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A New Type of Sodium Transport Inhibitor in the Toad Bladder

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Summary. A new synthetic antimicrobial agent, CM-55, that inhibits lipid synthesis was found to inhibit the transepithelial transport of sodium in the isolated toad urinary bladder and to inhibit the response of this tissue to vasopressin. During the first 40 to 60 min after CM-55 addition, spontaneous transepithelial potential fell and resistance increased without significant change in urea or thiourea flux, bulk water flow or the hydroosmotic response to vasopressin. After 60 min, transepithelial potential and membrane resistance both fell to virtually zero; there was a marked increase in the flux of urea, thiourea and ^{22}Na, and the hydroosmotic response to vasopressin was inhibited. Tissue ATP concentration and the rate of protein synthesis were not altered, but fatty acid synthesis was reduced by 43 per cent. Thus, this agent has a biphasic effect upon this tissue: an initial inhibition of transcellular Na^+ transport and a later increase in the permeability of the epithelial cells. The time course of the later effect coupled with the significant reduction in fatty acid synthesis produced by CM-55 raises the possibility that cellular permeability properties depend upon continual turnover of lipid components.

The transepithelial transport of sodium across the amphibian urinary bladder is stimulated by the steroid hormone, aldosterone [5, 7, 17]. Earlier work on the mode of action of this hormone led to the postulate that it acted by controlling gene expression in this tissue [8, 18]. However, recent studies in this laboratory have shown that aldosterone causes a change in the fatty acid composition of membrane phospholipids [12]. These results raised the possibility that alterations in lipid metabolism might be one of the bases for the action of this hormone. To investigate this possibility an analysis of the effects of various inhibitors of fatty acid and lipid metabolism

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has been undertaken. One of the compounds investigated was a new synthetic antimicrobial agent, CM-55, which has been shown to be an inhibitor of lipid synthesis in yeast.[1]

The present report describes some unexpected findings during these studies, which illustrate that this agent has a complex effect upon Na^+ transport. It suppresses transepithelial Na^+ transport but increases, after a delay of 40 to 60 min, the flux of urea, thiourea and sodium. These results suggest that this agent may be opening a "shunt" pathway for small molecules between epithelial cells on this tissue.

Materials and Methods

Female toads (*Bufo marinus*) were purchased from National Reagents Company, Bridgeport, Connecticut, and kept on moist Sani-cel bedding at 22 to 24 °C. The animals were placed in 0.6% NaCl for 6 hr to reduce endogenous aldosterone secretion, then pithed and the two hemi-bladders removed and incubated overnight at room temperature in substrate-free well-aerated Ling-Ringer's phosphate buffer [16] that contained 50 μg/ml of both penicillin G and streptomycin sulfate. The tissue was then employed for studies of short-circuit current, urea flux, thiourea flux, inulin flux or bulk H_2O flow.

For metabolic studies hemi-bladders from six to ten toads were cut into approximately four to six equal pieces and distributed into flasks containing Ling-Ringer's phosphate buffer with 4 mM glucose. The tissues were preincubated with gentle shaking for 2 hr at room temperature and then additions were made as indicated in the ensuing figure legends.

Since CM-55 is unstable in solution when stored for longer than 12 hr, it was freshly dissolved in ethanol for each set of experiments. Arginine vasopression (AVP) was used as the aqueous solution, Pitressin (Parke Davis). Incubations were stopped by immersing tissue in liquid N_2. Frozen tissue was powdered in a chilled mortar and the powder employed for analysis. A neutralized 5% perchloric acid extract was used to measure ATP content by a standard enzymatic-fluorometric method [22]. The incorporation of [^{14}C] leucine into tissue protein was determined on a 5% TCA extract of the tissue. The residue from the TCA extract was dissolved in 1 N NaOH. An aliquot of this solution was either analyzed for protein content by the Biuret reaction [13] or was employed for liquid scintillation counting. Tissue lipid was extracted and quantitated as has been described [9]. The weight of the lipid in the extract was determined by weighing dried aliquots of the extract. The lipid extract was saponified and extracted by the method of Hanahan and Wakil [14]. Fatty acids were assayed colorimetrically [15].

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1 CM-55 is a new synthetic antimicrobial agent which has the following chemical structure:

$$CH_3(CH_2)_7-\underset{\underset{O}{\|}}{C}-CH=CH-\overset{\overset{O}{\|}}{C}-N(CH_3)_2.$$

The chemical and biochemical characteristics of CM-55 will be reported in a separate paper, which will describe the inhibitory effects of CM-55 upon lipid synthesis in yeast.

For electrophysiological experiments, hemi-bladders were mounted in an Ussing-type voltage clamp, as described by Sharp and Leaf [17], in which each hemi-bladder is clamped in a double-chamber allowing independent measurement of short-circuit current (SCC) in the two halves of each hemi-bladder. Ling-Ringer's phosphate buffer containing 4 mM glucose was added to both mucosal and serosal sides of the tissue and SCC recorded continuously. Electrical resistance of the bladder was measured manually at 5- to 20-min intervals by imposing a 10-mV potential across the voltage-clamped tissue for 9 to 10 sec. The difference in SCC originating from this connection was used as the denominator for the calculation of the resistance. Potential differences across the bladder were recorded continuously under open-circuit conditions.

Osmotic flow of water was determined by the method of Bentley [1]. Urea, thiourea, inulin and bidirectional ^{22}Na fluxes were determined as follows: To an appropriate side of the bladder mounted in the double-chamber, [^{14}C] urea, [^{14}C] thiourea or [^{14}C] inulin with carrier or 0.57 μC of carrier-free [^{22}Na] was added after a 2-hr preincubation; at 20-min intervals the mucosal or serosal fluid was removed and replaced with fresh buffer. An aliquot of the removed fluid was used for [^{14}C] or [^{22}Na] determination.

Results

Biochemical Effects of CM-55

The effect of CM-55 on three different biochemical parameters of the toad bladder are summarized in Table 1. A dose of 10 μg/ml (4.18×10^{-5} M)

Table 1. Effects of CM-55 on ATP concentration, rate of protein synthesis and rate of lipid synthesis in toad urinary bladder

	Control	CM-55
ATP (nmoles/mg protein)	3.61 ± 0.06 (4)[a]	3.55 ± 0.12 (4)
^{14}C-Leucine incorporation (cpm/mg protein)	721 ± 26 (4)	731 ± 33 (4)
^{14}C-Acetate incorporation		
(*a*) into total lipid (cpm/mg lipid)	351 ± 19 (5)	282 ± 24 (5)[b]
(*b*) into fatty acid (cpm/mequiv palmitic acid)	2712 ± 240 (4)	1547 ± 206 (4)[c]

[a] Number of flasks
[b] $p < 0.05$
[c] $p < 0.01$

Hemi-bladders of six to ten toads were cut into four to six equal pieces and distributed into appropriate flasks. After a 2-hr preincubation in Ling-Ringer's phosphate buffer containing 4 mM glucose, CM-55 in ethanol, or ethanol alone, was added and the tissues incubated for 3 hr. At this point, 1-^{14}C-leucine, or 1,2-^{14}C-acetate with respective carrier, was added to give a final concentration of 0.1 μC/ml and 0.1 mM of carrier substrate. Tissues were incubated a further 30 min for ^{14}C-leucine incorporation or 1 hr for ^{14}C-acetate incorporation.

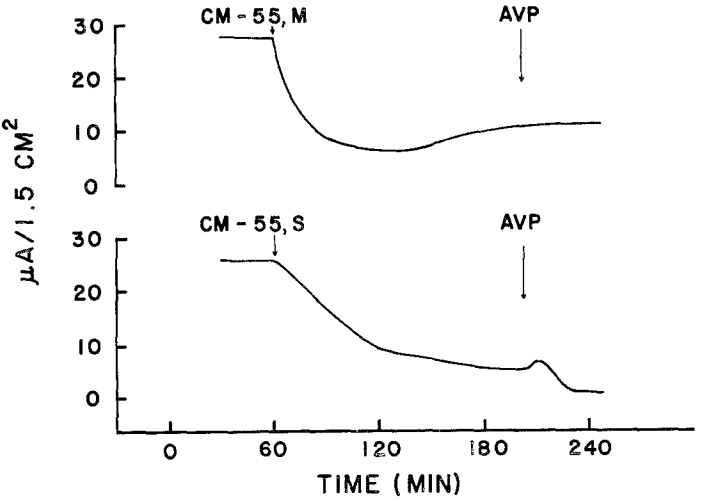

Fig. 1. Effect of CM-55 on short-circuit current. CM-55 was added either to the mucosal (*M*) (upper trace) or serosal (*S*) (lower trace) side of the toad bladder to a final concentration of 10 μg/ml (4.18×10^{-5} M). Vasopressin (AVP) was added to the serosal side to a final concentration of 80 mU/ml. This response is typical of the response seen in six separate experiments

was chosen since this concentration was the minimal dose necessary to induce a maximal effect on SCC (*see below*). As shown in Table 1, neither the ATP concentration nor the rate of protein synthesis was reduced after a 3-hr incubation with CM-55. However, both total lipid synthesis and fatty acid synthesis, examined by [^{14}C] acetate incorporation, were inhibited by 20% and 43%, respectively.

Effects upon Short-Circuit Current

On the basis of these results the effect of CM-55 on SCC was studied. CM-55 induced a rapid decrease in SCC as shown in Fig. 1. When CM-55 was added to the mucosal side of the bladder a more rapid decrease of SCC was observed than when CM-55 was added to the serosal side (Fig. 1). This suggested that the principal site of action of CM-55 was localized to the mucosal surface of the epithelial cells. Vasopressin did not increase SCC in the bladder previously treated with CM-55 on its mucosal side though a very small but consistent response to vasopressin was seen when CM-55 had been added to the serosal side (Fig. 1).

Effect of CM-55 upon Potential Difference and Resistance

As shown in Fig. 2, the addition of CM-55 to the mucosal medium led to a prompt fall in the potential difference (p.d.) across the tissue (Fig. 2*a*)

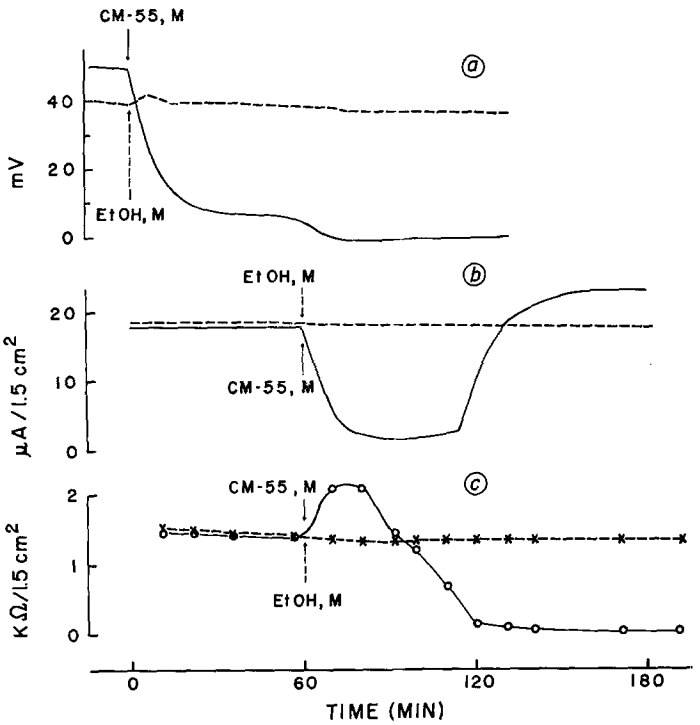

Fig. 2. Effects of CM-55 upon the (*a*) potential difference, (*b*) short-circuit current, and (*c*) resistance across the toad urinary bladder as a function of time. CM-55 (10 μg/ml) or ethanol was added at the indicated time. This experiment is representative of four similar experiments

associated with a fall in SCC (Fig. 2*b*) and a rise in resistance (Fig. 2*c*). Both p.d. and SCC remained low, but after the initial rise in resistance which lasted approximately 15 min, electrical resistance fell progressively to zero. As both resistance and potential difference fell to zero the SCC rose, but this rise was found to be an artifact of the electrical system for monitoring SCC and not indicative of a real increase in net transcellular Na^+ transport.

To test the possible reversibility of the effects of CM-55 measurements of resistance, potential difference and short-circuit current were carried out in a series of bladders to which 10 μg/ml of CM-55 was added to the mucosal medium for a period of 5 min and then its removal attempted by repeated changes of the bathing solution. Under these conditions CM-55 exerted the same time-dependent changes in the electrical properties of the tissue as bladders continually exposed to CM-55 (data not shown).

Effects of CM-55 upon Bulk H_2O Flow, Urea, Thiourea, Inulin and Bidirectional Na^+ Flux

To further characterize the effects of CM-55 upon transepithelial transport, osmotic water flow, urea, thiourea and inulin flux were examined across the bladders. As shown in Fig. 3 the hydroosmotic response to vasopressin was not altered by a 30-min exposure to CM-55 before hormone addition. After a 2-hr exposure to CM-55, however, there was a profound inhibition of the response to AVP.

When the mucosal-to-serosal fluxes of [^{14}C] urea and [^{14}C] thiourea were measured without an osmotic gradient (Fig. 4*a*, *b*) a dramatic increase was observed beginning 40 min after the addition of CM-55. This latent period corresponded to the time for complete loss of resistance and potential difference seen after CM-55 treatment (Fig. 2). Although the bladder is not normally permeable to thiourea the flux of thiourea was increased at the same time after CM-55 addition as an increase in urea flux was seen (Fig. 4*b*).

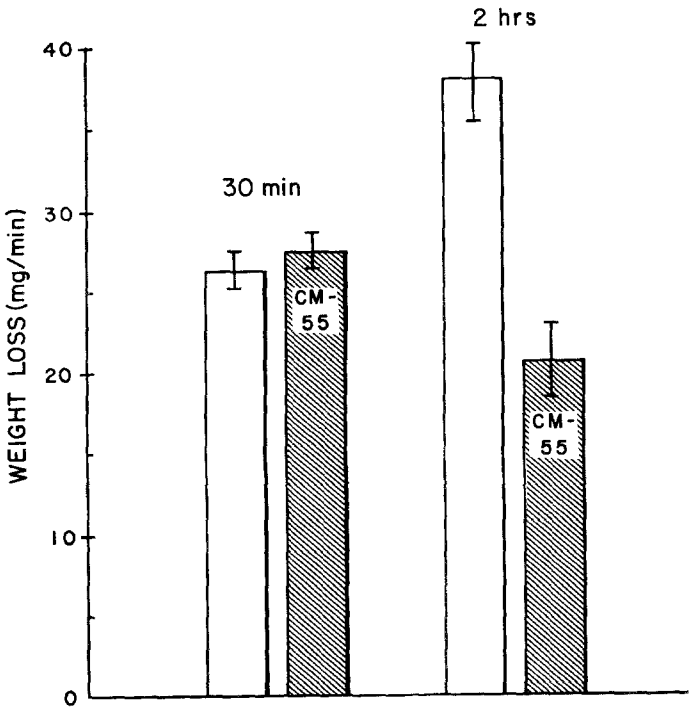

Fig. 3. The effect of CM-55 on the hydroosmotic response to vasopressin. Six pairs of bladders were incubated with 10 μg/ml of CM-55 for 30 min or 2 hr. Fresh mucosal bathing solution (one-fifth diluted Ling-Ringer's phosphate buffer) was added just before vasopressin (80 mU/ml) was added. The vertical bars express the standard error of the mean

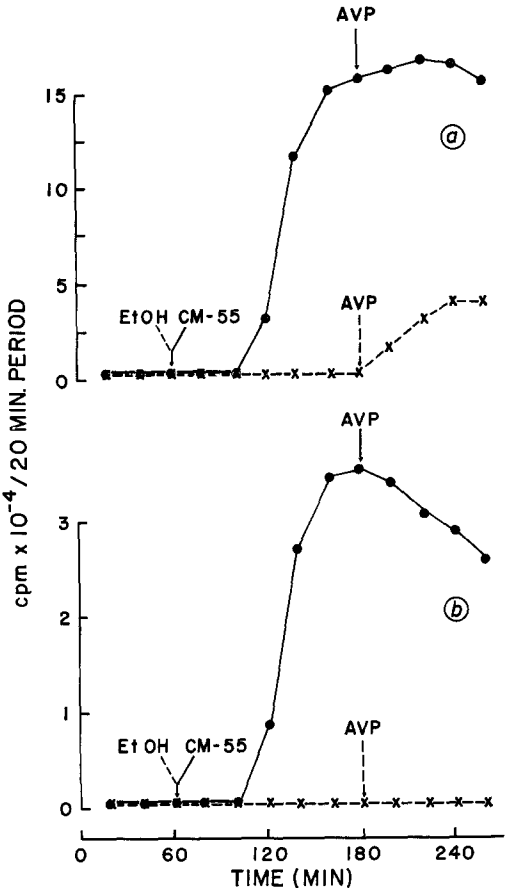

Fig. 4. The time course of the change in urea (*a*) and thiourea (*b*) fluxes after the addition of CM-55 (•—•) or ethanol (×---×) and the subsequent addition of vasopressin (AVP)

The fact that both urea and thiourea flux increased so dramatically, plus the fact that the electrical resistance and potential difference across the tissue both fell, led us to consider the possibility that CM-55 treatment led to an increase in solute transport between the epithelial cells. To gain further support for this concept the effect of CM-55 upon the bidirectional fluxes of ^{22}Na was examined. As shown in Fig. 5, approximately 1 hr after CM-55 addition there was a marked and progressive increase in the bidirectional flux of sodium across this tissue. This increase in tissue permeability to low molecular weight solutes was not, however, associated with increased [^{14}C]inulin permeability after up to a 3-hr exposure to CM-55 (data not shown).

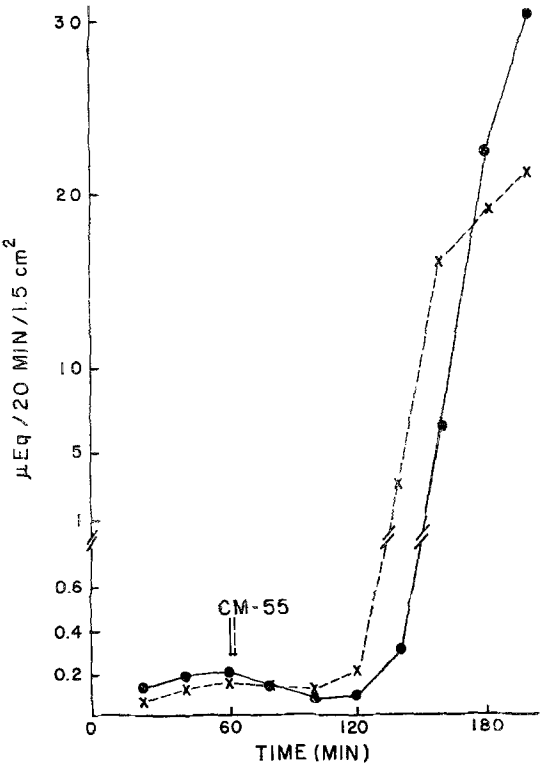

Fig. 5. Effect of CM-55 on the bidirectional flux of sodium across the toad urinary bladder. At the indicated time CM-55 was added to the mucosal bathing solution of one side of the chamber. ^{22}Na was present in either the mucosal or serosal bathing solution from the beginning of the incubation

Discussion

From the present results it seems clear that CM-55 has two sequential effects upon the transport properties of this tissue: an initial inhibition of Na^+ entry across the mucosal face of the epithelial cells and a later increase in the flux of ions and urea by an extra- or intracellular pathway or shunt.

The fact that the effect of CM-55 upon net Na^+ transport (SCC) was more rapid and profound when CM-55 was added to the mucosal compared to serosal medium, that trans-tissue electrical resistance rose initially and p.d. fell (Figs. 1 and 2) all argue that CM-55 had a direct and immediate effect upon the entry of Na^+ across the mucosal face of the epithelial cells forming the transporting layer in this tissue. The fact that ATP concentrations and rates of protein synthesis were not altered even after prolonged

exposure to CM-55 (Table 1) argues that CM-55 was not acting as a general metabolic poison.

The second phase of the response to CM-55 is characterized by a marked fall in electrical resistance, p.d. and SCC with a concomitant rise in bi-directional ^{22}Na fluxes and urea and thiourea fluxes (Figs. 4 and 5). These changes, coupled with the fact that ATP concentrations and the rate of protein synthesis were well maintained (Table 1), are consistent with an increase in the rate of solute transport via a shunt pathway which might operate between the epithelial cells.

The changes in electrical properties of this tissue after prolonged exposure to CM-55 are quite similar to those observed when the solute concentration of the mucosal medium is increased by the addition of sucrose, urea or mannitol [2, 10, 19, 20]. To account for this phenomenon Ussing and Windhager [21] have proposed the existence of a shunt pathway whose ion conductivities are altered by changes in the tonicity of the solution bathing the mucosal cell surface. Frömter and Diamond [11] have proposed that the conductivity of this shunt, or extracellular pathway, is determined by the properties of the "tight, or limiting, junctions" between the epithelial cells, and DiBona [6] has shown by electron-microscopy that in the toad bladder there is an inverse relationship between electrical resistance and deformed tight junctions with widened inter-membrane spaces. In addition, Civan, Kedem and Leaf [4], and Bentley [3] have presented evidence that heterogeneous pathways for sodium transport exist in this tissue.

In light of these observations the present data suggest that after prolonged exposure to CM-55 the permeability of the tight junctions linking the epithelial cells and/or the apical plasma membranes in the toad bladder is increased, leading to an increase in water and low molecular weight solute flux. However, this altered tissue permeability is not associated with increased inulin permeability or any morphological alteration at the level of resolution of the electron-microscope of either the apical plasma membrane or the tight junctions.

The permeability change is preceded by and associated with a significant inhibition of fatty acid synthesis. This association raises the possibility that alterations in lipid synthesis may be coupled to changes in the permeability properties of the cell membranes. If so, CM-55 is a new tool with which to explore this relationship.

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