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IRREVERSIBLE INHIBITION OF EPITHELIAL SODIUM CHANNELS BY ULTRAVIOLET IRRADIATION

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1 The effects of u.v. irradiation at 254 nm and 350 nm on sodium transport across frog skin epithelium have been investigated.

2 Irradiation at 254 nm but not at 350 nm produces a dose-dependent, functionally selective blockade of sodium transport. The effect is apparently due to the irreversible closure of apical sodium channels.

3 The amiloride-sensitive conductance was directly related to sodium transport as measured by short circuit current (SCC) both in normal and irradiated tissues, although both conductance and current were reduced in irradiated tissues.

4 The sensitivity of epithelia to irradiation at 254 nm was defined from the rate constants for the decline in SCC during three 2 min periods of irradiation at 1850 μW cm^{-2}. The rate constant for the initial 2 min irradiation was 0.093 ± 0.008 min^{-1}.

5 Lowering the sodium concentration to 5.5 mM from 110 mM increased the rate constant to 0.141 ± 0.014 min^{-1}, consistent with the view that more functional sodium channels exist at lowered sodium concentration.

6 Lowering the temperature to 7°C from 23°C reduced the rate constant to 0.032 ± 0.007 min^{-1} suggesting that blockade of channels is not due to a direct interaction with photons.

7 Using a variety of experimental protocols we were unable to demonstrate that bromamiloride or iodoamiloride can act as photoligands for sodium channels in the epithelium of *Rana temporaria*. This is in contrast to earlier reports with other epithelia.

Introduction

The amiloride-sensitive sodium channel of epithelia is widely distributed throughout the animal kingdom. Its properties are well conserved in different biological situations, in particular the sensitivity to blocking drugs (see for example Cuthbert, Fanelli & Scriabine, 1979). The rate controlling step of transepithelial sodium transport is normally the apical membrane, the domain in which sodium channel is located; thus the functioning of the channel is of considerable interest and importance. A view is emerging that regulatory and adaptive changes in the sodium permeability of the apical membrane is a vital component of homeostatic mechanisms for the benefit of the organism as a whole and for the integrity of the transporting tissues in particular (for a recent review, see Cuthbert, 1981).

The pharmacology of the epithelial sodium channel has received considerable attention. Two major groups of drugs, the pyrazine carboxamides and the aminopteridines, have proved to be specific, reversible blockers. Of the former group amiloride (Eigler, Kelter & Renner, 1967) is the best known while triamterene (Salako & Smith, 1971) is representative of the second. Structure-activity relationships for the reversible blockers have been described (Cuthbert & Fanelli, 1978). An irreversible blocker of the epithelial sodium channel would facilitate greatly exploration of the regulatory mechanisms exhibited by sodium transporting tissues. Thus far a straightforward irreversible ligand has not been found. However, there are two accounts of irreversible blockade by amiloride analogues following ultraviolet (u.v.) irradiation. Prolonged irradiation in the presence of bromamiloride apparently produces a modest (30%) irreversible inhibition (Benos & Mandel, 1978) while irradiation for a short period with tissues exposed to high concentrations of iodoamiloride gives complete irreversibility (Cobb & Scott, 1981). Irradiation in the presence of amiloride was found to be ineffective.

We have reinvestigated these phenomena and find that u.v. irradiation alone at 254 nm produces irreversible, and apparently, selective inhibition of transport. Ultraviolet irradiation in the presence of bromo- or iodoamiloride under simple defined conditions does not, we find, produce more inhibition than u.v. irradiation alone and our conclusion is that

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any reduction of transporting capacity achieved with the analogues is likely to be not related to the presence of the drugs themselves.

Methods

All experiments were carried out on the isolated ventral skin of the frog, *Rana temporaria*. Sodium transport across the epithelium of the skin was recorded as short circuit current (SCC) by conventional techniques using KCl-agar bridges leading via calomel cells to the input of the voltage clamp while current was passed through Ag-AgCl electrodes connected to the tissue baths by further KCl-agar bridges. The transepithelial voltage was clamped at 0 mV, that is the tissue was short circuited, the SCC being displayed on a pen recorder. Timing circuits within the clamp allowed the voltage to be held at a few mV above and below zero, alternately and at a chosen frequency and duration. The current pulses so generated and displayed on the recorder were used to measure transepithelial conductance.

The skin was mounted horizontally in a chamber of the type described previously (Cuthbert, 1973) with the apical surface uppermost. The apical surface could be irradiated through the apical bathing solution. The depth of this fluid was normally adjusted to 1 cm. The skin area when this type of chamber was used was 9.6 cm^2.

Most experiments were carried out at room temperature (23°C) but others were done at 7°C. To achieve this, fluid from a 11 reservoir maintained at 7°C was circulated through the mucosal bath via a roller pump. Temperature equilibration was established within 10–15 min.

During the progress of the work it became necessary to develop a double chamber so that records from the two halves of the same tissue could be recorded together. The electrical cross-talk which becomes a problem in double chambers when sensitive voltage clamps are used was avoided in the design developed. An exploded view of the chamber is shown in Figure 1. It has one serosal compartment and a double mucosal chamber. The serosal bath contains a single voltage sensing electrode which terminates just below the supporting bar. Current is passed through a platinum electrode coated with platinum black. After the double chamber was assembled with the tissue an electrode assembly was lowered into the upper chamber. This consisted of paired voltage-sensing KCl-agar electrodes connected to miniature calomel cells and paired platinum black electrodes for passing current. The assembly was lowered on a micromanipulator so that one voltage and one current passing electrode were correctly positioned in each half chamber. The output from the assembly was connected to the voltage clamp through a time sharing device such that each half of the tissue was short circuited alternately, usually for 15 s. Drugs added to the mucosal bath of one side did not affect the SCC of the other half. Only the mucosal baths were gassed by bubbling air through the solutions. Washing of the tissue was by aspiration and replacement of the fluid.

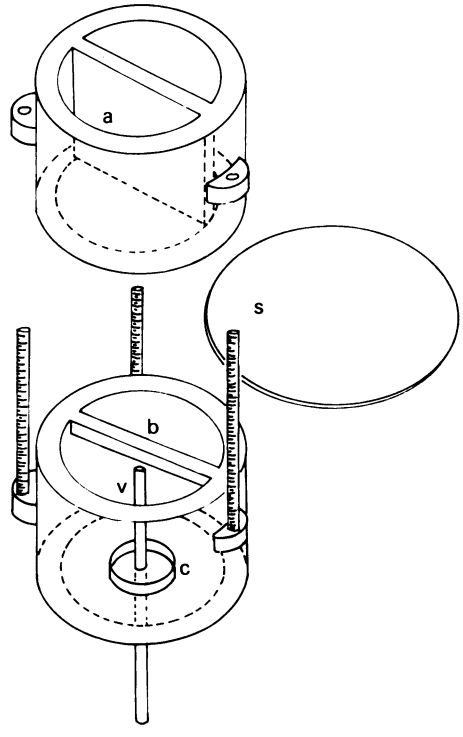

Figure 1 Exploded view of double chamber. The single serosal compartment (b) contains a potential sensing electrode (v) and a platinum current-passing electrode (c). The upper chamber (a) is split into two halves and was secured to the serosal chamber with three bolts with the skin (s) sandwiched between.

Ultraviolet irradiation was achieved from above the chambers with a Camag Universal UV Lampe TL-900/9 with two sources generating irradiation with a primary peak at either 254 nm or 350 nm. Irradiation doses were in the range 500–1850 $\mu W\,cm^{-2}$ and were monitored with a UVX digital radiometer. Short circuit recording could be monitored continuously during u.v. irradiation unless, as in some instances, the fluid was removed from the apical bath to leave only a thin covering layer above the tissue.

The bathing solution used in most experiments had the following composition (mM): NaCl 110, $CaCl_2$ 1, KCl 2, Tris buffer (pH 7.6), 5 and glucose 11.0. A low

sodium solution was used on a few occasions for the mucosal bath. This solution had an identical composition to that given above, except that NaCl concentration was reduced to 5.5 mM. A solution of pH 8.4 identical with that of Benos & Mandel (1978) was also used for some experiments. It had the following composition (mM): NaCl 110, $CaCl_2$ 1 and $KHCO_3$ 2.5. All solutions were equilibrated by gassing with air.

Results

Characteristics of the response to u.v. irradiation

Ultraviolet irradiation (254 nm) of the apical side caused a decline in SCC. The current fell rapidly at first for 8–10 min but afterwards more slowly. The transepithelial conductance decreased initially, particularly during the phase of rapid current decline but during the second and slower decline conductance increased dramatically, especially after the basal current was reduced to approximately 50% of its initial value (Figure 2).

Further characteristics of the effects of irradiation are shown in other figures. These include (a) cessation of the decline in SCC and of conductance when irradiation was stopped (Figure 6). However, after extensive irradiation conductance continued to increase despite interruption of u.v. irradiation, (b) complete sensitivity of the residual SCC to amiloride after irradiation (Figure 4) and (c) irreversibility of the effects of u.v. irradiation on SCC, at least on the time scale of several hours (Figure 7).

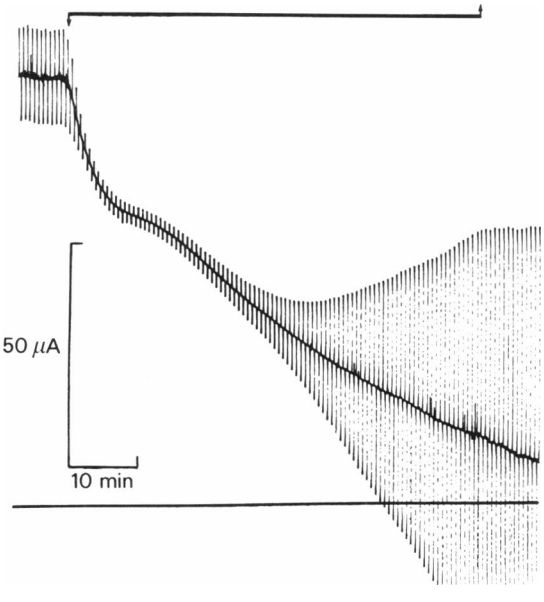

Figure 2 Effect of u.v. irradiation (254 nm) on SCC and conductance of frog skin. Area 9.6 cm^2. Voltage was clamped at ± 1 mV for 0.8 s every 20 s. Skin conductance was originally 9.8 mS, fell to a minimum of 2.3 mS and then increased to 52.0 mS. The horizontal line indicates the duration of irradiation (2300 $\mu W\ cm^{-2}$). Lower line indicates zero SCC in this and other figures.

Ultraviolet irradiation (254 nm) of the serosal side increased SCC and transepithelial conductance. It is unlikely that in this situation the irradiation penetrates to the epithelium as it would need to pass a thick connective tissue layer plus a layer of chromatophores (pigment cells) adjacent to the epithelium. In some instances skins were irradiated from the serosal side after which they were remounted in the chamber in reverse and re-exposed to u.v. from the apical side. For example a skin with an initial SCC of 144 μA (9.6 cm^2) was irradiated from the serosal side for 20 min by which time the current had reached 324 μA. After remounting in reverse the current was 290 μA but fell to 76 μA after irradiation from the apical side for 20 min. These preliminary results suggest that the fall in SCC following apical irradiation are due to an effect of u.v. on the transporting epithelium itself. The stimulant effects with serosal irradiation may be due to liberation of substances, such as prostaglandins (Hall, O'Donoghue, O'Regan & Penny, 1976), from the corium but this has not been further investigated.

Experiments were devised next to examine whether the primary lesion caused by apical irradiation was due to effects on passive entry processes or active exit processes for the sodium ion. If the major effect is on the normally rate limiting entry step then it is necessary to show that entry remains rate-limiting after irradiation. The polyene antibiotic, amphotericin B, is known to increase the passive permeability of the apical barrier when applied to that side. In frog skin the response is rather unreliable and varies considerably in a seasonal way. However, amphotericin B was able to increase SCC in irradiated skins in similar proportion to its effects in non-irradiated skins (Figure 3). Antidiuretic hormone applied in the serosal bathing solution also increases apical sodium permeability indirectly. This agent too increased SCC in irradiated skins (Figure 3). That this effect was due to increased sodium transport is shown by almost complete inhibition of SCC by amiloride after hormone. These results indicate therefore that one effect of irradiation is to reduce apical sodium permeability as agents which normally increase this also restore the current towards the pre-irradiation value.

Effects on sodium conductance

Not all of the conductance decrease seen during the

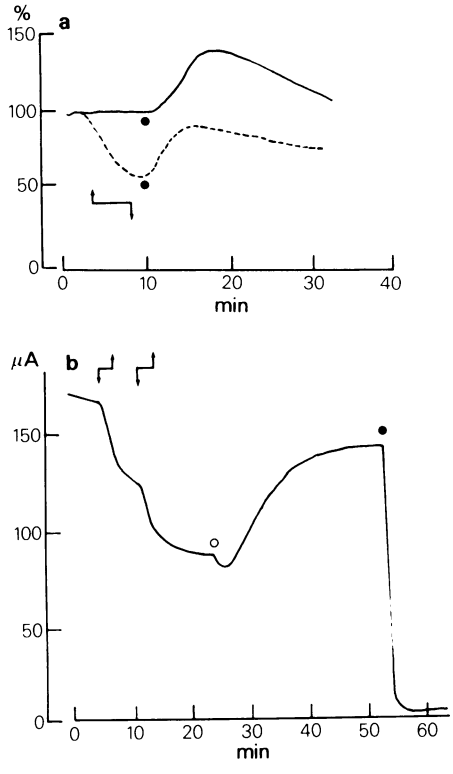

Figure 3 Effects of amphotericin and antidiuretic hormone on u.v. irradiated skins. (a) Shows percentage change in SCC in two skins (9.6 cm^2). One skin (dashed line) was irradiated at 254 nm and 1850 $\mu W\,cm^{-2}$ for 5 min, afterwards amphotericin (●), 0.6 mg/ml, was added to the mucosal bath. The other skin (continuous line) was not irradiated but amphotericin (●), 0.6 mg/ml was again added to the mucosal bath. (b) Shows SCC in skin (9.6 cm^2) irradiated at 254 nm, 900 $\mu W\,cm^{-2}$ for two periods each of 2 min. Antidiuretic hormone (○), 400 mu ml^{-1} was added to the serosal bath and after SCC stabilized at its new value amiloride (●), 0.1 mM was added to the mucosal bath.

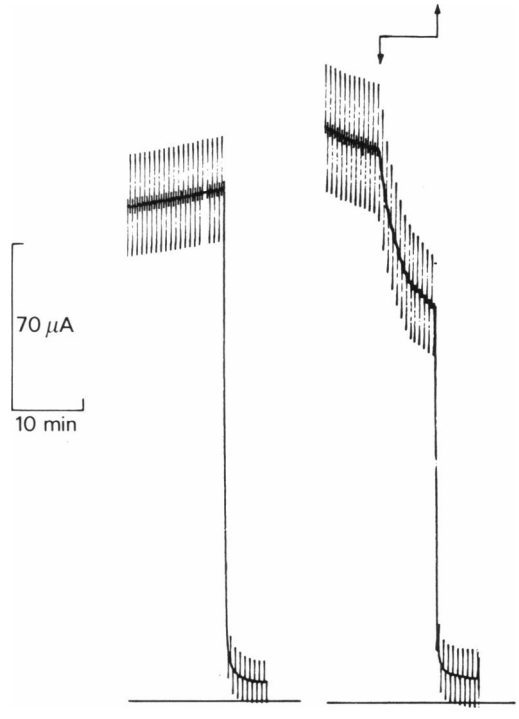

Figure 4 Procedure for measuring amiloride-sensitive conductance. Voltage was clamped at ±5 mV for 0.8 s every 20 s. Amiloride, 10^{-4} M, reduced SCC from 210 μA to 7 μA and reduced conductance by 2.4 mS. After amiloride was removed, SCC was 229 μA. Irradiation by u.v. at 254 nm and 1850 $\mu W\,cm^{-2}$ for 5 min reduced SCC to 162 μA. At this point amiloride, 10^{-4} M, reduced SCC to 7 μA and conductance by 1.8 mS. Skin area 9.6 cm^2.

phase of rapid current decline under u.v. irradiation is necessarily due to an effect on sodium entry sites and a method to determine that part of the conductance change associated with entry sites is required. The total transepithelial conductance approximates to that of the apical membrane plus the intercellular shunts. As amiloride blocks, specifically, the apical sodium conductance the difference in total conductance in the presence and absence of a high amiloride concentration (0.1 mM) gives a measure of the apical sodium conductance. The apical sodium conductance measured in this way is linearly related to the basal SCC, that is the sodium transporting capacity of the epithelium (Cuthbert & Shum, 1978). The procedure for estimating apical sodium conductance in this way is illustrated in Figure 4. Notice that in this example amiloride was unable to reduce conductance to quite the same extent after irradiation, indicating a possible effect of u.v. on non-sodium conductance even though SCC had fallen by only 30%.

In a series of experiments the apical sodium conductance was measured, using amiloride, both before and after irradiation (254 nm) from the apical side. Sodium conductance was correlated with SCC both in irradiated and non-irradiated skins. More importantly the correlation was equally precise when data from irradiated and non-irradiated skins was pooled (Figure 5). Thus irradiation fails to modify the relation between apical sodium permeability and SCC but simply reduces SCC, consistent with the view that irradiation inactivates apical sodium channels in a dose-dependent fashion without changing the channel characteristics of the non-inactivated sites.

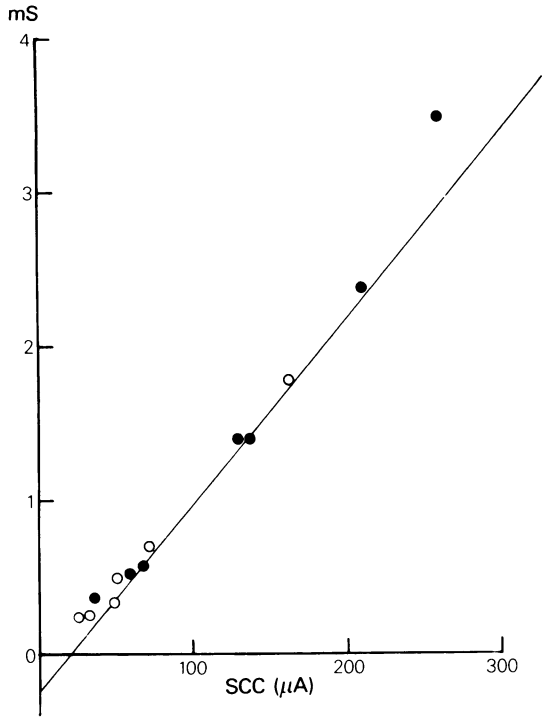

Figure 5 Correlation between SCC and amiloride-sensitive sodium conductance. SCC is plotted versus the conductance removed by amiloride, 10^{-4} M, in skins (9.6 cm^2) before irradiation (●), $n = 7$, and after irradiation at 254 nm for 5 min at 1850 $\mu W\,cm^{-2}$ (○), $n = 6$. Regression line has the characteristics $g = 0.0132\ SCC - 0.239$, $r = 0.99$, $P < 0.001$.

Effects of sodium concentration and of temperature

Prolonged irradiation from the apical side caused a biphasic decline in SCC as illustrated in Figure 2. As we suspected that the large increase in conductance associated with the second phase was due to tissue damage it was decided to confine further measurements to the early phase during which the conductance was declining. Incidentally, the massive conductance increases seen on prolonged irradiation were not reversed or prevented by amiloride, confirming that it was not a result of increased apical sodium permeability.

Even the first rapid decline in SCC on irradiation (254 nm) did not follow a simple exponential time course. Consequently we adopted a scheme using 2 min irradiation periods followed by 2 min rest periods for a series of exposures until the conductance began to increase. After this stage had been reached irradiation was continuous. An experiment of this type is illustrated in Figure 6. Notice that the decline in SCC ceases when irradiation is interrupted. We chose to measure the rate constants for current decline for the first three 2 min exposures to u.v. irradiation plus the rate constant for the decline in the final phase as characteristic of a tissue. Rate constants were obtained from semilog plots as shown in Figure 6 by extrapolation. They are defined as the reciprocal of the time taken for current to fall to 1/e of its original value. Rate constants were found to vary rather little between tissues (Table 1) but the initial rate of decline was faster than upon subsequent exposures.

When the temperature of the apical solution was reduced to 7°C from 23°C, the rate at which the SCC declined was reduced for the first three 2-min exposures (Table 1). In contrast to the effect of temperature when the sodium concentration was lowered to 5% of the normal value, there was a significant increase in the rate constants for SCC decline (Table 1). The transmittance of this dilute solution compared to the normal solution was only marginally (1%) greater and it is improbable therefore that this manoeuvre significantly affected the dose of irradiation reaching the epithelium.

Irradiation in the presence of potential photolysable blocking drugs

The sensitivity of the transport process to u.v. irradiation (254 nm) creates special problems in assessing

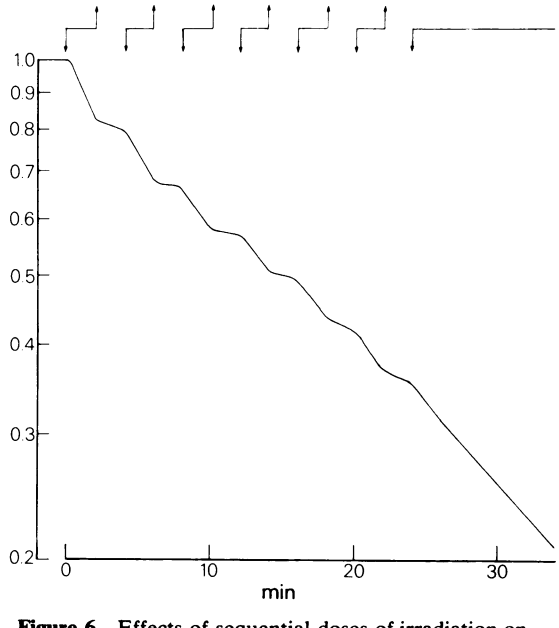

Figure 6 Effects of sequential doses of irradiation on SCC. Skin, 9.6 cm^2, was irradiated with u.v. at 254 nm, 1850 $\mu W\,cm^{-2}$ for 2 min periods followed by 2 min rest periods. Current is shown as a fraction of the initial SCC.

Table 1 Effects of u.v. irradiation (254 nm) on the rate constant for decline of SCC at different temperatures and sodium concentrations

	Irradiation period			
	0–2 min	4–6 min	8–10 min	20–30 min
Control				
23°C, 110 mM Na	0.093 ± 0.008	0.066 ± 0.005	0.052 ± 0.004	0.057 ± 0.002
$n = 4$				
	**	**	**	
7°C, 110 mM Na	0.032 ± 0.007	0.025 ± 0.006	0.031 ± 0.003	0.060 ± 0.011
$n = 3$				
	*	*	*	*
23°C, 5.5 mM Na	0.141 ± 0.014	0.107 ± 0.013	0.087 ± 0.01	0.150 ± 0.011
$n = 3$				

All values are rate constants (min^{-1}). Values marked with an asterisk are significantly different from the appropriate control values (using Student's t test ($^*P < 0.05$; $^{**}P < 0.01$). Irradiation was at 254 nm at 1850 $\mu W\,cm^{-2}$.

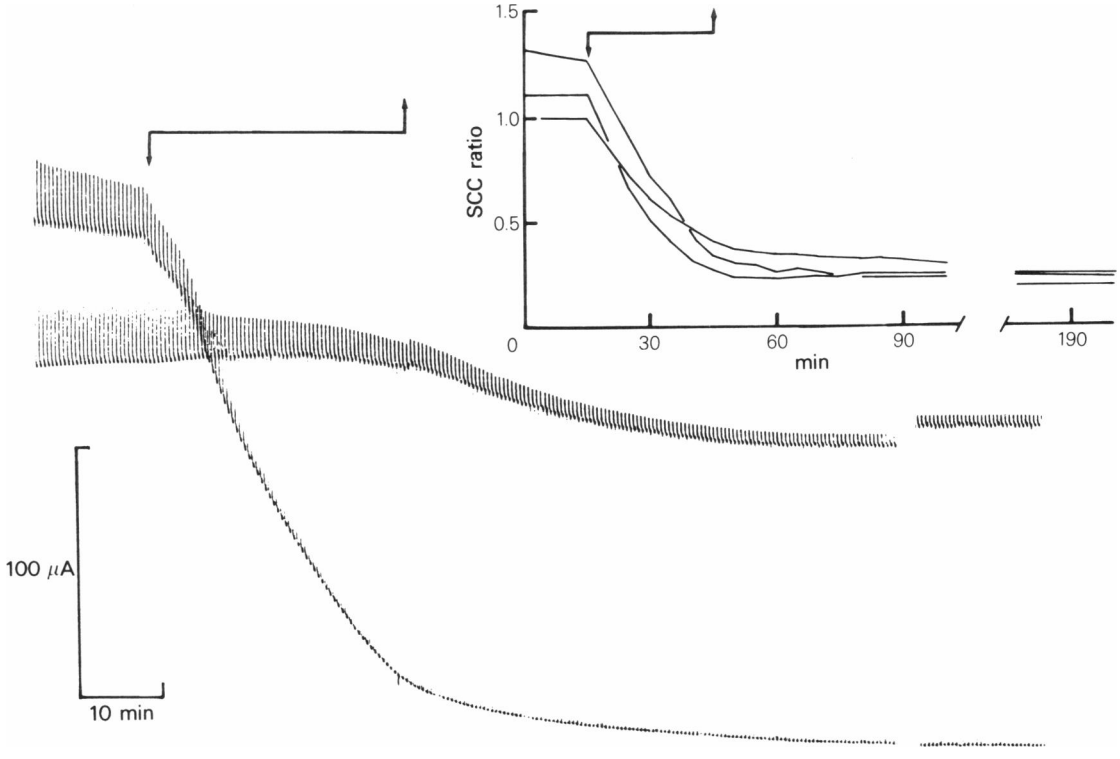

Figure 7 Irreversible effects of u.v. irradiation. Skin was mounted in double chamber (each half preparation 4.73 cm^2), one half was irradiated at 254 nm for 30 min at 1850 $\mu W\,cm^{-2}$. The two half preparations were voltage clamped alternately at 15 s intervals. Inset shows ratio of current in irradiated half compared to control for this and two other similar experiments. Note that the spikes shown here (and in Figures 8, 9 and 11) are equilibrium currents which flow when one half is clamped and the other unclamped. They should not be confused with the current spikes for conductance measurement as in Figures 2 and 4.

potential irreversible photoactivatable ligands. These problems are: (a) effects of u.v. irradiation itself have to be distinguished from inactivation by irreversible ligands; (b) ligands which absorb powerfully at 254 nm may effectively shield the tissue from irradiation and produce results which might be erroneously considered as protection and (c) ligands have inhibitory effects in the absence of irradiation which alter the state of the transporting mechanism and, more consequently, maybe its sensitivity to u.v. Added to these problems are problems of biological variation. All of these have been eliminated by using a special double chamber and suitable experimental protocols as described below.

In initial experiments only the tissue in one half of the chamber was irradiated (254 nm) while the other half was shielded. By expressing the ratio of SCC of the irradiated half to that in the non-irradiated side of the chamber allowance is made for variation of SCC with time which occurs during experiments of long duration. Figure 7 shows records from a sample experiment and results from 3 similar experiments. These findings show that it is possible to record separately from the two halves of the same tissue and that irradiation of one half has no effect on SCC of the other. In all three experiments the SCC ratio fell to about 0.2, that is the current in the irradiated skin was reduced to about 20% of control. The constancy of this ratio 2.5 h after irradiation was stopped indicates that the u.v. effect is irreversible, at least on the time scale of hours. Similar results are obtained with smaller doses of irradiation.

In a further series of experiments illustrated in Figure 8 both halves of the skin were irradiated (254 nm) but only one half was treated with amiloride during irradiation. The SCC ratio is expressed as the current in the side receiving amiloride to that in the untreated side. The concentration of

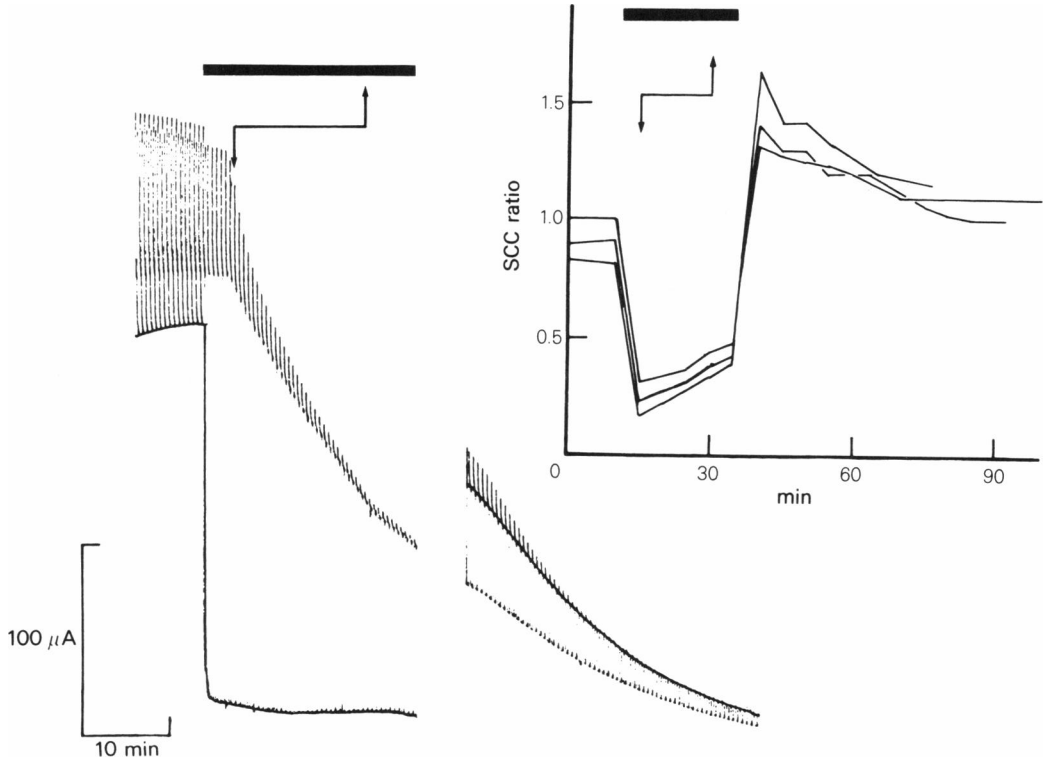

Figure 8 Protective effect of amiloride aganist u.v. irradiation. Double chamber was used and both halves of the tissue were irradiated (254 nm, 1850 W cm^{-2}) for the same time after amiloride (10^{-6}M) had been added to one chamber. For clarity a solid line has been drawn through the record for the amiloride-treated tissue. Inset shows ratio of current in the half treated with amiloride compared to the other half for this and two other similar experiments. The times during which drug was present and when the tissue was irradiated are indicated by the thick and thin bars.

amiloride used (1 μM) was sufficient to inhibit current by 80% and to reduce transmittance by 3.3%. Thus under these conditions the dose of irradiation is not significantly altered by the presence of drug. There are two indicators that amiloride affords a minor protective action against irradiation. First, the SCC remains more constant in the presence of amiloride, even if much smaller, during u.v. irradiation which is reflected in a slow increase in the SCC ratio from the initial value achieved after amiloride was added. Second, after the solution bathing the apical surface of both halves is replaced the SCC ratio increases to a value higher than the initial value, this increase being maintained for at least 1 h. This protective effect might be due to a reduction in the u.v.-sensitivity of the amiloride-channel complex compared to the free channel or alternatively is a consequence of the altered state of the transporting system.

We were particularly interested in examining the effects of bromo- and iodo-amiloride, both of which have been reported to be photoactivatable irreversible ligands for the epithelial sodium channel, while amiloride does not have this property (Benos & Mandel, 1978; Cobb & Scott, 1981). As control experiments had shown that amiloride had some protective effects of uncertain cause against u.v. irradiation (254 nm) (Figure 8) it was vital to ensure that the transport mechanism was in the same state in both control and test halves when potential photoactivatable ligands were tried. Consequently equiactive concentrations of amiloride and its analogue were added to separate halves so that the state of the transporting system in both halves was identical during the period of irradiation. It is known already that bromamiloride is equiactive with amiloride while iodoamiloride has one-tenth of this activity (Cuthbert & Fanelli, 1978). Thus when amiloride was compared with bromamiloride concentrations of 1 μM were used which cause virtually identical reductions of transmittance (Table 2) so that both halves of the tissue received identical doses of irradiation.

Table 2 Relative transmittance of solutions of amiloride analogues

		Transmittance 254 nm	350 nm
Amiloride	1 μM	96.7	96.6
	10 μM	85.8	71.4
	100 μM	22.2	2.4
Bromamiloride	1 μM	96.6	96.4
	10 μM	82.8	74.2
	100 μM	14.0	4.0
Iodoamiloride	1 μM	95.0	96.9
	10 μM	85.9	78.1
	100 μM	26.2	7.4

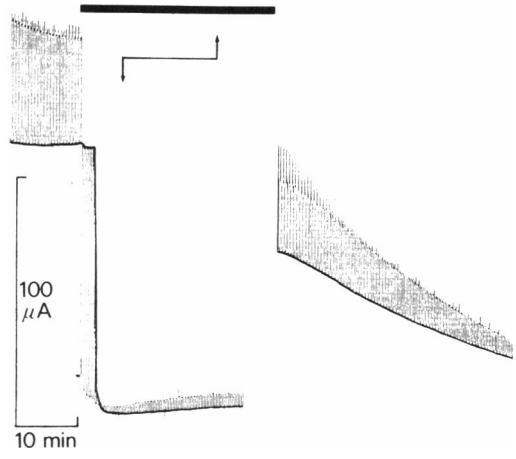

Figure 9 Comparison of the effects of irradiation in the presence of amiloride and iodoamiloride using the double chamber. Amiloride (1 μM) was added to one half of the preparation and iodoamiloride (10 μM) to the other. Note that an approximately equal degree of inhibition was achieved. Both halves were then irradiated at 254 nm and 1850 $\mu W cm^{-2}$ for 15 min. A solid line has been drawn through the current responses of the amiloride-treated preparation. Afterwards drugs were removed by washing and recording continued.

With iodoamiloride it was necessary to use a concentration of 10 μM to produce inhibition comparable to that with 1 μM amiloride and to keep the transporting mechanism in both halves in the same condition. However transmittance is reduced by 14.4% (Table 2) with 10 μM iodoamiloride compared to 3.3% with 1 μM amiloride. Thus in experiments with iodoamiloride the dose of irradiation (254 nm) will be somewhat less than with amiloride. Figures 9 and 10 show the results with these analogues. As before, the SCC ratio is given as the current in the half exposed to amiloride compared to the half exposed to the analogue. If either analogue acts as a photoaffinity label then the current will be less than that in the half exposed to amiloride after the drugs are removed. SCC ratios should therefore increase after irradiation if irreversible inhibition occurs. In all experiments the SCC ratios remained at their preirradiation values after removal of the drugs. It is concluded that in situations where care has been taken to ensure, as far as practicable, that the two halves of the same skin received the same dose of irradiation and where the transport mechanism was inhibited to the same extent no evidence for photoaffinity labelling was found.

As we found no evidence for photoaffinity labelling in spite of the earlier reports we carried out

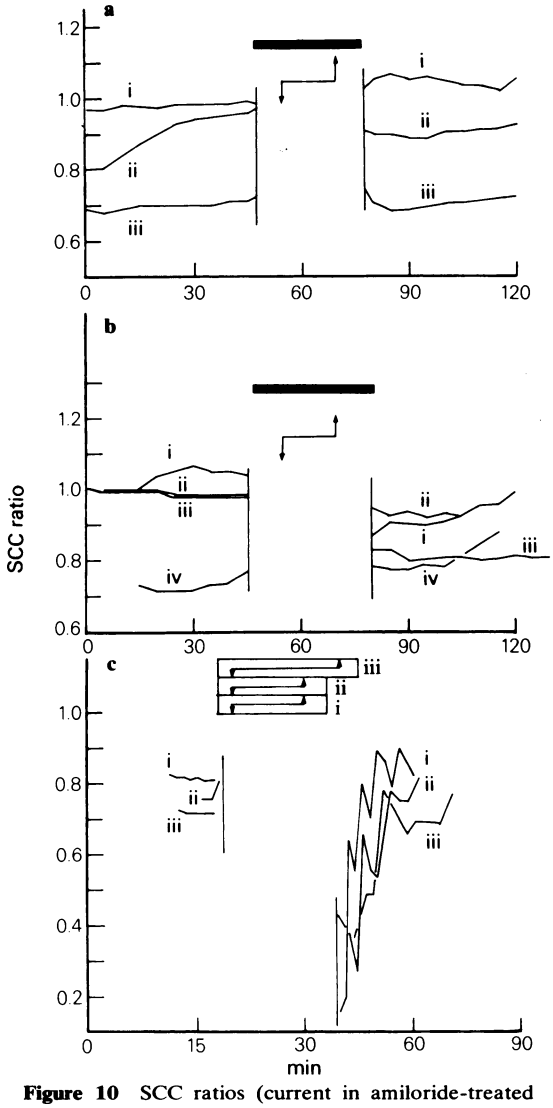

Figure 10 SCC ratios (current in amiloride-treated half compared to the other half) for 10 experiments in which the effects of irradiation in the presence of amiloride were compared to effects in the presence of analogues. (a) Comparison of amiloride (1 μM) and bromamiloride (1 μM). Presence of drugs shown by the thick bar and irradiation (254 nm, 1850 μW cm^{-2}) by the thin arrowed bar. (b) As (a) but a comparison of amiloride (1 μM) with iodoamiloride (10 μM). (c) Comparison of amiloride (100 μM) with iodoamiloride (100 μM) in bicarbonate Ringer at pH 8.4. Drug contact times and irradiation periods shown above as before: (i) irradiation at 254 nm, 950 μW cm^{-2}; (ii) irradiation at 350 nm, 1184 μW cm^{-2}; (iii) irradiation at 350 nm, 1184 μW cm^{-2} with bathing solution thickness reduced to 2 mm. With the high concentrations of drugs used in this series several washings were required leading to the step lide recovery back to the initial ratio. Note that the Scc ratios remained remarkably similar after treatment to the original values.

further experiments with different conditions, bearing in mind the conditions used by previous authors. These changes included (a) substituting a bicarbonate containing salt solution at pH 8.4 as used by Benos & Mandel (1978) for the Tris buffered solution at pH 7.6, (b) irradiation at around 330 nm, (c) use of ligand concentrations far in excess of those required to inhibit transport completely (b and c are conditions used by Cobb & Scott (1981)) and (d) irradiation of the tissue covered with a layer of only 0.2 cm thickness of drug containing solution. With procedure (d) it was not possible to monitor continually the SCC during irradiation.

Ultraviolet irradiation of the apical side at 350 nm in the absence of inhibitors for 20 min produced no effect on SCC. In none of the situations described above was evidence for irreversible inhibition of SCC obtained. For example when both halves of the tissue were exposed to 100 μM iodoamiloride but only one half irradiated at 350 nm for 20 min there was no indication that SCC recovered less well in the irradiated half. Figure 10 shows three experiments in which iodoamiloride, 10^{-4} M was compared with amiloride, 10^{-4} M. Because these concentrations of blocking agent are in excess of those required to inhibit transport completely it is assured that the transporting system was in an identical state in both halves and furthermore each half received approximately the same dose of radiation. The results show that irradiation at either 254 or 350 nm at pH 8.4 or irradiation at 350 nm at pH 8.4 with the apical solution thickness reduced to 0.2 cm had no effect on the SCC ratio of the two halves after the drugs were washed away. Unlike the earlier experiments with low drug concentrations the SCCs were fully restored to the original values after washing, showing that these high concentrations effectively shielded the tissue from the inhibitory effects of irradiation at 254 nm. Notice too that here the SCC ratio did not immediately attain its original value but progressed to it during a series of washes. This arises because the effect of the less active iodoamiloride is more quickly reversed by washing than amiloride and, as throughout, the SCC ratio expresses the current in the amiloride treated half to that in the other.

Throughout these experiments results which might erroneously be interpreted as irreversible inhibition have been obtained on two occasions. One of these experiments is shown in its entirety in Figure 11. Our reasons for so doing is that it may provide a clue to explain the discrepancies with other reports of successful photolabelling.

On both occasions amiloride and iodoamiloride, 10^{-4} M, were being compared and irradiation was for 20 min at 350 nm. On removal of the drugs the half treated with iodoamiloride recovered faster but by the third wash, equal recovery had occurred. Neither

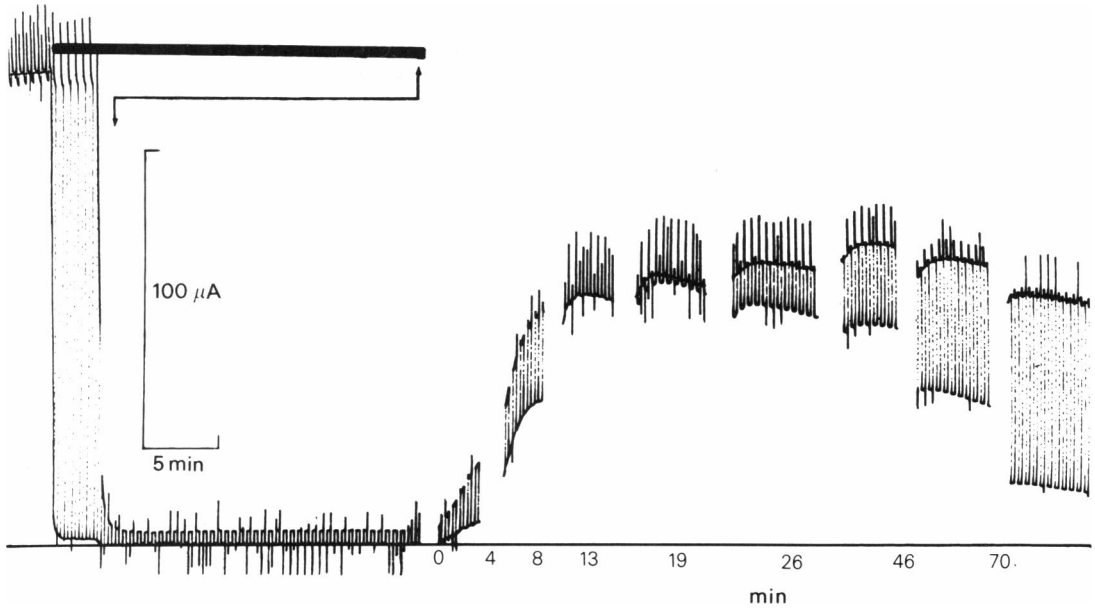

Figure 11 Apparently selective effect of iodoamiloride. Amiloride (100 μM) was compared with iodoamiloride (100 μM) with irradiation at 350 nm, 508 $\mu W\,cm^{-2}$ for 20 min using the double chamber. Both inhibitors caused almost complete abolition of SCC. Recovery after 8 wash periods is shown. A solid line has been drawn to indicate the amiloride-treated half. Note the SCC ratio (current in amiloride-treated half compared to current in other half) is originally low, reaches its original value at 13 min and then increases to 4.4 by 70 min after removal of the drugs.

half recovered its original SCC but the SCC of the half exposed to the iodo-derivative declined during the next hour until it was only 20% of that in the amiloride-treated half. We cannot be sure of the reasons for these effects but it is possible that products of u.v. irradiation exert a slow toxic effect on the tissue. Furthermore it is known that amiloride breaks down in a different way from its analogues during u.v. irradiation (Benos & Mandel, 1978). The differential effect on current cannot be due to photolabelling of the apical sodium channels with iodoamiloride as currents in the two halves were identical 10 min after irradiation ceased and after the inhibitors had been removed. We may well have considered ascribing the results to selective photoaffinity labelling by iodoamiloride if the SCC had not been monitored throughout or if a single tissue had been irradiated first in the presence of amiloride and subsequently with iodoamiloride.

Discussion

Ultraviolet irradiation at 254 nm of the apical surface of frog skin epithelium produces an apparently selective, irreversible inactivation of the sodium entry sites. The selectivity is not necessarily true at the molecular level but from a functional viewpoint irradiated tissues behave as if the apical sodium permeability is irreversibily reduced. The apical membrane remains the rate limiting step in transepithelial sodium ion movement in irradiated tissues since SCC is partially restored when apical permeability is increased either with antidiuretic hormone or amphotericin. Supporting evidence for this view is the reduced sodium conductance occurring concurrently with the reduction in SCC on irradiation, also the constancy of the relation between sodium conductance and SCC in irradiated and non-irradiated tissues.

The large, amiloride-insensitive increase in transepithelial conductance occurring on prolonged irradiation has not been investigated extensively but we consider it probably results from tissue damage.

This is the first account of u.v. effects on epithelial sodium channels although there are several on the irreversible effects of u.v. irradiation on voltage-sensitive sodium channels in excitable membranes (Fox & Stämpfli, 1971; Fox, 1974; Oxford & Pooler, 1975; Schwarz & Fox, 1977). The susceptibility of both types of channel is of interest since some similarities in the properties of these two types of membrane molecule have been noted (Cuthbert, 1976).

We cannot be sure of the reasons why Benos & Mandel (1978) saw no effect of u.v. irradiation of frog skin other than to point out that a different frog

species was used and that their irradiation intensities were low ($14\,\mu W\,cm^{-2}$), although tissues were exposed for prolonged periods (30 min). Thus in their experiments the total energy absorbed by the tissue was $25.2\,mJ\,cm^{-2}$, whereas in our experiments effects on current were seen with energies as low as $10.0\,mJ\,cm^{-2}$ delivered at a rate of $500\,\mu W$. This suggests that the irradiation effect is unlikely to be due to direct photon capture by sodium channels but rather is due to secondary effects (see later). One of us (D.D.F.) found, in preliminary experiments, that irradiation of toad bladder preparations at 254 nm also inhibited SCC. Irradiation of frog skin at 350 nm for prolonged periods had no discernible effect on SCC, as was found by Cobb & Scott (1981) with toad bladder preparations.

Ultraviolet irradiation produces multiple photoreactions such as photoperoxidation of lipids, photochemical events involving aromatic groupings such as tyrosine and tryptophan perhaps leading to crosslinking and the generation of free radicals. If photons interact directly with apical sodium channels the reaction should not be temperature-dependent and SCC should decay with a simple exponential time course. As neither of these criteria were fulfilled it is probable that the inactivation effect is indirect. Apparently the final phase of current decline (20–30 min) during which conductance was increasing was not temperature-sensitive (Table 1).

When the sodium concentration bathing the apical surface is lowered the density of conducting sodium channels is increased due to attenuation of the regulatory effect that sodium has on its own entry process (Aceves & Cuthbert, 1979; Van Driessche & Lindemann, 1979). Ultraviolet irradiation is expected to be more effective if the density of active sites is increased, whether or not the effect is due to a direct hit process or to a secondary effect. Thus the significant increases in the rate constants for SCC decline at reduced sodium concentration (Table 1) are consistent with the idea that irradiation produces its effects on current via an effect on sodium channels.

Specificity of photoaffinity labels depends on the conversion of the ligand to a covalently binding molecule while attached to the receptor or alternatively the conversion of free ligand in the environs of the receptor to an active form. We found that irradiation (254 nm) in the presence of amiloride produced a slight protective effect on SCC compared to control irradiated skins without amiloride. When amiloride was compared to either bromamiloride or to iodoamiloride, no differences were recorded in recovery after irradiation between amiloride and its analogues. In these experiments care was taken to ensure that the degree of inhibition caused by the inhibitors and the dose of irradiation received were the same in the test and control halves of the preparations. Thus we have been unable to confirm that bromamiloride can act as a photoaffinity label in frog skin (Benos & Mandel, 1978). These authors showed that during irradiation bromamiloride loses its halogen atom while this is not so for amiloride. It is probable the iodine is lost even more readily from iodoamiloride. Cobb & Scott (1981), using short periods of longer wavelength irradiation (330 nm), found iodoamiloride to be a more effective photoligand than bromamiloride. However, concentrations of ligand far in excess of those required to inhibit transport completely and to saturate the channels were used. This makes it uncertain that the effects were due to direct photolabelling with receptor attached ligand molecules.

A variety of other conditions were used in attempts to demonstrate photolabelling with iodoamiloride but all were unsuccessful. On two occasions apparently selective effects of iodoamiloride were obtained which may have been caused by slowly developing toxic effects due to the products of irradiation. Regrettably we have to conclude that bromamiloride and iodoamiloride are not useful as photoligands for epithelial sodium channels, at least not those in the skin epithelium of *Rana temporaria*.

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