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Inhibitory and Stimulatory Effects of Amiloride Analogues on Sodium Transport in Frog Skin

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Summary. Effects of amiloride analogues on Na transport were studied in isolated skins of the frog *Rana ridibunda*. The pattern of structure-activity relationship of these compounds showed that both the $-NH_2$ group at position 5 and Cl at position 6 of the pyrazine ring of the amiloride molecule were important for their biological activity. The paramount role of the groups at position 5 was further demonstrated by the striking properties of an analogue resulting from dimethylation of that $-NH_2$ group. A stimulation of Na transport, opposite to the effect of amiloride itself, was observed in this instance. The increase in Na transport could already be seen at 10^{-6} M and was equivalent to the measured increase in Na influx, reversible, dose-dependent, and additive to the natriferic action of oxytocin. Such characteristics resemble those reported with "external" agents like propranolol and La^{3+}. Furthermore, mutual inhibition was observed between the stimulatory effects of this analogue and those of propranolol or La^{3+}. These results suggest that the analogue may be considered as another "external" agent acting at sites of the external membrane distinct from those activated by cAMP but similar to the Ca sites described by Herrera and Curran (Herrera, F.C., Curran, P.F. 1963. *J. Gen. Physiol.* **46**:999).

In recent years, intensive work from several laboratories has been focused on the permeation processes occurring at the apical border of amphibian epithelia. According to current concepts, this portion of the plasma membrane is the main barrier to transepithelial Na and water movement and one of the loci where hormones exert their natriferic and hydrosmotic effects.

Amiloride, first presented as a potassium-sparing diuretic (Baer *et al.*, 1967), became a drug of choice for the study of Na transport in a variety of epithelia (Cuthbert, 1974; de Sousa, 1975). In view of its highly specific interaction with membrane Na-entry sites, much effort

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has been devoted to elucidate the mode of action of amiloride and to gain insight into the molecular structure of amiloride-sensitive pathways involved in the translocation of Na across biological membranes. In this endeavor, a variety of sophisticated approaches has been employed, including fluctuation analysis (Lindemann & Van Driessche, 1977), microelectrodes (Nagel, 1976; Helman & Fisher, 1977; Sudou & Hoshi, 1977) and C^{14}-amiloride binding studies (Cuthbert, 1973; Cuthbert & Shum, 1974).

The availability of structural analogues of amiloride provided an opportunity to join in such effort with the help of standard techniques utilized for the study of epithelial transport. In this report, effects of some amiloride analogues on Na transport in frog skin are presented. Particular emphasis was given to an analogue obtained by dimethylation of the amino group at position 5 of the pyrazine ring of amiloride. In contrast with the phenomenological inhibitory effect of the parent species, this compound exerted a stimulatory effect on Na transport. To investigate further the nature of the observed natriferic action, we also analyzed the interaction between the effects of this compound and those of three reference substances – oxytocin, propranolol, and La^{3+} – the natriferic action of which has been documented (de Sousa, 1975; Marguerat, 1975). A preliminary report of this work was presented elsewhere in abstract form (Li & de Sousa, 1977b).

Materials and Methods

The abdominal skin of *Rana ridibunda* was mounted as a flat sheet between Lucite chambers. Two types of chamber were used: a double and a single chamber.

The double chamber, with rectangular windows of 2.0 cm^2 and a volume of 5 ml, had been described previously (de Sousa & Grosso, 1973). With this device, two equal areas of the same skin were exposed to Ringer solutions contained in adjacent compartments. One area of the skin was used as control of the other, thus facilitating the execution of "cross experiment" protocols. At the right and the left extremities of the chamber, rectangular plates of Ag-AgCl were positioned for passing electrical current. Through the center of these electrodes, 3% agar in 3M KCl bridges were introduced into the solutions bathing the skin. The other end of the bridges was connected, via 3 M KCl solution, to calomel electrodes (Metrohm), for monitoring the electrical potential difference across the skin.

The single chamber, developed mainly for studying tracer flows and for eliminating Ag^+ contamination from the electrodes, was a modification of the set up previously used in the study of ion transport through artificial membranes (Li, de Sousa & Essig, 1974). It consisted of four cylindrical Lucite compartments having approximately a cross sectional area of 6 cm^2 and a volume of 16 ml. The skin was first mounted on rubber gaskets 0.6 cm in thickness and with a circular hole of 3.14 cm^2. This assembly was then clamped

between the two central compartments separated by metal spacers at a fixed distance so that a good seal was achieved with minimal clamping pressure to reduce "edge damage". The outer compartments were separated from the central experimental compartments by "tight" and highly anion permselective ion-exchange membranes (Ionics QZL-219) sealed with rubber O-rings. At the two ends of the outer compartments, circular metallic electrodes (Ag-AgCl or Ag-Ag_2SO_4) were placed and connected to the voltage clamp. The electrical potential difference across the skin was measured with calomel electrodes via two polyethylene bridges of tip diameter ca. 0.1 cm. They were filled with 3% agar in Ringer solution and entered the central compartments laterally so that the tips were closely apposing each other near the center of the exposed skin.

An effective stirring of the Ringer solution was achieved by means of rotating Lucite paddles mounted on magnetic bars located in the central compartments. The outer compartments were filled with Na solutions containing the same major anions (Cl^- or SO_4^{-2}) present in the Ringer solutions in contact with the skin. The ion-exchange membranes were pre-equilibrated with the corresponding Na solutions. This technique had two major advantages: (i) the resistance associated with the use of narrow agar bridges to pass electrical current was greatly reduced, thus circumventing technical difficulties due to the relatively low output voltage of some of our voltage clamps; (ii) the possible contamination with Ag^+ of the Ringer solutions in direct contact with the skin was avoided by intercalating the ion-exchange membranes between the skin and the silver electrodes. This precaution seemed necessary, in view of the effects of Ag^+ on frog skin, described elsewhere (Li & de Sousa, 1977a; Li & de Sousa, *unpublished*). Before each experiment, the single chamber was assembled and filled with Ringer solution in the absence of the skin. Correction was made for any potential difference greater than ± 0.01 mV. The resistance of the Ringer solution between the tips of the bridges was measured and introduced in the voltage clamp as a pre-set value to compensate for the potential drop at the solution layers during voltage-clamping of the skin.

Na Tracer Fluxes

Tracer flux measurements were performed in the single chamber with the skin continuously short circuited. When SCC had reached a steady value, carrier-free Na^{22} (NEN, NEZ-081) was added to the outer solution (approximately 10 μCi per ml of Ringer solution). After a period of 10–15 min to allow for tracer equilibration, 20- and 100-μl samples were drawn from the outer and the inner solutions, respectively, at time intervals varying from 5 to 10 min. Unidirectional Na fluxes were calculated from the values of tracer transfer rate from the outer to the inner solution. Since during the entire course of the experiment the tracer activity in the inner solution remained insignificant in comparison with that in the outer solution, no correction for the backflux of tracer was needed. Four or five samples were taken before and after treatment with the amiloride analogue and counted in a gamma spectrometer (Packard, Auto-gamma spectrometer, model 3002). Although the full effect of the analogue on SCC was rapidly attained in less than 5 min, evaluation of tracer fluxes was only started 10 to 15 min after the administration of the compound so that new steady-state values of SCC and Na influx, determined with Na^{22}, were compared.

Chemicals and Solutions

The composition of NaCl and Na_2SO_4 Ringer solutions were as follows (in mM): (i) NaCl, 112; $MgSO_4$, 1; KH_2PO_4, 1.2; $KHCO_3$, 2; $CaCl_2$, 1.3; pH 7.5; osmolality, 200–220 mosmol kg H_2O; (ii) Na_2SO_4, 56; K_2SO_4, 1.25; $CaSO_4$, 1; Tris, 5; pH 8.0; osmolality,

142–145 mosmol kg H_2O. Studies with La^{3+} were carried out in a modified Ringer solution described elsewhere (Marguerat, 1975). None of these Ringer solutions appeared to modify the responses of the skins to the agents tested in this work. Cl-Ringer was the solution most frequently employed.

All chemicals used were reagent grade. Oxytocin was purchased from Sandoz (Syntocinon) and propranolol from ICI. The amiloride analogues were a gift from Merck, Sharpe & Dohme.

Statistical analysis was done by using the Student's paired t test.

Results

Effects of Amiloride Analogues on SCC[1]

The effects of amiloride analogues on the SCC of isolated frog skins were examined by adding these compounds to the outer bathing solution up to the concentration of 10^{-4} M. At this concentration, amiloride itself usually blocks completely the entry of Na into the epithelial cells and brings SCC close to zero. The relative potency of the analogues could thus be evaluated by determining: (i) the degree of inhibition of SCC at 10^{-4} M; (ii) the inhibition of an eventual residual SCC by the subsequent addition of 10^{-4} M amiloride.

A typical experiment following this protocol is shown in Fig. 1. To help in visualizing structure-activity relationships, the molecular structure of amiloride and its analogues is schematized on the left-hand side of Fig. 2 and their effects on SCC are summarized on the right hand side of the same figure.

It is clear that none of the analogues tested was as effective as the parent amiloride molecule in inhibiting the SCC. Not only was the average percentage reduction of SCC induced by the analogues at 10^{-4} M less than that observed with amiloride, but also the remaining SCC was inhibitable, in each experiment, with 10^{-4} M amiloride.

Elongation of the guanidine side chain of the amiloride molecule by insertion of an additional $-NH$ group (analogue I) altered the effectiveness only slightly. In contrast, the replacement of the $-NH_2$ group at position 5 of the pyrazine ring with $-H$ diminished considerably the potency to inhibit SCC (analogue III). If the same substitution was made on analogue I the resulting analogue II was more potent than analogue III. Replacement of $-Cl$ with $-H$ at the position 6 of the pyrazine ring also significantly reduced the potency of the compound (analogue IV). Finally, when both the $-Cl$ (position 6) and the $-NH_2$

1 SCC = short-circuit current.

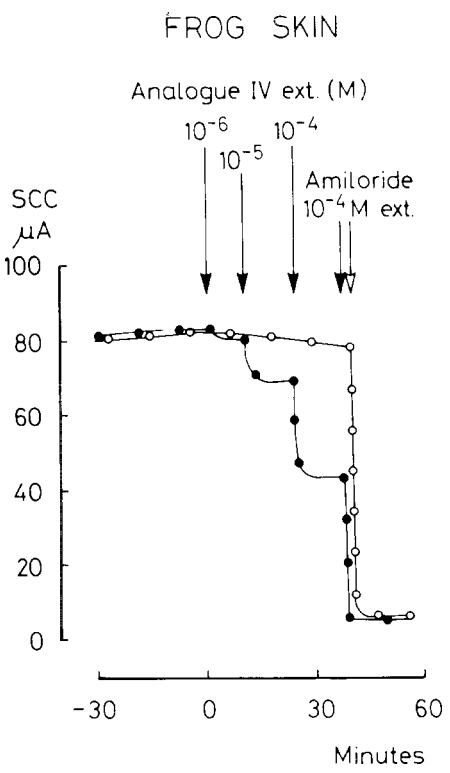
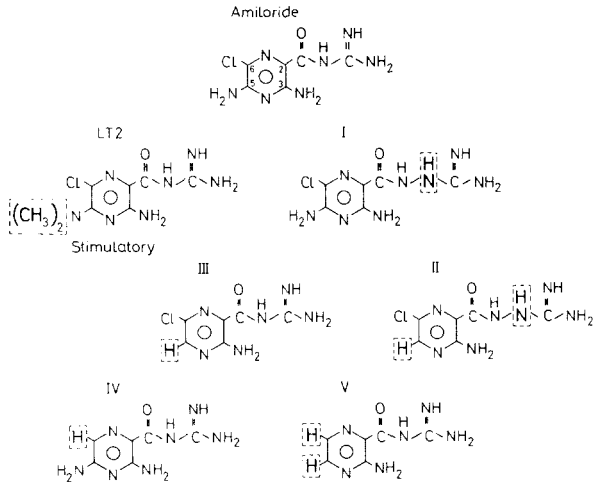


Fig. 1. Example of a typical experiment for evaluating the effect of amiloride analogues on Na transport. The SCC's of two segments of the same skin were recorded continuously as described in *Materials and Methods*. Note that addition of 10^{-4} M amiloride abolished the residual SCC in the segment pretreated with the analogue [●–●–●], as well as the basal SCC in the control paired segment [o–o–o]

group (position 5) were replaced with $-H$, the corresponding analogue V had hardly any inhibitory action on SCC.

So far, the changes made in the amiloride molecule resulted in compounds the effectiveness of which was less or practically nil when compared with the inhibitory effect of the parent species. In contrast, a striking qualitative change in the biological action of this family of compounds was seen when the $-NH_2$ group at position 5 of the ring was replaced with a $-N(CH_3)_2$ group. This analogue, coded LT2, did *stimulate* SCC when added to the outer solution bathing the frog skin.

In view of the fact that some agents can exert both stimulatory and inhibitory actions, depending on the concentration used, we performed a dose-response curve for LT2. As shown in Fig. 3, a stimulation of SCC was already noticeable in the presence of 1 μM of LT2.



Ana-logue	% Inhibition of SCC		
	10^{-6} M	10^{-4} M	N
I	42.1 ± 2.9	90.9 ± 1.4	10
II	7.7 ± 1.8	71.1 ± 3.4	7
III	2.0 ± 0.4	54.3 ± 3.0	5
IV	2.3 ± 0.6	47.6 ± 2.6	14
V	0	0	11

Fig. 2. Structure-activity relationship of amiloride and its analogues. Modifications on the parent amiloride molecule are marked with bold type and enclosed in dashed rectangles. The average percentage (±SEM) of inhibition of SCC is tabulated at right for five of the analogues at the concentrations 10^{-6} and 10^{-4} M. At 10^{-4} M, amiloride inhibited more than 95% of the basal SCC. From 10^{-6} to 10^{-3} M, the analogue LT2 stimulated the SCC. In this table and in the remaining figures, *N* denotes the number of skins tested

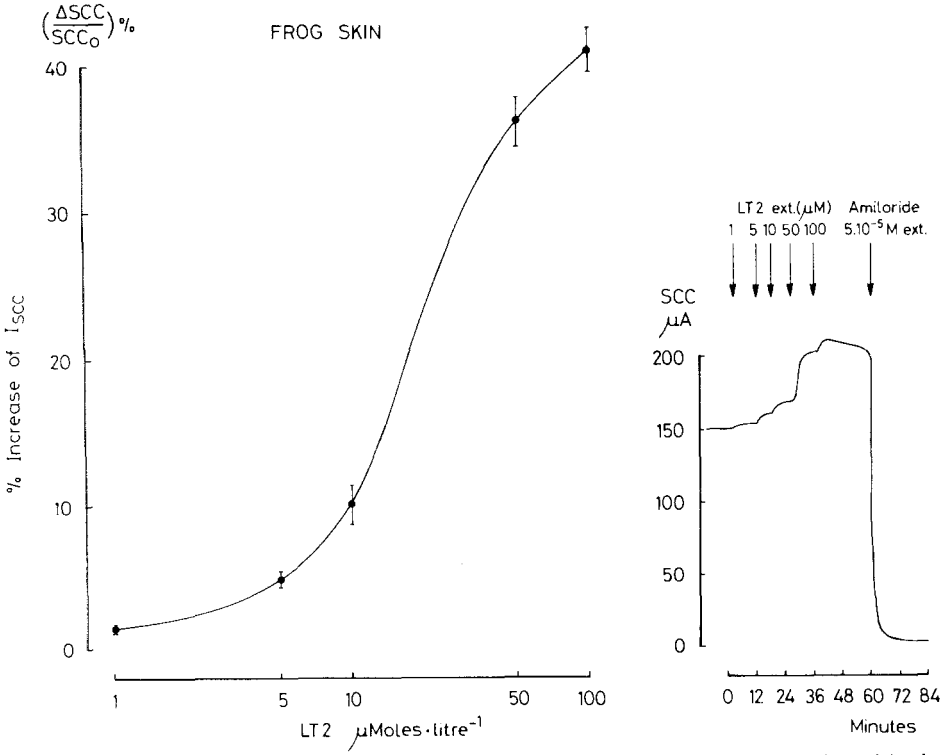


Fig. 3. Dose-response curve of LT2. The concentration of LT2 is plotted in a logarithmic scale; the response is expressed as percentage increase of the basal SCC value. At right, a typical dose-response experiment is shown; the curve at left was drawn from the average values of four such experiments. Note that both basal and LT2-induced SCC's were abolished by amiloride

This biological response was monotonically increasing with increasing concentration of LT2. In fact, up to 1 mM the action of LT2 remained stimulatory (records not shown). The rise of SCC had a rapid onset but was sustained and reversible; furthermore, it could be abolished by pretreatment of the skin with amiloride or ouabain.

Na^{22} Influx in the Presence of LT2

The quasi-equivalence between SCC and Na influx in frog skin has been well established, and a number of studies has demonstrated that the amiloride-sensitive SCC closely represents the net inward Na current. Since both the LT2-induced and the basal SCC were totally inhibited by amiloride (Fig. 3), we considered that, very likely, the increase in

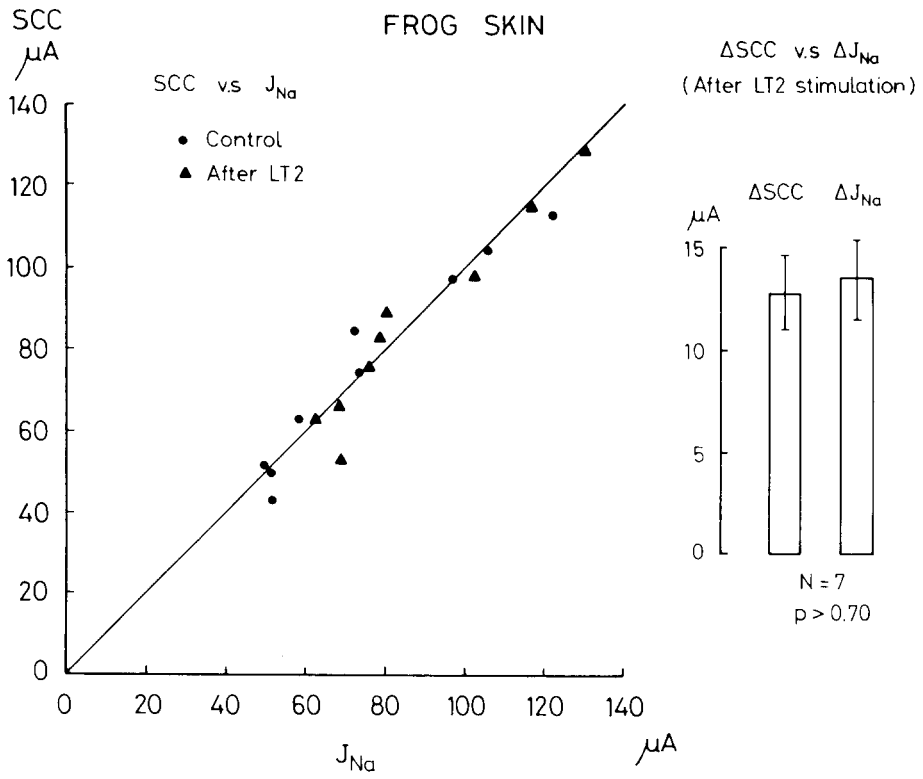


Fig. 4. Comparison of SCC and Na influx. Each point represents the values of SCC (ordinate) and the corresponding Na influx (abscissa) of the same preparation. Note that the points identifying measurements under control condition (●) and after LT2 stimulation (▲) fall on the line of unity slope. The results of the statistical analysis are summarized at the right

SCC induced by LT2 reflected also a similar increase in the net Na inward current. The analogue LT2, however, presented a unique peculiarity: despite being so similar to amiloride from the structural viewpoint, its biological effects were just opposite to those of the parent species. Consequently, it appeared essential to establish that SCC, in the presence of LT2, remained indeed a reliable measure of the Na current.

During the measurement of Na^{22} influx the skins were kept short circuited. The results are summarized in Fig. 4. Na influx, $\bar{J}_{Na}$, was plotted against SCC under two experimental conditions: control and after exposure to LT2. In both instances, Na influx and SCC showed little difference, as all the experimental points fell closely on the line of unity slope. Besides, when the increases in SCC and in Na influx were compared, the two averages were not statistically different. It could thus be concluded that the LT2-induced stimulation of SCC was closely correlated with a concomitant increase in Na influx; in addition, the passive efflux of Na in this preparation appeared insignificant, as reported previously (Li & de Sousa, 1977*c*).

Interaction of LT2 with Oxytocin, Propranolol and La^{3+}

The stimulatory action of LT2 on SCC was further investigated by examining the interaction of its biological effect with that of three other natriferic agents: oxytocin, propranolol, and La^{3+}. Figure 5 shows a typical example and the statistical analysis of "cross-experiments" performed with LT2 and oxytocin. Such protocol allowed for the comparison of the response of the skin to LT2 in two adjacent segments of the same skin, one under basal conditions, the other pre-exposed to a supramaximal concentration of oxytocin. Likewise, the effects of oxytocin could be compared in a tissue pre-exposed to LT2 and under basal conditions. The most salient feature of these experiments was the finding that SCC remained sensitive to LT2 stimulation despite a maximal stimulation by oxytocin. Comparison of ΔSCC values of LT2 before and after exposure to oxytocin yielded no significant difference. On the other hand, the average ΔSCC values induced by oxytocin appeared slightly enhanced in the presence of LT2 (Fig. 5).

The effect of LT2 in the presence of supramaximal concentrations of oxytocin has been observed with a series of "external" agents studied in this laboratory, namely, propranolol and La^{3+} (de Sousa, 1975; Marguerat, 1975). Consequently, it seemed to be of interest to examine the

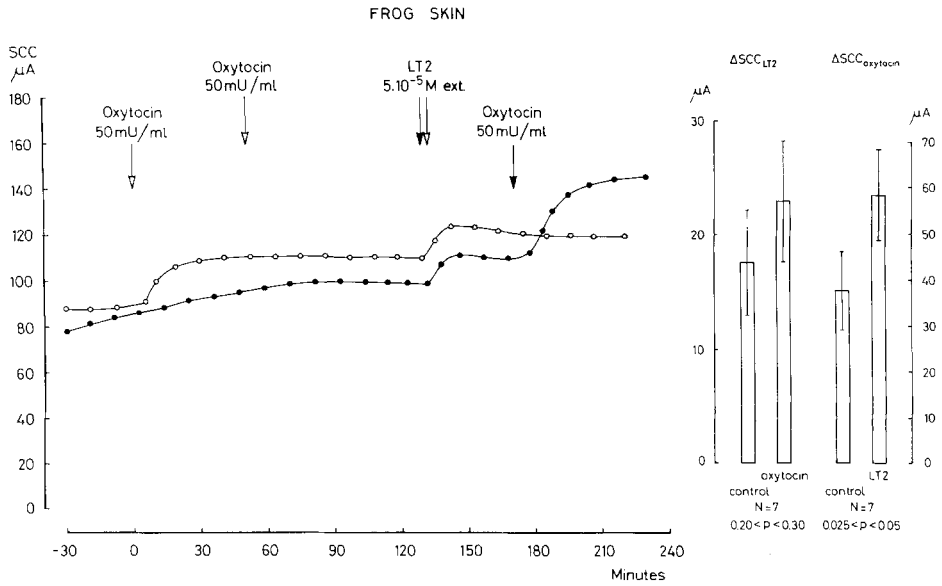


Fig. 5. A typical experiment and statistical results of the interaction between LT2 and oxytocin. Note that the segment having been exposed to a supramaximal concentration of oxytocin [o–o–o] remained sensitive to LT2 stimulation

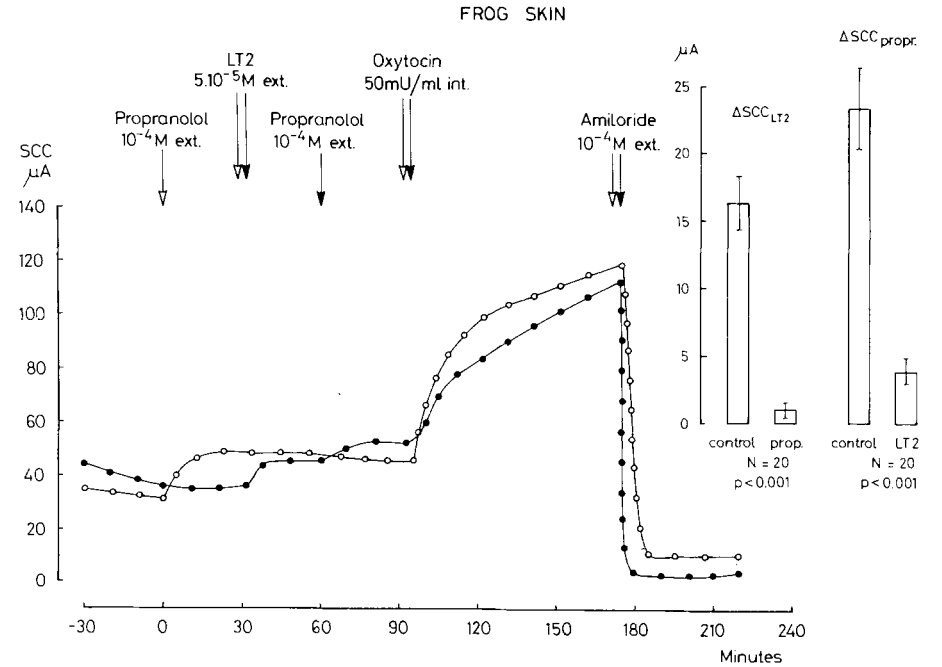


Fig. 6. A typical experiment and statistical results of the interaction between LT2 and propranolol. Note the absence of response to LT2 in the presence of propranolol [o–o–o], the reduction in the response to propranolol in the presence of LT2 [●–●–●], and the persisting actions of oxytocin and amiloride in the presence of both LT2 and propranolol

interactions between LT2 and propranolol and also between LT2 and La^{3+}.

Figure 6 shows the results obtained with propranolol. In 15 out of 20 paired skins tested, the response to LT2 was completely absent in tissues pre-exposed to propranolol; in the remaining 5, the effect was greatly reduced. Likewise, the response to propranolol was also markedly reduced after preexposure to LT2. Statistical analysis of the data is summarized on the right-hand side of Fig. 6 and suggests mutual inhibition.

Finally, the interaction between the effects of LT2 and La^{3+} exhibited a pattern similar to that observed between LT2 and propranolol. As shown in Fig. 7, the stimulatory action of LT2 was greatly reduced in the presence of La^{3+} and *mutatis mutandis* for La^{3+} after pre-exposure to LT2. In 6 out of 11 paired studies, SCC increased with La^{3+} but actually decreased upon the subsequent addition of LT2. However, since the reduction of SCC after addition of LT2 was never greater than

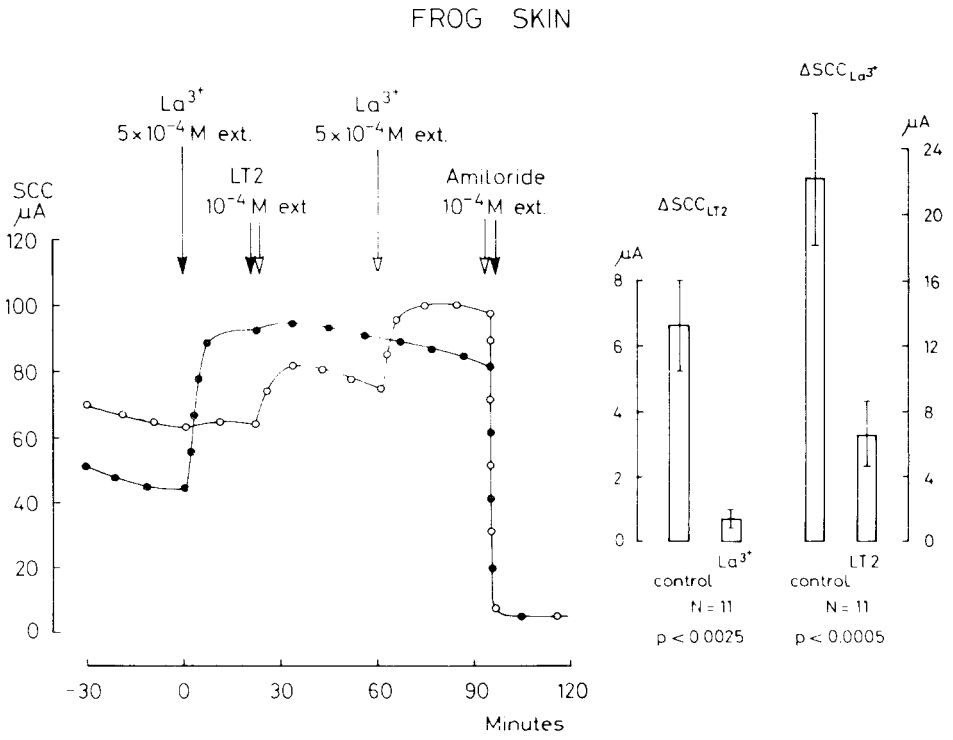


Fig. 7. A typical experiment and statistical results of the interaction between LT2 and La^{3+}. Note the mututal inhibition of the stimulatory effects of LT2 and La^{3+} and the persisting action of amiloride

the increase in SCC previously induced by La^{3+}, the response to LT2 was taken as zero in computing the statistics.

Discussion

Despite the plethora of studies on the effects of amiloride on a wide variety of epithelia (Cuthbert, 1974; de Sousa, 1975), little is known of the nature of the molecular interaction between this drug and biomembranes. The bulk of available data suggests that amiloride specifically and reversibly blocks the Na-entry sites located on the apical (or external) portion of the plasma membrane of the epithelial cells. Consequently, the study of structural analogues of this diuretic was, *a priori,* of potential interest to get further insights into the apical process of Na permeation and the mode of action of amiloride.

The effects on Na transport of a small but comparable series of amiloride analogues has been studied on dog and rat kidney (Cragoe *et al.*, 1967), on a model phospholipid membrane (Singer, 1974), and on the isolated skin of the frog *Rana pipiens* (Benos *et al.*, 1976). The work presented herein, conducted on the skin of another frog species (*R. ridibunda*) agrees in general and extends the observations of Benos *et al.* (1976), with particular relevance for the hitherto unreported natriferic action of the analogue LT2.

The primary structure of the amiloride molecule shows a substituted pyrazine ring and a guanidine side group attached to ring position 2. An interesting pattern of structure-activity relationships of amiloride analogues emerges from our results and those of Benos *et al.* (1976). The elongation of the guanidine side chain, by insertion of an additional $-NH$ group, affects only slightly the potency of the molecule to inhibit the SCC of the skin. However, the same type of elongation appears to reduce the loss of potency caused by the replacement of the amino group of ring position 5 with $-H$. Both the $-NH_2$ group at positions 5 and Cl at position 6 play a key role in the activity of amiloride. When either of these two groups of the parent molecule is replaced with $-H$, the subsequent loss in potency is about the same (50%). When both groups are replaced with $-H$, the resulting compound has hardly any inhibitory action on SCC.

This pattern, so far, is similar to the observations of Benos *et al.* (1976); a conspicuous difference was found, however, with analogue LT2. At 10^{-6} M, this compound was reported by these authors as having

a slight inhibitory effect on SCC; in contradistinction, at the same concentration we noticed a stimulation of SCC in our preparation.

Structure-function analysis of amiloride and analogue LT2 showed again the key role of the $-NH_2$ group attached to position 5 of the pyrazine ring of amiloride. Dimethylation of this group resulted in compound LT2, the effects of which on Na transport across the skin were opposite to those of amiloride. In view of the striking natriferic properties of LT2, a more detailed study of this analogue was undertaken in the hope of gaining some insight into its mode of action. Two approaches were taken: (i) use of higher concentrations of LT2; (ii) study of the interaction of the effects of LT2 with those of three reference substances: oxytocin, propranolol, and La^{3+}.

It has been reported that inhibitors of (Na+K)-ATPase such as ouabain (McClane, 1965) and harmaline (de Sousa & Grosso, 1978) can stimulate SCC at low concentrations. To determine if a biphasic effect on SCC could also be seen with LT2, skins were exposed to concentrations of the analogue up to 10^{-3} M. In every instance, a stimulation of SCC was observed; besides, a sigmoidal dose-response curve was obtained up to 10^{-4} M (Fig. 3).

The increase in SCC induced by LT2 had a fast onset, was sustained, reversible, amiloride-sensitive, and equivalent to the concomitant increment in Na influx (Figs. 3 and 4). These features were reminiscent of those reported by this laboratory with a series of substances acting on the external surface of frog skin, namely, diphenylhydantoin (DPH), lanthanides, propranolol, and psychotropic drugs (de Sousa, 1975; de Sousa & Grosso, 1973, 1978; Grosso & de Sousa, 1978). Two other important characteristics common to all these agents are: (i) the capacity to stimulate further SCC in skins pre-exposed to supramaximal concentrations of oxytocin; (ii) mutual inhibition among themselves.

With this background in mind we studied first the interaction between the effects of oxytocin and those of LT2. As shown in Fig. 5, pre-exposure of the skin to oxytocin did not modify statistically the subsequent stimulation of SCC by LT2. Thus, it appears that the two natriferic effects are additive, but a more complex interaction cannot be ruled out. The most distinct feature, however, is the possibility of increasing SCC with analogue LT2, after maximal stimulation of the skin by oxytocin.

Mutual inhibition was clearly found between LT2 and propranolol (Fig. 6) and between LT2 and lanthanum (Fig. 7). Taken together, these observations, as well as those made with oxytocin, suggest very strongly that analogue LT2 shares the same properties of the other "external"

agents previously described and possibly competes for the same Na-entry sites on the external membrane.

The nature of the putative Na-entry sites activated by LT2 and by other "external" agents remains speculative. As previously suggested (de Sousa, 1975; de Sousa & Grosso, 1973), they appear to be distinct from the sites activated by cyclic AMP but closely resemble the "Ca sites" described by Curran's group (Curran & Gill, 1962; Curran, Herrera & Flanigan, 1963; Herrera & Curran, 1963). Consistent with this view is the finding that DPH, La^{3+}, and propranolol all interact with membrane Ca^{++} in a variety of cell systems (Sohn & Ferrendelli, 1973; Weiss, 1974; Porzig, 1975). Moreover, direct evidence for an interaction between Ca^{++} and DPH at the external surface of frog skin was provided by Riddle, Mandel and Goldner (1975).

It should be noted that Zeiske and Lindemann (1974) and Garcia-Romeu (1974) reported stimulatory effects on SCC by another analogue of amiloride, benzoylimidazole-2-guanidine. From the structural viewpoint, however, that compound is not as similar to amiloride as is analogue LT2. On the other hand, amiloride itself has been shown to enhance Na permeability of the gills of *Anguilla anguilla* (Cuthbert & Maetz, 1972). These observations and the results reported here suggest two final comments. First, slight modifications of the amiloride molecule not only bring about significant changes in the potency of the inhibitory effect on Na transport, but may even result in a switch from inhibitory to stimulatory action. Second, the same molecule (e.g., amiloride) may induce opposite effects on Na transport in different epithelia, a phenomenon likely due to differences in the structure of the apical membrane of various epithelial cells.

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