Electrophysiological Effects of Basolateral $[Na^+]$ in *Necturus* Gallbladder Epithelium

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ABSTRACT In *Necturus* gallbladder epithelium, lowering serosal $[Na^+]$ ($[Na^+]_s$) reversibly hyperpolarized the basolateral cell membrane voltage (V_{cs}) and reduced the fractional resistance of the apical membrane (fR_a). Previous results have suggested that there is no sizable basolateral Na^+ conductance and that there are apical Ca^{2+}-activated K^+ channels. Here, we studied the mechanisms of the electrophysiological effects of lowering $[Na^+]_s$, in particular the possibility that an elevation in intracellular free $[Ca^{2+}]$ hyperpolarizes V_{cs} by increasing gK^+. When $[Na^+]_s$ was reduced from 100.5 to 10.5 mM (tetramethylammonium substitution), V_{cs} hyperpolarized from -68 ± 2 to a peak value of -82 ± 2 mV ($P < 0.001$), and fR_a decreased from 0.84 ± 0.02 to 0.62 ± 0.02 ($P < 0.001$). Addition of 5 mM tetraethylammonium (TEA^+) to the mucosal solution reduced both the hyperpolarization of V_{cs} and the change in fR_a, whereas serosal addition of TEA^+ had no effect. Ouabain (10^{-4} M, serosal side) produced a small depolarization of V_{cs} and reduced the hyperpolarization upon lowering $[Na^+]_s$, without affecting the decrease in fR_a. The effects of mucosal TEA^+ and serosal ouabain were additive. Neither amiloride (10^{-5} or 10^{-3} M) nor tetrodotoxin (10^{-6} M) had any effects on V_{cs} or fR_a or on their responses to lowering $[Na^+]_s$, suggesting that basolateral Na^+ channels do not contribute to the control membrane voltage or to the hyperpolarization upon lowering $[Na^+]_s$. The basolateral membrane depolarization upon elevating $[K^+]_s$ was increased transiently during the hyperpolarization of V_{cs} upon lowering $[Na^+]_s$. Since cable analysis experiments show that basolateral membrane resistance increased, a decrease in basolateral Cl^- conductance (gCl^-) is the main cause of the increased K^+ selectivity. Lowering $[Na^+]_s$ increases intracellular free $[Ca^{2+}]$, which may be responsible for the increase in the apical membrane TEA^+-sensitive gK^+. We conclude that the decrease in fR_a by lowering $[Na^+]_s$ is mainly caused by an increase in intracellular free $[Ca^{2+}]$, which activates TEA^+-sensitive maxi K^+ channels at the apical membrane and decreases apical membrane resistance. The hyperpolarization of V_{cs} is due to increases in: (*a*) apical membrane gK^+, (*b*) the contribution of the Na^+ pump to V_{cs}, (*c*) basolateral membrane K^+ selectivity (decreased gCl^-), and (*d*) intraepithelial current flow brought about by a paracellular diffusion potential.

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INTRODUCTION

Ion movements across the apical membrane of *Necturus* gallbladder epithelium have been extensively characterized (for review, see Reuss, 1989), but only partial information is available on the ion pathways across the basolateral membrane. Basolateral Cl^- extrusion is via both electroneutral KCl cotransport (Corcia and Armstrong, 1983; Reuss, 1983) and a conductive mechanism (Stoddard and Reuss, 1988*a*, 1989*b*). If present, basolateral $gHCO_3^-$ seems to be small, since decreasing basolateral solution $[HCO_3^-]$ from 10 to 1 mM at constant CO_2 decreases intracellular pH (pH_i) by only 0.03 units in 3 min, and increasing $[HCO_3^-]$ to 50 mM at constant pH does not reduce the depolarization of basolateral membrane voltage (V_{cs}) produced by elevating serosal $[K^+]$ (Stoddard and Reuss, 1989*b*).

Basolateral Na^+ extrusion is via the Na^+ pump (Reuss et al., 1979; Rose and Nahrwold, 1980; Ericson and Spring, 1982; Reuss, 1989); it is unclear whether there are pathways for inward Na^+ movement across the basolateral membrane (see Weinman and Reuss, 1984).

Decreasing basolateral $[Na^+]$ ($[Na^+]_s$) in gallbladders incubated in 10 mM HCO_3^-/1% CO_2 hyperpolarizes V_{cs} (Stoddard and Reuss, 1989*b*). This hyperpolarization is by itself consistent with a sizable basolateral gNa^+, but estimations of gK^+ and gCl^- indicate that these alone could account for the basolateral conductance under control conditions (Stoddard and Reuss, 1989*b*). In addition, partial gNa^+ of the basolateral membrane must be small because the zero-current voltage of the basolateral membrane (E_b) ($\simeq -70$ mV) is far removed from the Na^+ equilibrium potential (E_{Na}) ($\simeq 60$ mV; Reuss and Weinman, 1979; Weinman and Reuss, 1984; Reuss, 1989).

Previous results indicate that there are Ca^{2+}-activated K^+ channels at the apical and basolateral membranes (Bello-Reuss et al., 1981; García-Díaz et al., 1983; Stoddard and Reuss, 1988*b*, 1989*b*; Reuss, 1989; Segal and Reuss, 1990*a*). Here we study the mechanisms of the electrophysiological effects of lowering $[Na^+]_s$, in particular the possibility that an elevation in intracellular free $[Ca^{2+}]$ hyperpolarizes V_{cs} by increasing gK^+.

METHODS

Mudpuppies (*Necturus maculosus*) purchased from Kons Scientific Co., Inc. (Germantown, WI) or Nasco Biologicals (Ft. Atkinson, WI) were maintained in aquariums at 5–10°C. Anesthesia was accomplished by immersion of the animals in a 1 g/liter tricaine methanesulfonate solution. The gallbladder was excised, opened, washed, and mounted in a modified Ussing chamber (Altenberg et al., 1990). Tissues were mounted serosal side up, a patch of subepithelial connective tissue was removed by dissection (Stoddard and Reuss, 1989*b*), and impalements with microelectrodes were done across the basolateral membrane. Bathing solutions contained (mM): 90 NaCl, 10 $NaHCO_3$, 2.5 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, and 0.5 Na_2HPO_4, gassed with 1% CO_2/99% air, pH 7.66. Low-Na^+ solutions were prepared by isomolar replacement of Na^+ with tetramethylammonium (TMA^+) or *N*-methyl-D-glucammonium ($NMDG^+$); for high K^+ solutions, Na^+ was replaced with K^+ mole by mole. In some experiments 5 mM tetraethylammonium (TEA^+) was added, replacing Na^+ or TMA^+ mole by mole.

Electrophysiological Techniques

Transepithelial voltage (V_{sm}, referred to the mucosal bathing solution, i.e., the bottom half-chamber) and cell membrane voltages (apical = V_{mc}, basolateral = V_{cs}, referred to the adjacent bathing solutions) were measured as previously described (Reuss and Finn, 1975*a*, *b*; Altenberg et al., 1990). The ground electrode was an Ag-AgCl pellet separated from the mucosal bathing solution by a short Ringer-agar bridge. The serosal bathing solution electrode was a flowing, saturated KCl bridge in series with a calomel half-cell. Hence, corrections for liquid junction potentials upon changes in the serosal solution were not required. The transepithelial resistance, R_t ($\Delta V_{ms}/I_t$), and the apparent fractional resistance of the apical membrane, fR_a ($\Delta V_{mc}/\Delta V_{ms} = R_a/(R_a + R_b)$, where the subscripts a and b denote apical and basolateral membranes, respectively, were determined from the voltage deflections at 600 ms after the onset of a DC pulse of 50 $\mu A/cm^2$ and 2-s duration, applied across the tissue through Ag-AgCl electrodes. The voltage deflections were corrected for series resistances.

To estimate changes in the apical (R_a) and basolateral (R_b) membrane resistances produced by lowering $[Na^+]_s$, we used two-point cable analysis (Petersen and Reuss, 1985; Stoddard et al., 1990). Two cells were simultaneously impaled with microelectrodes; current (I_0) was injected through one of the microelectrodes and the cell membrane voltage changes (ΔV_x) were measured in the second cell before, during, and after reducing $[Na^+]_s$. Results obtained at several interelectrode distances were pooled and normalized to a Bessel function, choosing the parameters that yield the average equivalent resistance of R_a and R_b in parallel (R_z) and also fR_a under the same conditions (gallbladders incubated in 10 mM HCO_3^-/1% CO_2; see Stoddard and Reuss, 1988*a*). By this procedure, the normalized distance (x) assigned to each measurement is retained for the analysis of all data obtained from the same pair of impalements. The values of ΔV_x under experimental conditions were fit to the Bessel function K_0 by a nonlinear, least-squares routine. The experimental values of A and the space constant (λ) were determined and used to calculate R_z [$R_z = R_aR_b/(R_a + R_b)$] according to Frömter (1972):

$$R_z = 2\pi A\lambda^2/I_0 \quad (1)$$

R_a and R_b were calculated from R_z and fR_a. I_0 varied between 7 and 20 nA, and ΔV_x was normalized to an I_0 of 10 nA (Stoddard and Reuss, 1988*a*).

Intracellular Na^+ activity (aNa_i) and pH (pH_i) were measured with ion-sensitive microelectrodes. Intracellular conventional and pH-sensitive microelectrodes and double-barreled Na^+-selective microelectrodes were constructed and calibrated as previously described (Stoddard et al., 1989*a*; Altenberg et al., 1990). Validation of impalements was as described before (Weinman and Reuss, 1984; Altenberg et al., 1990). Intracellular pH was measured with simultaneous impalements with single-barreled pH-sensitive and conventional microelectrodes (Weinman and Reuss, 1982; Altenberg et al., 1990). Intracellular Na^+ was measured with double-barreled microelectrodes.

Fluorescence Techniques

Attempts to load the epithelial cells of *Necturus* gallbladder with the acetoxymethyl (AM) ester form of the Ca^{2+}-sensitive dyes fura-2, fluo-3, and quin-2 were unsuccessful. We tested cell loading with different dye concentrations from 5 to 100 μM, by mucosal, serosal, or bilateral exposure, varying exposure times from 0.5 to 4 h using 2 mM probenecid, and subjecting the preparation to hyposmotic shock during exposure to the free acids of fura-2 or fluo-3. None of these procedures worked. Finally, we microinjected individual cells of the preparation with dextran-bound fura-2 (see below). The gallbladder was pinned to a cork ring, serosal side up,

the connective tissue was dissected away, and then the tissue was mounted mucosal side up in the modified Ussing chamber used for microelectrode studies (Altenberg et al., 1990). Glass pipettes with internal fiber, similar to those used to measure cell membrane voltages, were back-loaded with 2–3 μl of a solution containing 1.7 or 5 mM dextran-bound fura-2 (10,000 mol wt; Molecular Probes, Inc, Eugene, OR) in 100 mM KCl. The tip was filled and then broken to an outer diameter of ≃1 μm by pressing the connective tissue near the edge of the preparation. Well-dissected areas were selected and cells were injected manually by pressure using a plastic syringe (Graessmann and Graessmann, 1983). The glass pipette was withdrawn either when the cell diameter increased by ~10% or when the nucleus became clearly visible by an increase in contrast between nucleus and cytoplasm (Graessmann and Graessmann, 1983). In each preparation, 5–30 cells were microinjected. The plastic ring with the gallbladder was removed from the modified Ussing chamber (Altenberg et al., 1990) and then placed in a Leiden chamber (Leiden microincubator; Medical Systems Corp., Greenvale, NY) sealed at the bottom with a glass coverslip 110 μm thick. A droplet of control Ringer solution was placed on the coverslip, and the tissue, mucosal side down, was secured in place with dental wax. The serosal solution was replaced by gravity at a rate of ≃20 ml/min, while a static layer of fluid 50–100 μm thick remained in contact with the apical surface of the epithelium. Fluorescence determinations were carried out with a Deltascan system (Photon Technology International Inc., South Brunswick, NJ). The light source was a 75-W xenon lamp chopped between two monochromators set at wavelengths of 340 ± 4 and 380 ± 4 nm. Monochromatic light, carried by fiber optics, was reflected with a dichroic mirror (405 nm; Omega Optical Inc., Brattleboro, VT). An inverted microscope (Nikon Diaphot; Nikon, Tokyo, Japan) and a high numerical aperture objective (NA = 1.3, 40×, Nikon #78820) were employed. Light emitted from a single cell was filtered with a band-pass filter (510 ± 20 nm, Omega Optical) and measured with a photomultiplier (Photon Technology International Inc.). Data were acquired at 1-s intervals. The microinjected cells appeared homogeneously fluorescent under microscopic observation at 400×. There was no evidence of dye leak. Experiments were started 1–2 h after the microinjection, but the dye remained inside the cells for at least 4 h. At the end of the experiments 10–20 μM ionomycin was added to the control Ringer to obtain saturating free $[Ca^{2+}]_i$ and then 1–2 mM $MnCl_2$ was added, in the continuous presence of ionomycin, to quench the dye. Free $[Ca^{2+}]_i$ was estimated according to

$$\text{free } [Ca^{2+}] = [(R - R_{min})/(R_{max} - R)]K_D S_{f2}/S_{b2} \quad (2)$$

where K_D (190 ± 12 nM) is the apparent dissociation constant of the Ca^{2+}-dextran-bound fura-2 complex, and S_{f2}/S_{b2} is the ratio of fluorescence at 380 nm at 0 Ca^{2+} and at saturating Ca^{2+} levels (10.5 ± 0.7). R_{max} (18.9) and R_{min} (0.74) are the 340/380 nm wavelength ratios at saturating and 0 free $[Ca^{2+}]$. Calibration solutions contained (mM): 120 KCl, 20 NaCl, 1.0 $MgCl_2$, and 10 HEPES; these were titrated to pH 7.35 with KOH. The Ca^{2+} buffer EGTA (Fluka Chemical Corp., Ronkonkoma, NY) was used at a concentration of 5 mM (Tsien and Rink, 1981; Harrison and Bers, 1989), and $CaCl_2$ was added to each solution to obtain free $[Ca^{2+}]$'s of 10^{-9} to 10^{-5} M.

The microinjections did not result in significant membrane damage. Cell membrane voltages, fR_a, and apical membrane K^+ selectivity were similar in injected and noninjected cells from the same tissue.

Statistical Analysis

Results are given as mean ± SEM or SD, as indicated. Statistical comparisons were done by *t* tests for paired or unpaired data, as appropriate. A value of $P < 0.05$ was considered significant.

RESULTS

Effects of Lowering [Na⁺]ₛ on Membrane Voltages and fR_a

Fig. 1 illustrates the effects of lowering $[Na^+]_s$ from 100.5 to 10.5 mM (TMA^+ replacement) on transepithelial and cell membrane voltages and fR_a. Decreasing $[Na^+]_s$ caused a serosa-positive change in V_{sm} which reached a plateau in 1–2 min.

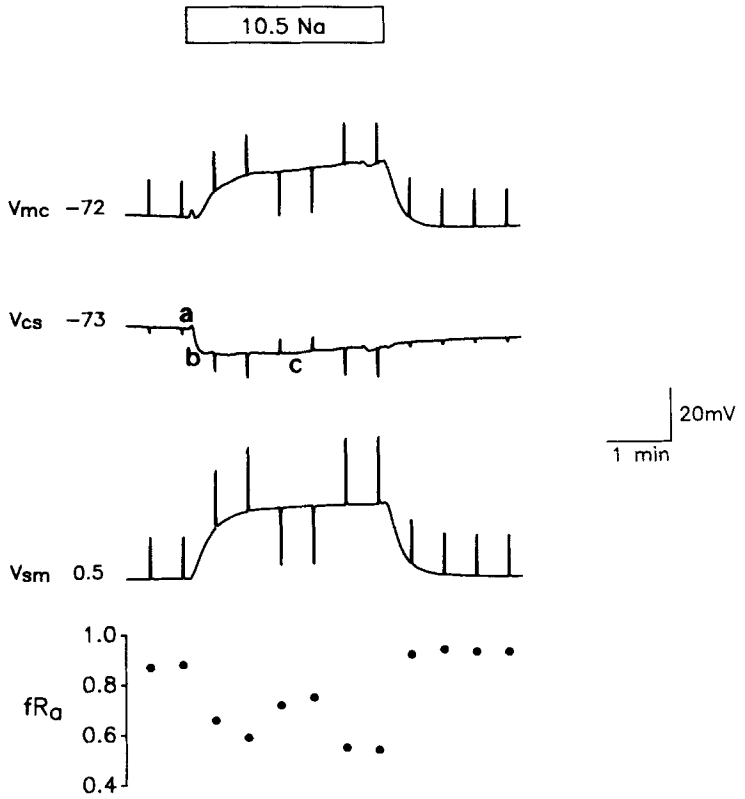

FIGURE 1. Effects of lowering serosal $[Na^+]$ ($[Na^+]_s$) on transepithelial (V_{sm}) and cell membrane voltages (apical = V_{mc}, basolateral = V_{cs}) and the apparent fractional resistance of the apical membrane (fR_a). Initial voltages are indicated at the beginning of the traces. V_{sm} was referred to the mucosal solution, and V_{cs} and V_{mc} were referred to the adjacent bathing solutions. Upward deflections indicate more positive voltage values. The spikes in the voltage records are the result of positive or negative transepithelial current pulses (50 $\mu A/cm^2$ and 2-s duration at 1-min intervals). Note the hyperpolarization of V_{cs} and the decrease in fR_a.

This voltage change is due to a paracellular biionic potential ($P_{Na} \gg P_{TMA}$). The changes in V_{cs} and V_{mc} were more complex and could be arbitrarily divided into three phases, a, b, and c (see Fig. 1). Both V_{cs} and V_{mc} initially depolarized by 0.1–4.2 mV (phase a). Over the next 10–20 s V_{cs} and V_{mc} hyperpolarized (phase b). In all cases the hyperpolarization of V_{cs} was larger than that of V_{mc} and persisted for the duration of

the experiment (phase c), although in most cases it decreased slowly toward control values (see also Figs. 2, 5, and 6). During phase c V_{mc} was always depolarized.

The small initial depolarization of V_{cs} (phase a) is an artifact.[1] The ensuing changes in membrane voltages are complex. The serosa-positive paracellular diffusion potential depolarizes V_{mc} and hyperpolarizes V_{cs} because of intraepithelial current flow; the change in V_{mc} is larger than that in V_{cs} because most of the transcellular electrical resistance is located at the apical membrane (fR_a is 0.84 in control and 0.62 in low-Na^+ Ringer; see Table I). Consistent with this notion, after the initial 20–30 s the change in V_{mc} resembles that of V_{sm}, while V_{cs} appears to be largely independent of V_{sm}. However, the hyperpolarization of V_{cs} during phase b is too large and the concomitant change in V_{mc} is in the wrong direction to be explained by the paracellular diffusion potential. Therefore, these voltage changes must result from changes in the ion transport properties of one or both cell membranes. The effects of decreasing $[Na^+]_s$ on membrane voltages are summarized in Table I.

TABLE I

Effects of Lowering $[Na^+]_s$ on Transepithelial and Cell Membrane Voltages, Fractional Resistance of the Apical Membrane, and Transepithelial Resistance

	V_{sm}	V_{cs}	peak ΔV_{cs}	V_{mc}	fR_a	R_t
	mV	*mV*	*mV*	*mV*		*Ω·cm⁻²*
Control	0.0 ± 0.1	−68 ± 2		−68 ± 1	0.84 ± 0.02	152 ± 6
Low $[Na^+]_s$	15.3 ± 0.7*	−78 ± 2*	−14.2 ± 0.9*	−62 ± 1*	0.62 ± 0.02*	209 ± 9*
Recovery	0.1 ± 0.1	−69 ± 2		−69 ± 1	0.83 ± 0.02	150 ± 7

V_{sm} is the transepithelial voltage, V_{cs} and V_{mc} are the apical and basolateral membrane voltages, respectively, fR_a is the fractional resistance of the apical membrane, and R_t is the transepithelial resistance. Values are means ± SD of n = 67 tissues, except for R_t (n = 65) and fR_a (n = 64). $[Na^+]_s$ was reduced from 100.5 to 10.5 mM (TMA^+ substitution). Peak ΔV_{cs} is the peak hyperpolarization of V_{cs} 15–25 s after reducing $[Na^+]_s$. All the other parameters in low $[Na^+]_s$ were determined at 1 min after the ionic substitution. Recovery values were determined 3–8 min after reexposure to control Ringer. *P < 0.001 compared with control and recovery periods. This table includes the control data from all experiments performed.

As shown in Fig. 1 and summarized in Table I, reducing $[Na^+]_s$ decreases fR_a reversibly. Since R_a is voltage sensitive (García-Díaz et al., 1983; Stoddard and Reuss, 1988*b*) the depolarization of V_{mc} may contribute to the change in fR_a. The magnitude of the apparent change in fR_a depends on the polarity of the transepithelial current pulses (Stoddard and Reuss, 1988*b*), but fR_a decreases with both hyperpolarizing and depolarizing pulses. Further, in ~10% of the experiments in Table I, fR_a was reduced at a time when V_{mc} was still hyperpolarized. In most of the experiments TMA^+ was used to replace Na^+, but similar results were obtained using $NMDG^+$.

[1] The artifact results from the subtraction of V_{sm} (an average measurement of the voltage across the entire preparation) from V_{mc} (the voltage of a single cell in a "patch" of the gallbladder with virtually no adjacent subepithelial tissue). The average change in V_{sm} is less than the change ideally expected in the vicinity of the impaled cell because of subepithelial diffusive delays in the rest of the preparation. Supporting this interpretation, the small depolarization of V_{cs} disappears when the ground electrode is positioned in the serosal bath, allowing for direct measurement of V_{cs}.

The changes in V_{cs} upon lowering $[Na^+]_s$ from 100.5 mM to 70.5, 40.5, 25.5, or 10.5 mM were studied in four tissues (Fig. 2). The magnitudes of the peak hyperpolarization of V_{cs} and the decrease in fR_a were proportional to the extent of the reduction in $[Na^+]_s$.

The hyperpolarization of V_{cs} could be due, in principle, to an increase in gK^+ at the apical and/or basolateral membranes, to the presence of a conductive pathway for Na^+ at the basolateral membrane, to a decrease in basolateral gCl^-, and/or to stimulation of the Na^+ pump. The decrease in fR_a could be explained by a decrease in R_a, an increase in R_b, or a combination of both. The experiments below were

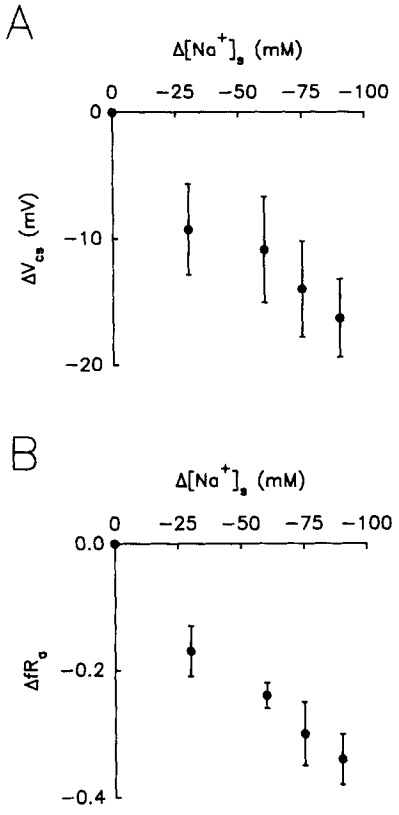

FIGURE 2. Dependence of fR_a and the peak hyperpolarization of V_{cs} on $[Na^+]_s$. The experimental protocol was similar to that in Fig. 1, but $[Na^+]_s$ was reduced to 70.5, 40.5, 25.5, or 10.5 mM. Data at $[Na^+]_s < 100.5$ mM were obtained at the peak hyperpolarization, usually observed 15–25 s after lowering $[Na^+]_s$. Values are means ± SEM ($n = 4$). Symbols as in Fig. 1. All values at low $[Na^+]_s$ are significantly different from control ($P < 0.05$).

aimed to ascertain the mechanisms of the initial hyperpolarization of V_{cs} (phase b) and of the decrease in fR_a produced by lowering $[Na^+]_s$. In addition, we explored the possibility that an elevation in free $[Ca^{2+}]_i$ could play a role in the electrophysiological effects of reducing $[Na^+]_s$.

Effects of TEA⁺

To explore the possible involvement of the TEA^+-sensitive apical membrane conductance in the response to lowering $[Na^+]_s$, we carried out the experiments illustrated in Fig. 3 *A* and summarized in Table II. As shown, TEA^+ reduces the hyperpolarization

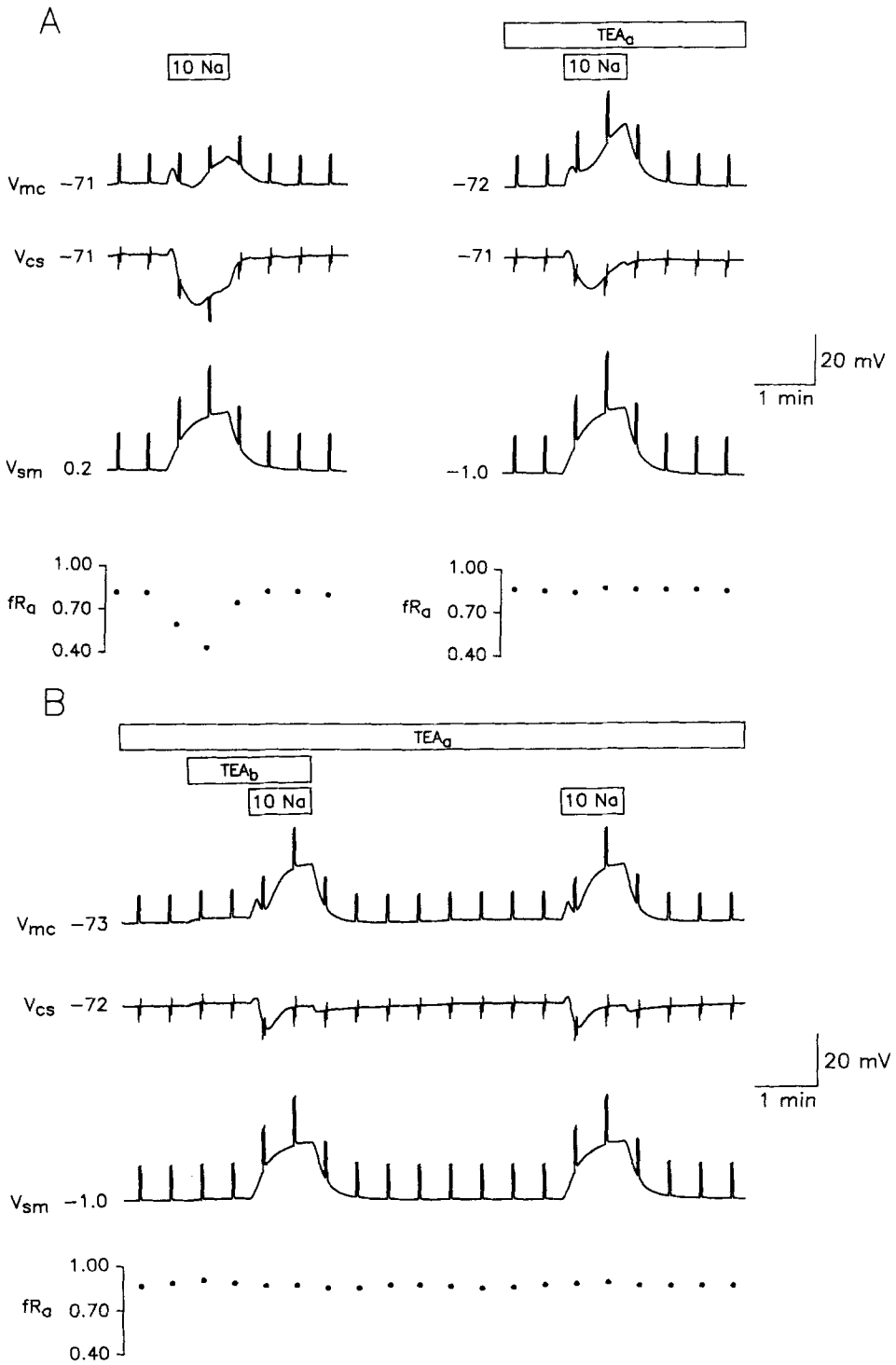
A
10 Na
TEA$_a$
10 Na
V_{mc} −71
−72
V_{cs} −71
−71
20 mV
1 min
V_{sm} 0.2
−1.0
1.00
fR_a 0.70
0.40
1.00
fR_a 0.70
0.40
B
TEA$_a$
TEA$_b$
10 Na
10 Na
V_{mc} −73
V_{cs} −72
20 mV
1 min
V_{sm} −1.0
1.00
fR_a 0.70
0.40

FIGURE 3.

of V_{cs} and abolishes the decrease in fR_a upon reducing $[Na^+]_s$. These results indicate that a TEA^+-sensitive apical membrane conductance is the main contributor to the initial fall in fR_a and accounts for a sizable portion of the hyperpolarization of V_{cs} elicited by lowering $[Na^+]_s$. It must be pointed out that since TEA^+ increases R_a (see below), the intraepithelial current flow is reduced, as well as the contribution of this current to the hyperpolarization of V_{cs}; for the same reason, in TEA^+ the magnitude of the depolarization of V_{mc} increases (see Fig. 3 *A*).

Inasmuch as the change in V_{cs} is not abolished by mucosal exposure to TEA^+, we studied the possibility of a contribution of a TEA^+-sensitive basolateral gK^+ to the V_{cs} hyperpolarization produced by lowering $[Na^+]_s$. To avoid apical membrane effects of serosal TEA^+, we carried out these experiments with 5 mM TEA^+ on the mucosal solution. In this series, lowering $[Na^+]_s$ in the presence of TEA^+ reduced fR_a from 0.85 ± 0.02 to 0.82 ± 0.02 (n = 6). Although this change was significant ($P < 0.05$), its magnitude was small compared with that observed in the absence of TEA^+ (Table

TABLE II

Effects of TEA^+ Addition to the Mucosal Solution on the Electrophysiological Responses to Lowering $[Na^+]_s$

	V_{sm}	V_{cs}	peak ΔV_{cs}	V_{mc}	fR_a	R_t
	mV	*mV*	*mV*	*mV*		*Ω·cm⁻²*
Control	−0.2 ± 0.2	−66 ± 2		−66 ± 2	0.84 ± 0.02	182 ± 15
Low $[Na^+]_s$	15.8 ± 1.4*	−76 ± 2*	−15.4 ± 1.8*	−60 ± 2*	0.58 ± 0.02*	250 ± 21*
Control	0.2 ± 0.2	−68 ± 3		−69 ± 3	0.87 ± 0.03	193 ± 18
TEA_a^+	−0.6 ± 0.2*	−68 ± 2		−68 ± 2	0.91 ± 0.01	188 ± 16
TEA_a^+/Low $[Na^+]_s$	16.2 ± 1.4*	−71 ± 2*‡	−10.5 ± 1.7*‡	−55 ± 3*‡	0.90 ± 0.01‡	273 ± 26*

Values are means ± SEM of n = 16 except for control vs. TEA_a^+ (n = 11). For abbreviations and experimental protocol see Table I and Fig. 4, respectively. Low $[Na^+]_s$ denotes low $[Na^+]$ Ringer (10.5 mM). TEA^+ (5 mM) was added to the mucosal solution (TEA_a^+) 10 min before the measurements. Peak ΔV_{cs} is the peak hyperpolarization of V_{cs} 15–25 s after reducing $[Na^+]_s$. All the other parameters were determined after 1 min of exposure to low $[Na^+]$ Ringer. *$P < 0.05$ or better compared with the preceding condition; ‡$P < 0.05$ compared with the effect of lowering $[Na^+]_s$ under control conditions.

I). Fig. 3 *B* shows that addition of 5 mM TEA^+ to the serosal solution produces a small depolarization of cell membrane voltages (ΔV_{cs} = 1.2 ± 0.4 mV, $P < 0.05$; ΔV_{mc} = 1.8 ± 0.4 mV, n = 6, $P < 0.02$), suggesting that a small TEA^+-sensitive basolateral gK^+ is present under control conditions. Lowering $[Na^+]_s$ in the continuous presence of serosal TEA^+ causes a hyperpolarization of V_{cs} not significantly different from that in its absence. These results rule out that a basolateral membrane

FIGURE 3. (*opposite*) Effects of mucosal and serosal exposure to 5 mM TEA^+ on the responses to lowering serosal $[Na^+]$. All traces were obtained from the same impalement. (*A*) Effects of lowering $[Na^+]_s$ under control conditions and with TEA^+ added to the mucosal solution (TEA_a) 10 min before lowering $[Na^+]_s$. (*B*) Effects of lowering $[Na^+]_s$ in the presence of TEA^+ on both sides and on the mucosal side only. Exposure to mucosal TEA^+ reduces the hyperpolarization of V_{cs} and prevents the decrease in fR_a. Serosal TEA^+ (TEA_b) produces a small depolarization of cell membrane voltages but does not affect the response to lowering $[Na^+]_s$.

TEA^+-sensitive gK^+ contributes to the change of V_{cs} upon lowering $[Na^+]_s$. The small changes in V_{sm} produced by application of mucosal (Table II) and serosal TEA^+ (not shown) are most probably the result of paracellular biionic potentials.

Effects of Ouabain

In *Necturus* gallbladder epithelium bathed with 10 mM HCO_3^-/1% CO_2, exposure to 10^{-4} M ouabain on the serosal side depolarizes cell membrane voltages by $\simeq 3$ mV in $\simeq 15$ s (Baerentsen et al., 1982; Stoddard and Reuss, 1989*a*), suggesting that the Na^+ pump is electrogenic and/or that its inhibition leads to a rapid change in transmembrane ionic gradients, i.e., a fall in E_K (Stoddard and Reuss, 1989*a*). Since the activity

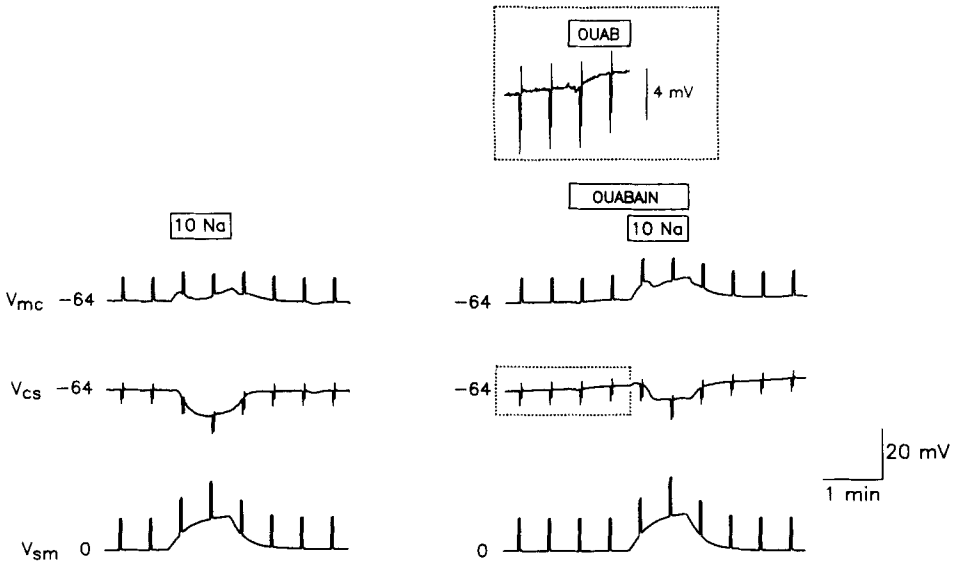

FIGURE 4. Effects of serosal exposure to 10^{-4} M ouabain on the responses to lowering serosal $[Na^+]$. (*Left*) Control. (*Right*) Exposure to ouabain from 1 min before lowering $[Na^+]_s$. Traces are from the same cell. Ouabain depolarized V_{cs} (see amplified trace, top right) and reduced the hyperpolarization of V_{cs} upon lowering $[Na^+]_s$.

of the Na^+ pump is increased by decreasing extracellular $[Na^+]$ (Garrahan and Glynn, 1967; Nakao and Gadsby, 1989), the Na^+ pump could contribute to the hyperpolarization of V_{cs} upon lowering $[Na^+]_s$. Therefore, we tested this possibility by measuring the ouabain-induced depolarization of V_{cs} under control conditions and upon lowering $[Na^+]_s$. Fig. 4 shows that 10^{-4} M ouabain depolarizes V_{cs} by a few millivolts. When $[Na^+]_s$ was reduced after 1 min of exposure to ouabain (in the continuous presence of the steroid), the peak hyperpolarization of V_{cs} was significantly less than in the absence of ouabain. In contrast, the change in fR_a was not affected (Table III). To confirm the significance of the small effect of ouabain on the peak hyperpolarization of V_{cs}, we compared the ouabain-induced depolarization in control and low-Na^+ media. In 11 experiments, lowering $[Na^+]_s$ hyperpolarized V_{cs} as described above. After 2 min, a 1-min addition of 10^{-4} M ouabain to the low-$[Na^+]_s$ Ringer depolarized

V_{cs} from −74 ± 2 to −69 ± 2 mV (ΔV_{cs} = 5.0 ± 1.5 mV, $P < 0.01$). In the same tissues, addition of ouabain to the control Ringer depolarized V_{cs} less than in low-$[Na^+]_s$ Ringer, from −71 ± 2 to −69 ± 2 mV (ΔV_{cs} = 1.3 ± 0.4 mV, $P < 0.05$ compared with low-$[Na^+]_s$ Ringer). In these experiments, the 1-min exposure to ouabain was reversible as assessed from the absence of continuous depolarization that was observed with longer exposure times.

The effects of ouabain and mucosal solution TEA^+ appear to be produced by different mechanisms, since TEA^+ prevents the effect of lowering $[Na^+]_s$ on fR_a, whereas ouabain does not. To test this hypothesis directly, the combined effects of both agents were studied. In 10 experiments apical solution TEA^+ alone reduced the peak hyperpolarization of V_{cs} by 25 ± 4% (4.7 ± 1.7 mV), whereas apical TEA^+ and ouabain reduced the voltage change significantly more, by 44 ± 8% (8.2 ± 2.6 mV, $P < 0.05$ compared with TEA^+ alone).

TABLE III

Effects of Serosal Addition of Ouabain on the Electrophysiological Responses to Lowering $[Na^+]_s$

	V_{sm}	V_{cs}	Peak ΔV_{cs}	V_{mc}	fR_a	R_t
	mV	*mV*	*mV*	*mV*		*Ω·cm⁻²*
Control	0.0 ± 0.1	−71 ± 2		−71 ± 2	0.79 ± 0.03	136 ± 9
Low $[Na^+]_s$	14.3 ± 1.3*	−78 ± 2*	−12.4 ± 2.2*	−64 ± 2*	0.61 ± 0.02*	191 ± 19*
Control	−0.2 ± 0.1	−71 ± 2		−72 ± 2	0.78 ± 0.03	137 ± 10
Ouabain	−0.2 ± 0.1	−70 ± 2*	1.7 ± 0.3*	−71 ± 2*	0.78 ± 0.03	141 ± 10
Ouabain/low $[Na^+]_s$	14.7 ± 1.4*	−75 ± 2*‡	−10.0 ± 2.5*†	−61 ± 2*‡	0.64 ± 0.02*‡	199 ± 19*‡

Values are means ± SEM of n = 11 tissues. For abbreviations and details on experimental protocol see Table I and Fig. 5, respectively. Ouabain (10^{-4} M) was added to the serosal solution and after 1 min $[Na^+]_s$ was reduced in the continued presence of ouabain. Peak ΔV_{cs} is the peak change of V_{cs} 15–25 s after reducing $[Na^+]_s$ or adding ouabain as indicated. All the other values were measured 1 min after reducing $[Na^+]_s$. *$P < 0.05$ compared with the preceding condition; †$P < 0.05$ or better compared with the effect of lowering $[Na^+]_s$ in the control condition.

Effects of Amiloride and Tetrodotoxin

To test for a possible contribution of an amiloride-sensitive basolateral membrane Na^+ conductance to the V_{cs} hyperpolarization by lowering $[Na^+]_s$, we evaluated the effects of reducing $[Na^+]_s$ in the presence and absence of 10^{-5} M amiloride on the serosal side. At this concentration, amiloride is a highly specific blocker of epithelial Na^+ channels (Benos, 1982, 1988). Fig. 5 *A* shows that amiloride did not affect membrane voltages and did not change the V_{cs} and fR_a responses to lowering $[Na^+]_s$.

Tetrodotoxin (TTX) blocks Na^+ channels in excitable cells and recently TTX-sensitive Na^+ channels have been described in epithelia (Fain and Farahbakhsh, 1989). Therefore, we tested for their possible existence and potential role in the hyperpolarization of V_{cs} upon lowering $[Na^+]_s$. Fig. 5 *B* shows that serosal addition of 10^{-6} M TTX did not affect V_{cs} or its response to lowering $[Na^+]_s$.

Effects of Lowering $[Na^+]_s$ on the V_{cs} Response to Changes in Serosal Solution $[K^+]$

Since basolateral gK^+ seems to be increased by elevating cytosolic free $[Ca^{2+}]$ (Bello-Reuss et al., 1981), we explored the possible contribution of an increase in the partial gK^+ of the basolateral membrane to the hyperpolarization of V_{cs}. This was

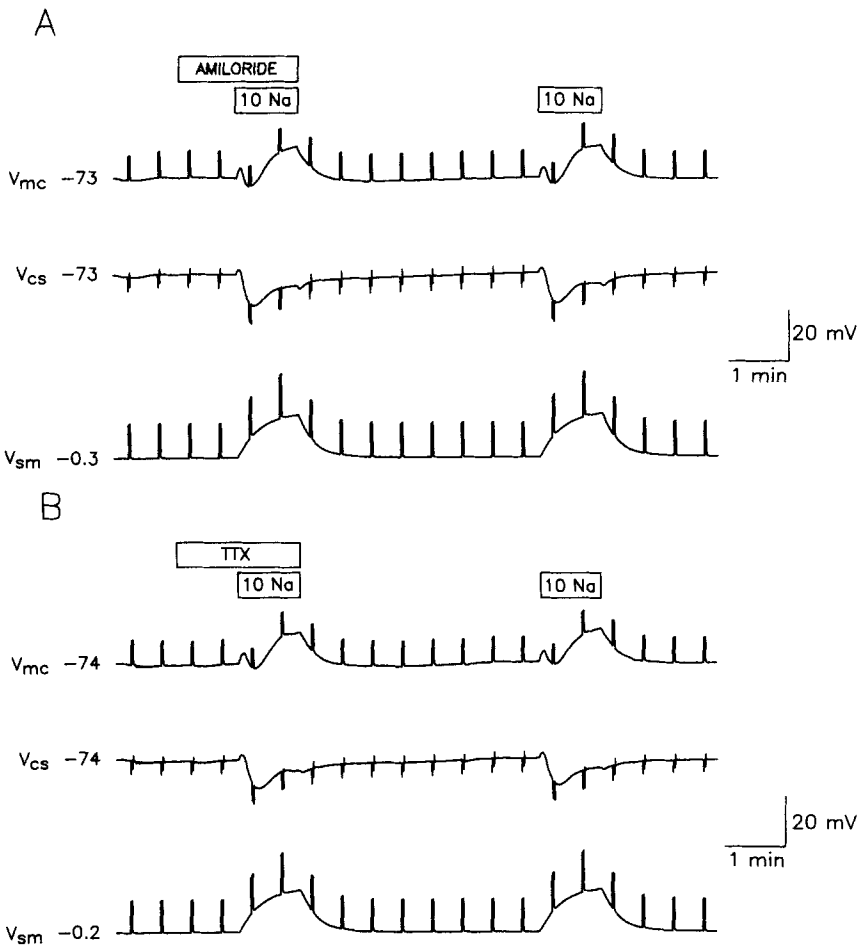

FIGURE 5. Effects of 10^{-5} M amiloride (*A*) and 10^{-6} M TTX (*B*) on the electrophysiological responses to lowering $[Na^+]_s$. Both drugs were added to the serosal side only. All traces were obtained from the same impalement. Amiloride and TTX had no effects under control conditions or on the response to lowering $[Na^+]_s$. Similar results were obtained in 10 experiments with amiloride and 6 experiments with TTX.

tested by measuring the depolarization of V_{cs} produced by a 10-fold increase in serosal solution $[K^+]$. To prevent changes in apical membrane gK^+, 5 mM TEA^+ was added to the apical side. Under these conditions, fR_a remains high (see Table II) and V_{cs} approaches E_b (see Reuss and Finn, 1975*b*; Stoddard et al., 1990). In these experiments, $[Na^+]_s$ was first reduced from 100.5 to 10.5 mM and then serosal $[K^+]$

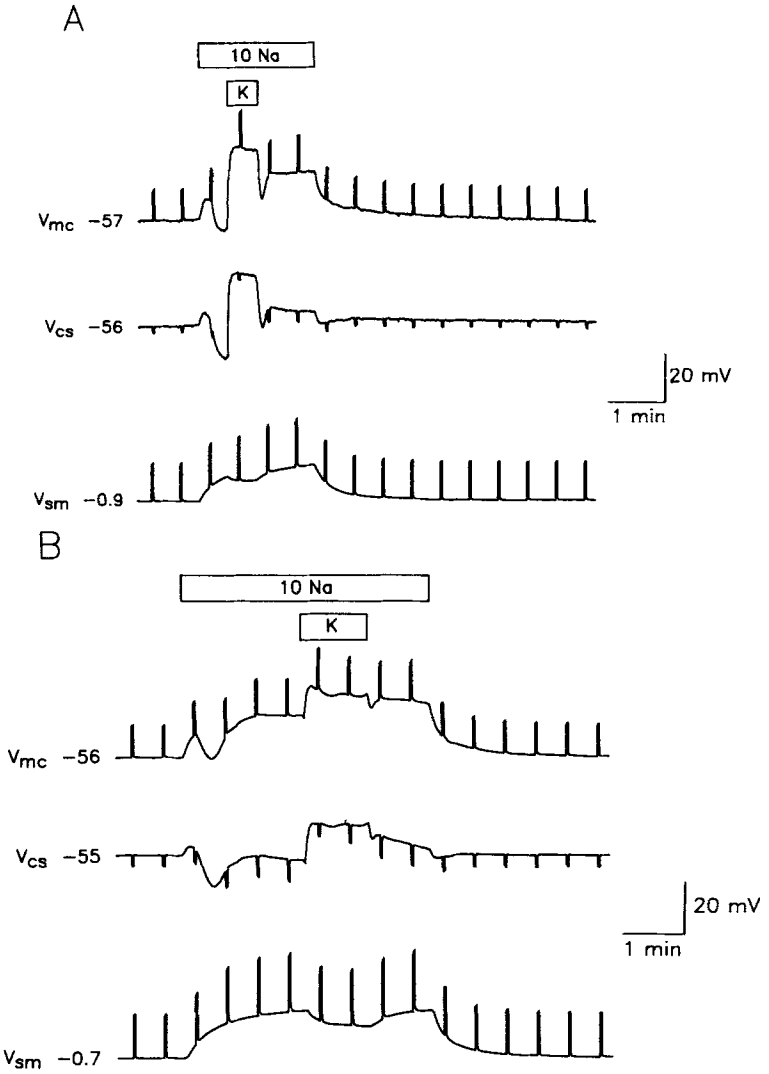

FIGURE 6. Effects of lowering $[Na^+]_s$ on the membrane voltage changes upon elevating serosal solution $[K^+]$. 5 mM TEA^+ was present throughout in the mucosal solution to minimize the changes in fR_a (see text). (*A*) Serosal $[K^+]$ was increased from 2.5 to 25 mM at the peak of the hyperpolarization of V_{cs}. (*B*) The experimental protocol was similar to that in *A*, but serosal $[K^+]$ was elevated 2 min after reducing $[Na^+]_s$. Note that the depolarization of V_{cs} upon increasing $[K^+]$ is greater at the peak hyperpolarization than $\simeq 1.5$ min later, when V_{cs} is closer to the control value.

was increased from 2.5 to 25 mM, either at the peak hyperpolarization (usually 15–25 s after lowering $[Na^+]_s$) or once V_{cs} had repolarized to a value close to that in control Ringer (~ 2 min after lowering $Na^+]_s$). Fig. 6 shows that the V_{cs} depolarization upon increasing serosal $[K^+]$ was larger at the peak of V_{cs} hyperpolarization

compared with the value obtained 2 min after reducing $[Na^+]_s$. In four tissues, the depolarization of V_{cs} produced by increasing $[K^+]$ at the peak of the hyperpolarization was 33 ± 5 mV, a value significantly larger than that obtained 2 min after lowering $[Na^+]_s$ (20 ± 3 mV, $P < 0.05$). In four additional experiments no difference was found between the depolarization of V_{cs} produced by elevating serosal $[K^+]$ to 25 mM in control conditions and 2 min after lowering $[Na^+]_s$ (ΔV_{cs} = 30 ± 2 and 30 ± 3 mV, respectively).

Effects of Lowering $[Na^+]_s$ on Cell Membrane Resistances

At the basolateral membrane, gK^+ and gCl^- are the main conductances (Stoddard and Reuss, 1989*b*). To determine which was the dominant change in conductance responsible for the changes in selectivity shown above, we performed two-point cable

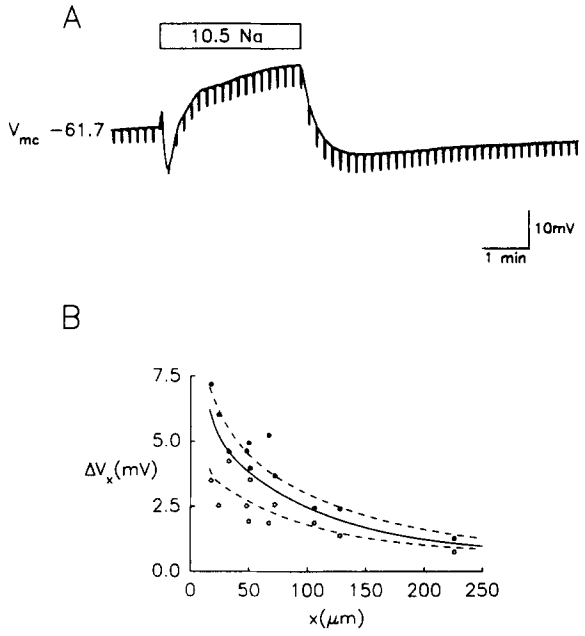

FIGURE 7. Effect of lowering $[Na^+]_s$ on intraepithelial current spread. (*A*) Negative pulses of 11 nA and 1-s duration were injected into a cell, and the changes in cell membrane voltages (ΔV_x) induced by the applied current (I_0) were measured in another cell ≈70 μm distant. Note that ΔV_x reversibly falls at the peak of the hyperpolarization, and later increases above control values. (*B*) Data were obtained from traces such as that in *A*. Lines are fits to the Bessel function K_0. Control data were fit to a curve characterized by parameter A = 2.1 mV and space constant λ = 266 μm (solid line); the normalized distance x was thus assigned to each point (see text). Data obtained 20 s (open circles) and 180 s (filled circles) after lowering $[Na^+]_s$ (dashed lines) were fit to the same function and yielded A = 1.2 mV and λ = 408 μm at 20 s, and A = 2.3 mV and λ = 304 μm at 180 s. Changes in cell membrane voltage were reduced by 28 ± 5% at 20 s ($P < 0.001$), and increased by 20 ± 5% at 180 s ($P < 0.005$).

analysis experiments (see Methods). The trace in Fig. 7 *A* was obtained from a cell ≈70 μm away from the current injection point, and illustrates the changes in V_x produced by exposure to low-$[Na^+]_s$ Ringer. 11 such observations at varying distances in seven tissues were pooled and normalized to average results under the same conditions (see Methods). The voltage deflections (ΔV_x) under control conditions, 20 and 180 s upon reducing $[Na^+]_s$, are shown in Fig. 7 *B* with their corresponding fits to the Bessel function K_0 (Frömter, 1972). The trace in Fig. 7 *A* shows that ΔV_x initially fell, later increased above control, and finally returned slowly to control levels upon

TABLE IV

Effects of Lowering [Na⁺]ₛ on Apical (R_a) and Basolateral (R_b) Membrane Resistances, and the Equivalent Resistance of the Apical and Basolateral Membrane Resistances in Parallel (R_z)

	Control	Low Na⁺		Recovery
		20 s	180 s	
R_a, $\Omega \cdot cm^2$	7,150	3,680	2,810	7,250
R_b, $\Omega \cdot cm^2$	1,070	1,810	2,590	1,080
R_z, $\Omega \cdot cm^2$	930	1,210	1,350	940
fR_a	0.87	0.67	0.57	0.87

Data were obtained during continuous impalements across the basolateral membrane before, during, and after exposure to 10.5 mM [Na⁺]ₛ (7 tissues, 11 measurements). Values of R_z were calculated from the fit of the two-dimensional cable equation to the data (see text). Control data were fit to a curve characterized by A = 2.1 mV and λ = 266 μm. The distance from the current injection site was thus normalized for each impalement. R_a and R_b were calculated from R_z and fR_a. fR_a values were taken from the experiments in Table I.

reexposure to control Ringer. The changes in ΔV_x were $-28 \pm 5\%$ ($P < 0.001$) and $20 \pm 5\%$ ($P < 0.005$) 20 and 180 s after lowering $[Na^+]_s$, respectively. From R_z and fR_a, R_a and R_b were estimated according to: $R_a = R_z/(1 - fR_a)$, and $R_b = R_z/fR_a$. The observed decrease in R_a (see Table IV) supports our conclusion of a role for apical maxi K^+ channels in the response to lowering $[Na^+]_s$. As indicated above, at the peak of V_{cs} hyperpolarization ΔV_x is reduced, λ is increased from 266 to 408 μm, and parameter A is reduced from 2.1 to 1.2 mV. The latter effect suggests an increase in cell-to-cell coupling resistance, perhaps due to the increase in free $[Ca^{2+}]_i$ (De Mello, 1987; see below).

Effect of Lowering [Na⁺]ₛ on Intracellular Free [Ca²⁺]

Changes in intracellular free $[Ca^{2+}]$ (free $[Ca^{2+}]_i$) were determined in six cells from different gallbladders microinjected with 1.7 mM dextran-bound fura-2 (see Methods). Shortly after lowering $[Na^+]_s$, the 340/380- nm excitation ratio rises and reaches a peak at 38 ± 5 s. Thereafter, the ratio returns to lower values, but always remains above control levels. Two examples are presented in Fig. 8. In four of the six cells

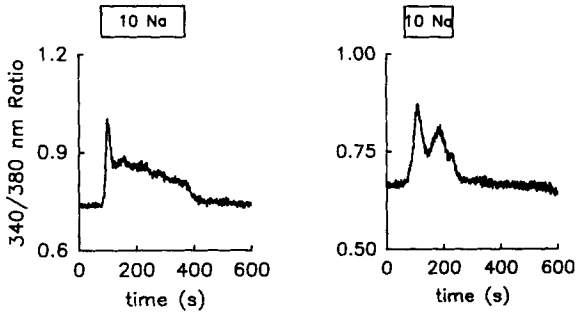

FIGURE 8. Effect of lowering $[Na^+]_s$ on the 340/380 nm excitation ratio of dextran-bound fura-2. Two traces are shown as examples of the increase in the 340/380 nm ratio upon lowering $[Na^+]_s$. Note that there is a rapid increase in the ratio followed by partial recovery toward the control value. In most experiments, increases in the 340/380 nm ratio were observed after the initial peak (left trace). For additional details see text.

studied the ratio underwent a second peak or actually oscillated. Based on the calibration in vitro of dextran-bound fura-2 and using a viscosity correction factor of 20% (Rink, 1988), free $[Ca^{2+}]_i$ increased from 41 ± 13 nM to a peak value of 81 ± 13 nM ($P < 0.005$), and remained above control 180 s after lowering $[Na^+]_s$ (54 ± 12 nM, $P < 0.005$). After 1–3 min in control Ringer, free $[Ca^{2+}]_i$ was similar to the levels determined before reducing $[Na^+]_i$ (41 ± 13 nM). In addition to the known limitations of the method (Grynkiewicz et al., 1985; Rink, 1988), we acknowledge several problems with our free $[Ca^{2+}]_i$ estimations: (*a*) The calculated free $[Ca^{2+}]$, 41 ± 13 nM, is lower than the values reported using Ca^{2+}-sensitive microelectrodes (Palant and Kurtz, 1987). (*b*) The preparation exhibits considerable autofluorescence when excited with ultraviolet light. This was somewhat reduced by dissecting away most of the subepithelial connective tissue, but autofluorescence remained a problem; the signal to autofluorescence ratio under control conditions was similar at 340 and 380 nm, and averaged 2.6 ± 0.3. This ratio could be increased by microinjecting more dextran-bound fura-2 (5 mM, see Methods), but under these conditions the 340/380 nm excitation ratio peaked later (64 ± 11 s, $n = 6$, $P < 0.05$ compared with microinjection of 1.7 mM dextran-bound fura-2) and no oscillations were observed, suggesting that Ca^{2+} was buffered by the microinjected dye. (*c*) The estimated changes in free $[Ca^{2+}]_i$ upon adding 10–20 μM ionomycin were less than expected. The 340/380 nm excitation ratio increased from 0.91 ± 0.10 to 1.73 ± 0.23, which corresponds to a free $[Ca^{2+}]_i$ of 170 ± 37 nM ($n = 10$). This result suggests that ionomycin does not equilibrate intra- and extracellular free $[Ca^{2+}]$ and cannot be used to perform an in situ calibration of the Ca^{2+}-sensitive dye. Because of the uncertainty on the actual free $[Ca^{2+}]_i$ levels, we emphasize only the qualitative response to lowering $[Na^+]_s$.

As indicated in Methods, only one side of the tissue could be superfused. Inasmuch as the *Necturus* gallbladder epithelium has a high paracellular permeability, it is possible that changes in the $[Na^+]$ in the static mucosal fluid layer can be responsible for the increase in free $[Ca^{2+}]_i$. Therefore, in two experiments we superfused the mucosal side, keeping a static thin layer of solution on the serosal surface. Under these conditions, lowering mucosal $[Na^+]$ produces a late (>50 s latency), and very slow increase in free $[Ca^{2+}]_i$ of 12 nM at 180 s after lowering $[Na^+]$. These results indicate that the rapid elevation in free $[Ca^{2+}]_i$ upon lowering $[Na^+]_s$ is specific for the serosal side.

Effects of Lowering $[Na^+]_s$ on aNa_i and pH_i

Lowering $[Na^+]_s$ might produce changes in intracellular Na^+ activity (aNa_i) and/or pH_i that could be responsible for the increases in apical gK^+ and Na^+ pump activity (Eaton et al., 1984; Oberleithner et al., 1988; Copello et al., 1991). If there are significant basolateral membrane Na^+ pathways sensitive to $[Na^+]_s$, then aNa_i should be reduced. In fact, as illustrated in Fig. 9 *A*, lowering $[Na^+]_s$ reduces aNa_i. After 3 min of exposure to low Na^+, aNa_i fell by 1.8 ± 0.3 mM ($P < 0.02$, see Table V). Fig. 9 *B* depicts the effect of lowering $[Na^+]_s$ on pH_i. Intracellular pH fell over 3 min by 0.06 ± 0.02 ($P < 0.02$, see Table V). The mechanisms responsible for these changes in aNa_i and pH_i were not studied further, but decreases in aNa_i and pH_i would inhibit

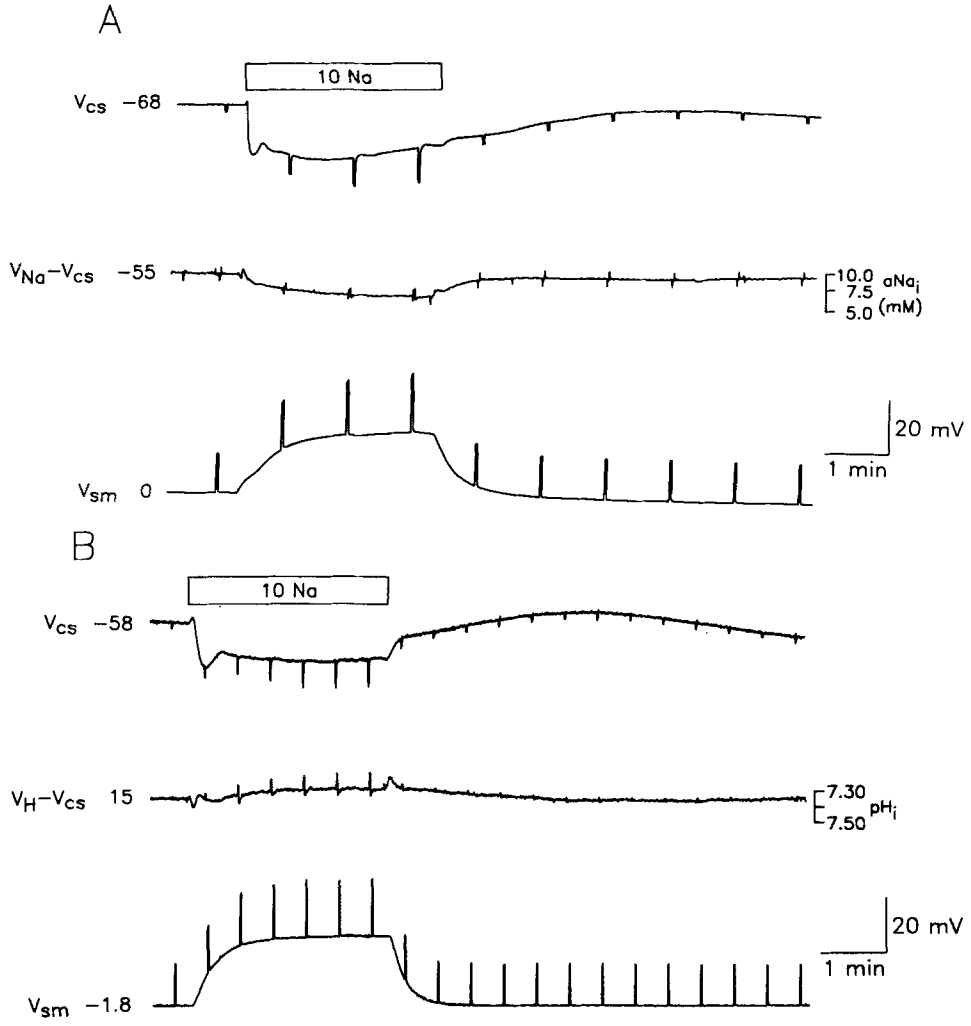

FIGURE 9. Effects of lowering $[Na^+]_s$ on intracellular Na^+ activity (aNa_i, panel *A*), and intracellular pH (pH_i, panel *B*). $V_{Na} - V_{mc}$ is the difference between the voltages measured with the Na^+-sensitive barrel and the conventional barrel of the microelectrode, and $V_{pH} - V_{mc}$ is the difference between the voltage output of the pH-sensitive and the conventional microelectrodes. The experiments were done in different tissues. Lowering $[Na^+]_s$ reduced both aNa_i and pH_i.

the Na^+ pump and the cell acidification would reduce, not increase gK^+ (Eaton et al., 1984; Oberleithner et al., 1988; Copello et al., 1991).

DISCUSSION

These studies show that reducing $[Na^+]_s$ results in complex changes in membrane voltages in *Necturus* gallbladder epithelium. Regardless of some differences from

tissue to tissue, attributable to the several mechanisms involved, a pattern is evident. Lowering $[Na^+]_s$ increases free $[Ca^{2+}]_i$, hyperpolarizes V_{cs}, decreases R_a, and increases R_b.

Evidence against Basolateral Na^+ Channels

Stoddard and Reuss (1989*b*) calculated that in *Necturus* gallbladder epithelial cells bathed with 10 mM HCO_3^-/1% CO_2 Ringer solution, gCl^-, and gK^+ contribute ≃50% each to the basolateral membrane conductance. Further, E_{Na} is far removed from E_b, suggesting that if a basolateral Na^+ membrane conductance exists, it is very small (Stoddard and Reuss, 1989*b*). The experiments with pharmacological inhibitors of Na^+ channels support this idea. Neither amiloride nor TTX had any effects on cell membrane voltages or on the hyperpolarization of V_{cs} elicited by lowering $[Na^+]_s$. We conclude that it is unlikely that basolateral Na^+ channels contribute to the membrane voltage under control conditions, or to the hyperpolarization of V_{cs} produced by lowering $[Na^+]_s$.

TABLE V

Effect of Lowering $[Na^+]_s$ on Intracellular Na^+Activity (aNa_i) and Intracellular pH (pH_i)

	V_{sm}	V_{cs}	aNa_i	pH_i
	mV	*mV*	*mM*	
Control	−0.2 ± 0.3	−65 ± 4	11.2 ± 3.1	7.37 ± 0.03
Low $[Na^+]_s$	18.4 ± 1.0*	−80 ± 4*	9.3 ± 2.8*	7.31 ± 0.03*
Recovery	−0.4 ± 0.3	−68 ± 4	10.9 ± 2.8	7.36 ± 0.03

Values in low $[Na^+]_s$ Ringer were determined after 3 min of reducing $[Na^+]$ from 100.5 to 10.5 mM (TMA^+ substitution). Recovery values were measured 4–8 min after reexposure to control Ringer. aNa_i and pH_i were measured in different tissues (n = 4 and n = 7, respectively). Voltage values were not different between both series of experiments and were pooled. Values are means ± SEM. *P < 0.05 or better compared with control and recovery periods. For abbreviations and experimental protocols see Table I and Fig. 9, respectively.

Mechanism of the Decrease in fR_a

Lowering $[Na^+]_s$ produces a dramatic decrease in fR_a. The two-point cable analysis experiments indicate that R_a decreases and R_b increases. The decrease in R_a is the dominant mechanism, at least initially. The contribution of the increase in R_b is evident at 20 s and much greater at 180 s after lowering $[Na^+]_s$. The decrease in fR_a upon lowering $[Na^+]_s$ is substantially reduced by 5 mM TEA^+, suggesting that the change in fR_a is brought about by activation of apical membrane maxi K^+ channels (Segal and Reuss, 1990*a*, *b*). The simplest explanation is that when serosal solution Na^+ is partially replaced by TMA^+ or $NMDG^+$, the serosa-positive paracellular diffusion potential causes apical membrane depolarization and hence activates the apical maxi K^+ channels. However, two arguments indicate that depolarization of V_{mc} is not the main mechanism accounting for the decrease in fR_a: First, the fall in fR_a is also observed in experiments in which V_{mc} is near control values, or even hyperpolarized at the time of the fR_a measurements (see Results). Second, when similar V_{mc}

depolarizations are produced by lowering serosal solution pH or increasing serosal $[K^+]$, a much smaller decrease in fR_a is observed (i.e., 0.05 ± 0.02 by lowering HCO_3^-, $n = 6$ [data not shown], and 0.19 ± 0.03 by lowering $[Na^+]_s$ in 10 mM HCO_3^--Ringer [see Stoddard and Reuss, 1989*b*]). Elevations of cytosolic free $[Ca^{2+}]$ also activate apical membrane maxi K^+ channels (Segal and Reuss, 1990*a*) and increase apical membrane gK^+ (Bello-Reuss et al., 1981; García-Díaz et al., 1983). Since lowering $[Na^+]_s$ increases free $[Ca^{2+}]_i$, it seems reasonable to conclude that the latter is the main cause of the initial decrease in R_a. The depolarization of V_{mc} may contribute to the fall in R_a, especially when free $[Ca^{2+}]_i$ is below peak values (Fig. 1, phase c).

In several cell types, lowering external $[Na^+]$ raises free $[Ca^{2+}]_i$ by reducing Ca^{2+} efflux through the Na^+/Ca^{2+} exchanger (Grinstein and Erlij, 1978; see also Chase, 1984 and Lorenzen et al., 1984) and/or by Ca^{2+} release from intracellular stores (Smith et al., 1989). We have not studied the mechanism of the elevation in free $[Ca^{2+}]_i$, but it was recently reported that replacing serosal solution Na^+ with $NMDG^+$ in *Necturus* gallbladder produced an increase in free $[Ca^{2+}]_i$ qualitatively similar to the change reported here (Dillard and Finn, 1991). Based on its dependence on the extracellular $[Ca^{2+}]$, the early rise in free $[Ca^{2+}]_i$ was thought to be due to release from intracellular stores, and the sustained elevation to Ca^{2+} influx (Dillard and Finn, 1991).

Mechanism of the Hyperpolarization of V_{cs}

The present results indicate that the mechanism of the hyperpolarization of V_{cs} by lowering $[Na^+]_s$ is complex and that the relative contributions of different factors vary with time. The late phase of the hyperpolarization of V_{cs} (phase c) is mainly caused by: (*a*) the fall in fR_a and the elevation in intraepithelial current flow (due to both the change in V_{sm} and the decrease in transcellular resistance), and (*b*) the increase in TEA^+-sensitive apical gK^+. This conclusion is based on the effects of apical TEA^+, which reduces the plateau hyperpolarization of V_{cs} by $\simeq 70\%$ while reducing the peak hyperpolarization by only $\simeq 30\%$. The mechanisms of the peak hyperpolarization of V_{cs} (phase b) are discussed below.

The TEA^+-sensitive apical K^+ channels (via increases in E_a and intraepithelial current flow, and a decrease in fR_a) account for about one-third of the hyperpolarization of V_{cs}; therefore, their activation clearly is not the only mechanism involved. Addition of ouabain during exposure to low $[Na^+]_s$ medium caused a larger depolarization than under control conditions. An increased contribution of the Na^+ pump current to V_{cs} is expected even in the absence of pump stimulation because of the increase in R_b, but stimulation of the pump (Nakao and Gadsby, 1989) could also contribute to the hyperpolarization of V_{cs}. During exposure to low-$[Na^+]_s$ in the continuous presence of mucosal TEA^+, the K^+ selectivity of the basolateral membrane increases transiently. The increase in basolateral K^+ selectivity, together with an increase in R_b, strongly suggests that a decrease in basolateral gCl^- is the dominant mechanism of the change in K^+ selectivity. The possibility of a concomitant increase in basolateral gK^+, brought about by the elevation in free $[Ca^{2+}]_i$, seems reasonable since A23187 and cyanide increase basolateral gK^+ in *Necturus* gallbladder epithelial cells (Bello-Reuss et al., 1981). 2 min after lowering $[Na^+]_s$ basolateral K^+ selectivity

returns to control and R_b is even greater than at the peak of the hyperpolarization of V_{cs} (Table IV), suggesting that, at the basolateral membrane, both gK^+ and gCl^- are reduced. The mechanism of the late fall in basolateral gK^+ is unknown. The effects of low $[Na^+]_s$ on basolateral membrane ionic conductances could be related to a decrease in cell volume produced by KCl efflux (via apical maxi K^+ channels and basolateral Cl^- channels). However, preliminary results indicate that there are no measurable changes in cell volume 20 s after lowering $[Na^+]_s$. Alternatively, the decrease in basolateral gCl^- may be due to the hyperpolarization of V_{cs} or the increase in free $[Ca^{2+}]_i$. Cl^- channels inactivated by hyperpolarization and elevation in free $[Ca^{2+}]_i$ have been described (Akaike, 1990; Weiss and Magleby, 1990), but the properties of the Cl^- conductive pathway(s) at the basolateral membrane of *Necturus* gallbladder epithelium are not known.

Summary and Conclusions

Lowering $[Na^+]_s$ produces a marked decrease in fR_a and hyperpolarization of V_{cs}. The dominant effect accounting for the decrease in fR_a is a rapid reduction in R_a due to activation of TEA^+-sensitive maxi K^+ channels at the apical membrane; there is also the contribution of an increase in R_b, which becomes more important at later times. The increase in free $[Ca^{2+}]_i$ seems to play a central role in the activation of apical membrane maxi K^+ channels. Several factors participate in the hyperpolarization of V_{cs}: (*a*) activation of the TEA^+-sensitive maxi K^+ channels responsible for the decrease in fR_a, (*b*) increase in the ouabain-sensitive fraction of V_{cs}, (*c*) transient TEA^+-insensitive increase in the K^+ selectivity of the basolateral membrane mainly due to a decrease in basolateral gCl^-, and (*d*) increase in intraepithelial current flow due to the serosa-positive change in V_{sm}.

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