Br. J. Pharmacol. (1988), **94**, 155–163

Inhibition by chloride channel blockers of anion secretion in cultured epididymal epithelium and intact epididymis of rats

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1 Anion secretion by primary monolayer cultures of rat epididymal cells was studied by the short circuit current technique.

2 Monolayers had a transepithelial potential difference of 1.34 mV, apical side negative and a short circuit current of 2.45 $\mu A\,cm^{-2}$. The transepithelial resistance was 504 $\Omega\,cm^2$.

3 Addition of anthracene-9-carboxylate (9-AC) to the apical side caused a biphasic response, a decrease followed by an increase in the short circuit current (SCC) which then returned to the basal level. Addition of 9-AC to the basolateral side also caused a biphasic response but the increase in current was sustained.

4 Addition of diphenylamine-2-carboxylate (DPC) and 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB) caused an inhibition of the SCC when added to the apical or basolateral side.

5 When the epithelium was stimulated with adrenaline (0.23 μM, basolaterally), the SCC rose to a peak value of 10.36 $\mu A\,cm^{-2}$ and then stabilized at 3.82 $\mu A\,cm^{-2}$ after 15 min. Addition of 9-AC to the apical side caused a triphasic response: a decrease, reversal to the original level followed by a slow inhibition which was sustained. The inhibition achieved at the steady state was concentration-dependent with an apparent IC_{50} value of 2.51 mM. Addition of 9-AC to the basolateral side produced a similar response but a time lag of 20 s was observed.

6 DPC and NPPB also caused the SCC to decrease when added to the apical side of the monolayers stimulated with adrenaline. The IC_{50} values were 0.148 mM and 0.049 mM for DPC and NPPB, respectively. Basolateral application also caused an inhibition but higher concentrations were required to inhibit 50% of the adrenaline stimulated SCC.

7 Serosal to mucosal flux of chloride was studied in intact epididymis luminally perfused *in vivo*. 9-AC added to the luminal perfusion solution inhibited the Cl flux in a dose-dependent manner with an IC_{50} value similar to that observed in cultured epithelium *in vitro*.

8 It is concluded that anion secretion by the epididymal cells may conform to models proposed for other Cl secreting epithelia and may involve anion diffusion across the apical channels which are blocked by Cl channel blockers.

Introduction

Primary monolayer cultures of epithelial cells from the rat cauda epididymis secrete chloride and bicarbonate by an electrogenic transport process that appears to have characteristics in common with other anion secreting epithelia. Secretion is stimulated by kinins, prostaglandins (Cuthbert & Wong, 1986) and β-adrenoceptor agonists (Wong & Chan, 1987). Anion secretion induced by β-adrenoceptor agonists and possibly other secretagogues was mediated by a rise in intracellular adenosine 3′:5′-cyclic monophosphate (cyclic AMP) as the second messenger. Anion secretion in cultured epididymal epithelium was inhibited by addition of bumetanide, amiloride, 4-acetamido-4-′isothiocyanostilbene-2,2′-disulphonic acid (SITS) and acetazolamide to the basolateral side and it was proposed that anion secretion involves a number of transporters at the basolateral membrane; these include a Na/2Cl/K symport, a Cl/HCO_3, and a Na/H exchange (Wong & Chan, 1987).

In many other anion secreting epithelia, Cl transport across the apical membrane is through chloride conductive channels blocked by Cl channel blockers, anthracene-9-carboxylic acid (9-AC) (Oberleithner *et*

al., 1983; Welsh 1984), diphenylamine-2-carboxylate (DPC) (Di Stefano *et al.*, 1985) and 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB) (Wangemann *et al.*, 1986). Regulation of the apical Cl conductance determines, in part, the rate of transepithelial Cl secretion (Welsh & Liedtke, 1986). In this study we examined the ability of 9-AC, DPC and NPPB to inhibit anion secretion in cultured epididymal epithelium and in the intact cauda epididymis of anaesthetized rats.

Methods

Short circuit current measurement in cultured monolayers

Primary monolayer cultures of rat cauda epididymis were grown on millipore filters coated with collagen by methods described previously (Cuthbert & Wong, 1986; Wong & Chan, 1987). After 4 days of culture, the monolayers became confluent and were ready for the measurement of electrogenic ion transport using the short circuit current technique.

Epididymal monolayers (area 0.4 or 0.6 cm^2) were clamped between the two halves of an Ussing chamber with a 0.7 cm^2 window. The tissue was short circuited (voltage clamped at zero potential) using W-P Instruments Dual Voltage-Current Clamp Amplifier (DVC 1000) and the short circuit current displayed on a pen recorder (Kipp and Zonen). The clamp can be used to set the potential, intermittently, at a voltage displaced from zero (usually 0.3 mV), the resulting current change allowing calculation of the resistance from the ohmic relationship. Very often, the 2 channels of the amplifier were used simultaneously on parallel monolayers so that studies could be made in the same batch of cells under control and experimental conditions.

In most situations, monolayers were incubated on both sides with Krebs-Henseleit solution (20 ml), gassed with 95% O_2 and 5% CO_2 and warmed to 32°C. Drugs were added to either the apical or basolateral side of the epithelium. Delivery of drugs to cell surface was instantaneous. Experiments were designed to avoid excessive washing of the cell monolayers.

Luminally perfused rat cauda epididymis

In another set of experiments, serosal to mucosal Cl flux (J_{s-m}) was measured in the luminally perfused cauda epididymis in anaesthetized rats *in vivo*. Male rats weighing approximately 350 g were anaesthetized with pentobarbitone sodium (60 mg kg^{-1}, i.p. injection: Sagatal, May & Baker Ltd, Dagenham, Essex, U.K.). The left jugular vein and the right femoral artery were cannulated for intravenous injection of drugs and collection of blood samples, respectively. The epididymis was then prepared for luminal perfusion as described previously (Wong & Yeung, 1978; Au & Wong, 1980; Qiu & Wong, 1985). After cannulation of the epididymal duct and the vas, spermatozoa were flushed out by luminal perfusion of the epididymal duct with Krebs-Henseleit solution at a rate of 9 $\mu l\,min^{-1}$ using a slow infusion pump (Harvard). After the epididymis had been cleared of sperm, the sperm-free segment (about 20 cm long) of the epididymal duct was perfused with Krebs-Henseleit solution at a rate of 1 $\mu l\,min^{-1}$. After an equilibration period of 60 min, ^{36}Cl ($Na^{36}Cl$, 7.5 μCi, specific activity: 150 $\mu Ci\,ml^{-1}$) was injected intravenously. Thirty min later blood samples were taken from the femoral artery and the epididymis was perfused intermittently using a 'stop-flow' method (Qiu & Wong, 1985). The perfusate was retained in the epididymis for varying periods (ranging from 1 to 30 min). At the end of the stop-flow, 50 μl of fluid were flushed out from the cauda epididymis and ^{36}Cl activity was measured by scintillation counting using a Packard Tri-Carb liquid scintillation analyser (Model 2000CA). When ^{36}Cl activities in the perfusates were plotted against the time of stop-flow, the relationship was found to be linear up to 7 min stop-flow. The ^{36}Cl activity in the 5 min stop-flow sample was therefore taken as a measure of blood to lumen flux (serosal to mucosal flux, J_{s-m}) of chloride. The surface area of the 20 cm length epididymal duct was calculated from the mean diameter of the epididymal lumen (determined from histological section). J_{s-m} Cl was expressed as $\mu mol\,cm^{-2}\,min^{-1}$. In the measurement of J_{s-m} Cl, two experimental protocols were adopted. In the first protocol, the epididymis was perfused with Krebs-Henseleit solution containing 0.5% DMSO (0 mM 9-AC) for 10 min and then perfusion was stopped for 5 min during which time the serosal to mucosal Cl flux was measured. The epididymis was then perfused with solution containing increasing concentrations of 9-AC (1 mM to 7 mM) and Cl flux was measured in the same manner. The epididymis was exposed to each concentration of 9-AC for 15 min but measurement was made over the last 5 min stop-flow period. This method allowed the dose-response relationship to be studied for each epididymis and gave the results depicted in Table 5. In the second protocol, the effect of different concentrations of 9-AC on Cl flux was followed with time but each epididymis was exposed to only one concentration of the drug. This method gave results shown in Figure 7.

Solutions and drugs

The Krebs-Henseleit solution used had the following composition (mM): NaCl 117, KCl 4.7, $CaCl_2$ 2.56, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 24.8, glucose 11. The solution had a pH of 7.4 when bubbled with 95% O_2 and 5% CO_2. In some experiments, a Cl-free solution was used in which NaCl was replaced by Na gluconate or isethionate, KCl was substituted by K gluconate and $CaCl_2$ by $CaSO_4$.

Anthracene-9-carboxylic acid (9-AC) was obtained from Aldrich. Diphenylamine-2-carboxylate (DPC) and 5-nitro-2-(3-phenylpropylamino)-benzoate (NPPB) were gifts from Professor R. Greger. They were dissolved in dimethyl sulphoxide (DMSO) before each experiment. The final concentrations of DMSO used ranged from 0.05 to 0.5%. In the cultured epithelium the blockers were added to either the apical or basolateral solution in a cumulative manner so as to avoid washing of the tissue. In the luminally perfused epididymis, these drugs were added to the luminal perfusion solution which was filtered before use. Adrenaline was obtained from David Bull Laboratories.

Statistical analysis

Comparisons between groups of data were made by Student's *t* test.

Results

When epididymal monolayers were first clamped in the Ussing chambers they had a potential difference of about 2 to 4 mV, apical side negative. The potential soon declined and stabilized at 1.34 ± 0.10 mV ($n = 70$) with a short circuit current of $2.45 \pm 0.15\,\mu A\,cm^{-2}$ ($n = 70$). They had a transepithelial resistance of $504 \pm 27.2\,\Omega cm^2$ ($n = 70$) when calculated from the current change resulting from clamping the tissue at 0.3 mV (see Methods).

Addition of 9-AC to the apical side of the epididymal monolayers resulted in a transient biphasic response in the SCC. There was first a decrease followed by an increase before the current returned to the basal level. There was no change in tissue resistance. The magnitude of the biphasic change was dose-dependent (Figure 2a and Table 2). Addition of DMSO (0.1 and 0.5% final concentration) to the apical side did not affect the SCC (Figure 1 and Table 1). When 9-AC was added to the basolateral side, the SCC also showed a biphasic change, a slight inhibition preceding a rise which appeared to be sustained (Figure 2b). At higher doses (4 and 9 mM), the transepithelial resistance decreased (Figure 2b and Table 3). DMSO (0.1 and 0.5% final concentration) added to the basolateral side had no apparent effect on the SCC (Figure 1 and Table 1).

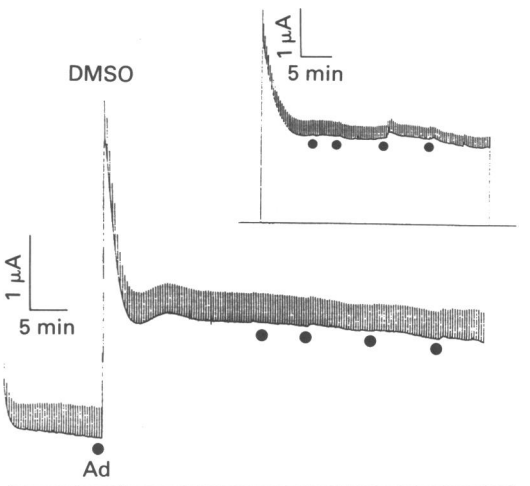

Figure 1 Effect of DMSO on the basal (inset) and adrenaline (Ad, 0.23 μM added basolaterally) stimulated short circuit currents (SCC) in 2 epididymal monolayers, area 0.4 cm^2. At the dots DMSO was added to produce the following final concentrations: 0.1% apical, 0.5% apical, 0.1% basolateral, 0.5% basolateral. Transient current pulses were the result of intermittently clamping the potential of 0.3 mV. The horizontal lines indicate zero SCC. Each record is representative of 4 different experiments.

Table 1 Effect of DMSO on the basal and adrenaline (0.23 μM) stimulated short circuit current (SCC)

	Basal		*Stimulated*	
DMSO	*Apical*	*Basolateral*	*Apical*	*Basolateral*
0.1%	0.000 ± 0.008	-0.010 ± 0.015	-0.005 ± 0.013	-0.005 ± 0.012
0.5%	-0.003 ± 0.009	-0.045 ± 0.050	-0.006 ± 0.012	-0.002 ± 0.011

Results are expressed as the change in current, ΔSCC ($\mu A\,cm^{-2}$) measured before and 10 min after addition of DMSO.
Each value shows the mean ± s.e. mean of 4 experiments. Negative sign indicates a decrease. All data were not significantly different from zero (paired *t* test). In all cases there was no change in resistance.

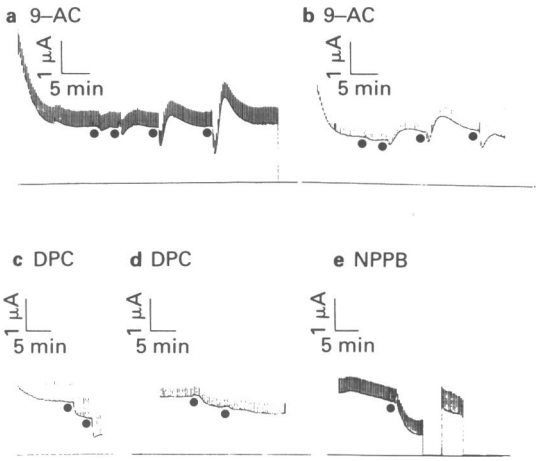

Figure 2 Short circuit currents (SCC) of 5 separate monolayers, area 0.4 cm^2. (a) Anthracene-9-carboxylate (9-AC) was added to the apical and (b) basolateral side to produce the following final concentrations: 0.25, 1.5, 4 and 9 mM. (c) Diphenylamine-2-carboxylate (DPC, 0.1 and 1.1 mM) was added to the apical side. (d) DPC (0.1 and 0.6 mM) was added to the basolateral side. (e) 5-Nitro-2-(3-phenylpropylamino)-benzoate (NPPB, 0.33 nM) was added to the apical side. At the gap in the record the tissue was washed 7 times with Krebs-Henseleit solution. Transient current pulses were the result of intermittently clamping the potential at 0.3 mV. The horizontal lines indicate zero SCC. Each record is representative of 3–4 different experiments.

DPC (0.1 mM) added to the apical side reduced the SCC from $2.62 \pm 0.11\,\mu A\,cm^{-2}$ ($n = 4$) to $1.87 \pm 0.9\,\mu A\,cm^{-2}$ ($n = 4$) ($P < 0.005$). Addition of a higher dose (1.1 mM) further reduced the current to $0.83 \pm 0.09\,\mu A\,cm^{-2}$ ($n = 4$) ($P < 0.0001$ compared with the basal value). However, the resistance was unchanged (Figure 2c). Addition of DPC (0.1 mM) to the basolateral side also caused a decrease of the SCC from the basal value of $2.75 \pm 0.14\,\mu A\,cm^{-2}$ ($n = 4$) to $2.40 \pm 0.10\,\mu A\,cm^{-2}$ ($n = 4$). Addition of a higher dose (0.6 mM) further reduced the current to $2.02 \pm 0.13\,\mu A\,cm^{-2}$ ($n = 4$) ($P < 0.05$ compared with the basal value). In both cases there was no change in tissue resistance (Figure 2d).

NPPB also caused an inhibition of the SCC when added to the apical side (Figure 2e). The currents before and after NPPB (0.33 mM) were $2.17 \pm 0.36\,\mu A\,cm^{-2}$ ($n = 4$) and $1.17 \pm 0.17\,\mu A\,cm^{-2}$ ($n = 4$), respectively. The difference was statistically significant, $P < 0.05$. The inhibition was reversible upon washing out the inhibitor (Figure 2e). Unlike 9-AC, no stimulation of the current was observed with DPC and NPPB.

Table 2 Effect of apical application of anthracene-9-carboxylate (9-AC) on the basal short circuit current (SCC)

9-AC (mM)	*Initial decrease*	*Delayed increase*
0.25	-0.17 ± 0.03^a	0
1.5	-0.59 ± 0.12^a	0
4	-1.00 ± 0.12^c	$+0.50 \pm 0.07^b$
9	-1.33 ± 0.07^c	$+1.20 \pm 0.10^c$

The biphasic response (see Figure 2a) was separated into 2 phases, the initial decrease and the delayed increase and expressed as the difference in current, ΔSCC ($\mu A\,cm^{-2}$) measured before and at the peak response after drug addition.
Each value shows the mean ± s.e. mean of 4 experiments. Negative sign indicates a decrease and positive sign an increase in current. $^aP < 0.01$, $^bP < 0.005$ and $^cP < 0.001$ when compared to zero by use of paired t test. In all cases there was no change in tissue resistance.

Adrenaline (0.23 μM) added to the basolateral side caused a rise of the SCC to a peak value of $10.36 \pm 1.61\,\mu A\,cm^{-2}$ ($n = 32$) which then stabilized at $3.82 \pm 0.24\,\mu A\,cm^{-2}$ ($n = 32$) after about 15 min (Figure 3). Addition of 9-AC to the apical side caused a dose-dependent decrease of the adrenaline-induced SCC (Table 4 and Figure 3). The response to 9-AC consisted of 3 phases, a rapid decrease, then reversal to the original level followed by a slow but sustained inhibition. The concentration of 9-AC required to inhibit 50% of the response to adrenaline (IC_{50}) was 2.51 mM (geometric mean, $n = 4$, 95% confidence limits = 2.27, 2.78). There was no change

Table 3 Effect of basolateral application of anthracene-9-carboxylate (9-AC) on the basal short circuit current (SCC) and resistance

9-AC (mM)	*SCC* ($\mu A\,cm^{-2}$)	*R* ($\Omega\,cm^2$)
0.25	-0.11 ± 0.01^c	0
1.5	$+0.35 \pm 0.05^a$	0
4	$+0.97 \pm 0.03^d$	-156 ± 29^b
9	$+0.28 \pm 0.02^a$	-435 ± 18^d

Results are expressed as the difference in current, ΔSCC ($\mu A\,cm^{-2}$) or resistance, ΔR ($\Omega\,cm^2$) between the basal values and the values recorded 5 min after drug application. The initial decrease in current after 1.5 and 4 mM 9-AC (see Figure 2b) was not measured.
Each value shows the mean ± s.e. mean of 3 experiments. Negative sign indicates a decrease and positive sign an increase in current. $^aP < 0.01$, $^bP < 0.02$, $^cP < 0.005$ and $^dP < 0.001$ when compared to zero by use of paired t test.

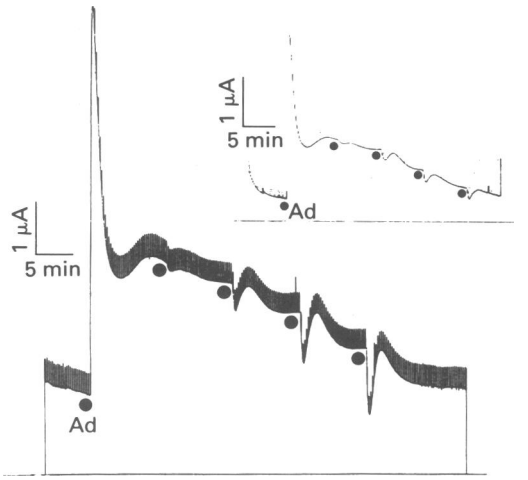

Figure 3 Short circuit currents (SCC) of 2 separate epididymal monolayers, area 0.6 cm^2, recorded simultaneously. The monolayers were challenged with adrenaline (Ad, 0.23 μM) added to the basolateral sides. When the currents had stabilized, anthracene-9-carboxylate (9-AC) was added to the apical or basolateral (inset) bathing solution at the time shown by the dots to produce the following final concentrations: 0.25, 1.5, 4 and 9 mM. Transient current pulses were the result of intermittently clamping the potential at 0.3 mV. The horizontal lines indicate zero SCC. These records are representative of 4 different sets of experiments.

in resistance at all concentrations of 9-AC used (Figure 3 and Table 4). Addition of 9-AC to the basolateral side caused an inhibition similar to that observed after apical application with an IC_{50} of 2.10 mM (geometric mean, $n = 4$, 95% confidence limits = 1.90, 2.32), except that the resistance was significantly decreased at 4 and 9 mM 9-AC (Figure 3 and Table 4). There was a time lag of about 20 s before an inhibitory effect on the current was seen. Addition of DMSO (0.1 and 0.5% final concentration) to the apical or basolateral side of the stimulated monolayers had little effect on the SCC and tissue conductance (Figure 1 and Table 1).

The effects of DPC and NPPB on the adrenaline stimulated SCC are shown in Figures 4 and 5. Addition to the apical side of the epithelium inhibited the SCC with IC_{50} values of 0.148 mM (geometric mean, $n = 4$, 95% confidence limits = 0.126, 0.173) and 0.049 mM (geometric mean, $n = 4$, 95% confidence limits = 0.040, 0.061) for DPC and NPPB, respectively (Table 4). The inhibition was reversible on washing (Figures 4 and 5). Basolateral application also produced an inhibition but higher concentrations were required to produce 50% inhibition (Table 4).

In Cl-free solution (solution containing 25 mM HCO_3), adrenaline (0.23 μM) added to the basolateral side caused a rise in the SCC but the response was about 34% of normal (when Cl was present). The net

Table 4 Effects of Cl channel blockers on the adrenaline (0.23 μM) stimulated short circuit current (SCC) and resistance (R) in cultured epididymal epithelium

	Apical application			*Basolateral application*		
	SCC ($\mu A\,cm^{-2}$)	% *inhibition*	*R* ($\Omega\,cm^2$)	*SCC* ($\mu A\,cm^{-2}$)	% *inhibition*	*R* ($\Omega\,cm^2$)
9-AC (mM)*						
0**	3.75 ± 0.43		443 ± 39	3.70 ± 0.20		500 ± 15
0.25	3.50 ± 0.26	7	441 ± 39	3.30 ± 0.17	10.8	499 ± 13
1.5	2.40 ± 0.28[a]	36	439 ± 39	2.05 ± 0.15[d]	44.6	500 ± 16
4.0	1.23 ± 0.34[c]	67	439 ± 44	1.00 ± 0.28[d]	73.0	174 ± 11[d]
9.0	0.35 ± 0.22[d]	90	421 ± 59	0.37 ± 0.09[d]	90.0	74 ± 9[d]
DPC (mM)*						
0**	5.00 ± 0.20		476 ± 80	4.75 ± 0.16		445 ± 54
0.01	4.25 ± 0.18[a]	15	476 ± 80	—	—	—
0.11	3.00 ± 0.20[d]	40	470 ± 86	3.75 ± 0.15[c]	21	447 ± 55
1.11	0	100	466 ± 88	2.50 ± 0.18[d]	47	338 ± 53
NPPB (mM)*						
0**	4.00 ± 0.24		563 ± 49	4.75 ± 0.26		506 ± 50
0.003	3.50 ± 0.20	15	563 ± 49	—	—	—
0.036	2.50 ± 0.16[c]	40	552 ± 41	3.75 ± 0.16[b]	17	489 ± 38
0.37	0	100	512 ± 27	1.75 ± 0.14[d]	61	338 ± 22[b]

Each value shows the mean ± s.e. mean of 4 experiments.
* SCC and resistance measured at steady state (15 min after 9-AC, DPC or NPPB addition).
** Values measured after the response to adrenaline had stabilized at the plateau level.
[a]$P < 0.05$, [b]$P < 0.02$, [c]$P < 0.005$ and [d]$P < 0.001$ when compared to 0 mM 9-AC, 0 mM DPC or 0 mM NPPB.
9-AC = anthracene-9-carboxylate, DPC = diphenylamine-2-carboxylate and NPPB = 5-nitro-2-(3-phenylpropylamino)-benzoate.

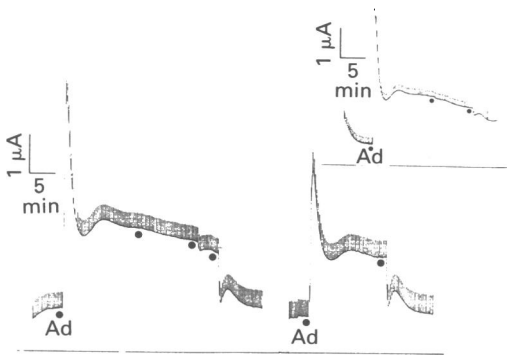

Figure 4 Short circuit currents (SCC) of 2 separate epididymal monolayers, area 0.4 cm^2, recorded simultaneously. The monolayers were challenged with adrenaline (Ad, 0.23 μM, basolaterally). Diphenylamine-2-carboxylate (DPC) was added to the apical or basolateral (inset) bathing solution to produce the following final concentrations: 0.01, 0.11 and 1.1 mM (apical) or 0.1 and 1.1 mM (basolateral). At the gap in the record the tissue was washed 7 times with Krebs-Henseleit solution and rechallenged with adrenaline followed by DPC (1 mM) added to the apical side. The horizontal lines indicate zero SCC. These records are representative of 4 different sets of experiments.

charge movements in the first 10 min following addition of adrenaline (0.23 μM) were 3764 ± 425 μCoulomb cm^{-2} epithelium ($n = 6$) and 1283 ± 159 μCoulomb cm^{-2} epithelium ($n = 6$) in monolayers incubated in normal and Cl-free solutions, respectively ($P < 0.005$). When the SCC became steady, 9-AC (2.5 mM) added to the basolateral side caused a reduction of SCC from 1.37 ± 0.03 $\mu A\,cm^{-2}$ ($n = 3$) to 0.53 ± 0.03 ($n = 3$) ($P < 0.001$) but with no change in tissue resistance (before, $506 \pm 6.8\,\Omega cm^2$; after, $507 \pm 6.7\,\Omega cm^2$). Further addition of 9-AC (5 mM) to the apical side immediately reduced the SCC to zero, also with no change in resistance ($509 \pm 9.3\,\Omega cm^2$) (Figure 6). NPPB (0.22 mM) added to the apical side of the monolayer incubated in Cl-free solution reduced the adrenaline stimulated SCC from 0.93 ± 0.07 $\mu A\,cm^{-2}$ ($n = 3$) to zero. There was no change in resistance (before, $451 \pm 59\,\Omega cm^2$; after, $423 \pm 67\,\Omega cm^2$) (Figure 6).

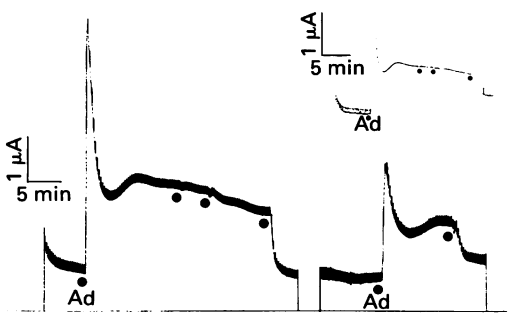

Figure 5 Short circuit currents (SCC) of 2 separate epididymal monolayers, area 0.4 cm^2, recorded simultaneously. The monolayers were challenged with adrenaline (Ad, 0.23 μM, basolaterally). 5-Nitro-2-(3-phenylpropylamino)-benzoate (NPPB) was added to the apical or basolateral (inset) bathing solution to produce the following final concentrations: 0.003, 0.036 and 0.366 mM. At the gap in the record the tissue was washed 7 times with Krebs-Henseleit solution and rechallenged with adrenaline followed by NPPB (0.165 mM) added to the apical side. The horizontal lines indicate zero SCC. These records are representative of 4 different sets of experiments.

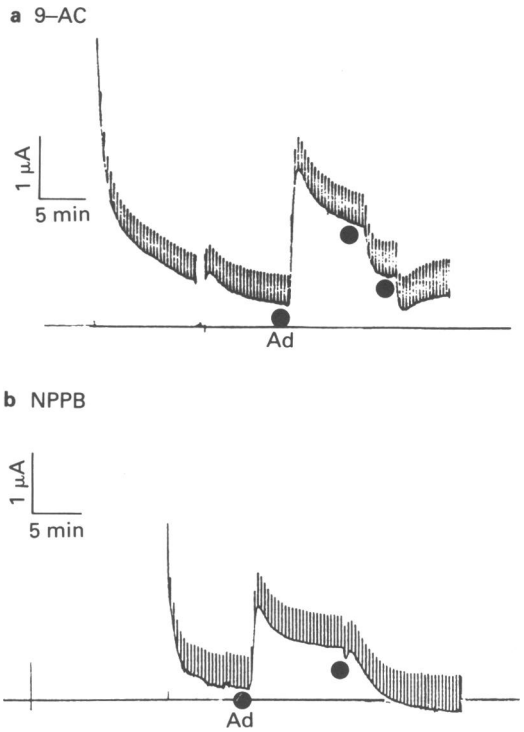

Figure 6 Short circuit currents (SCC) of 2 separate epididymal monolayers, area 0.6 cm^2 (a) and 0.4 cm^2 (b), recorded simultaneously. The monolayers were incubated both sides in Cl-free solution (Cl replaced by gluconate and sulphate). Both tissues were challenged with adrenaline (Ad, 0.23 μM) applied basolaterally. When the SCC had stabilized, anthracene-9-carboxylate (9-AC, a) was first added to the basolateral side at a concentration of 2.5 mM and then to the apical side (5 mM). (b) 5-Nitro-2-(3-phenylpropylamino)-benzoate (NPPB, 0.22 mM) was added to the apical side. The horizontal lines indicate zero SCC. These records are representative of 3 different sets of experiments.

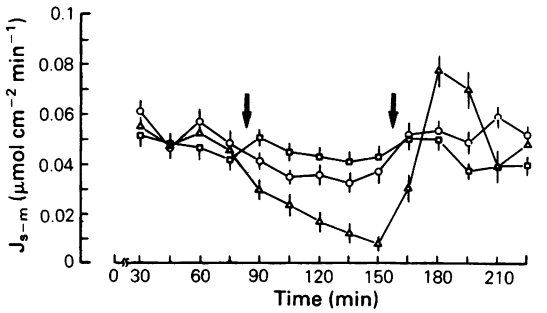

Figure 7 Serosal to mucosal chloride flux (J_{s-m}) across the rat cauda epididymis. At the first arrow, anthracene-9-carboxylate (1 mM, ○, or 5 mM, △) or DMSO (0.5%, □) was added to the luminal perfusion solution and was removed at the second arrow. Each point shows the mean of 3 to 4 determinations; vertical lines indicate s.e. mean.

Effect of anthracene-9-carboxylic acid on $J_{(s-m)}$ in intact epididymis

Under basal conditions, the serosal to mucosal flux of chloride $J_{(s-m)}$ was 0.054 ± 0.003 μmol cm^{-2} min^{-1} ($n = 5$ experiments). Addition of 9-AC to the luminal solution reduced the $J_{(s-m)}$ in a dose-dependent manner (Figure 7, Table 5) with an apparent IC_{50} value of 1.99 mM (geometric mean, $n = 5$, 95% confidence limits = 1.85, 2.13). The effect of 9-AC on chloride flux was reversible upon removal of the drug from the perfusion solution (Figure 7). DMSO added to the luminal perfusion solution did not affect the $J_{(s-m)}$ Cl flux (Figure 7).

Discussion

In previous studies of muscle membrane, Palade & Barchi (1977) found that a variety of aromatic carboxylic acids which are analogues of benzoic acid reduced the membrane Cl conductance of rat diaphragm. The most potent of the series was anthracene-9-carboxylic acid. The inhibition was initially rapid, reaching a stable value by 10 min. Inhibition was reversible and concentration-dependent. The conclusion that 9-AC blocks Cl conductance was also made by Bryant & Morales-Aguilera (1971) from electrophysiological studies of goat intercostal muscles and by Hayward & Barchi (1980) from measurement of ^{36}Cl efflux from rat skeletal muscle.

Table 5 Effect of anthracene-9-carboxylate (9-AC) on basal serosal to mucosal Cl flux (J_{s-m}) of the intact cauda epididymis of the rat

9-AC (mM)	J_{s-m} (μmol cm^{-2} min^{-1})	*% Inhibition*
0	0.054 ± 0.003 ($n = 5$)	
1.0	0.037 ± 0.003^a ($n = 5$)	31.5
2.5	0.024 ± 0.007^a ($n = 5$)	55.5
5.0	0.014 ± 0.003^b ($n = 5$)	74.1
7.0	0.019 ± 0.002^b ($n = 5$)	64.8

Each value shows the mean ± s.e. mean with the number of experiments shown in parentheses. The epididymis was exposed to the drug for a total of 15 min and flux measurement was made over the last 5 min (see Methods).
$^aP < 0.005$ and $^bP < 0.001$ when compared to 0 mM 9-AC.

9-AC has been shown to inhibit Cl reabsorption in the diluting segment of the frog kidney (Oberleithner *et al.*, 1983) and block Cl secretion in canine tracheal epithelium (Welsh, 1984). In many secretory epithelia, including the tracheal epithelium (Welsh, 1983), the salivary gland (Case *et al.*, 1984), the colonic epithelium (Dharmsathaphorn *et al.*, 1985) and the rectal gland of the dogfish (Greger *et al.*, 1986), anion secretion is thought to be mediated by Cl entry via a Na/2Cl/K symport located at the basolateral membrane. This co-transport system is energized by the Na-K pump present at the same membrane. Exit of Cl at the apical side is passive and through diffusible channels which are controlled by cyclic AMP (Frizzell *et al.*, 1986; Welsh & Liedtke, 1986). It is known that these Cl conductive channels are blocked by 9-AC, DPC (Di Stefano *et al.*, 1985) and NPPB, which has been found to be the most potent Cl channel blocker to date (Wangemann *et al.*, 1986).

Anion secretion in the epididymis may conform to the general model of anion secretion described above. Anion secretion is electrogenic in that secretion is associated with a transepithelial potential difference (Cuthbert & Wong, 1986; Wong & Chan, 1987). Although direct measurement of Cl fluxes in the cultured epididymal monolayers is difficult, indirect evidence has suggested that a net anion secretion contributes to the short circuit current. First, the SCC responses to secretagogues were reversed by inhibitors affecting anion transport (Wong & Chan, 1987). Secondly, the SCC responses were dependent on the presence of Cl and HCO_3 (Cuthbert & Wong, 1986). The second messenger mediating the secretory responses appears to be cyclic AMP (Wong & Chan, 1987). Anion secretion in the cultured rat epididymal cells may involve a Na/2Cl/K symport, a Cl/HCO_3 exchange and a Na/H exchange located at the basolateral membrane. Application of bumetanide, SITS and amiloride to the basolateral side partially reversed the SCC response to adrenaline when these agents were added individually, but when present together, they completely reversed the adrenaline-induced SCC (Wong & Chan, 1987).

The exit of anions at the apical membrane may be through anion conductive channels. This contention was supported by the present observation that 9-AC,

DPC and NPPB added to the apical side inhibited the adrenaline stimulated SCC in the primary cultures of rat cauda epididymal cells. The IC_{50} values show the order of inhibitory potency: NPPB > DPC > 9-AC, which are consistent with findings in the thick ascending limb of the loop of Henle (Oberleithner *et al.*, 1983; Wangemann *et al.*, 1986). 9-AC also decreased $J_{(s-m)}$ chloride flux in the intact epididymis. The concentration-response relationships in the intact epididymis and the cultured epithelium were similar, suggesting an inhibition of $J_{(s-m)}$ chloride flux by 9-AC as the cause of the inhibition of the SCC by 9-AC.

Addition of the inhibitors to the basolateral side of the epithelium also caused a decrease in the SCC response to adrenaline. For 9-AC, basolateral application was as effective as apical application (Figure 3 and Table 4), although for the former a time lag of 20 s was observed. For DPC and NPPB basolateral application was less effective (Figures 4 and 5). Concentrations of one or more order of magnitude higher were required on the basolateral side to inhibit 50% of the SCC (Table 4). As these inhibitors are highly lipophilic the effects of basolateral application may still reflect an effect on the apical anion channels (Wangemann *et al.*, 1986). However it is also possible that these blockers have other effects. For example, 9-AC has been shown to affect cell metabolism. DPC and NPPB may also act on the Na/K/Cl co-transporter and the Cl/HCO_3 antiport at the basolateral membrane, since their molecular structures share some common features with those of the loop diuretic, frusemide and blockers of band-3-protein in red cells (see Wangemann *et al.*, 1986).

The biphasic response observed, i.e. inhibition followed by stimulation of the SCC when 9-AC was applied either apically or basolaterally to the unstimulated epithelium (Figure 2) may be due to two independent ionic events. Basolateral application produced a higher stimulating effect (Figure 2), whereas inhibition (transient) was more marked when the drug was added apically. The ionic mechanisms underlying the stimulatory effect of the SCC is unknown but the inhibition may be due to the blockage of anion channels at the apical side. The triphasic response (decrease, reversal to the original level and a slow sustained decrease) (Figure 3) to 9-AC in the adrenaline stimulated epithelium may also be due to the operation of two opposing events. Under stimulated conditions, the inhibition produced by 9-AC predominated over the stimulation so that the SCC was lowered at the steady state. The decrease in current measured at the steady state was dependent on the dose of 9-AC (Table 4). The dual effects of 9-AC were not seen with DPC and NPPB which produced only an inhibitory effect on the SCC when added basolaterally or apically and under basal or stimulated conditions (Figures 2, 4 and 5).

In the absence of Cl, the SCC response to adrenaline (0.23 μM) was reduced by 65%; the remaining current was due to HCO_3 secretion since the current was inhibited by acetazolamide, a carbonic anhydrase inhibitor (Cuthbert & Wong, 1986). In tissues secreting HCO_3 only, 9-AC and NPPB added to the apical or basolateral solution inhibited the SCC (Figure 6). As in Cl containing solution, basolateral application required 20 s for 9-AC to act. In contrast, the effect of apical application was immediate. The time lag may be explained by the time required for 9-AC to penetrate the epididymal cells and diffuse toward the apical side where it exerts its effect. Similar observations have been made with regard to the inhibition by 9-AC of Cl secretion in canine tracheal epithelium (Welsh, 1984).

In the intact epididymis, 9-AC added to the luminal perfusion solution caused a dose-dependent decrease in the serosal to mucosal Cl flux (Figure 7) with an IC_{50} value similar to that observed in cultured epithelium stimulated with adrenaline. The epididymis which is heavily innervated with sympathetic nerves (El-Bedwai & Schenk, 1967) is constantly under sympathetic nerve stimulation. It is conceivable that even under basal conditions, the tissue is under the influence of noradrenaline released from sympathetic nerves and anion secretion is taking place. Evidence to support this contention is that propranolol (3 mg) injected intravenously into rats caused a decrease in J_{s-m} Cl flux (unpublished), whereas addition of propranolol (1.7 μmol l^{-1}, final concentration) to the cultured epithelium devoid of nerve fibres did not affect the basal SCC (Wong & Chan, 1986).

In conclusion, anion secretion by the epididymal cells may conform to the general model proposed for other anion secreting epithelia and may involve Cl and HCO_3 diffusion across apical channels which are sensitive to Cl channel blockers.

This work was supported by the World Health Organization's Special Programme of Research, Development and Research Training in Human Reproduction under Project 85009 and the University of Hong Kong. I am grateful to Professor R. Greger for the gifts of DPC and NPPB.

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(Received July 7, 1987
Revised November 17, 1987
Accepted December 3, 1987)