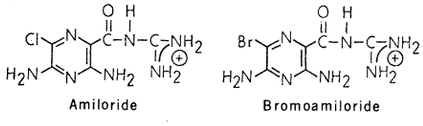
Irreversible Inhibition of Sodium Entry Sites in Frog Skin by a Photosensitive Amiloride Analog

Abstract. *A photosensitive binding reaction is described in which an analog of amiloride is bound to sites that control sodium entry into frog skin. This reaction results in irreversible inhibition of net sodium transport.*

Amiloride is a relatively new, potassium-sparing diuretic that inhibits sodium transport across amphibian skin as well as in renal distal tubules, colon, salivary ducts, caudal epididymis, toad urinary bladder, fish gills, and erythrocytes (*1*). This action is extremely specific for sodium, completely inhibiting active sodium transport in frog skin at concentrations as low as $10^{-5}M$. Amiloride is effective in frog skin only when present in the external solution. Its speed of action and rapid reversibility are indicative of an interaction with sodium entry sites located on the external aspect of the frog skin (*2*). This site has properties that render its classification as a "receptor" justifiable, namely, saturation kinetics, modulation of transport rate by various external influences such as *p*H, divalent cation concentration and hormones, competition with other cations, and finally drug inhibition (*2-4*). Evidence has been presented that this site is a protein (*4*) having characteristics identified with those of a channel (*5*).



The specificity of amiloride's action has already provided information regarding the functional nature of its interaction (or receptor) site, especially through the use of chemical analogs (*4*). The rapid reversibility of amiloride's inhibitory effect has proved invaluable in these and other similar studies. Additional information concerning both the functional and chemical attributes of this entry site could be obtained if an analog of amiloride were found that would both inhibit sodium transport in frog skin as well as bind irreversibly to the entry site. For example, the binding of tritiated ouabain to intact cells and tissues has promoted a greater understanding of the distribution and physiological operation of the sodium-potassium active transport system (*6*). In order to perform similar studies on the sodium entry site in frog skin and other amiloride-sensitive epithelia, we initiated a search for an analog of amiloride with the aforementioned characteristics. We now report that one analog of amiloride, bromoamiloride (Br-A), can irreversibly inhibit sodium transport across frog skin after irradiation with ultraviolet light.

Binding of Br-A to frog skin was accomplished by the following procedure. The ventral skin of the bullfrog (*Rana catesbiana*) was mounted in a modified Ussing chamber equipped with a circular quartz window, and then equilibrated in 110 m*M* NaCl Ringer solution under short-circuit conditions (*7*). A paired skin was usually mounted in a conventional chamber. A trace reproducing the short-circuit current (I_{sc}) as a function of time in a typical experiment is shown in Fig. 1. After equilibration, the external surface of the experimental skin was first irradiated for 30 minutes to ensure that ultraviolet exposure by itself had no untoward effects on I_{sc}. In this experiment, ultraviolet irradiation of the external skin surface in the absence of drug resulted in a slight increase in I_{sc} (Fig. 1A). Thereafter, the reversibility of amiloride was tested with and without ultraviolet irradiation. The effect on I_{sc} of a 30-minute exposure to $10^{-6}M$ amiloride in the absence of ultraviolet was completely reversible after thorough washing with 0.5 to 1 liter of Ringer solution (Fig. 1B). Photoirradiation in the presence of $10^{-6}M$ amiloride likewise caused no irreversible inhibition of I_{sc} (Fig. 1C). This result contrasts with the irreversible I_{sc} inhibition (16 percent) shown in Fig. 1D after a 30-minute period of ultraviolet exposure in the presence of $10^{-6}M$ Br-A. Exposure of the paired skin to $10^{-6}M$ Br-A for repeated 30-minute periods in the absence of ultraviolet light resulted in no irreversible inhibition of I_{sc} (Table 1). A second exposure of ultraviolet irradiation in the presence of $10^{-6}M$ Br-A produced additional irreversible inhibition (26 percent), as shown in Fig. 1E (*8*). The last panel in Fig. 1 again demonstrates that ultraviolet irradiation in the pres-

Table 1. Reversibility and irreversibility of I_{sc} under various experimental conditions. Photoirradiation and drug additions were to the external surface of the skin. Results are expressed as the mean percentage of the initial I_{sc} remaining (± 1 standard error of the mean) subsequent to exhaustive washing after the experimental manipulation indicated. Abbreviations; *N*, number of skins; UV, ultraviolet irradiation.

Experimental condition	Percent of initial I_{sc} remaining
UV irradiation (30 minutes)	99.1 ± 1.6 (N = 22)
$10^{-6}M$ amiloride exposure (30 minute; no UV)	99.8 ± 3.0 (N = 13)
$10^{-6}M$ amiloride exposure (30 minute; no UV)	102.0 ± 3.1 (N = 3)
$10^{-6}M$ Br-A exposure (30 minute; no UV)	115.7 ± 3.1 (N = 5)
$10^{-6}M$ Br-A plus UV (30 minutes)	66.3 ± 3.2 (N = 12)
$10^{-4}M$ amiloride and $10^{-6}M$ Br-A plus UV (30 minutes)	103.4 ± 5.9 (N = 4)

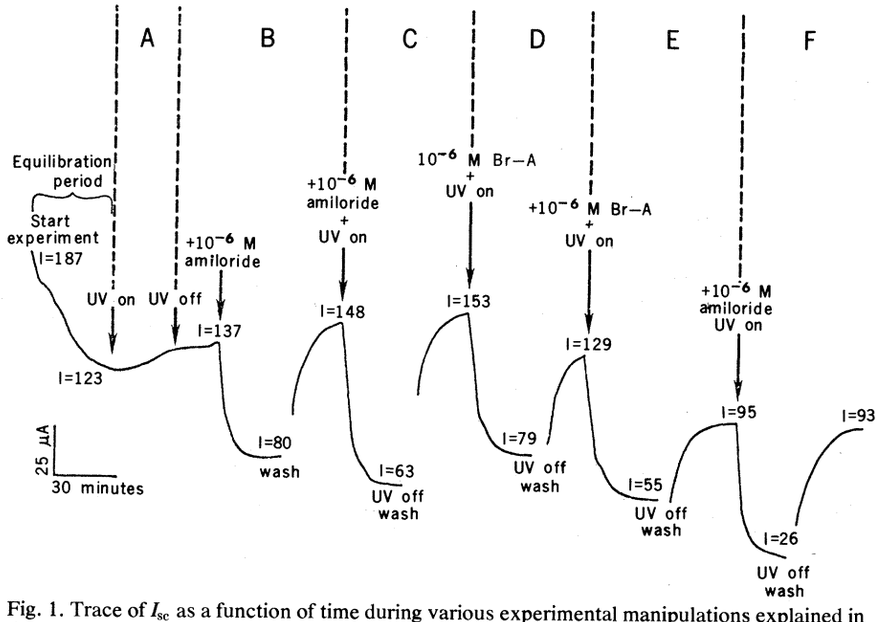


Fig. 1. Trace of I_{sc} as a function of time during various experimental manipulations explained in the text. Current is given in microamperes; the exposed surface area of bullfrog skin epithelium was 1.77 cm². Amiloride and Br-A were added to the external bathing solutions only.

0036-8075/78/0317-1205$00.50/0

ence of amiloride does not elicit any irreversible effects on I_{sc}.

A 30-minute exposure of the frog skin to either ultraviolet by itself, $10^{-6}M$ amiloride (without ultraviolet), $10^{-6}M$ Br-A (without ultraviolet), or $10^{-6}M$ amiloride plus ultraviolet results in no permanent change in I_{sc} (Table 1). However, only a 66.3 percent recovery of I_{sc} is obtained after a 30-minute exposure to $10^{-6}M$ Br-A in the presence of ultraviolet. Further irreversible inhibition of I_{sc} can be obtained with additional ultraviolet and Br-A exposure periods. We interpret the inactivated portion of I_{sc} to be the result of the irreversible binding of Br-A to sodium entry sites on the external surface of the frog skin (*9*).

We also designed experiments to test whether the binding of Br-A to the sodium site is specific (*10*) (Table 1). The observed irreversible loss of transport activity may be due to (i) Br-A binding to the active site or (ii) secondary conformational effects having nothing to do with site labeling. Since amiloride was found to be unreactive and since it presumably interacts at the same active site as does Br-A, amiloride was chosen as a specific site "protector" agent. The presence of $10^{-4}M$ amiloride significantly depresses (indeed prevents) the inactivation of I_{sc} produced by $10^{-6}M$ Br-A upon photolysis (*11*). This observation strongly supports the contention that the photosensitive reaction between Br-A and the sodium entry site is specific.

The mechanism of this Br-A, sodium entry site reaction is unknown, but it may proceed by at least two possible routes: free radical generation or photonucleophilic substitution (or both) (*12*). Elucidation of the reaction mechanism is complicated, as evidenced by similar ultraviolet absorption spectra of Br-A and amiloride solutions before and after photoirradiation (Fig. 2), and by the observation that irradiated solutions of Br-A and amiloride fail to inhibit I_{sc}, even at $10^{-4}M$. These facts may indicate that absorption of ultraviolet light may cause rearrangement or breakage of the pyrazine ring (*13*).

Regardless of the mechanism, the photoreaction of Br-A and the frog skin probably involves the 6-pyrazine ring carbon atom. This conclusion is based on the following experiment. Solutions (10 mM) of Br-A and amiloride were prepared in distilled water and samples of each were placed in quartz cuvettes. These solutions were then irradiated for 30 minutes under the conditions described earlier. All solutions were subsequently measured for free halide (*14*). The free halide concentration of the non-

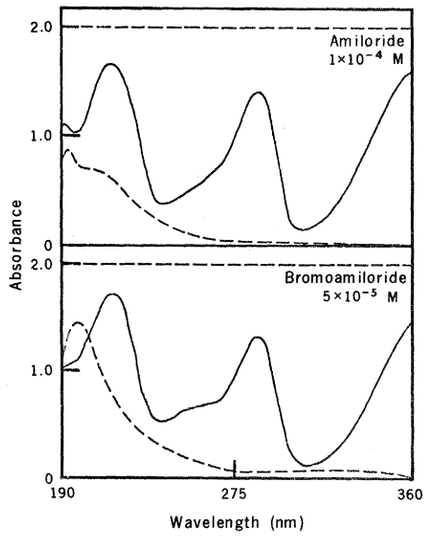


Fig. 2. Ultraviolet absorption spectra of amiloride (*N*-amidino-3,5-diamino-6-chloropyrazine carboxamide) and bromoamiloride (*N*-amidino-3,5-diamino-6-bromopyrazine carboxamide) before (solid line) and after (broken line) 30 minutes of ultraviolet irradiation. Compounds were dissolved in distilled water at the concentrations indicated and placed in a quartz cuvette; the ultraviolet spectra were determined with a Beckman DB-GT grating spectrophotometer. Photoirradiation was accomplished with an Oriel low-pressure mercury lamp (energy output, ~14 μW/cm^2 at 3 cm; major wavelength, 254 nm).

irradiated, 10 mM stock solutions of Br-A and amiloride was determined to be, respectively, 0 and 10.6 mM (amiloride is prepared commercially as the hydrochloride whereas Br-A is not). After irradiation, the halide concentration in the Br-A solution increased to 8.3 mM, while that of the irradiated amiloride solution remained as 10.7 mM. These results indicate that bromide is liberated from the pyrazine ring during photolysis, and that the 6-pyrazine ring carbon atom may be the site of covalent attachment of the Br-A molecule to its receptor group on the frog skin.

In conclusion, we have found that Br-A can act as a specific and irreversible blocking agent of sodium transport in frog skin after photoirradiation, due to covalent binding to the sodium entry site. This photoreaction may be exploited for use as a tool for numerous experiments involving the sodium entry step in amiloride-sensitive epithelia, such as determining the number and distribution of these sites, and could lead to the eventual isolation of these transport proteins.

D. J. BENOS
L. J. MANDEL

Department of Physiology, Duke University Medical Center, Durham, North Carolina 27710

References and Notes

1. M. S. Glitzer and S. I. Steelman, *Nature (London)* **212**, 191 (1966); J. E. Baer, C. B. Jones, S. A. Spitzer, H. F. Russo, *J. Pharmacol. Exp. Ther.* **157**, 472 (1967); P. J. Bentley, *J. Physiol. (London)* **195**, 317 (1968); E. N. Ehrlich and J. Crabbé, *Pfluegers Arch.* **302**, 79 (1968); J. P. Guignard and G. Peters, *Eur. J. Pharmacol.* **10**, 255 (1970); W. Nagel and A. Dörge, *Pfluegers Arch.* **317**, 84 (1970); L. A. Salako and A. J. Smith, *Br. J. Pharmacol.* **39**, 99 (1970); L. H. Schneyer, *Am. J. Physiol.* **219**, 1050 (1970); F. C. Herrera, *ibid.* **222**, 499 (1972); L. B. Kirschner, L. Greenwald, T. H. Kerstetter, *ibid.* **224**, 832 (1973); L. J. Barratt, *Pfluegers Arch.* **361**, 251 (1976); P. Y. D. Wong and C. H. Yeung, *Br. J. Pharmacol.* **58**, 529 (1976); A. W. Cuthbert and W. K. Shum, *Nature (London)* **266**, 468 (1977).
2. T. U. L. Biber, *J. Gen. Physiol.* **58**, 131 (1971).
3. P. F. Curran and J. R. Gill, *ibid.* **45**, 625 (1962); T. U. L. Biber and P. F. Curran, *ibid.* **56**, 83 (1970); L. J. Mandel and P. F. Curran, *ibid.* **62**, 1 (1973); L. J. Mandel, *ibid.* **65**, 599 (1975).
4. D. J. Benos, S. A. Simon, L. J. Mandel, P. M. Cala, *ibid.* **68**, 43 (1976).
5. B. Lindemann and W. Van Driessche, *Science* **195**, 292 (1977).
6. P. B. Dunham and J. F. Hoffman, *J. Gen. Physiol.* **58**, 94 (1971); P. F. Baker and J. S. Willis, *J. Physiol. (London)* **224**, 441 (1972); C. H. Joiner and P. K. Lauf, *J. Membr. Biol.* **21**, 99 (1975); J. W. Mills, S. A. Ernst, D. R. Dibona, *J. Cell Biol.* **73**, 88 (1977); P. M. Cala, N. S. Cogswell, L. J. Mandel, *J. Gen. Physiol*, in press. The cardiac glycoside ouabain binds to and inhibits a membrane bound, Na,K-activated enzyme, adenosine triphosphatase. This enzyme has several characteristics that support the notion that it may be, at the very least, a part of the active Na,K transport system. This (Na,K) adenosine triphosphate is located on the serosal border of the frog skin.
7. A new frog skin chamber permitting ultraviolet irradiation and rapid solution changes without requiring dismantling was conceived, designed, and constructed in collaboration with R. Overaker of Duke University. This chamber has a circular quartz window with the same area as that of the mounted, exposed skin namely, 1.77 cm^2. Each chamber half is fitted with circular platinum current electrodes; the membrane potential is monitored by calomel electrodes connected to the chamber by way of agar bridges. The volume of each compartment is 1.25 ml. The skin is held in place with Sylgard washers. Teflon leur fittings are present to open or close the aeration and solution-change conduits. The chamber body is made of polyvinyl chloride. The Ringer solution used in all these studies consisted of 110 mM NaCl, 1.0 mM $CaCl_2$, and 2.5 mM $KHCO_3$, pH 8.4. Photoirradiation was achieved with a low-pressure mercury lamp (Oriel Optics Corp., Stamford, Conn., model C-13-61; emission maximum, 254 nm; energy output, 14 μW/cm^2; lamp distance, 3 cm).
8. Figure 1, D and E, show that progressive inhibition occurs with new Br-A solutions and new irradiation periods. This is typical of other systems that undergo photolysis. See B. Haley and J. F. Hoffman, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 3367 (1974); B. Haley, personal communication.
9. Several experiments were performed in which I_{sc} after the 30 minute exposure to $10^{-6}M$ Br-A and ultraviolet light was monitored for periods as long as 2 hours. We found no change in I_{sc}, even with periodic washings. We conclude that the inhibition of I_{sc} by Br-A was not reversible, at least for this length of time.
10. S. J. Singer, *Adv. Prot. Chem.* **22**, 1 (1967).
11. Complete recovery of I_{sc} after exposure of the skin to $10^{-4}M$ amiloride while photoirradiating for 30 minutes was noted.
12. D. C. Neckers, *Mechanistic Organic Photochemistry* (Reinhold, New York, 1967); F. Pietra, *Q. Rev. Chem. Soc.* **23**, 504 (1969).
13. M. Mousseron-Camet and J.-C. Mami, *Photochemie et Réactions Moleculaires* (Dunod, Paris, 1969).
14. Free halide was determined with a Radiometer CMT 10 chloride titrator (Radiometer, Copenhagen). Standard solutions of NaCl and NaBr were run, the results being indistinguishable.
15. Supported by grants AM 05624 and AM 16024 from the National Institute of Arthritis, Metabolism, and Digestive Diseases. We thank Dr. E. J. Cragoe, Jr., of Merck Sharp & Dohme, for supplying us with amiloride and bromoamiloride, and D. G. Shoemaker, R. S. Balaban, and Dr. P. K. Lauf for criticisms of the manuscript.

29 July 1977; revised 7 September 1977