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Conversion of sodium channels to a form sensitive to cyclic AMP by component(s) from red cells

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1 Sodium transport has been measured in the isolated epithelium from colons of male Sprague-Dawley rats.

2 Sodium transport in colons was induced by pretreating the animals with dexamethasone ($6\ mg\ kg^{-1}$) which caused the appearance of an amiloride-sensitive short circuit current within a few hours.

3 Forskolin, a diterpene, which activates adenylate cyclase, was found to increase the cyclic adenosine monophosphate (cyclic AMP) content of rat colons and also to increase short circuit current at the same time. However, measurements of chloride and sodium fluxes across the epithelium indicated that forskolin activates chloride secretion but has no effect on sodium transport.

4 In confirmation of (3) it was found that the amiloride-sensitive short circuit current was unchanged after the short circuit current had been increased by forskolin under a variety of conditions.

5 The behaviour of the mammalian colon as indicated in (3) and (4) is unlike that of amphibian sodium transporting epithelia. It is shown that in toad urinary bladder forskolin increases amiloride-sensitive short circuit current.

6 Procedures were investigated which might make sodium transport in the mammalian colon sensitive to cyclic AMP. Exposing the apical surface to sonicated suspensions of nucleated red cells (frog, toad and duck), followed by washing, gave preparations with amiloride-sensitive short circuit currents which were increased by forskolin or dibutyryl cyclic AMP.

7 It would appear that the sodium channel in the mammalian colon, unlike that of amphibian tissues, has lost the ability to have its properties modified by cyclic AMP. Incubation of colons with sonicated suspensions of nucleated red cells apparently modifies the tissues such that sodium transport across the tissue becomes sensitive to the nucleotide.

Introduction

Although epithelial sodium channels are widely distributed in nature they are absent, normally, from the colonic epithelium of the rat, the tissue chosen for this study. Amiloride, a specific blocker of electrogenic epithelial sodium ion transport has no effect on normal rat colon (Will, Lebowitz & Hopfer, 1980; Cuthbert & Margolius, 1982). However, by feeding rats on a low sodium diet, giving mineralocorticoids or high doses of glucocorticoids, electrogenic sodium transport is induced which is amiloride-sensitive (Binder, 1978; Will, Delisle, Cartright & Hopfer, 1981; Cuthbert & Margolius, 1982).

In the rabbit the situation is somewhat different in that amiloride-sensitive sodium transport is present normally (Schultz, Frizzell & Nellans, 1977) but nevertheless, can be increased by mineralocorticoids (Frizzell & Schultz, 1978). The avian coprodaeum behaves rather as the rat in that amiloride-sensitive sodium transport appears only after salt deprivation (Choshniak, Munck & Skadhauge, 1977; Bindslev, Cuthbert, Edwardson & Skadhauge, 1982).

A number of agents, notably cyclic adenosine monophosphate (cyclic AMP), prostaglandins (Frizzell & Heintze, 1979) and kinins (Cuthbert & Margolius, 1982), increase short circuit current (SCC) in mammalian colon. The increased current is not due to enhanced sodium absorption but rather to chloride secretion. Measurements of ion fluxes have shown, for the rabbit, that sodium transport is insensitive to cyclic AMP (Frizzell & Heintze, 1979) and we show here that this is true too for the rat colon. Alone, the failure of sodium transport to be modified by cyclic AMP is unremarkable until it is recalled that, in other systems, cyclic AMP and hormones which act via this

second messenger can modify sodium transport. For example, vasopressin increases sodium transport in frog skin and toad urinary bladder via cyclic AMP as suggested first by Orloff & Handler (1967). Recent work has shown that vasopressin recruits electrically silent channels without affecting the mean life-time or unit conductance of individual channels (Li, Palmer, Edelman & Lindemann, 1981). In this way the recruitment of silent channels accounts for the macroscopic increase in sodium SCC.

In this study we have used a variety of protocols designed to increase intracellular cyclic AMP in rat colons taken from animals pretreated with dexamethasone and exhibiting amiloride-sensitive SCC. Sodium transport was not modified by these procedures; however, if the apical surface of the colon was exposed to sonicated red cell suspensions from either toads, frogs or ducks, sensitivity of the sodium transporting process to cyclic AMP was conferred. The inference is that in mammalian colon, sodium channels are in some way deficient compared to those in, say, toad urinary bladder. This result provides a novel way to investigate sodium channel mechanisms.

Methods

Short circuit current recording

Most experiments were carried out on colons of male Sprague-Dawley rats; however, a few experiments were also performed with toad (*Bufo marinus*) urinary bladders. Colons were opened longitudinally and the longitudinal and circular muscle dissected away, such preparations being referred to as stripped colons. Urinary bladders were used without further preparation.

Tissues were voltage clamped at zero potential (short-circuited) with compensation for the series resistance between the potential electrodes and the tissue. Potential electrodes were filled with the bathing solution whereas the current passing electrodes contained KCl-agar. Short circuit currents (SCCs) were displayed continuously on pen recorders. The methods used for mounting and maintaining the tissue were conventional and details can be found in earlier papers (Cuthbert, 1976; Cuthbert & Margolius, 1982).

Flux measurements

Fluxes of sodium and chloride across the colonic epithelium were measured using trace amounts of either ^{22}Na or ^{36}Cl in short circuited tissues. We decided not to use the method devised by Cuthbert & Margolius (1982) for, although it produces very accurate measurements of flux, it is not possible to obtain paired preparations from single descending colons. As an alternative, flux measurements of either ^{36}Cl or ^{22}Na were made in paired preparations of only 0.6 cm^2 taken from the same tissue. In these cases the isotope was added to the mucosal bath of one preparation and to the serosal bath of the other. Twenty minutes were allowed for the tissue to achieve isotopic equilibrium, following which 4 samples were taken in pairs at 30 min intervals from the bath opposite to that to which the isotope was added. The first pair was taken before drug addition and the second pair after the drug effect was established. Thus net fluxes before and during drug effects were obtained by subtracting the mucosal to serosal flux from that in the opposite direction.

Measurement of cyclic AMP generation

Colons were opened longitudinally, washed in Krebs-Henseleit solution and blotted. The epithelial cells were scraped from the mucosal surface using a glass microscope slide. Cells were placed in Krebs-Henseleit solution containing isobutylmethylxanthine (IBMX) 5 mM, gentamycin 600 $\mu g\,ml^{-1}$ and trasylol 200 $\mu g\,ml^{-1}$ and homogenized by hand. To a sample of this suspension (250 μl), prewarmed to 30°C, was added 1 ml of prewarmed reaction mix containing ATP (0.8 mM), creatine phosphate (20 mM), phosphocreatine kinase (1 $mg\,ml^{-1}$), Tris HCl, pH 7.5 (2.5 mM) and bovine serum albumen (4 $mg\,ml^{-1}$) together with forskolin (10 μM) where appropriate. The reactions were stopped by placing the incubation tubes in a boiling water bath for 5 min. After cooling, the tubes were centrifuged and the supernatant removed and stored frozen. Cyclic AMP in the supernatant solutions was assayed by a standard radio-immunoassay. Protein concentrations were measured by the method of Lowry, Rosebrough, Farr & Randall (1951). Occasional checks were made to show that the amount of cyclic AMP measured varied linearly with the volume of solution assayed and that known amounts of cyclic AMP added to the extracts could be accounted for quantitatively on assay.

Induction of sodium transport

Sodium transporting activity in rat colons was induced by following the procedures developed by Will *et al.* (1981). Usually dexamethasone (Decadron Shock-Pak, Merck Sharp & Dohme) was given by a single intraperitoneal injection (6 $mg\,kg^{-1}$). Occasionally dexamethasone was given by continuous infusion for periods of up to 1 week using implanted osmotic minipumps (Alzet Model 2001) delivering approximately 120 $\mu g\,kg^{-1}\,h^{-1}$. The minipumps were

inserted between the skin and underlying muscle layers in the flank.

Sonicated red cell suspensions

Blood was collected from either pithed toads (*Bufo marinus*) or frogs (*Rana temporaria*) or by venepuncture from ducks and human volunteers. Collection was made into heparinised vessels and red cells separated by centrifugation. The plasma and buffy coat were discarded and the red cells washed, three times, in Krebs-Henseleit solution. The cells were sonicated in an MSE instrument at 0°C. In some instances the Krebs-Henseleit solution in which the red cells were sonicated contained 50% polyethylene glycol (mol. wt. 1000).

Solutions

Krebs-Henseleit solution, gassed with 95% O_2 and 5% CO_2 at 37°C and pH 7.4 was used for the colon preparations. The composition was (mM) NaCl 117, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, $NaHCO_3$ 24.8, KH_2PO_4 1.2 and glucose 11.1.

Toad bladders were mounted in a Tris buffered solution at pH 7.6 at room temperature (circa 20°C) and gassed with air. The composition of this solution was (mM) NaCl 110, KCl 2, $CaCl_2$ 1, Tris HCl 5 and glucose 11.1.

Drugs

Amiloride was a gift from Dr G. Fanelli of the Merck Institute for Therapeutic Research and Piretanide from Dr S. Dombey of Hoechst, U.K. Other drugs were from the usual suppliers as follows: amphotericin (Squibb), dexamethasone (Merck Sharp & Dohme), dibutyryl cyclic AMP and kallidin (Sigma), forskolin (Calbiochem), gentamycin (Kirby-Warwick Ltd) and IBMX (Aldrich).

Results

Effect of dexamethasone on amiloride sensitivity

An experiment, illustrated in Figure 1, was carried out to confirm that dexamethasone induced amiloride-sensitivity in the colon. Illustrated is maximum change in SCC caused by amiloride following application of a supramaximally effective concentration (100 μM) to the mucosal bath. By way of controls some tissues were taken from animals given a single intraperitoneal injection of solvent, in this instance water, or of canrenoate (20 mg kg^{-1}, MCP Pharmaceuticals), an aldosterone antagonist. The latter was an extra control in case solvent alone caused

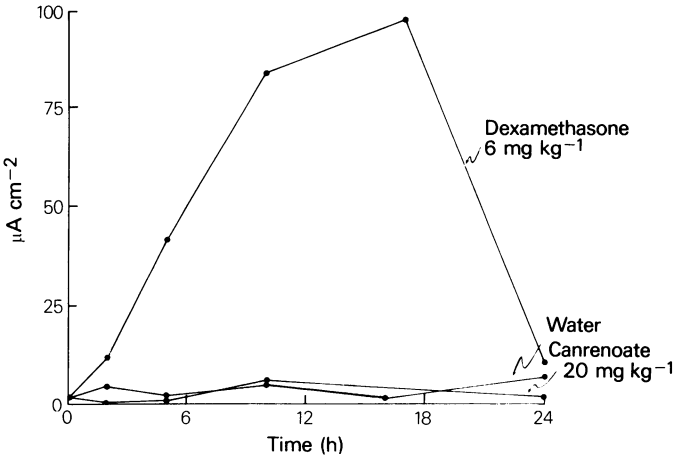

Figure 1 Experiment with tissues from 18 rats, 6 each were given intraperitoneal injections of dexamethasone (6 mg kg^{-1}), canrenoate (20 mg kg^{-1}) or water (0.6 ml) at zero time. At appropriate times, three preparations were set up from each animal and the amiloride-sensitive short circuit current (ASCC) determined by adding 100 μM amiloride to the mucosal bath. Each point is the mean of three separate measurements.

significant release of endogenous aldosterone, giving a false positive result. Combining the results given in Figure 1 with those of a larger, but less complete, series of observations, it is concluded that a definite amiloride-sensitive short circuit current (ASCC) was measurable in isolated tissues 2 h after dexamethasone was given to rats. This increased considerably by 5 h (46.0 ± 14.3 μA cm^{-2}, $n = 9$). Following a single injection of dexamethasone (6 mg kg^{-1}) the peak value of ASCC occurred at approx. 17 h (129.5 ± 21.5 μA cm^{-2}, $n = 11$) but this high value waned by 24 h. In most of the other experiments described in this paper we have used colons from rats given a single dexamethasone injection (6 mg kg^{-1}) between 17–22 h previously.

Concentration-response curves to amiloride were measured in paired preparations of dexamethasone-treated colons. In one of the pair the SCC was first stimulated by forskolin, 10 μM, added to both the mucosal and serosal bath. Forskolin, which increases cyclic AMP generation is of crucial significance for the arguments to be presented later in this paper and it is important to know if this agent affected amiloride-sensitivity. Figure 2 shows that the half maximally effective concentration of amiloride was 0.25 ± 0.02 μM and that this increased to 0.47 ± 0.07 μM after treatment with forskolin. However, near maximal inhibition of SCC was obtained with amiloride concentrations between 10 and 100 μM both in the absence and presence of forskolin. The mean value of the Hill slopes before forskolin

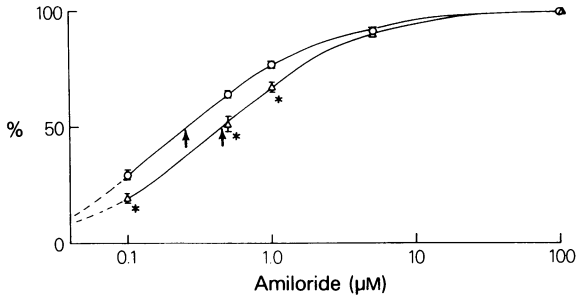

Figure 2 Concentration-responses curves to amiloride in dexamethasone-treated rat colons. Maximal inhibition was taken as that achieved with amiloride, 100 μM, added to the mucosal bath. (○) Control values; (△) results after tissues were treated with forskolin, 10 μM. Values marked with an asterisk are significantly different from controls at $P<0.05$, using a paired t test. For all points, $n = 5$.

was 0.84 ± 0.07 and afterwards 0.95 ± 0.07. Six of the ten values had Hill slopes greater than 0.9 but the mean values were influenced by a few low values before and after forskolin. In three experiments the Hill slope was increased after forskolin and in two experiments the reverse was true.

Amiloride-sensitive short circuit current in dexamethasone-treated colons

In this section we shall assume ASCC is a measure of the sodium transporting capability of the colon and that this is directly related to the sodium permeability of the apical face; this reasoning is justified later on. In order to ascertain whether or not agents which increase SCC affect sodium transport, the value of ASCC was measured before and after application of

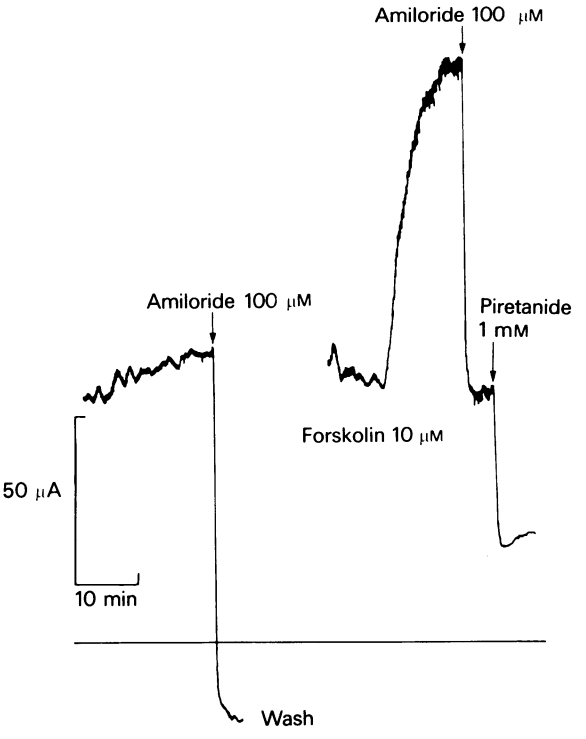

Figure 3 ASCC was measured in a dexamethasone-treated colon (0.6 cm^2) by addition of amiloride, 100 μM to the mucosal bath. The blocking drug was removed by washing and the SCC regained its original value. Forskolin, 10 μM, was then added to both sides of the preparation when SCC increased to a new, elevated, steady state at which time ASCC was remeasured. In the presence of amiloride, a further fall in SCC occurred on addition of piretanide, 1 mM, to the serosal bath. Horizontal line indicates zero SCC.

Table 1 Amiloride-sensitive short circuit current (ASCC) in dexamethasone-treated colons before and after stimulation of SCC

Expt.	*Dexamethasone treatment* (h)	*Stimulating agent*	*SCC'* (μA)	*ASCC'* (μA)	*SCC"* (μA)	*ASCC"* (μA)	*Sig*	n
1	15–22	Forskolin, 15′	62.4 ± 13.0	69.4 ± 14.9	139 ± 11.9	57.4 ± 14.8	NS	8
2	5	Forskolin, 15′	38.0 ± 8.6	29.0 ± 12.8	202 ± 29.0	23.5 ± 12.7	NS	6
3	12.5–20	Forskolin, 60′	181.7 ± 20.6	182.3 ± 22.1	268 ± 41.2	188.7 ± 23.1	NS	6
4	17–21	Kallidin, 15′	70.2 ± 20.4	73.6 ± 20.6	132 ± 23.6	61.6 ± 18.0	NS	5
5	160	Forskolin, 15′	63.0 ± 26.1	41.8 ± 14.2	209 ± 39.1	32.8 ± 13.2	NS	4
6	16–22	Equilibration	177 ± 34.9	139.5 ± 33.7	48.5 ± 19.0	46.2 ± 20.5	<0.05	6

All currents are in μA 0.6 cm^{-2}. Dexamethasone was given as a single intraperitoneal injection at 6 mg kg^{-1} except in group 5 where it was administered continuously at 120 μg kg^{-1} h^{-1}. Fortskolin (10 μM) was applied in the mucosal and serosal baths. Amiloride was used at 100 μM in the mucosal bath and kallidin at 1 μM in the serosal bath. Sig refers to P values using Student's t test of ASCC" versus ASCC'. In groups 1 and 4, ASCC" was significantly smaller than ASCC' using a paired t test. SCC' and ASCC' are values measured before SCC was stimulated while SCC" and ASCC" were measured after stimulation. In all experiments the mean values of SCC' are significantly different ($P<0.05$) from those for SCC".

the stimulating drugs. These two measurements of ASCC were relatively easily made as the blocking action of amiloride is rapidly reversed by washing. Five groups of experiments with this protocol were carried out. In the first, ASCC was determined before and after forskolin (Figure 3). About 15 min were allowed for the forskolin response to peak before the second determination of ASCC was made. Even though there was a substantial increase in SCC, the absolute amount of current which can be removed by addition of amiloride is unchanged. The current remaining after forskolin in the presence of amiloride is certainly due in part to chloride secretion as shown by its sensitivity to piretanide (Cuthbert & Spayne, 1982). Table 1 (Expt 1) shows the results of 8 similar experiments with rat colons taken from animals pretreated with dexamethasone 15–22 h previously. There was no statistical difference between the value of ASCC determined before or after forskolin unless a paired *t* test was used, when it was shown that ASCC was slightly smaller ($P<0.05$) after forskolin.

In a second group of experiments the same protocol was used but in this instance the colons were taken from animals that had been treated with dexamethasone only 5 h previously, a time at which ASCC had not fully developed. From Table 1 it is again found that the ASCC was not increased by forskolin in these tissues.

In the first two groups of experiments the second measurement of ASCC was made immediately the forskolin had reached its peak value, usually 10–15 min after addition of the terpene. In a further group of experiments, forskolin was allowed to act for 1 h before the second determination of ASCC was made. It was considered that prolonged elevated

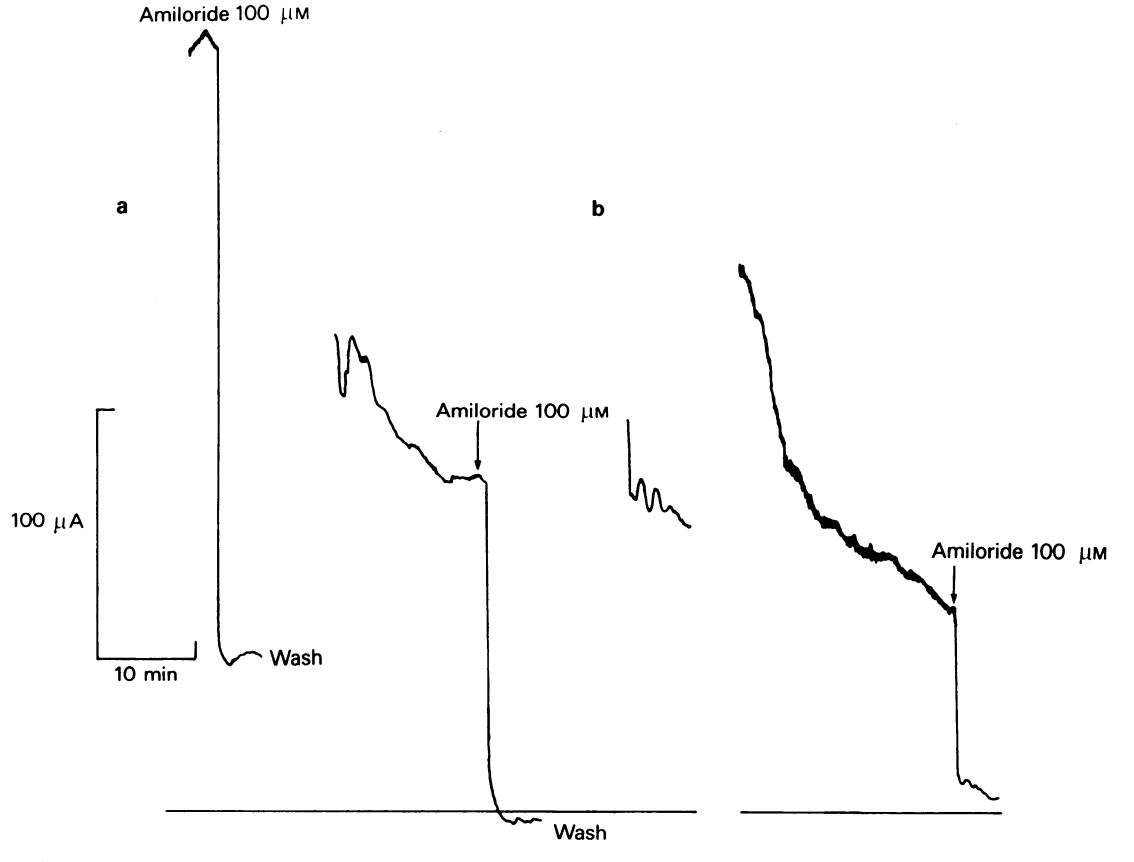

Figure 4 Traces illustrate an experiment with paired pieces of rat colon from a dexamethasone-treated rat (0.6 cm^2). In (a) ASCC was measured as soon as practicable after the preparation had been mounted. The blocking drug was washed away and ASCC determined a second time after SCC had been allowed to reach its steady state value. Note its value was less than with the original determination. Preparation (b), set up at the same time as (a) was allowed to equilibrate and ASCC determined only once simultaneously with the second determination in (a). Horizontal line indicates zero SCC.

cyclic AMP levels might produce an effect on the transport mechanism. However, ASCC was not significantly changed by this procedure (Table 1).

In normal rat colons, kallidin causes a large increase in SCC. It has a similar effect in dexamethasone-treated tissues and it was of considerable interest to know if this agent would affect ASCC. As with the three previous groups of experiments, ASCC was not affected even though SCC was approximately doubled with kallidin (Table 1).

Although dexamethasone causes the appearance of an amiloride-sensitive sodium transport within a few hours, it is possible that the sensitivity of the transport mechanism to cyclic AMP develops more slowly. Consequently we administered dexamethasone to rats, continuously for 1 week at approximately 120 $\mu g\,kg^{-1}\,h^{-1}$, using implanted osmotic minipumps. Thus the duration of the exposure to steroid is greater than the lifetime of the epithelial cells (Cremaschi, James, Meyer, Peacock & Smith, 1982). Yet again the value of ASCC was not increased after forskolin (Table 1).

In a final set of experiments the protocol was somewhat different. We had noticed that immediately after setting up dexamethasone-treated colons, the SCC was initially high but declined rapidly to a lower steady state during 15 min or so after setting up the preparation. In this final set ASCC was determined immediately after setting up and again after the SCC had reached steady state; the value of ASCC was significantly greater ($P<0.05$) on the first determination than the second (Figure 4, Table 1). Notice from Figure 4 that, in the pre-equilibrium determination of ASCC, amiloride fails to reverse the SCC but does so after equilibration. This may indicate that not only does amiloride-sensitive sodium transport decline during equilibration but processes associated with non-amiloride-sensitive SCC also decline. This last group of experiments proves that tissues are capable of a greater degree of sodium transport than is shown at steady state. Yet increasing cellular cyclic AMP levels with forskolin, or adding kallidin to increase SCC, does not increase ASCC, that is, the changes occurring during equilibration are not reversed.

The behaviour of the rat colon as shown in Table 1 is in distinct contrast to the behaviour of amphibian tissues that are described in the next section. Here we have shown, using a variety of protocols, that the sodium transporting capacity, measured as ASCC, cannot be increased by procedures assumed to increase cyclic AMP.

Comparative experiments in amphibian tissues

In amphibian tissues, such as frog skin and toad urinary bladder, all of the SCC is accounted for by electrogenic sodium transport inwards through the tissue. SCC in frog skin is stimulated by forskolin and the whole of the increase plus the basal current is abolished by amiloride, indicating that forskolin increases sodium transport in this tissue (Cuthbert &

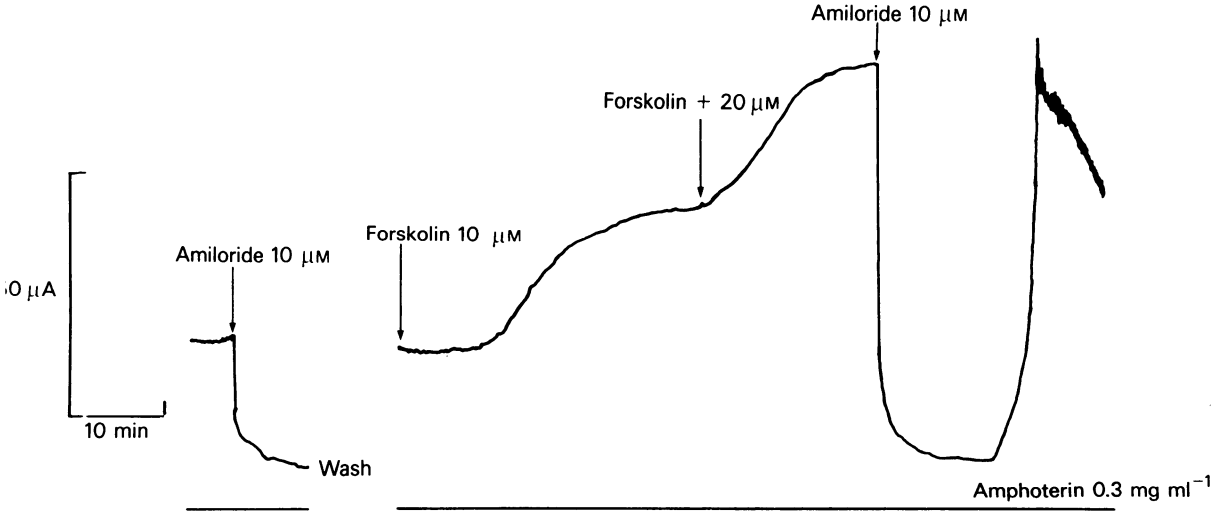

Figure 5 SCC record from a toad urinary bladder. ASCC was measured and the blocking drug removed so that the SCC was restored to the original value. Addition of two concentrations of forskolin to the mucosal and serosal baths increased SCC to new steady state values. Addition of amiloride abolished all of the SCC generated in the presence of forskolin. In the continued presence of amiloride, amphotericin caused SCC to increase. Horizontal line indicates zero SCC. Tissue area 7 cm^2.

Spayne, 1982). Here we show that the same is true for another amphibian tissue, the toad urinary bladder. Figure 5 is typical of several experiments and shows that amiloride reduces SCC to exactly the same value both before and after transport was stimulated with forskolin. After sodium transport was blocked with amiloride, it could be restored by making the apical membrane leaky to ions, including sodium, indicating that the block cannot be explained by an effect on the sodium pump located on the basolateral aspect of the cells.

Na^+ and Cl fluxes in response to forskolin

Thus far we have shown that dexamethasone causes the appearance of electrogenic sodium transport in rat colon which is apparently insensitive to forskolin or kallidin. However, SCC does increase with both of the latter and ion fluxes were measured to determine which ions are responsible for the current increase.

Using 6 sets of paired preparations, the mucosal to serosal flux (J_{ms}) and the J_{sm} flux of Na^+ was measured during a control period and following treatment with forskolin in tissues from dexamethasone-treated animals. As the preparations were paired we may assume that they had similar characteristics, so that net fluxes can be obtained by combining J_{ms} and J_{sm} measurements. From Table 2 it can be seen that dexamethasone-treated colons show net sodium absorption (J_{ms}^{net}) of approximately 14 μEq cm^{-2} h^{-1} and that this is not significantly altered by forskolin. Theoretically both J_{ms} and J_{sm} fluxes might increase by the same amount in the presence of the terpene without affecting J_{sm}^{net}; however, the table shows that neither are changed by the treatment. On the other hand, the SCC increased significantly after forskolin by approximately 5 μEq cm^{-2} h^{-1} but this could not have been due to sodium absorption. At the end of the second flux period the value of ASCC was measured and found to be 214.7 ± 47.1 μA cm^{-2} ($n = 6$). This corresponds to around 8 μEq cm^{-2} h^{-1}, indicating that not the whole of the sodium absorbed is by an amiloride-sensitive, electrogenic process.

In a further set of 6 paired experiments the effect of forskolin on chloride fluxes was investigated. In all experiments forskolin increased J_{sm} and reduced J_{ms} so that there was an increase in the net flux of chloride in the serosal to mucosal direction (Table 3). When the areas under the forskolin SCC responses were integrated, it was found that the current responses were not significantly different from increase in net chloride secretion (Table 3). It can be concluded therefore that the responses to forskolin in dexamethasone-treated colons are to a large extent due to chloride secretion and that any inequality which may exist between chloride secretion and the SCC responses cannot be accounted for by increased

Table 2 Sodium fluxes and associated biophysical parameters in dexamethasone-treated colons: effects of forskolin

J_{ms}^{net}			$\Delta J_{forskolin}$			SCC		
Control	*Forskolin*	ΔJ_{ms}	ΔJ_{sm}	ΔJ_{ms}^{net}	*Control*	*Forskolin*	ΔSCC	*ASCC*
14.65 ± 1.64	14.03 ± 2.74	-0.13 ± 1.73	0.49 ± 0.35	-0.62 ± 1.47	8.08 ± 1.53	12.76 ± 2.24	4.68 ± 0.97	8.01 ± 1.76

Values are μEq cm^{-2} h^{-1}. Forskolin was used at a concentration of 10 μM. Rats were treated with dexamethasone, 6 mg kg^{-1}, 16–21 h before use. $\Delta J_{forskolin}$ is the difference in fluxes between the control period and during the action of forskolin. Net fluxes were obtained from the two unidirectional fluxes in paired preparations. All flux values are the means ± s.e. for six observations while measurements of SCC are means ± s.e. for 12 observations.

Table 3 Chloride fluxes and associated SCC responses in dexamethasone-treated rats: effects of forskolin

ΔJ_{sm}	$\Delta J_{forskolin}$ ΔJ_{ms}	ΔJ^{net}	ΔSCC
3.83±0.69	−6.27±1.54	10.10±1.58	8.67±0.67

Values are $\mu Eq\,cm^{-2}\,h^{-1}$. Forskolin was used at a concentration of 10 μM. Rats were treated with dexamethasone, $6\,mg\,kg^{-1}$, 16–21 h before use. $\Delta J_{forskolin}$ is the difference in fluxes between the control period and during the action of forskolin. Net fluxes were obtained from the two unidirectional fluxes in paired preparations. During the control period the net flux of chloride was in the inward direction ($J^{net} = 5.03 \pm 2.48$ $\mu Eq\,cm^{-2}\,h^{-1}$). For measurements of flux $n = 6$ and for SCC $n = 12$.

sodium absorption. In other experiments (data not shown), forskolin was found to cause a similar increase in chloride secretion in normal rat colons.

Other inferences can be drawn from the results given in Tables 2 and 3. Under control conditions there was a net inward sodium movement of $14.7\,\mu Eq\,cm^{-2}\,h^{-1}$ of which only $8\,\mu Eq\,cm^{-2}\,h^{-1}$ was electrogenic, that is sensitive to amiloride (Table 2). In the other set of control measurements (Table 3) there was a net influx of chloride of $5\,\mu Eq\,cm^{-2}\,h^{-1}$, which is not very different from the non-electrogenic sodium absorption ($14.7-8.0 = 6.7$ $\mu Eq\,cm^{-2}\,h^{-1}$), although it must be realized that the two sets of measurements are not strictly comparable as they derive from different sets of tissues. It seems reasonable to propose that dexamethasone-treated tissues can absorb NaCl by an electrically neutral process while at the same time absorbing sodium by an electrogenic, amiloride-sensitive mechanism.

Effect of forskolin on adenylate cyclase

The effect of forskolin on the activity of adenylate cyclase was measured in broken cell preparations prepared from colons as described under Methods. Essentially, homogenized epithelial cells were added to a reaction mixture containing ATP in which cyclic AMP was generated and subsequently assayed by radioimmunoassay. The reaction mixture was similar to that described by Beubler (1981) but we omitted ethylenediamine tetraacetate. The cell homogenate was produced in Krebs-Henseleit buffer containing IBMX (to inhibit phosphodiesterase), gentamycin (as an antibacterial) and trasylol (as a general protease inhibitor), but even so the generation of cyclic AMP remained linear for only a few minutes. This may have been due to incomplete inhibition of phosphodiesterase, as in separate experiments (data not shown) we showed that the amount of cyclic AMP generated in a fixed time continued to increase as the concentration of IBMX was increased up to 5 mM. It is probable too that autolysis was occurring in broken cell preparations due to the presence of digestive enzymes and bacteria, even though trasylol and gentamycin were added to inhibit these.

To show the effects of forskolin on adenylate cyclase, identical aliquots with or without forskolin (10 μM) were incubated for a given time and the reaction stopped by boiling. The effect of forskolin was taken as the difference in cyclic AMP content found in the incubates in the presence and absence of the terpene. Figure 6 gives the results from two experiments in which normal and dexamethasone-treated colons were used. It is seen that forskolin stimulates adenylate cyclase in both types of tissue and apparently stimulation is greater in the dexamethasone-treated tissues. We are not able to say from these results whether the difference is due to increased enzyme activity or to increased amounts of enzyme. Furthermore, the data are presented as cyclic AMP generated per mg protein and we cannot be sure that the ratio of surface area to protein content is the same for colonic epithelia in the two conditions,

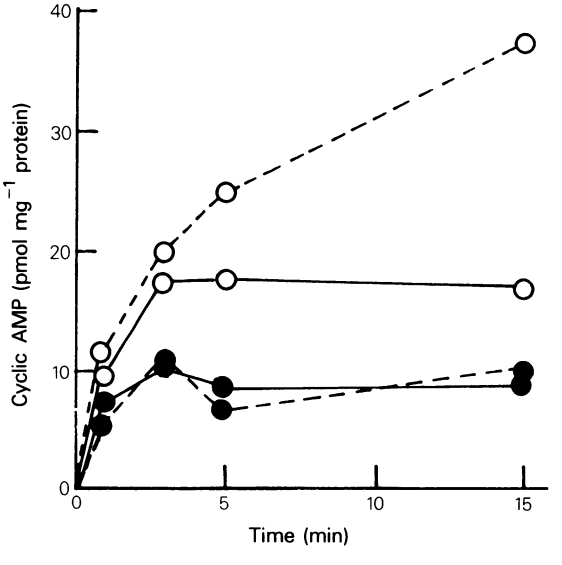

Figure 6 Effects of forskolin on adenylate cyclase. The values plotted are the differences in cyclic AMP content of identical tissue samples incubated in the presence and absence of forskolin, 10 μM. Two separate experiments are shown (continuous or dotted lines) in which tissues from normal animals (●) were compared with those from dexamethasone-treated ($6\,mg\,kg^{-1}$, 17 h) animals (○). Each point is the mean of duplicate determinations.

although gross differences of this kind would have been apparent. However, it is clear that adenylate cyclase is stimulated by forskolin in the colon and this provides a biochemical basis for the chloride secretory activity of forskolin in intact epithelia.

Effects of sonicated red cell suspensions on amiloride-sensitive short circuit current

The results given in an earlier section and in Table 1 demonstrate conclusively that when the SCC is increased by either forskolin or kallidin in dexamethasone-treated rats the ASCC, and hence the transport of sodium, is not enhanced in contrast to the results with amphibian bladders. We shall assume at this point that the sodium channels in mammalian and amphibian tissues are similar, indeed their affinity for amiloride is almost identical, but that some component necessary for channel modification in response to cyclic AMP is missing from the mammalian channel.

For reasons given in the discussion, we considered such a missing component might be present in nucleated red cells and therefore set out to interact vesicles prepared from washed red cells, sometimes in the presence of a fusogenic agent, with the apical surface of rat epithelia. After exposing to sonicated suspensions of red cells the epithelia were washed and treated according to the method used for the experiments in Table 1. We have found that exposure to sonicated suspensions of toad, frog or duck erythrocytes confers the sensitivity typical of amphibian tissues.

Figures 7 to 10 show representative experiments in which ASCC is increased following exposure and removal of sonicated suspensions of erythrocytes. Furthermore, it is immaterial whether SCC is increased with forskolin, dibutyryl cyclic AMP or kallidin. On average, a 70% increase in ASCC is found (Table 4) and on occasion ASCC is more than doubled.

Figure 7 shows one example using paired preparations of dexamethasone treated colons in which SCC was stimulated by forskolin. This agent produced a comparable increase in SCC in both tissues, although the time courses were different. The tissue pre-exposed to a toad red cell suspension showed, after forskolin, an ASCC 2.2 times larger than the original value, while the preparation not exposed to red cells showed exactly the same ASCC before and after

Table 4 Summary of the effects of exposure to sonicated red cell suspensions on amiloride-sensitive short circuit current (ASCC)

Group 1 *Agent*	*ASCC'*	*ASCC''*	*ΔASCC*	*Red cells*
Forskolin	54	72	18	Toad (P)
Forskolin	51	83	32	Toad
Forskolin	25	56	31	Toad
Forskolin	100	216	116	Duck (P)
db cyclic AMP	66	143	77	Toad
db cyclic AMP	46	75	29	Duck
db cyclic AMP	97	115	18	Duck
Kallidin	41	66	25	Duck (P)
Kallidin	44	69	25	Duck (P)
Kallidin	80	142	62	Frog
	60.3 ± 7.9	103.7 ± 15.9	43.1 ± 10.1	
		$P < 0.05$		
Group 2				
Forskolin	40	44	4	Toad (serosal)
Forskolin	9	9	0	Nil
db cyclic AMP	80	87.5	7.5	Human
db cyclic AMP	18	27	9	Human
db cyclic AMP	70	72.5	2.5	Human
Kallidin	26	50	24	Human

Agents used to increase SCC are indicated together with the source of the red cells. Concentrations of drugs used were forskolin, 10 μM; db cyclic AMP, 1 mM; kallidin, 1 μM and amiloride, 10 μM. (P) indicates that polyethylene glycol was present during exposure to red cells. All values are μA 0.6 cm^{-2}.

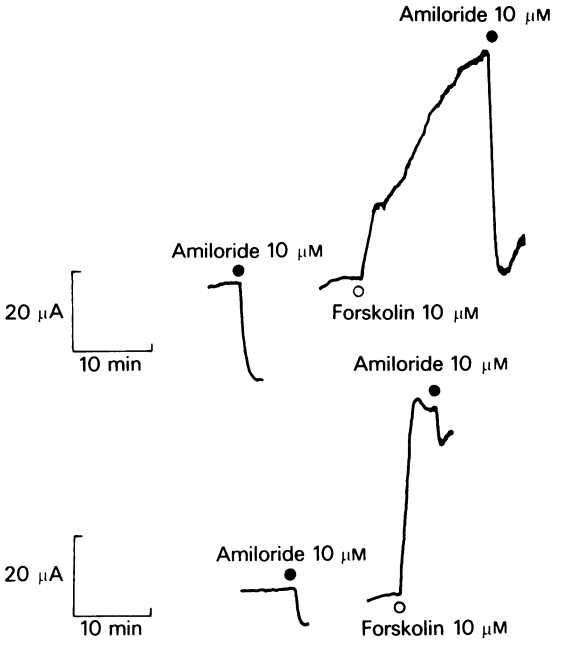

Figure 7 SCC records from two preparations taken from a dexamethasone-treated rat. The preparation represented by the upper records was treated with a sonicated toad red cell suspension for 30 min on the apical side while the other preparation received no treatment. The red cell suspension had no effect on SCC. Both preparations were then rinsed with fresh K-H solution and the ASCC determined in each using 10 μM amiloride (●). The amiloride was removed and SCC reassumed its former value. Each preparation was treated with forskolin, 10 μM (○). At the peak of the response to forskolin, ASCC was again measured in each preparation. Notice in the upper trace ASCC was increased by 2.2 times while the untreated preparation showed no change in ASCC.

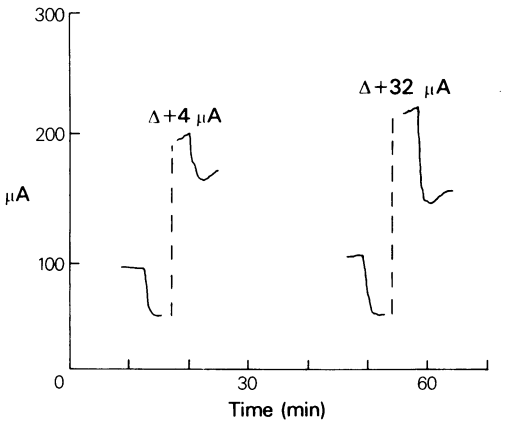

Figure 8 SCC records from two preparations taken from the same dexamethasone-treated rat. Both preparations were exposed to aliquots of the same suspension of sonicated toad red cells (final haematocrit approximately 5%). The preparation represented by the left hand tracings was exposed on the basolateral side, while the tracings on the right represent the other preparation which was exposed on the apical surface. Both preparations were exposed for 30 min after which the red cell suspension was removed. ASCC was then measured in both preparations both before and after treatment with forskolin (10 μM) using amiloride, 10 μM. The figure shows the amiloride responses (ASCC). In the left hand traces values of ASCC were 40 μA and 44 μA, respectively, before and after forskolin. On the right the corresponding values were 51 μA and 83 μA.

forskolin. This latter result is in keeping with the earlier results. Figure 8 illustrates a similar experiment but in this instance both preparations were exposed to aliquots of the same sonicated red cell suspension. One preparation was exposed on its basolateral surface while the other was exposed from the apical side. After forskolin only the preparation exposed on the apical surface showed a substantial increase in ASCC.

Two paired preparations were also used for the experiment illustrated in Figure 9. One preparation was exposed to a sonicated red cell suspension of human origin while the other was exposed to an equivalent suspension of toad material. Both preparations were exposed on the apical face, but only the preparation treated with toad material showed a substantial increase in ASCC. In this instance SCC was increased using dibutyryl cyclic AMP (db cyclic AMP) rather than forskolin, again suggesting that it is a cyclic nucleotide-sensitive event which is permitted in tissues exposed to toad red cells.

Parts of two separate experiments are shown in Figure 10. In each the apical surface was exposed to sonicated red cells, duck cells in Figure 10a and frog cells in Figure 10b. SCC was stimulated by db cyclic AMP in the former experiment while kallidin was used in the latter. Thus it does not seem to matter whether the cells are derived from frog, toad or duck, or whether SCC is stimulated by forskolin, db cyclic AMP or kallidin.

We have collected all the results of the experiments where red cells have been used into Table 4. In group 1 are listed the experiments using nucleated red cells and in which SCC was stimulated with either forskolin, db cyclic AMP or kallidin. In every instance ASCC was increased by these procedures and the mean ASCC″ was significantly increased ($P<0.05$) over ASCC′. Group 2 consists of control experiments which were carried out in paired preparations alongside some of the experiments listed in Group 1. It is difficult to decide if sonicated human erythrocytes do have the property shown by nuc-

leated cells but to a lesser extent. In all four instances in which human cells were used, the response was considerably smaller than the corresponding paired preparation treated with a nucleated red cell suspension, although in one experiment the potentiation by human material was substantial (Table 4). The activity of sonicated human erythrocytes has not been further explored as it is clear that the potentiating effect, if real, is weak in relation to the effect of the nucleated cells. We have also found that vesicle suspensions prepared from purified toad erythrocyte membranes can also confer sensitivity of the sodium current to cyclic AMP. Purified membrane preparations are currently being used to discover the mechanism of potentiation.

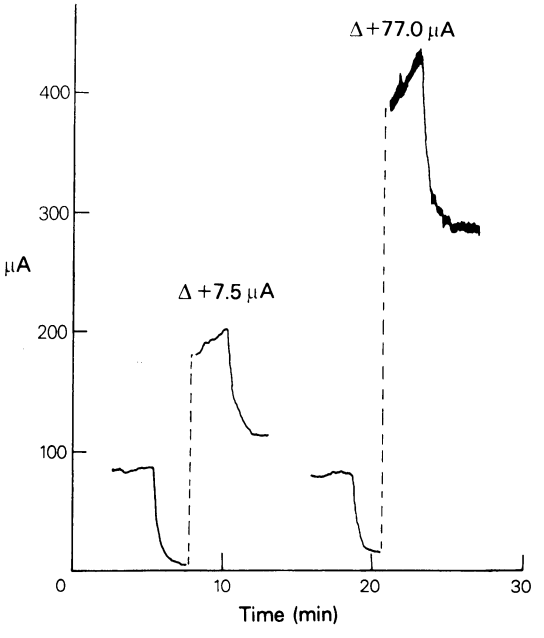

Figure 9 SCC records from two preparations taken from the same dexamethasone-treated rat. Both were exposed to a sonicated red cell suspension on the apical face for 30 min. The preparation represented by the left hand tracings was exposed to a human red cell suspension while the one represented by the right hand traces was exposed to a toad cell suspension. Both erythrocyte suspensions were used at the same final haematocrit (≏5%) and were sonicated under the same conditions. Red cell suspensions were removed by washing with fresh K-H solution after which ASCC was measured with amiloride, 10 μM. After amiloride was removed by washing and SCC stimulated by adding dibutyryl cyclic AMP (1 mM) to the serosal bath, ASCC was redetermined with amiloride. In the left hand traces the values of ASCC were 80 μA and 87.5 μA, respectively, before and after dibutyryl cyclic AMP. The corresponding values for the right hand traces were 66 μA and 143 μA.

Finally, incubation of normal rat colon with sonicated toad red cell suspensions neither affected the basal SCC nor induced any amiloride-sensitivity into the tissue. It is clear, therefore, that the manipulation does not simply introduce amphibian sodium channels into the apical membrane and the effects reported above must result from a modification of the properties of channels already present in the tissue.

Discussion

Our findings confirm earlier reports (Binder, 1978; Will *et al.*, 1981) that amiloride-sensitive sodium transport can be induced by dexamethasone in rat colon. Sodium transport, sensitive to amiloride, is found in the colon of many species, for example rabbit (Schultz *et al.*, 1977) and toad (Cuthbert, 1973), while this property only appears in the rat after salt deprivation or steroid treatment. The sensitivity of the sodium transporting mechanism to amiloride is rather constant throughout nature, for example the value found here for the rat for half-maximal inhibition (0.25 μM) is similar to that for amphibian skin (0.18 μM) (Cuthbert & Fanelli, 1978). There can be little doubt that sodium entry through the apical face of the colon is by a channel mechanism rather than by carrier mediation. Current fluctuation analysis in mammalian colon has revealed channel mechanisms with microscopic parameters similar to those of other tight epithelia (Zeiske, Wills & Van Driessche, 1982). The Hill slope for the amiloride effect on rat colon has a value of less than 1.0, unlike some other amiloride-sensitive tissues (Bindslev *et al.*, 1982) and this may reflect heterogeneity in the receptor population.

Forskolin, a diterpene, activates adenylate cyclase probably by a direct effect on the catalytic subunit or possibly on the nucleotide regulatory or coupling components (Insel, Stengel, Ferry & Hanoune, 1982). Here we have shown that forskolin activates adenylate cyclase in colonic epithelia, from both normal and dexamethasone-treated animals. We showed earlier that this agent stimulates SCC in normal rat colon and frog skin (Cuthbert & Spayne, 1982). The toad urinary bladder is, not unexpectedly, similar in behaviour to frog skin, showing an increase in ASCC after forskolin. More importantly we have demonstrated in this paper that forskolin causes a net chloride secretion in dexamethasone-treated colons which matches the SCC responses. Strikingly, forskolin had no effect on net sodium absorption in dexamethasone-treated colons. The conclusion must be that the forskolin effect on SCC must be due entirely to increased chloride secretion even though the tissues are simultaneously absorbing sodium.

Amiloride is effective only from the apical side in

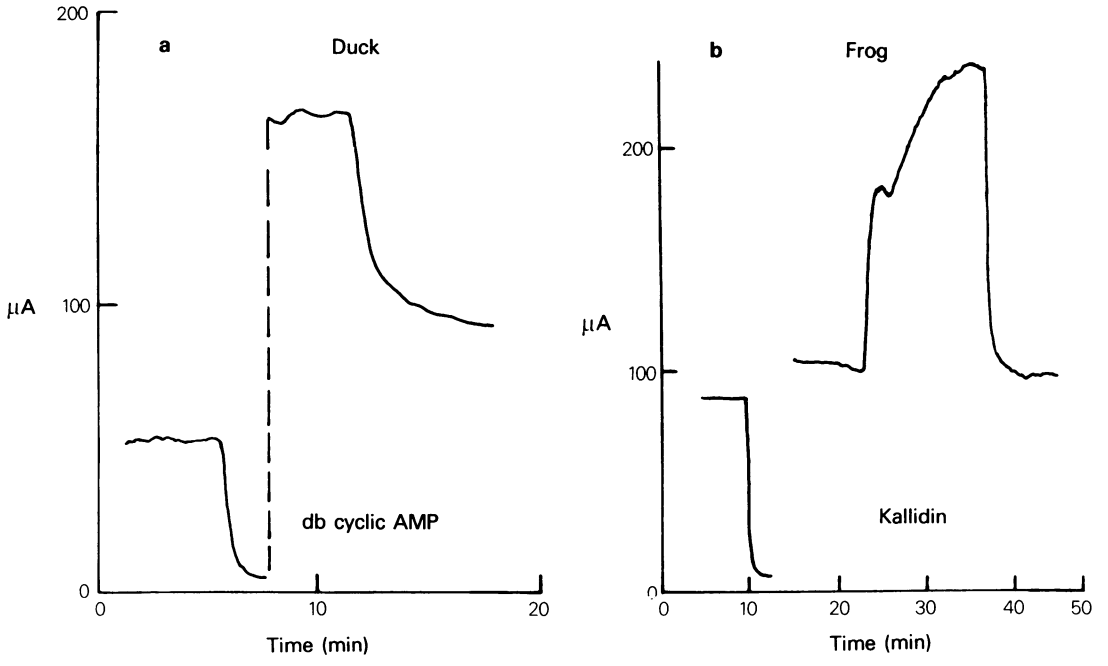

Figure 10 SCC records from two separate experiments. Both experiments illustrate that ASCC in response to amiloride (10 μM) is increased after pre-exposure of the apical surface to sonicated red cells. The details for each experiment are as follows. (a) Duck red cell suspension was used and SCC was increased with dibutyryl cyclic AMP (db cyclic AMP, 1 mM). ASCC increased from 46 μA to 75 μA. (b) Frog cell suspension was used and SCC was increased with kallidin (1.0 μM). ASCC increased from 80 μA to 142 μA.

the dexamethasone-treated colon where it reduces SCC, transepithelial potential and conductance. It follows therefore that apical sodium permeability and electrogenic sodium absorption will be reduced to zero in the presence of high concentrations of amiloride, the change in SCC being the amiloride-sensitive SCC. In an extensive series of experiments it proved impossible to increase ASCC after the SCC was increased with forskolin. This result is entirely compatible with those from measurements of sodium flux and we conclude that electrogenic sodium transport is insensitive to cyclic AMP, a situation quite different from that obtaining in the toad urinary bladder.

It is important to consider if ASCC should remain constant after SCC has been increased with forskolin even though sodium transport is unaltered. Figure 11 gives a schematic diagram of a chloride secreting, sodium absorbing cell. Sodium ions enter the cell through the apical membrane by way of amiloride-sensitive ion channels. They are then subsequently removed into the serosal bath by the Na-K-ATPase located in the basolateral domain. Chloride ions, on the other hand, move uphill into the cell using the energy available from the sodium gradient and employing a frusemide-sensitive cotransport mechanism (Frizzell, Field & Schultz, 1979). Under short circuit conditions the Na^+ ions return to the serosal bath via the Na-K-ATPase while the chloride ions leak across the apical membrane into the mucosal bath. Agents which increase intracellular cyclic AMP also increase chloride secretion by an effect on the apical exit step. There are two sites of potential competition between the two transport processes; at the sodium pump and at the apical membrane. In the latter situation both ions compete to use the same transapical gradient. Therefore it would be expected that increasing chloride secretion would reduce sodium absorption and vice-versa. The evidence from these studies suggest that reciprocal coupling between Na^+ and Cl^- transport is not prominent in this tissue, since forskolin does not affect sodium absorption, neither is the ASCC much reduced. In other intestinal preparations, for example rabbit colon, a similar situation obtains where cyclic AMP increases chloride secretion without affecting sodium absorption (Frizzell & Heintze, 1979). In some of the experiments shown in Table 1, ASCC was indeed marginally but significantly smaller after forskolin if a paired *t* test was used. This may indicate a low level of coupling between the

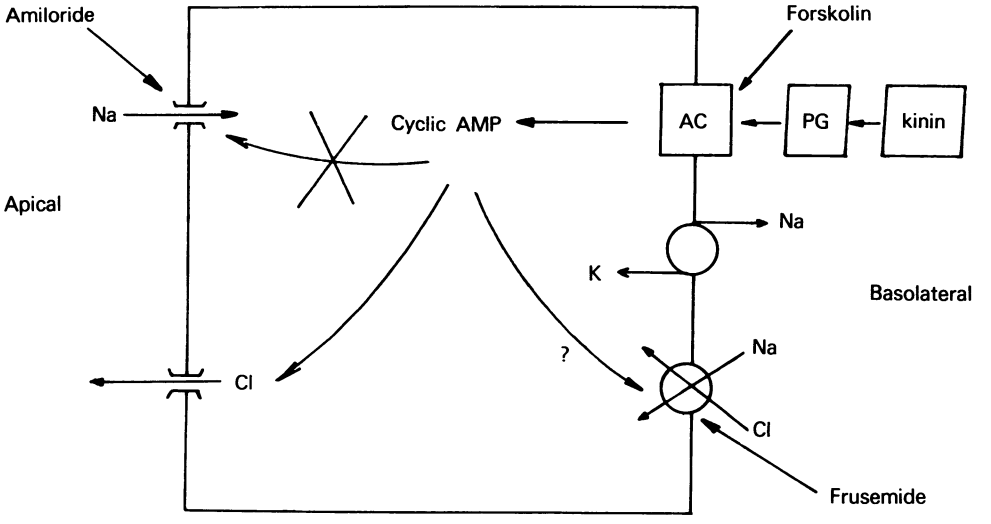

Figure 11 Hypothetical diagram of a cell which is both secreting chloride and absorbing sodium by electrogenic processes. For sodium absorption, sodium ions enter the cell through the apical face via amiloride-sensitive channels and are subsequently removed across the basolateral membrane by the sodium pump. Chloride ions enter through the basolateral face using a frusemide-sensitive Na-Cl cotransport mechanism. The sodium ions are returned via the sodium pump to the basolateral bath. Chloride ions leave the cell via the apical face through chloride channels (?). The apical chloride permeability is increased by cyclic AMP or indirectly by stimulating adenylate cyclase with forskolin. Kinins stimulate the formation of prostaglandins which may act on adenylate cyclase or in other ways. Apparently cyclic AMP cannot affect sodium permeability unless the apical surface is first exposed to a red cell suspension. In an alternative model sodium and chloride transport may occur in different cell types.

two transport processes but is more likely, in our view, to be due to the slow fall in ASCC which occurs with time, an effect which occurs spontaneously whether or not Cl^- secretion is stimulated with forskolin. There is evidence in the gut that the secretory and absorption processes are located at morphologically distinct parts of the epithelium (see, for example, Field, 1981) in which case the absorptive and secretory processes take place in different cells. In other situations, such as the epithelium lining the mammalian trachea, it would appear that both chloride secretion and sodium absorption are carried out by the same cells (Frizzell, Welsh & Smith, 1981). Thus while we remain uncertain of the extent of coupling in the rat colon we have no evidence to suggest that ASCC can be increased by raising the concentration of intracellular cyclic AMP as is normal in amphibian tissues.

It is pertinent to consider at this stage the way cyclic AMP increases sodium transport in amphibian epithelia. In these tissues sodium channels show off-on kinetics but no situation has been found in which either an increase in channel conductance or channel lifetime can explain the increase in sodium transport. Rather, electrically silent channels are recruited with exactly the same characteristics as existing channels so that the increase in macroscopic sodium transport results from an increase in the number of active channels contributing to the current noise (Li *et al.*, 1981). One might explain the present results with colon by proposing there are no channels available for recruitment. However, this explanation is untenable in view of results given in group 6, Table 1, where we have shown the ASCC is much larger immediately after mounting the tissue than 15–20 min later when a steady state is reached. The reason why the current declines in this way is uncertain but in the rabbit colon, which also shows a similar decline with a half-time of 6 min, it is suggested that an increase in intracellular sodium exerts a negative feedback on the sodium permeability of the apical membrane (Turnheim, Frizzell & Schultz, 1978). A similar phenomenon has also been reported for the toad urinary bladder (Cuthbert & Shum, 1978). Whatever the explanation for the current decline, if channels are switched off, or even internalized, this process cannot apparently be reversed by elevating cyclic AMP within the cells.

We have shown that it is possible to make sodium transport in the colon sensitive to cyclic AMP by incubation with vesicles prepared from sonicated red cells. The choice of this unusual procedure requires some explanation. First, it was reasonable to choose cells of amphibian origin, particularly ones not

known to have sodium channels, and toad red cells were an obvious choice. Further, amphibian cells exhibit a Na^+-H^+ exchange flux which is sensitive to cyclic AMP and blocked by high concentrations of amiloride (10^{-4} M) (Siebens & Kregenow, 1980; Palfrey, Stapleton, Alper & Greengard, 1980). On the other hand, avian red cells have a Na^+-K^+-Cl^- co-transport system which is also sensitive to cyclic AMP and is blocked by frusemide-like drugs (Palfrey, Feit & Greengard, 1980). We were concerned that we might be introducing amphibian-type sodium channels in our experiments but found that alone, the application of sonicated red cell suspensions had no effect on SCC in dexamethasone-treated colons, neither was amiloride-sensitivity conferred on non-treated colons. We do not know whether or not the vesicles actually fuse with the apical membrane or whether they are taken into the cell. The failure to confer sensitivity to cyclic AMP when applied to the basolateral side would perhaps support the first view.

Obviously our efforts are now directed to understanding how addition of a component or components from an amphibian or avian red cell to a mammalian epithelium confers sensitivity of the sodium transporting process, and in particular the sodium permeability of the apical membrane, to cyclic AMP. What we seem to have achieved is the conversion of a mammalian type of sodium channel to one characteristic of lower phyla. Our latest findings that the phenomenon is repeatable using purified membrane preparations from red cells suggests it is a membrane component that is important, although we cannot exclude the possibility of minor contamination from adherent intracellular material.

Although our present attempts at membrane engineering are not sophisticated, the results are so clear cut that we might be allowed a little speculation. In general, cyclic nucleotides are believed to operate by activating a variety of protein kinases. For example, the effect of cyclic AMP on chloride secretion may be exerted via a protein kinase associated with the chloride channel (Figure 11). Biochemical evidence for a cyclic AMP-dependent protein kinase activation of gastric anion (Cl^-) transport has been obtained (Soumarmon, Abastado, Bonfils & Lewis, 1980). If a protein kinase is associated with the chloride channel in rat colon it is unable to operate the sodium channel induced by dexamethasone. It seems possible at this stage that cyclic AMP-dependent protein kinase normally not present in rat colon, but present in avian and amphibian erythrocytes and which can activate electrically silent sodium channels is a likely candidate for the effects we have described. In *Aplysia* neurones, microinjection of the catalytic subunit of protein kinase mimics the action of 5-hydroxytryptamine or cyclic AMP on K-channels (Klein & Kandel, 1980; Kaczmarek, Jennings, Strumwasser, Nairn, Walter, Wilson & Greengard, 1980). Similarly the catalytic subunit can influence calcium channel activity in cardiac myocytes (Osterreider, Brum, Hescheler, Trautwein, Flockerzi & Hofmann, 1982). In human erythrocytes Na-K cotransport is inhibited by cyclic AMP, the reverse of the situation in the avian red cell (Garay, 1982). However, we are not able to assert that our failure to get consistent sensitization to cyclic AMP with human erythrocytes is related to this or is for some other reason, for example failure to fuse or be taken up by the colon.

Finally, whatever the reasons for the effects we describe, it seems that during evolution the mammalian cell has retained the amiloride-sensitive sodium channel but has, under normal conditions, lost the ability for modulation by hormones acting through cyclic AMP.

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