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LOCALIZATION OF SODIUM PUMP SITES IN FROG URINARY BLADDER

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SUMMARY

[^{3}H]Ouabain binding in frog and toad urinary bladder was investigated by short-circuit current (SCC), scintillation counting and autoradiographic techniques. SCC data and analysis of tissue digests following serosal exposure to ouabain showed that ouabain binding and inhibition of Na^+ transport was completely reversible in toad bladder whereas, in frog bladder, [^{3}H]ouabain was tightly bound and Na^+ transport remained suppressed even after a 60-min washout. Mucosal exposure of frog bladder to [^{3}H]ouabain or serosal exposure after preincubation with unlabeled ouabain led to a marked reduction in binding. Specificity of binding was assessed further by adjusting the concentration of certain (Na^+–K^+)-ATPase ligands (K^+, ATP) to levels known to reduce ouabain binding. High K^+ concentrations and depletion of endogenous ATP by incubation under anoxic conditions resulted in a significant drop in [^{3}H]ouabain binding. Autoradiographic analysis showed that grains are localized primarily to the basolateral plasma membranes of the granular cells, providing direct morphological evidence for the location of Na^+ pumps at these sites. Although autoradiographs did not provide sufficient resolution to rule out unequivocally ouabain binding to the mitochondria-rich cell, morphological evidence suggests that grain densities are significantly higher between adjacent granular cells than between granular cell–mitochondria-rich cell interfaces.

INTRODUCTION

The amphibian urinary bladder is employed extensively in the study of Na^+ transport. Evidence gathered from short-circuit current (SCC) [1] and microelectrode [2] studies support the hypothesis that the Na^+ pump is located at the basolateral plasma membrane of the mucosal epithelial cells. However, since the mucosal epithelium is composed of more than one cell type [3, 4], the specific cell type(s) responsible for transepithelial Na^+ transport is not resolved. This problem could be approached by the use of a technique, developed by Stirling [5], for the autoradiographic localization of [^{3}H]ouabain. As is the case with other transport tissues, ouabain depresses

Abbreviation: SCC, short-circuit current.

Na^+ transport across the amphibian urinary bladder [6] and also specifically inhibits (Na^+–K^+)-ATPase [6], an enzyme thought to be intimately associated with, if not identical to, the Na^+ pump [7]. Therefore, high resolution autoradiographic localization of [^{3}H]ouabain binding sites would provide morphological evidence for the actual subcellular site of the Na^+ pump as well as a method for investigating the involvement of the various cell types in transepithelial Na^+ transport.

Since most of the research on urinary bladder Na^+ transport employs the toad bladder, this species would seem to be the logical choice for [^{3}H]ouabain binding studies. However, the success of such a study depends on showing that membrane-specific ouabain binding is essentially irreversible, since unbound label must be washed from the tissue. In this regard, a recent study [8] indicates that inhibition of Na^+ transport by ouabain in toad bladder, but not in frog bladder, is reversed by washing. This suggests that ouabain binding in the latter may be irreversible and therefore amenable to autoradiographic analysis.

MATERIALS AND METHODS

Hemibladders from toads (*B. marinus*), or whole bladder from frogs (*R. catesbeiana*), were mounted in lucite chambers (1.8 cm^2) and SCC was monitored by an automatic voltage clamp [9]. The bladders were exposed on the mucosal and serosal sides to isotonic amphibian Ringer's solution [10]. After a steady-state SCC was reached, 5 μCi/ml of [^{3}H]ouabain was added to the serosal side of each chamber. After a 60-min incubation period, the chambers were drained and the tissues were washed with ouabain-free Ringer's solution at 20-min intervals for 1 h. Then the chambers were opened and the tissue rapidly cut out, blotted, weighed and dissolved in 1 ml of NCS (Amersham-Searle). 10 ml of Dimilume (Packard Instruments) were added to this digest prior to liquid scintillation counting.

For autoradiographic analysis of ouabain binding sites, bladders were exposed to [^{3}H]ouabain as described above. Following the 60-min washout, the bladders were rapidly frozen and then prepared for autoradiography following the method of Stirling [5]. Sections (1 μm) were coated with NTB-2 emulsion (Kodak), exposed for 45 days at 4 °C developed for 2.5 min with Dektol developer and stained with methylene blue.

Specificity of binding was assessed by adjusting the concentration of certain (Na^+–K^+)-ATPase ligands to levels known to suppress ouabain binding (high concentrations of serosal K^+, endogenous ATP depletion) and by serosal exposure to unlabeled ouabain (10^{-4} M) prior to washing and exposure to label.

RESULTS AND DISCUSSION

Preliminary binding experiments were designed to determine if the degree of reversibility of ouabain inhibition of Na^+ transport in toad and frog bladders correlates with the degree of reversibility of [^{3}H]ouabain binding to these tissues. As expected from data showing that large concentrations of ouabain (10^{-4} M) are required to depress Na^+ transport across the toad bladder by 50 % [6, 11], a 60-min exposure of this tissue to $4.4 \cdot 10^{-7}$ M ouabain had no effect on SCC (Table I). After the 60-min washout period only 0.008 pmoles of [^{3}H]ouabain were bound per mg wet

TABLE I

EXPOSURE OF FROG AND TOAD URINARY BLADDER TO OUABAIN: COMPARATIVE EFFECTS ON [^{3}H]OUABAIN BINDING AND SCC WITH VARIOUS CONDITIONS OF INCUBATION

[^{3}H]Ouabain binding is expressed in pmoles/mg wet wt. SCC is expressed as the fractional change after a 60-min exposure to ouabain (SCC at t = 60 min/SCC at t = 0 min). Numbers in parenthesis are the number of experiments performed. Standard errors are given for N > 2. Anoxia: N_2 continuously bubbled through incubation media. Anoxia → air: Anoxic bladder reexposed to air before addition of ouabain.

Condition	Frog		Toad	
	SCC	[^{3}H]Ouabain binding	SCC	[^{3}H]Ouabain binding
Serosal exposure	0.75 ± 0.02 (9)	0.154 ± 0.023 (9)	0.96 (2)	0.008 (2)
Mucosal exposure	1.06 ± 0.06 (6)	0.004 ± 0.001 (6)		
Serosal exposure: preincubation with 10^{-4} M unlabeled ouabain	0.0 (1)	0.004 (1)		
Serosal exposure: K^+-Ringer's solution		0.035 ± 0.011 (3)		
Serosal exposure: anoxia		0.061 (2)		
Serosal exposure: anoxia → air	0.71 (2)	0.150 (2)		

wt (Table I). In contrast, under the same conditions, SCC across the frog bladder was reduced by 25% and 0.154 pmoles of [^{3}H]ouabain was bound per mg wet wt (Table I). This figure was essentially the same after an additional 30-min washout, whereas the binding in the toad bladder was reduced to zero. In addition, ouabain binding in frog bladder was demonstrated in dual label experiments with [^{14}C]inulin at the same specific activity as [^{3}H]ouabain. At the end of the 60-min wash, the ouabain/inulin distribution space was 7 : 1. Therefore, [^{3}H]ouabain binding in the frog bladder is irreversible and correlates with the irreversibility of inhibition of SCC.

Autoradiographs of frog bladder exposed to [^{3}H]ouabain on the serosal side show a specific localization of grains to the basal and lateral plasma membranes of the cells comprising the more superficial portion of the epithelium (Fig. 1). This area of the mucosa is composed of two cell types [4]: granular cells and a few darkly staining mitochondria-rich cells. All of the granular cells are heavily labeled (Figs 1–3). Critical studies are now underway to determine if differential ouabain binding exists between these two cell types since preliminary examination of autoradiographs (Figs 2 and 3) indicates that a higher grain density is associated with the plasma membranes of adjacent granular cells than with the interface between granular cells and mitochondria-rich cells. Moreover, in favorable sections where the intercellular space between mitochondria-rich cells and granular cells is expanded, grains often appear to be associated preferentially with the granular cell plasmalemma (Fig. 3).

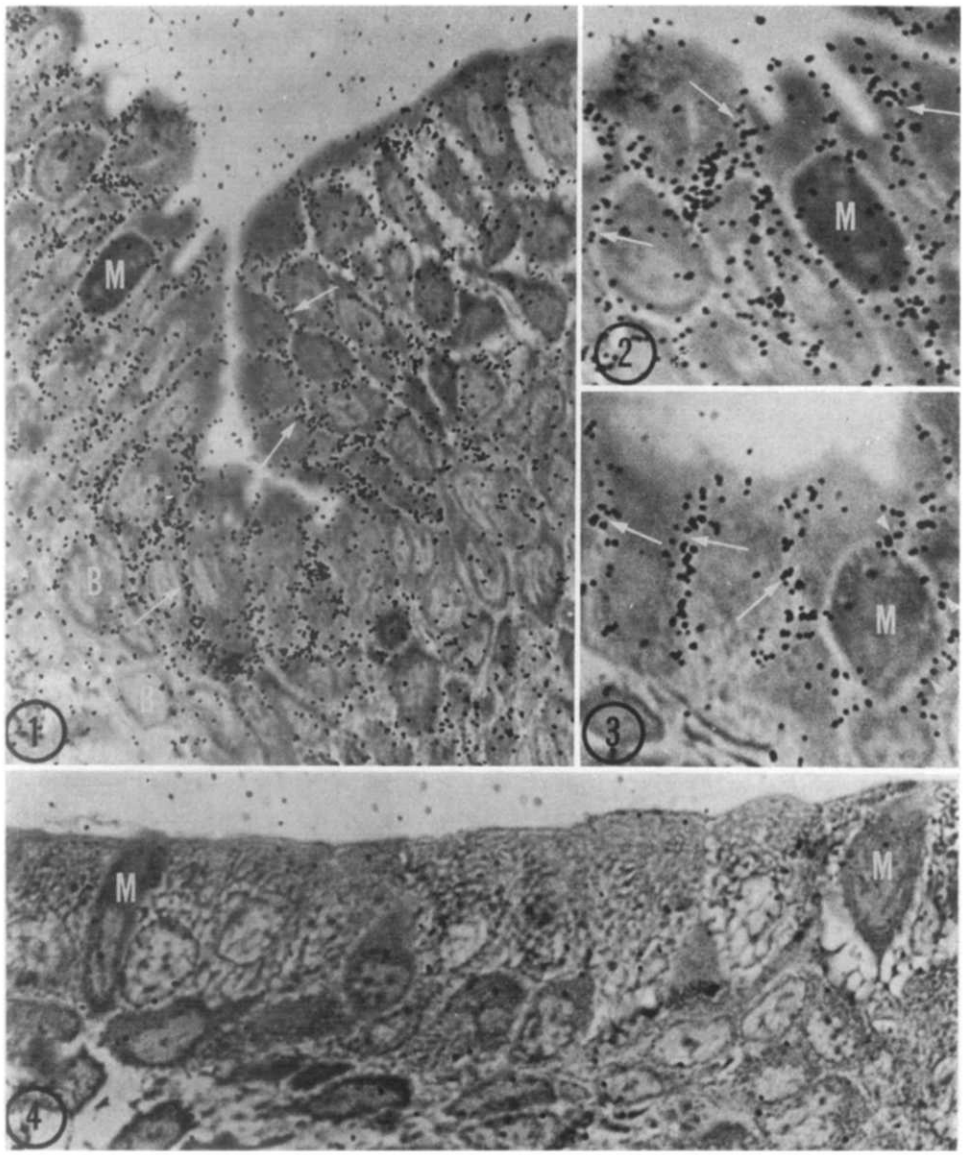

Fig. 1. Exposure of frog urinary bladder to [^{3}H]ouabain from the serosal side. Autoradiographic grains are localized predominantly to the basolateral plasma membranes (arrows) of adjacent granular cells. Basal cells (B) are only lightly labeled. Serosal connective tissue (not shown) showed a similar low grain density. A single darkly staining mitochondria-rich cell (M), is present at the upper left and is shown at higher magnification in Fig. 2. × 1120.

Fig. 2. High magnification of the mitochondria-rich cell (M) seen in Fig. 1. Interfaces between granular cells (arrows) appear more heavily labeled than those between the mitochondria-rich cell and the adjacent granular cells. The differential binding to granular cells is seen to even greater advantage in Fig. 3. × 1820.

Fig. 3. Serosal exposure to [^{3}H]ouabain. This high magnification of bladder mucosa shows the

In order to determine the specificity of ouabain binding to the mucosal cells, frog bladders were exposed to [^{3}H]ouabain from the mucosal side. As shown in Table I, SCC was unaffected and binding was minimal. Alternatively, when serosal exposure to [^{3}H] ouabain was preceded by incubation with 10^{-4} M unlabeled ouabain, SCC fell to zero and only 0.004 pmoles of [^{3}H]ouabain were bound per mg wet wt (Table I). Binding specificity was also tested by exposing the serosal side to [^{3}H]ouabain in a Ringer's solution in which all of the NaCl was replaced by KCl. High K^+ concentrations are known to reduce [^{3}H]ouabain binding [5, 10, 12] and (Na^+–K^+)-ATPase inhibition by ouabain [7]. Addition of K^+–Ringer's solution alone caused a precipitous drop in SCC which precluded measurements of this parameter during subsequent exposure to [^{3}H]ouabain. However, Table I shows that K^+–Ringer's solution effected a more than 75% decrease in ouabain binding. This reduction in binding was reflected by reduced grain densities in autoradiographs (Fig. 4).

Specificity of in situ ouabain binding was further assessed by investigating the effect of endogenous ATP depletion on binding efficiency, since ATP is required for maximal binding of ouabain to partially purified (Na^+–K^+)-ATPase preparations [12]. Depletion of ATP was accomplished by subjecting both control and experimental bladders to anoxic conditions by bubbling N_2 gas through the chambers. In toad urinary bladder, this procedure was shown to reduce the ATP concentration by 30% after a 40-min incubation [13]. After SCC declined to a steady-state value, air was bubbled into the control chamber. Following the increase in control SCC to a new steady state, the anoxic and reoxygenated bladders were exposed to serosal [^{3}H]ouabain for 60 min. The total time of exposure of the anoxic bladder to N_2 prior to the addition of [^{3}H]ouabain was 90 and 135 min, respectively, for the two experiments reported in Table I. No significant change in the already depressed level of SCC across the anoxic bladder was apparent following exposure to ouabain, whereas the fractional change in SCC across the reoxygenated bladder was 0.71 (Table I). Similarly, binding of [^{3}H]ouabain to the anoxic bladder was reduced to 40% of that bound to the reoxygenated control (Table I). Finally, the values for SCC and pmoles of ouabain bound for the reoxygenated bladders were the same as that measured for bladders never subjected to an anoxic period (Table I). Therefore, it seems reasonable to conclude that the reduction in these values under anoxic conditions is due to ATP depletion rather than to tissue damage.

The results reported here demonstrate that the frog urinary bladder, in contrast to the toad bladder, binds ouabain irreversibly. Furthermore, this binding is highly specific and is restricted to the basolateral cell membranes of mucosal epithelial cells, thereby providing direct morphological evidence for the presence of the Na^+ pump

heavy distribution of grains to the opposing lateral plasma membranes of adjacent granular cells (arrows). The interfaces between the mitochondria-rich cell (M) and the surrounding granular cells show only a few grains. Many of these grains appear to be associated with the plasmalemma of the granular cells (arrowheads) which lie adjacent to the mitochondria-rich cell. × 1820.

Fig. 4. This micrograph shows a portion of a bladder exposed to serosal [^{3}H]ouabain in a Ringer's solution in which all of the NaCl was replaced by KCl. The grain density is reduced to background levels. M, mitochondria-rich cell. × 1680.

at these sites. This localization in a non-mammalian reabsorptive epithelium supports the original findings of Stirling [5] in the rabbit small intestine and is directly contradictory to the mucosal localization reported by Cassidy [14] in hamster small intestine using a similar technique.

Grain distributions in the urinary mucosa indicate that the majority of the Na^+ pump sites are associated with the granular cell. This observation is in agreement with earlier reports suggesting that the granular cell is probably the cell type responsible for the transepithelial transport of Na^+ [15, 16]. However, other studies [17, 18] indicate that the mitochondria-rich cell may play a significant role in Na^+ transport. The autoradiographic data acquired from whole bladders, although highly suggestive of differential binding to granular cells, does not allow unequivocal determination of cell-specific binding sites when grains are seen in the intercellular spaces between two different cell types. Therefore, this problem may be solved by employing the cell separation technique developed by Scott et al. [18] in conjunction with autoradiography of [^{3}H]ouabain binding sites. Studies along this line are underway in our laboratory.

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