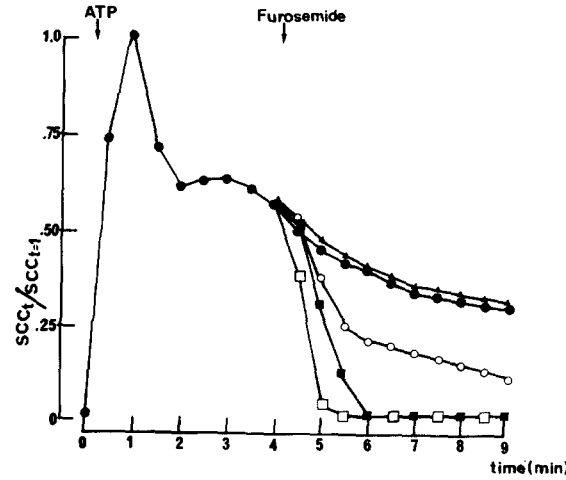

Fig. 3. Action of furosemide upon the ATP-mediated increase in short-circuit current. ATP was added to the basal bathing solution at $t = 0$. Furosemide was added at $t = 4$ min. All records were normalized to the maximum short-circuit current observed prior to furosemide addition; ●———●, control no additions; ▲———▲, 0.1 mM furosemide was added to the apical bathing solution. ○———○, $1 \cdot 10^{-6}$ M furosemide basal bathing solution; ■———■, $1 \cdot 10^{-5}$ M furosemide basal bathing solution; □———□, $1 \cdot 10^{-4}$ M furosemide basal bathing solution. All points are the mean of at least three determinations.

monolayer resistance and increased transepithelial p.d. Basal furosemide inhibits the electrogenic component of ATP action in all five separate experiments; the epithelial conductance with furosemide is unchanged compared to ATP alone, but is considerably increased compared to control conductance in the absence of ATP ($P < 0.01$ for paired values).

Preincubation of epithelial monolayers with basal furosemide (0.1 mM) (Fig. 4) has a similar inhibitory effect upon the ATP-dependent short-circuit current as observed above. However, despite a 10-min furosemide preincubation a significant ATP-stimulated short-circuit current remains. The peak mean short-circuit current at 10 min was 8.2 ± 2.1 ($n = 4$) $\mu A \cdot cm^{-2}$ compared to 16.4 ± 3.2 ($n = 4$) $\mu A \cdot cm^{-2}$ for control values, this suggests that ATP alters furosemide sensitivity (compare Figs. 4 and 3). Furosemide has no effect upon the basal short-circuit current (Fig. 4), in agreement with previous data [23].

ATP-stimulated net Cl^- secretion is abolished by furosemide (Table I) since net Cl^- flux in the presence of ATP and furosemide does not differ signifi-

TABLE IV

EFFECT OF $1 \cdot 10^{-4}$ M FUROSEMIDE APPLIED TO THE BASAL BATHING SOLUTION UPON THE ATP-STIMULATED INCREASE IN TRANSEPITHELIAL CONDUCTANCE AND p.d.

ATP was added to the basal bathing solution at $t = 1$ min, and furosemide at $t = 6$ min. Resistance determinations were made by $2\ \mu A \cdot cm^{-2}$ hyperpolarizing current pulses.

Expt.	Resistance ($k\Omega \cdot cm^2$)			p.d. (mV) (basal solution electropositive)		
	Control $t = 0$	+ATP ($1 \cdot 10^{-4}$ M) $t = 5$ min	+ATP ($1 \cdot 10^{-4}$ M) +furosemide ($1 \cdot 10^{-4}$ M) $t = 10$ min	Control $t = 0$	+ATP ($1 \cdot 10^{-4}$ M) $t = 5$ min	+ATP ($1 \cdot 10^{-4}$ M) +furosemide ($1 \cdot 10^{-4}$ M) $t = 10$ min
1	2.17	1.90	1.87	2.0	7.2	1.81
2	2.00	1.66	1.22	1.7	6.7	2.0 (5)
3	2.11	1.80	1.66	0.5	4.8	0.9
4	5.63	4.12	4.86	1.4	18.9	2.1
5	5.10	3.0	3.20	1.2	23.2	1.90
Mean ± S.E.	3.40 ± 0.81	2.50 ± 0.47	2.56 ± 0.67	1.4 ± 0.3	12.2 ± 3.7	1.8 ± 0.2

cantly from net Cl^- flux with furosemide ($P > 0.5$). Furosemide increases the bi-directional Cl^- fluxes indicating non-specific actions with long incubations (1 h).

(e) The action of certain pharmacological agents upon the ATP-stimulated short-circuit current

Amiloride, a potent inhibitor of Na^+ transport in various epithelia [29], at 10^{-4} M in the apical solution is without inhibitory effect upon the ATP-dependent increase in short circuit current (Table V). 4-Aminopyridine inhibits K^+ transport in nerves [31]; but is without inhibitory effect upon the ATP-stimulated short-circuit current at $2 \cdot 10^{-3}$ M (Table V). Phloretin is an inhibitor of Cl^- transport in human blood cells [32]. 0.1 mM phloretin added to either apical or basal bathing solutions is an effective inhibitor of the ATP-stimulated short circuit current. This contrasts with the action of furosemide where an inhibitory action is observed only from the basal epithelial surface (see above). The stilbene SITS [25] is ineffective as an inhibitor of the ATP-dependent increase in short-circuit current when applied at 10 μM to both epithelial surfaces (Table V).

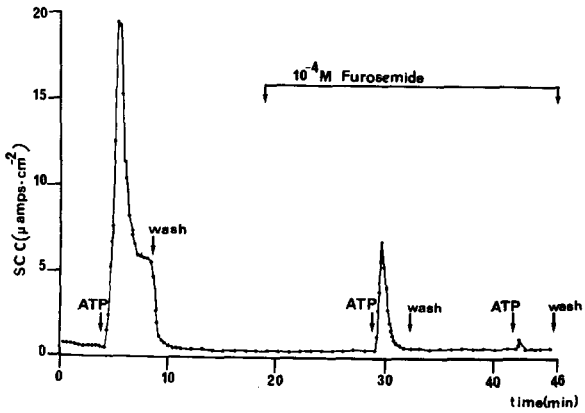

Fig. 4. Effect of preincubation of an epithelial monolayer with $1 \cdot 10^{-4}$ M furosemide upon the ATP mediated increase in the short-circuit current. A control response to ATP ($1 \cdot 10^{-4}$ M basal addition) precedes addition of furosemide. Note the absence of effect of furosemide upon the basal short-circuit current.

Ethacrynic acid ($1 \cdot 10^{-4}$ M) and piretanide ($1 \cdot 10^{-4}$ M) had a similar action to furosemide. Both were effective only when applied to the basal bathing solution and although a significant reduction in the ATP-stimulated short-circuit current was observed (Table V) no effect of ethacrynic acid or piretanide was observed upon the tissue resistance as compared to ATP-stimulated tissues (Table V).

10 mM thiocyanate inhibits the ATP-stimulated short-circuit current when applied to either apical or basal bathing solution (Table III). This action of thiocyanate is similar to that observed for the secretory process in shark rectal gland [4]. The inability of

TABLE V

THE ACTION OF INHIBITORS OF ANION AND CATION TRANSPORT UPON THE 10^{-4} M ATP-MEDIATED PEAK INCREMENT IN THE SHORT-CIRCUIT CURRENT (ΔSCC PEAK)

SITS, amiloride and 4-aminopyridine were applied for a 20-min preincubation period. Thiocyanate and phloretin were added 10 and 5 min prior to ATP, respectively. Ethacrynic acid and piretanide were applied 5 min subsequent to ATP stimulation. Control measurements and values of short circuit current in the presence of piretanide or ethacrynic acid were made 10 min subsequent to the addition of ATP. Measurements of resistance were made prior to the addition of ATP where appropriate and are included to show the absence of drug effects on monolayer integrity. a and b denote apical or basal additions, respectively. Measurements of resistance for the ethacrynic acid/piretanide experiments were made 10 min subsequent to ATP addition. In the case of SCN^- thiocyanate was substituted for Cl^- in an equimolar fashion. ATP was added to the basal bathing solution in all experiments. Errors are ± S.E.

Treatment	*n*	Resistance ($\Omega \cdot cm^2$)	*P*	ΔSCC peak ($\mu A \cdot cm^{-2}$)	*P* vs. control
Control	24	2482 ± 672		5.55 ± 1.73	
4-Aminopyridine (a + b) $2 \cdot 10^{-3}$ M	4	2951 ± 1250	n.s.	8.75 ± 1.75	n.s.
Amiloride (a) $1 \cdot 10^{-4}$ M	4	2943 ± 254	n.s.	12.00 ± 4.84	n.s.
Phloretin (b) $1 \cdot 10^{-4}$ M	3	4374 ± 1444	n.s.	0.35 ± 0.08	<0.05
Phloretin (a) $1 \cdot 10^{-4}$ M	3	2150 ± 1273	n.s.	0.39 ± 0.24	<0.005
*SCN^- (b) 10^{-2} M	3	1969 ± 842	n.s.	1.04 ± 0.41	<0.005
*SCN^- (a) 10^{-2} M	3	1940 ± 1182	n.s.	2.06 ± 0.89	<0.05
SITS $1 \cdot 10^{-5}$ M (a + b)	4	2642 ± 782	n.s.	7.42 ± 1.20	n.s.
Control	5	1270 ± 100		7.90 ± 1.50	
Ethacrynic acid (a) $1 \cdot 10^{-4}$ M	3	920 ± 280	n.s.	9.50 ± 2.50	n.s.
Ethacrynic acid (b) $1 \cdot 10^{-4}$ M	3	1210 ± 280	n.s.	4.13 ± 0.26	<0.05
Piretanide (a) $1 \cdot 10^{-4}$ M	4	1460 ± 220	n.s.	8.70 ± 0.30	n.s.
Piretanide (b) $1 \cdot 10^{4}$ M	4	1110 ± 180	n.s.	1.3 ± 0.45	<0.01

thiocyanate to substitute for Cl^- in the secretory process (Table III) and the inhibition observed with thiocyanate in the presence of Cl^-, suggests that the relative affinities of anions for binding to the membrane components involved in the rate-limiting step of anion transport, form a different order from that of their transport rates.

(f) Effect of high K^+-containing media

In tight-epithelia such as frog skin and urinary bladder the basal lateral membranes have a high resting K^+ conductance; replacement of the serosal bathing medium NaCl by KCl should result in a depolarization of the basal membranes [33–35]. Elevation of the basal bathing solution K^+ reduces the transepithelial spontaneous p.d. without affecting resistance (Table I). Apical K^+ elevation, is in contrast, without affect upon these electrical parameters (Table I). A similar pattern is observed for the ATP-stimulated short-circuit current. Basal elevation of the K^+ markedly reduces the ATP-stimulated short-circuit current (Tables I and II) by significantly reducing the ATP-stimulated p.d. without affecting the ATP-stimulated reduction in monolayer resistance (Table I). Apical K^+ elevation has no effect upon the ATP-stimulated p.d. Both apical and basal K^+ elevation result in a decreased monolayer resistance but only in the presence of ATP (Table I).

High K^+ media in the basal solution abolishes the net Cl^- secretion seen with exogenous ATP; net Cl^- flux plus ATP does not differ significantly from that in control (high K^+) media ($P > 0.5$ Table II).

(g) The effect of non-zero voltage clamping upon the ATP-dependent short-circuit current

The current-voltage relationship for high-resistance MDCK epithelial layers is linear and time-independent up to 80 mV for hyperpolarizing current passage [23]; for depolarizing current passage excepting for the 'initial' values of potential, the current-voltage plot is non-linear and dependent upon the time of current passage [23]. This is seen in Table I where

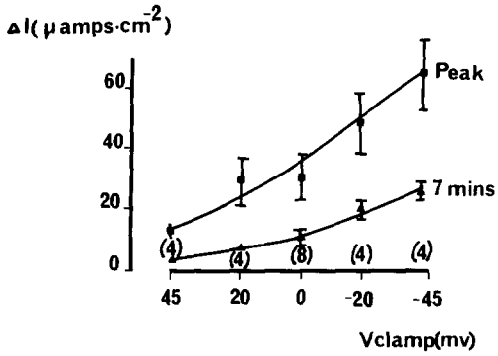

Fig. 5. Summary of the effect of voltage clamp at non-zero levels upon the ATP-dependent increase in short-circuit current. ■———■, peak values; ▲———▲, values at 7 min subsequent to ATP addition. Data from four separate monolayers. Hyperpolarization decreases the short-circuit current and accelerates the rate of decline to prestimulation levels. The converse is true of depolarization.

prolonged voltage clamping at depolarised potentials for 5 min decreased control monolayer resistance minus ATP from 1.5 ± 0.3 k$\Omega \cdot$ cm^2 to 0.90 ± 0.1 k$\Omega \cdot$ cm^2 ($P < 0.01$ for paired values). Hyperpolarization, in contrast, has no effect upon resistance (Table I). In control tissues, voltage clamped to zero p.d. addition of ATP stimulates the short-circuit current to a value of 39 ± 5.7 ($n = 4$) μA $\cdot$ cm^{-2} and tissue resistance falls to 0.6 ± 0.05 k$\Omega \cdot$ cm^2 ($n = 4$). Hyperpolarization (Table I) decreases the ATP-stimulated current and accelerates the decay of the current to pre-ATP levels (Table I, Fig. 5). The resistance change (1.4 ± 0.2 to $0.7(5) \pm 0.1$ k$\Omega \cdot$ cm^2) is similar to that observed in control tissues. Depolarization as noted above, decreases the control epithelial resistance; ATP stimulation results in an elevated current response (Table I) compared to the response observed at zero p.d. The resistance change due to ATP stimulation (0.90 ± 0.1 to 0.70 ± 0.1 k$\Omega \cdot$ cm^2) is reduced due to the introduction of parallel current-induced pathways [23].

Fig. 5 shows mean results of voltage-clamping four epithelial monolayers at various non-zero potential differences upon the ATP-stimulated current. The electrical gradient is clearly important in determining both the initial magnitude and duration of the ATP-stimulated current.

Discussion

This paper deals with the effects of exogenous ATP upon monolayers of MDCK cells which form an epithelium of high electrical resistance [18,22,23]. That exogenous ATP applied to cell monolayers results in a rheogenic Cl^- secretion from basal to apical surfaces of the cell monolayers is evident from the following data: (a) exogenous ATP though stimulating the short-circuit current has no effect upon transepithelial net Na^+ flux; (b) Cl^- secretion from basal to apical cell surfaces is stimulated by exogenous ATP to a sufficient magnitude to account for the observed increase in short-circuit current; (c) furosemide markedly reduces the ATP-stimulated short-circuit current and abolishes the net Cl^- secretion observed plus exogenous ATP, and (d) medium Cl^- substitution by the isethionate anion abolishes the ATP-stimulated short circuit current whereas Na^+ replacement by choline$^+$ does not.

ATP stimulation of Cl^- secretion is most probably the result of ATP interaction with a purine (P2) receptor [17,19,20]. Purinergic stimulation of various physiological functions has been demonstrated [19, 20] and purinergic stimulation is known to alter both cellular cyclic nucleotide and Ca^{2+} levels [19,20]. It is of interest to note that exogenous ATP has a stimulatory effect upon the short-circuit current in mammalian small intestine [37] that is Na^+-independent [37] and is similar to the data reported here for MDCK epithelium.

Certain features of the ATP-stimulated secretory response are similar to those observed in other Cl^- secreting epithelia: (a) Net Cl^- secretion in MDCK epithelia is accompanied by an increased tissue conductance. In theophylline-treated small intestine the increased tissue conductance observed in the presence of triaminopyrimidine is Cl^--dependent [9,24]. Similarly prostaglandin induced Cl^- secretion in corneal epithelium is associated with an increased transcellular conductance [2]. (b) Cl^- secretion in many systems (cornea, [2], fish gill [3], shark rectal gland [4], mammalian small intestine [6–9]) is inhibited by loop diuretics such as furosemide and ethacrynic acid [3,4,30]. Cl^- secretion in MDCK epithelium is inhibited by basal applications of furosemide, piretanide and ethacrynic acid. Phloretin and thiocyanate are also effective inhibitors in the MDCK systems. (c)

The ATP-stimulated short-circuit current is a linear function of Cl^- concentration in the bathing solution, similar to Cl^- secretion in fish-gill [3] and theophylline-treated mammalian intestine [24]. In MDCK epithelium replacement of bathing medium NaCl by choline chloride has only modest effects upon the ATP-stimulated short-circuit current, whereas in other secretory systems Na^+ is necessary [[3,7–9]. K^+ may be the co-ion for Cl^- secretion in MDCK epithelium. (d) The observed sequence for anions to be able to support the ATP-stimulated short circuit current is similar to type 4, 5 or 6 of Wright and Diamond [28], suggesting that the rate-limiting transport pathway in anion transport is hydrophobic. A similar conclusion has been reached for secretory mammalian small intestine [8].

In natural epithelia displaying a high electrical resistance 60–70% of tissue resistance is accounted for by the muscosal or apical membranes of the epithelial cells [38,39]. Junctional resistance in preparations mounted without edge-damage is thought to be greater than the apical membrane [38,39]. Also the largest resistances are associated with small transepithelial p.d.'s where the apical membrane conductance comprises over 95% of the total tissue resistance [38, 40]. MDCK epithelia display a high resistance/low transepithelial p.d. profile [23] suggesting that apical membrane resistance is high relative to that of the basal-membrane [38,39]. Clearly, if this is so, the ATP-stimulated transepithelial conductance will reflect an increased conductance of the apical membrane. Additionally current passage across the epithelial layer will predominantely effect the apical trans-membrane p.d. (providing shunt pathways are of small magnitude). The marked voltage dependence of the ATP-stimulated current may, therefore, reflect a potential sensitive transport step at the apical membrane. The inhibition of the ATP-stimulated Cl^- secretion by basal furosemide does not abolish the ATP-increased conductance.

Though phloretin and thiocyanate inhibit the ATP-dependent short-circuit current from either epithelial surface, furosemide, piretanide and ethacrynic acid are only effective from the basal solution. Taken together, these results are consistent with two separate Cl^- transport mechanisms, a furosemide sensitive Cl^- transport at the basal-lateral membranes and an ATP-induced Cl^- conductance at the apical membranes.

The effect of basal solution K^+ elevation upon the ATP-stimulated Cl^- secretion suggests that the furosemide-sensitive basal membrane Cl^- transport is potential sensitive and therefore not electroneutral in its overall stoichiometry. This particular result, however, must be regarded as tentative until confirmation of the effect of elevated K^+ upon the cell to basal solution electrical membrane potential is determined by microelectrode measurements, and the nature of the coupling of Cl^- movement to cations such as Na^+ and K^+ is elucidated.

The pharmacological sensitivity of Cl^- secretion reported here, (ethacrynic acid, furosemide, piretanide sensitive, SITS insensitive) is reminiscent of the diuretic sensitive $Na^+ + K^+ + Cl^-$ cotransport observed in both human red-cells and ascites tumor cells [26, 27,43]. We have recently demonstrated a Na^+-dependent, Cl^--dependent, furosemide-sensitive K^+ influx into MDCK cells whose properties closely resemble those of the red-cell system [41].

Cl^- transport across the basal-lateral membranes in other secretory epithelia is envisaged to be mediated

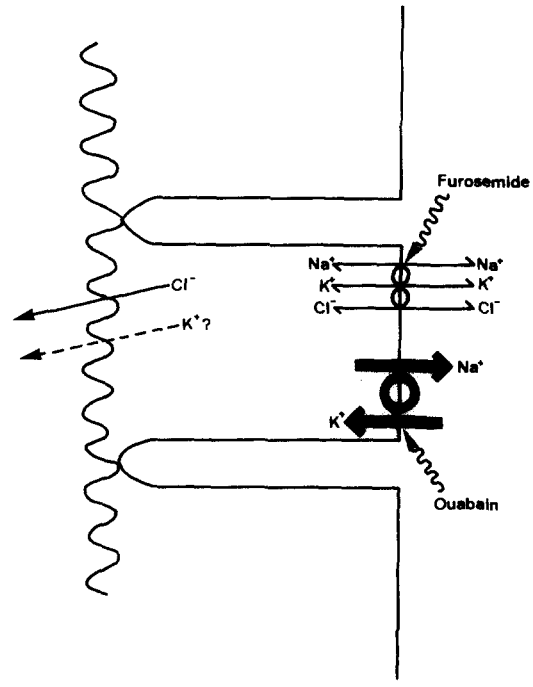

Fig. 6. A possible model of Cl^- secretion across MDCK epithelium. Thin lines represent passive ion movements, thick lines are pump-mediated ion movements.

by a coupled $Na^+ + Cl^-$ cotransport system [4,44]; a $Na^+ + Cl^-$ cotransport system has been identified in basal-lateral membrane vesicles from shark rectal gland [44]. Cl^- is then accumulated above its electrochemical equilibrium by utilising the energy inherent in the Na^+ gradient. Transepithelial Cl^- secretion is thus achieved by downhill movement of Cl^- across the apical membrane.

For MDCK epithelium Cl^- movement across the basal-lateral membranes may be driven uphill against the Cl^- electrochemical gradient by the furosemide-sensitive coupled $(Na^+ + K^+) + Cl^-$ cotransport system similar to that found in human red-blood cells [26, 27] (Fig. 6). Outwards K^+ movement or inwards Na^+ movement could provide the energy for Cl^- movement as envisaged by Shindo and Spring [45] for necturus basal-lateral Cl^- transport. Na^+ extrusion from the cell and a normal K^+ influx are driven by the $(Na^+ + K^+)$-ATPase localised in the MDCK cell to the lateral intercellular cell-membranes [42] (Fig. 6). Stimulation of net Cl^- secretion by exogenous ATP would then simply involve an increased apical membrane conductance for Cl^- (Fig. 6). Although in ascites cells the $Na^+ + K^+ + Cl^-$ cotransport is thought to be electroneutral [43] the sensitivity of transepithelial Cl^- transport to basal K^+ elevation may indicate that this is not true for the putative MDCK cell system.

Clearly measurements of intracellular Cl^- activities within MDCK cytoplasm, to verify the major features of the proposed mechanism, are necessary. It is also pertinent to note that Cl^- activities above electrochemical equilibrium values are sometimes not observed in epithelial tissue [47].

The inability to demonstrate a significant Na^+ dependence to the ATP dependent short-circuit current, seems to contradict an Na^+-gradient linked model of Cl^- secretion; however the K_m for Na^+ stimulation of the passive ouabain-insensitive anion-dependent K^+ influx in MDCK cells is <10 mM Na^+ (unpublished observations). Low Na^+ conditions adjacent to the cells in the lateral intercellular spaces will thus be difficult to establish experimentally.

The model proposed for Cl^- secretion suggests that net Cl^- secretion will be dependent upon a normally functioning (Na^+, K^+)-pump to maintain normal cellular cation gradients. In a companion paper [46] the action of ouabain upon the ATP-stimulated Cl^- secretion is investigated.

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