BBA 75 305

SLOW *CIS*-STIMULATION OF SODIUM TRANPORT ACROSS ISOLATED URINARY BLADDERS OF THE FRESH-WATER TURTLE, *PSEUDEMYS SCRIPTA*

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(Received January 29th, 1969)
(Revised manuscript received April 3rd, 1969)

SUMMARY

1. Isolated hemibladders of the fresh-water turtle, *Pseudemys scripta*, were bathed on both surfaces by an oxygenated Na^+-free choline–Ringer solution (17 mM HCO_3^-–Krebs buffer) and were maintained in a short-circuited state. Forward (mucosal to serosal) clearance of $^{22}Na^+$ was measured. Clearance (μl/h) was defined as the forward flux (nmoles/h) per unit of mucosal Na^+ concentration (mM). When serosal $[Na^+] < 0.5$ mM and mucosal $[Na^+] < 0.1$ mM, clearance progressively and spontaneously decreased at an average rate of 29%/h.

2. During the period of progressive decline, nonradioactive $^{23}Na^+$ was added only to the mucosal fluid to a final concentration ranging between 1 and 10 mM. Following this addition, clearance changed from a state of spontaneous decline to one of progressive increase. The average rate of increase was 37%/h.

3. The period of progressive increase (slow *cis*-stimulation) lasted an average of 2 h; afterwards clearance spontaneously began to decrease progressively.

4. On the average, the peak value of clearance was 2.2 times the value of clearance at the time $^{23}Na^+$ was added.

5. Even during the period of decline, the clearance did not return to the level at which it would have been if Na^+ had not been added to the mucosal fluid.

6. Washout of $^{22}Na^+$ from the tissue pool subsequent to the addition of mucosal $^{23}Na^+$ was eliminated as the explanation for the observed increase in clearance.

7. Concomitant changes in short-circuiting current independently confirmed the validity of the slow *cis*-stimulation but also suggested that the movement of ions other than Na^+ may have been affected by slow *cis*-stimulation.

Abbreviations: Φ (nmoles/h), forward (mucosal to serosal) flux of Na^+; C (μl/h), forward (mucosal to serosal) clearance of Na^+ = forward flux per unit of mucosal Na^+ concentration; G_t ($m\Omega^{-1}$) total membrane conductance; G_{Na^+} ($m\Omega^{-1}$), moiety of G_t assigned to transmembrane movement of Na^+; G_x ($m\Omega^{-1}$), stimulated moiety of G_t; I_{sc} (μA), short-circuiting current; I_{Na^+} (μA), moiety of I_{sc} assignable to transmembrane movement of Na^+; I_x (μA), stimulated moiety of I_{sc}; m, mucosa or mucosal; s, serosa or serosal; m → s, the forward direction, from mucosa to serosa; s → m, the back direction, from serosa to mucosa; PD (mV), transbladder potential difference.

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INTRODUCTION

Urinary bladders of *Pseudemys scripta* prepared *in vitro* have been shown to transport Na^+ (refs. 1–7) and Cl^- (refs. 3, 4, 8) actively from mucosa to serosa (m → s) against their respective electrochemical gradients and to acidify mucosal media by either removal of HCO_3^- (refs. 9, 10) or possibly by secretion of H^+ (refs. 6, 11).

A study of the m → s flux (Φ) and clearance (C) as a function of $[Na^+]_m$ was undertaken. At all times $[Na^+]_s$ was maintained at less than 0.5 mM to minimize accelerative exchange diffusion. At $[Na^+]_m$'s which were well below the transport K_m (15–25 mM by unpublished observations of H. R. WYSSBROD AND D. E. GENTILE), C increased when $[Na^+]_m$ was increased from less than 0.1 mM to between 1 and 10 mM; *i.e.*, a *cis*-stimulation of m → s Na^+ movement was observed. Since Michaelis–Menten kinetics predicts a *cis*-inhibition of C for all ranges of $[Na^+]_m$, the present study was undertaken to elucidate the more complex kinetics governing Na^+ movement at low $[Na^+]_m$'s.

MATERIALS AND METHODS

Solutions

Choline–Ringer solution. The serosal surface of each hemibladder was bathed at all times by a modified Krebs buffer system in which choline was substituted for Na^+. The solution had the following composition (in mM): 101 choline$^+$; 4.8 K^+, 2.0 Ca^{2+}, 0.8 Mg^{2+}, 92 Cl^-, 17 HCO_3^-, 0.07 $H_2PO_4^-$, 0.73 HPO_4^{-2}, 0.80 SO_4^{-2}, 0.33 CO_2, and 11 D-glucose. $[Na^+] < 0.05$ mM. Osmolality was 221 mosmoles/kg, and the ionic strength was 0.116. During the experiment, the isolated preparation was continuously replenished with O_2 by bubbling the serosal medium with a mixture of O_2–CO_2 (99:1, v/v) presaturated with water vapor. The pH varied between 7.4 and 7.8.

Choline sulfate solution. The mucosal surface was bathed by choline–Ringer solution in experiments designated Group A and by unbuffered 50 mM choline sulfate solution in those designated Group B. Choline sulfate was selected because it is devoid of the three known transportable ions: Na^+, Cl^- and HCO_3^-. To maintain an equal chemical potential of water on both sides of the bladder, the nonpenetrating[3] solute, sucrose was added to the 50 mM choline sulfate so that its osmolality approximated that of the choline–Ringer solution (221 mosmoles/kg). The solution had the following composition (in mM): 100 choline$^+$, 50 SO_4^{2-}, and 86 sucrose. $[Na^+] < 0.05$ mM. Ionic strength was 0.150. The solution was bubbled continuously throughout the experiment with 100% O_2. By the addition of small amounts of 1 M KOH or 0.5 M H_2SO_4, adjustments of the mucosal pH were made to prevent a transbladder gradient of pH.

Na^+–Ringer and Na_2SO_4 solutions. Na^+–Ringer and Na_2SO_4 solutions were identical to choline–Ringer and choline sulfate solutions, respectively, except that Na^+ replaced choline$^+$ mole for mole. In Group A, Na^+ was added to the mucosal medium in the form of Na^+–Ringer solution while in Group B in the form of Na_2SO_4 solution.

Analyses

Chemical measurements of Na^+, Cl^-, K^+, HCO_3^-, pH and osmolality were performed routinely on all stock choline$^+$ and Na^+ solutions by analytical techniques described previously [3,9,12]. Analyses for Na^+ and K^+ were performed using an Eppen-

dorf flame photometer. Standards were made from weighed, predried NaCl or KCl. Using distilled water to set the zero, the dial readings were proportional to various concentrations set by the standards even in the most sensitive range (0–0.1 mM).

Samples of urine and bathing media (before addition of mucosal Na^+) were routinely taken for [Na^+] analysis.

Tissue preparation

Both male and female turtles (*Pseudemys scripta*), weighing 0.75–1.5 kg and possessing a carapace 15–20 cm wide were obtained from the Lemberger Co., Oshkosh, Wisc. Most experiments of Group A were performed between August and October and those of Group B between October and February.

Turtles were sacrificed by decapitation. Urinary bladders were excised and placed in 100 ml of choline–Ringer solution. As much blood as possible was massaged out of the cut ends of the peritoneal blood vessels. The bladder was transferred to another bowl containing 100 ml of fresh solution. A sample of urine was removed from the bladder for chemical analysis of [Na^+]. An incision was made from one lobe to the other. Urine was removed, and the bladder was placed in another bowl containing 350 ml of fresh solution. The bladder was transformed into the shape of a diaphragm by a technique described previously[8]. After a 15-min elution in the Na^+-free Ringer solution to minimize the tissue content of Na^+, the bladder was transferred to another bowl containing 350 ml of fresh solution. After another interval of 15 min, the bladder was ready for mounting in a double-barreled chamber (modified after USSING AND ZERAHN[13]). If one of the mucosal surfaces was to be bathed by choline sulfate solution, the mucosa was first rinsed with 40 ml of choline sulfate solution to remove superficital HCO_3^- and Cl^-. See ref. 8 for the mounting technique and for the structure and function of the Lucite chamber.

The double-barreled chamber permitted two hemibladders to be used simultaneously as separate experiments. Each side of each hemibladder was bathed by 12 ml of the appropriate solution. All bathing media were continuously mixed and circulated past the bladder. Each hemibladder had a surface area of 1.5 cm^2 exposed to the bathing solutions, a dry weight of 10.4 $\pm$ 5.1 mg and a tissue-water content of 51.3 $\pm$ 21.9 μl (values shown are the mean $\pm$ S.D.).

All experiments were performed at 22–25°.

Short-circuiting technique

Isolated bladders were maintained in the short-circuited state using the technique of USSING AND ZERAHN[13]. The short-circuiting current (I_{sc}) and transbladder potential difference (PD) were measured by techniques described previously[8]. Membrane conductance was measured as follows: every 5 or 10 min, the short-circuiting current was interrupted for about 10 sec. During this time a calibrated pulse of current (ΔI) ranging between 10 and 100 μA was sent through the bladder. G_t was taken as ΔI divided by the increment in PD resulting from the sending of ΔI. A small correction was made for the series resistance of the solution (35 Ω). The following sign reference was adopted: cations (*viz.*, Na^+) moving in the forward (m $\rightarrow$ s) direction contributed a positive moiety of I_{sc} and anions (*viz.*, Cl^- and HCO_3^-) a negative moiety. Thus, I_{sc} was negative when the bladder was bathed on both sides by Na^+-free choline–

Ringer solution and increased in the positive sense when Na^+ was consequently added to the mucosal medium.

An Esterline–Angus model E1102S potentiometric strip chart recorder with a high input-impedance (100 kΩ) was used to measure PD (Esterline-Angus Co., Indianapolis, Ind.).

All values of I_{sc} and G_t are per hemibladder (*i.e.*, per 1.5 cm^2).

Isotopic technique

Na^+ clearance (C) was measured by use of tracer amounts of $^{22}Na^+$ and was calculated as follows:

$$C(\mu l/h) = \frac{\text{total } ^{22}Na^+ \text{ appearing on the serosal side per unit of time (counts/min per h)}}{\text{mucosal } ^{22}Na^+ \text{ per unit of volume (counts/min per } \mu l)} \quad (1)$$

Note that the evaluation of C does not depend upon a knowledge of the concentration or movement of $^{23}Na^+$.

The steady-state flux (Φ) and Na^+ current (I_{Na^+}) were calculated as follows:

$$\Phi(\text{nmoles/h}) = C(\mu l/h) \cdot [Na^+]_m \text{ (mM)} \quad (2)$$

$$I_{Na^+}(\mu A) = \frac{0.0268\ \mu A}{1\ \text{nmole/h}} \cdot \Phi(\text{nmoles/h}) \quad (3)$$

When $t = 30$ min, 20 μC of $^{22}Na^+$ (carrier-free, New England Nuclear) was added to each mucosal reservoir which contained 12 ml of solution. Isotopic samples were taken every 25 min, starting when $t = 125$ min.

All values of Φ and C are per hemibladder (*i.e.*, per 1.5 cm^2).

RESULTS

The following results comprised part of the Ph. D. thesis of the author[14].

Representative experiment for changes in clearance (Fig. 1)

Fig. 1 presents a plot of values for the logarithm of C *vs.* t in min from an experiment which was selected because values of data from it approximated the average values of data from all experiments.

After 100 min of incubation in the Na^+-poor media, samples of $^{22}Na^+$ were taken every 25 min.

During the initial period, C decreased progressively from 380 to 260 μl/h over a 2-h period. The average rate of decrease of C was 22%/h.

When $t = 231$ min, $^{23}Na^+$ was added to the mucosal bathing fluid to a final concentration of 2.2 mM, after which C changed from a state of progressive decrease to one of progressive increase. The average rate of increase of C was 37%/h. The state of progressive increase lasted for 1.5 h before C reached a maximal value of 440 μl/h. The period of progressive increase in C represents the slow *cis*-stimulation.

After achieving its peak value, C changed from the state of progressive increase to one of progressive decrease. The average rate of decrease was 14%/h. The state of progressive decrease continued for 2.5 h during the final decline period before termination of the experiment.

The results depicted in Fig. 1 represent a single example from 24 experiments

from which the results will be shown collectively in tabular form. In all 24 experiments choline–Ringer solution (containing 17 mM HCO_3^- and 90 mM Cl^-) bathed the serosal surface of each hemibladder. In Group A (12 of the 24 experiments), choline–Ringer solution bathed each mucosal surface. In Group B (the remaining 12 experiments), choline sulfate solution (poor in actively transported ions) bathed each mucosal surface.

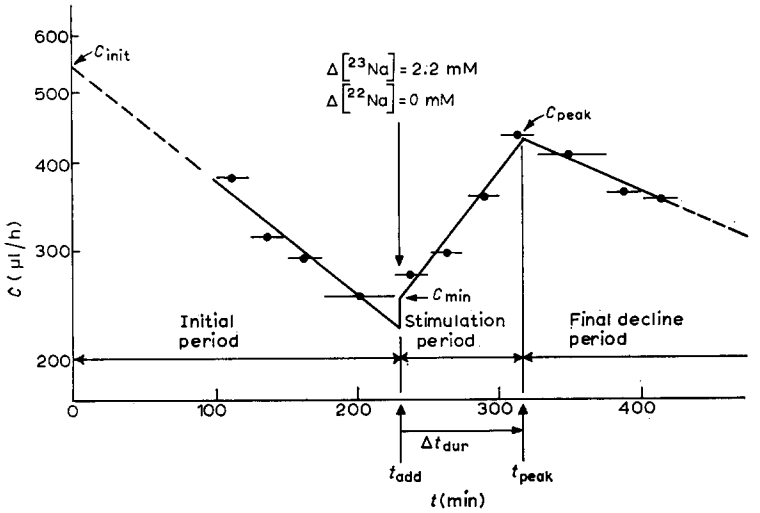

Fig. 1. Clearance as a function of time for a representative experiment (Expt. CGR): choline–Ringer solution on both sides. Horizontal lines represent values of C between sampling times; ●, the point (logarithm of average C, midtime of isotopic sample interval). See text for further description. Note that when $t = 231$ min, the mucosal concentration of nonradioactive $^{23}Na^+$ was increased by 2.2 mM; but the concentration of tracer, $^{22}Na^+$, was not changed.

Magnitude of clearance (Table I)

For each of the three distinct periods (initial, stimulation and final decline), a separate regression line was drawn through the points (logarithm of average C, midtime of isotopic sample interval). C_{init} was the C-intercept of the regression line for the initial period with the ordinate drawn when $t = 0$; *i.e.*, C_{init} was an extrapolated value which provided a rough estimate of the C at the beginning of the experiment. C_{min} was the C-intercept of the regression line for the stimulation period with an ordinate drawn when $t = t_{add}$; *i.e.*, C_{min} was the extrapolated minimal C at the beginning of the stimulation period. C_{peak} was the C-intercept of the regression line for the stimulation period with the regression line for the final decline period; *i.e.*, C_{peak} was the estimated peak C resulting from the addition of Na^+.

Table I presents average values for initial clearance (C_{init}), minimal clearance (C_{min}), peak clearance (C_{peak}) and C_{peak}/C_{min}. When $t = t_{add}$, Na^+ was added to the mucosal bathing solution to a final concentration between 1 and 10 mM. (No correlation could be found between the final concentration and the magnitude of the slow *cis*-stimulation.) In 11 cases, the experiments were terminated prior to the onset of the final decline period; in such cases, one could not be certain that the peak level of C had been reached. Only 12 (rather than 13) experiments were used to calculate C_{peak}/C_{min} since one value was more than 2 S.D.'s above the mean.

Table I shows that during the stimulation period, C increased significantly ($P <$

TABLE I

FORWARD Na^+ CLEARANCE BEFORE AND AFTER STIMULATION

Group A were bathed on the mucosal surface by choline–Ringer solution; Group B, by choline sulfate solution. Data are given as the mean ± S.E. The number of experiments is given by n.

Group	*Period*			
	Initial	*Stimulation*	*Final decline*	
	$t = o$	$t = t_{add}$	$t = t_{peak}$	*Peak to min. clearance*
	C_{init} (μl/h)	C_{min} (μl/h)	C_{peak} (μl/h)	C_{peak}/C_{min} (*unitless*)
A	770 ± 120 (n = 12)	260 ± 50 (n = 12)	620 ± 160 (n = 6)	
B	290 ± 40 (n = 12)	130 ± 20 (n = 12)	350 ± 40 (n = 7)	
A + B				2.2 ± 0.2 (n = 12)
P (A = B)	< 0.001	< 0.05	< 0.1	> 0.9

TABLE II

TIME RATE OF CHANGE OF FORWARD Na^+ CLEARANCE BEFORE AND AFTER STIMULATION

Data given as the mean ± S.E. The number of experiments is given by n. A P value relates to the statistical comparison of its respective rate of change with zero.

Group	*Period*					
	Initial	*Stimulation*		*Final decline*		
	α (%/h)	β (%/h)	$\beta - \gamma$ (%/h)	γ (%/h)	$\gamma - \alpha$ (%/h)	$\gamma - \beta$ (%/h)
A + B	−29 ± 3 (n = 24)	+37 ± 4 (n = 24)	+66 ± 6 (n = 24)	−19 ± 4 (n = 13)	+8 ± 5 (n = 13)	−59 ± 7 (n = 13)
P	< 0.001	< 0.001	< 0.001	< 0.001	> 0.1	< 0.001

0.02) from a mean of 260 to one of 620 μl/h in Group A, and from 130 to 350 μl/h in Group B ($P < 0.01$). The lower panel of the table presents a statistical comparison between the parameters of Groups A and B. Inasmuch as means for C_{min}, C_{init} and C_{peak} in Group A were significantly different from those of Group B, pooling of such data was not justified; however, the mean for C_{peak}/C_{min} of Group A was statistically indistinguishable from that of Group B. Consequently, the data for C_{peak}/C_{min} were pooled for subsequent statistical evaluation. The pooled mean for C_{peak}/C_{min} was 2.2; *i.e.*, the addition of $^{23}Na^+$ to the mucosal bathing fluid resulted in a mean increase of 120% in C.

Rate of change of clearance (Table II)

The average rate of change of C during each period corresponded to the slope of the requisite regression line.

Table II presents average values for rates of change of C during the initial period

(α), during the stimulation period (β) and during the final decline period (γ) for the same 24 hemibladders represented in Table I. The mean values of each parameter in Group A (*e.g.*, α, β, *etc.*) were compared statistically with the corresponding mean in Group B. In no case was any significant difference detected (P ranged between 0.2 and 1); *i.e.*, the rate of change of C in any of the three periods (initial, stimulation and final) was independent of the type of choline solution bathing the mucosal surface. Therefore the data of Groups A and B were pooled for statistical evaluation.

Table II shows that the mean rate of decrease of C during the initial period (α) was 29%/h, that the mean rate of increase of C during stimulation (β) was 37%/h, that the mean rate of decrease of C during the final period (γ) was 19%/h and that all three rates were significantly different from zero. Moreover, α was significantly different from β ($P < 0.001$), and β was significantly different from γ ($P < 0.001$). The average values of the relative rates ($\beta - \alpha$) and ($\gamma - \beta$) indicate the abrupt nature of the change in rates at the boundaries of the periods (*viz.*, when $t = t_{add}$ and $t = t_{peak}$, respectively). The high value of P for ($\gamma - \alpha$) indicates that, on the average, the rate of decrease of C during the final decline period could not be distinguished from that during the initial period (*i.e.*, before stimulation).

Duration of slow cis-stimulation

Intersection of the regression line for the final decline period with that for the stimulation period provided a value of time (t_{peak}) at which the peak clearance was estimated to have occurred. The duration of the stimulation period (Δt_{dur}) was taken as t_{peak} *minus* t_{add}, the time at which the Na^+ was added to the mucosal medium. The mean value for Δt_{dur} in Group A was not significantly different ($P > 0.1$) from that in Group B, and the pooled mean and S.E. for 13 hemibladders was 125 $\pm$ 15 min.

Washout of $^{22}Na^+$ into the serosal bathing fluid

Bladder containing mucosal cells, connective tissue and interstitium was found to take up $^{22}Na^+$ from the mucosal bathing fluid. When $[Na^+]_s < 0.5$ mM and $[Na^+]_m < 0.1$ mM, the concentration of $^{22}Na^+$ in the bladder (amount of $^{22}Na^+$ per unit volume of tissue water) was on the average 1.5 times that in the mucosal bathing fluid. Since the mean value for the content of tissue water in 1.5 cm^2 of bladder tissue was approx. 50 μl, the bladder contained on the average the same amount of $^{22}Na^+$ as contained in 75 μl of mucosal bathing fluid. Therefore, if Na^+ transport from the mucosal bathing fluid into the hemibladder was terminated suddenly and if the release of $^{22}Na^+$ from the hemibladder into the serosal bathing fluid continued such that the entire content of $^{22}Na^+$ was disgorged over the ensuing 25 min (the interval between isotopic samples), the apparent C would be

$$\frac{75\ \mu l}{25\ min} \cdot \frac{60\ min}{1\ h} = 180\ \mu l/h \qquad (4)$$

Back ($s \rightarrow m$) clearance of Na^+

$^{22}Na^+$ was introduced into the serosal fluid, and its rate of appearance in the mucosal fluid was measured in 4 hemibladders bathed by choline$^+$ solutions ($[Na^+]_s < 0.5$ mM; and $[Na^+]_m$ ranged between 1 mM and 6 mM). In no case was back clearance greater than 25 μl/h. The mean value of back clearance was 10 μl/h.

Representative experiment for changes in electrical parameters (Fig. 2)

Fig. 2 presents plots of short-circuiting current I_{sc} (Inset A) and of conductance G_t (Inset B) *vs.* time t in the same experiment shown in Fig. 1.

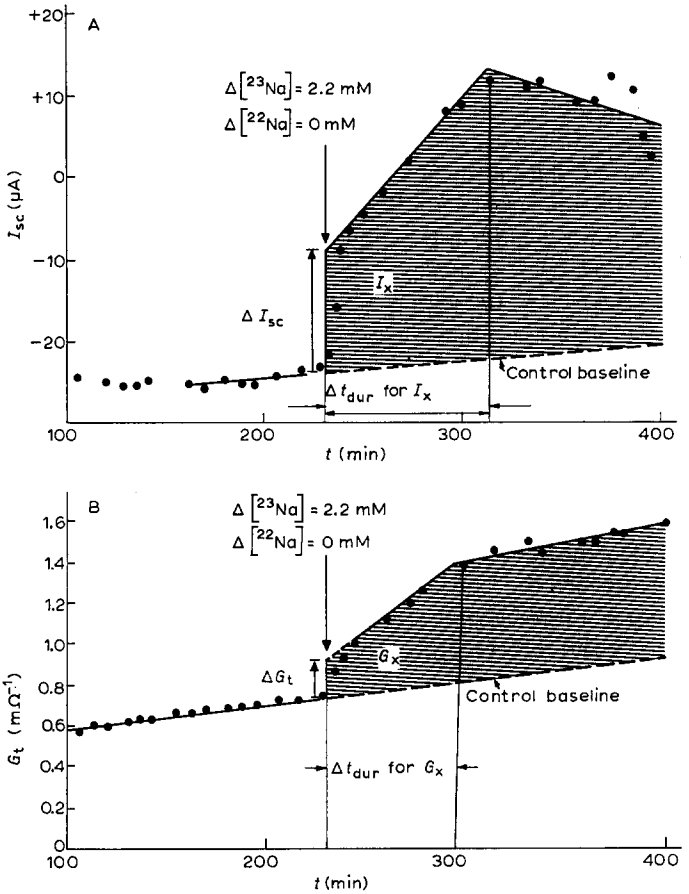

Fig. 2. Short-circuiting current and total membrane conductance as a function of time for a slow *cis*-stimulation experiment (Expt. CGR): choline–Ringer solution for both sides. See text for description. Note that when $t = 231$ min, the mucosal concentration of nonradioactive $^{23}Na^+$ was increased by 2.2 mM; but the concentration of tracer, $^{22}Na^+$, was not changed. The new moieties of I_{sc} and G_t (*viz.*, I_x and G_x, respectively) are indicated by the hatched areas, and are thought to represent the current carried by Na^+ (I_{Na^+} is approximately equal to I_x) and the specific transmembrane conductance for Na^+ (G_{Na^+} is approximately equal to G_x), respectively.

During the initial period, the value of G_t increased in magnitude while that of I_{sc}, a negative quantity, decreased in magnitude. When $t = 231$ min, $^{23}Na^+$ was added to the mucosal fluid to a final concentration of 2.2 mM. It can be seen that I_{sc} increased sharply from −24 to −10 μA and G_t from 0.75 to 0.88 mΩ^{-1}. The values of −10 μA and 0.88 mΩ^{-1} were obtained by the backward extrapolation of postaddition values to the instant of addition of Na^+ (see Fig. 2) and were used to define the "immediate" changes in I_{sc} and G_t, respectively. No values of I_{sc} and G_t were used for the first 15 min after t_{add} in the calculation of the backward extrapolation, since the transport

mechanism was adjusting to the mass introduction of mucosal Na^+ during this time interval.

The regression lines of I_{sc} and G_t in the initial period were extrapolated through the periods of stimulation and final decline (see Fig. 2) to provide base lines for estimating the increments in both parameters. Thus, I_x was defined as the measured I_{sc} at any time ($t > t_{add}$) *minus* the extrapolated baseline evaluated at that time. G_x was defined in an analogous manner.

The demarcation between stimulation and final decline periods for I_x was indicated by the first maximum in I_{sc} following the addition of Na^+. However, G_t did not achieve a maximum following the addition of Na^+; therefore, there was no sharp demarcation between stimulation and final decline periods for G_x. The postaddition period for G_t was separated into two periods by the graphical intersection of two straight lines arbitrarily drawn to fit the data. Once the intersection had been estimated, two separate regression lines were determined for points on each side of the intersection. The time corresponding to the intersection of the two regression lines was used for the estimation of Δt_{dur} for G_x.

During the stimulation period, the rates of change of G_x and I_x were +113 and +68%/h, respectively. The duration of the stimulation period for G_x was 65 min while that of I_x was 80 min. After reaching its peak value, I_x began to decrease progressively at a rate of 23%/h. G_x increased slightly at the rate of 8%/h during the final period.

The results depicted in Fig. 2 represent a single example from 22 experiments from which the electrical and flux data will be shown collectively in tabular form. The 22 experiments comprise a subset composed of the same 24 experiments cited in previous tables of this paper.

Immediate changes in I_{sc} and I_{Na^+}

The net m → s current carried by Na^+ is designated I_{Na^+}. After addition of $^{23}Na^+$ to final mucosal concentrations of 1–10 mM, I_{sc} increased rapidly to its immediate postaddition value (see ΔI_{sc} in Fig. 2). The immediate change in I_{Na^+} (ΔI_{Na^+}) was estimated by substitution of C_{min} (C at time of addition) and the change in $[^{23}Na^+]_m$ in Eqns. 2 and 3. In all 22 cases, the addition of Na^+ was followed by an increase in I_{sc} and I_{Na^+}. The mean value of $\Delta I_{Na^+}/\Delta I_{sc}$ in Group A was not found to differ significantly from that in Group B ($P > 0.3$). Consequently, the ratios from Groups A and B were pooled for subsequent statistical evaluation. The pooled mean value of $\Delta I_{Na^+}/\Delta I_{sc}$ was equal to 1.1 and was not significantly different from unity ($P > 0.4$); *i.e.*, the immediate change in I_{sc}, an electrical parameter, could not be distinguished from the estimated immediate change in movement of Na^+.

Immediate change in G_t

In all 22 cases, the addition of Na^+ to the mucosal bathing fluid was followed by an immediate increase in G_t. These increases in membrane conductance ranged between 0.01 and 0.47 $m\Omega^{-1}$.

Rates of change of G_x, I_x, and C (Table III)

Table III presents values for the duration of the stimulation period, the rates of change during the stimulation and final decline periods for each parameter: G_x, I_x and C. The mean values of each parameter (G_x, I_x and C) in the stimulation period in

Group A were compared statistically with the corresponding mean values in Group B. In no case was any significant difference detected ($P > 0.5$). Since the mean value for the rate of change of each of the three parameters was independent of the type of choline solution bathing the mucosal surface, data of Groups A and B were pooled for statistical evaluation.

TABLE III

COMPARISON OF CONDUCTANCE, CURRENT, AND CLEARANCE RATES OF CHANGE

Data are given as the mean ± S.E. The number of experiments is given by n. P values are explained in the text.

		β (%/h)	γ (%/h)	Δt_{dur} (min)
A	for G_x	85 ± 14	−22 ± 12	80 ± 10
B	for I_x	63 ± 8	−25 ± 6	90 ± 5
C	for C	46 ± 6	−17 ± 5	100 ± 5
D	P (A–B = 0)	$P > 0.7$	$P > 0.7$	$P > 0.1$
E	P (B–C = 0)	$P < 0.01$	$P > 0.3$	$P < 0.02$
F	P (C–A = 0)	$P < 0.05$	$P > 0.6$	$P < 0.02$
		$n = 13$	$n = 6$	$n = 6$

Table III presents means with S.E's and statistical evaluations for the parameters mentioned above. The rows designated A, B and C correspond to the parameters G_x, I_x and C, respectively. The columns designated β, γ and Δt_{dur} correspond to the rates of change during the stimulation period, rates during the final decline period, and duration of the stimulation period, respectively. Statistical comparisons, using the paired t-test, yielded the following results: row D shows the P value for the comparison of data of row A with those of row B; row E, for the comparison of data of row B with those of row C; and row F, for the comparison of data of row C with those of row A.

During the stimulation period, the mean rates of change (β's) of the electrical parameters (G_x and I_x) were not significantly different. However, the mean rate of change for each electrical parameter differed significantly from that for C. Note that the rate of change of C (46%/h) was less than that for either I_x (63%/h) or G_x (85%/h).

The mean durations of the stimulation period (Δt_{dur}) of the electrical parameters were not significantly different from one another. However, the mean duration for each electrical parameter differed significantly from that for C. Note that the mean value for the duration of the stimulation period for C (100 min) was longer than that for either I_x (90 min) or G_x (80 min).

Nonspontaneous nature of slow cis-stimulation

The main finding of the present work is the progressive increase of C following the addition of Na^+ to the mucosal medium bathing a hemibladder in which C had been steadily decreasing. Evidence for the nonspontaneous nature of stimulation is as follows:

(1) In all 24 experiments reported herein, the rates of change of C did not alter their orientation until Na^+ addition (t_{add}), even though t_{add} ranged from 2 to 7 h in the various experiments.

(2) In one experiment, Na^+ was added to the mucosal fluid of one hemibladder 2 h before it was added to that of its paired hemibladder. In neither hemibladder did C increase before Na^+ was added.

(3) In one experiment, addition of Na^+ to the mucosal fluid of one hemibladder resulted in stimulation. $Choline^+$, rather than Na^+, was added to the mucosal fluid of the paired hemibladder in which C progressively declined before and after addition of $choline^+$.

Failure to evoke slow cis-stimulation

In 23% of the cases (7 out of 31 hemibladders), the rate of change of C did not increase measurably for 2–4 h after addition of Na^+ to the mucosal bathing fluid. The mean and S.E. for α (the rate of change of C before the addition of Na^+) was -8 ± 3 %/h, and the mean and S. E. for β (the rate of change of C after the addition) was -10 ± 4%/h. The mean and S. E. for $(\beta - \alpha)$ was -2 ± 2%/h. For the 7 "failures", the value for $(\beta - \alpha)$ ranged between -13 and $+5$%/h. (When the value of $(\beta - \alpha)$ for a particular experiment exceeded $+20$%/h, that experiment was included as one of the slow *cis*-stimulation experiments reported in Tables I, II and III; otherwise, the experiment was listed as a so-called "failure".)

The mean value for the magnitude of C at the time of addition of Na^+ was greater for the 7 "failures" than for the 24 slow *cis*-stimulation experiments reported herein.

It is pertinent to note that immediate changes in I_{sc} and G_x were observed for the "failures" upon the addition of Na^+, but no progressive increase in either parameter was observed during the postaddition period. ΔI_{Na^+}, calculated by substitution of C evaluated at the time of addition and change in $[^{23}Na^+]_m$ in Eqns. 2 and 3, was matched by the observed ΔI_{sc}.

Analysis for Na^+ in the bathing media

Samples of bathing media were routinely taken before the addition of musosal $^{23}Na^+$. In all experiments, $[Na^+]_s < 0.5$ mM and $[Na^+]_m < 0.1$ mM.

Mucosal Na^+ in relation to slow cis-stimulation

Concentration of Na^+ in the urine. Na^+ analysis in urine taken from 25 different bladders shortly after sacrifice of the turtle showed that $[Na^+] < 0.1$ mM in 48% of the cases, < 0.2 mM in 72% of the cases and < 1 mM in 84% of the cases. The maximum concentration measured was 2.6 mM. Therefore, the choline solution bathing the mucosal surface during the initial period provided a continuation of the Na^+-poor mucosal state for about 72% of the bladders.

The previous data suggest that the failure of evoking stimulation could be related to a pre-stimulated state occasioned by previous exposure of the mucosal surface to Na^+ at concentrations in excess of 0.2 mM. However, high $[Na^+]$ in the urine did not correlate with failure to be stimulated by the addition of Na^+.

Threshold. Data of the present work indicated that the stimulation phenomenon could be evoked by increasing $[Na^+]_m$ from 0.1 to 1.0 mM. However, these data were inadequate for revealing a minimal value of concentration which precisely demarcated the response.

Functional relationship. An attempt was made to correlate the magnitude of the response (stimulation) with the increment in concentration of the Na^+ added to the

mucosal bathing fluid, but no correlation between the two parameters was evident in the data.

Maintenance of the level of clearance. After addition of Na^+, C increased progressively but finally reached a peak value (see C_{peak} in Table I). Thereafter, C progressively decreased during the final decline period (see γ in Table II). The rate of decrease of C during the final decline period was generally about the same as that during the initial period. The rates of decrease of C during the initial and final decline periods were not significantly different ($P > 0.1$ by the paired t-test) and may reflect the normal aging of the isolated system (see α and γ in Table II). However, apart from the decline rate the magnitude of C during the final decline period did not return to the "control level"; *i.e.*, did not return to the level defined by the line extrapolated from the plot of C *vs.* t during the initial period (see Fig. 1). In other words, the level of C did not return to where it would have been if no $^{23}Na^+$ had been added to the mucosal bathing fluid.

DISCUSSION

Differences between Group A and Group B

Although rates of change of C were similar in Groups A and B, the magnitude of C in Group A was significantly larger than that in Group B (see Table I). Present data is not sufficient to determine whether this difference can be ascribed to the difference between the two mucosal bathing solutions or to the seasonal difference between the two groups of turtles (since the experiments of Group A were performed between August and October and those of Group B, between October and February).

Discrepancy between the stimulation of clearance and that of the electrical parameters

During the stimulation period, I_x increased progressively at a significantly greater rate than C (see the β's in Table III). This discrepancy suggests that the hemibladder serves, at least during the stimulation period as a sink* for $^{22}Na^+$ following the addition of $^{23}Na^+$. Therefore, the rate of progressive increase of C which reflects the movement of $^{22}Na^+$ from the hemibladder to the serosal bathing fluid would be less than that of I_x which reflects the movement of both $^{23}Na^+$ and $^{22}Na^+$ from the mucosal bathing fluid into the hemibladder. The mean value of the observed duration of the progressive increase of C was greater than that for the electrical parameters (see the Δt_{dur}'s in Table III). This discrepancy suggests that time is required for the transported $^{22}Na^+$ to diffuse through the tissue pool of Na^+ into the serosal bathing fluid. Whereas the tissue pool can explain the differences in time-dependent responses of the electrical and flux parameters, it cannot account for the increment of C following addition of Na^+. In other words, the observed slow *cis*-stimulation cannot be due to an artifact representing washout of $^{22}Na^+$.

An alternate explanation for the discrepancy is as follows: addition of Na^+ to the mucosal medium results in a gradual change in movement of ions other than Na^+. For example, the discrepancy can be explained if Na^+ addition results (i) in an increase

* It is not surprising that $[^{22}Na^+]$ could increase in the interstitium on the serosal side of the Na^+ pump following stimulation, for movement of $^{22}Na^+$ from the interstitium into the serosal medium is probably *via* free diffusion. To maintain the larger C resulting from stimulation, a larger gradient of $[^{22}Na^+]$ in the interstitium would be required. Thus, total tissue content of $[^{22}Na^+]$ might well increase following stimulation.

in net m→s movement of cations or (ii) in an increase in net s→m movement of anions.

Present data is not sufficient to distinguish between the explanations suggested above for the discrepancy.

Washout of $^{22}Na^+$ into the serosal bathing medium

Eqn. 4 shows that on the average if all $^{22}Na^+$ were suddenly disgorged from the whole bladder tissue into the serosal medium over a 25-min period, the apparent increase in C would be 180 μl/h. Actually, the contribution of washout to C would be less than the maximal value of 180 μl/h, and consequently much less than the C-increment resulting from the addition of Na^+.

Furthermore, electrical parameters which were probably associated primarily with the transport of Na^+, *viz.*, I_x and G_x, were "stimulated" by the addition of Na^+ to the mucosal bathing fluid. If washout had been solely responsible for the observed stimulation of C, there would have been no concomitant stimulation of I_x and G_x which are dominated by the transport of $^{23}Na^+$.

Possible physiological significance of slow cis-stimulation

If the electrochemical gradient across the bladder becomes so large that it results in the loss of a substrate which should be conserved, the pre-stimulated state (high resistance to transbladder movement of that substrate) would be favored. Upon the appearance of sufficiently high substrate concentration in the urine, conservation of that substrate would be enhanced by the stimulated state (low resistance to movement).

Failure to evoke slow cis-stimulation

Since both I_{sc} and G_t increased sharply without a change in C upon addition of mucosal Na^+ in the group of "failures", this group was not a failure with respect to its ability to transport Na^+ when available on the mucosal side. Since the mean value for the magnitude of C at the time of addition of Na^+ was greater for the 7 "failures" than for the 24 slow *cis*-stimulation experiments, hemibladders listed as "failures" might have been in some state of stimulation before the addition of Na^+ and, consequently, might have been refractory to an additional stimulus (*viz.*, the addition of mucosal Na^+).

ACKNOWLEDGMENTS

This study was supported in part by National Institutes of Health Research Grant AM 13037, in part by National Science Foundation Research Grant GB-7764 and in part by National Aeronautics and Space Administration Research Grant 33-171-(001).

The author is indebted to Mr. Thomas I. Moxley for his technical assistance.

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