

# Behavioral and Neural Responses of Toads to Salt Solutions Correlate with Basolateral Membrane Potential of Epidermal Cells of the Skin

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## Abstract

Dehydrated toads initiated water absorption response (WR) behavior and absorbed water from dilute NaCl solutions. With 200–250 mM NaCl, WR behavior and water absorption were both suppressed. With 200–250 mM Na-gluconate, WR initiation was significantly greater than with NaCl but water loss was greater. Neural recordings from spinal nerve #6 showed a greater integrated response to 250 mM NaCl than to 250 mM Na-gluconate, whereas a larger rinse response was seen with Na-gluconate. Studies with isolated epithelium showed a large increase in conductance ( $G_t$ ) when 250 mM NaCl replaced NaCl Ringer's as the apical bathing solution that was accompanied by depolarization of the transepithelial potential ( $V_t$ ) and basolateral membrane potential ( $V_b$ ). Depolarization of  $V_b$  corresponded with the neural response to 250 mM NaCl. When 250 mM Na-gluconate replaced Ringer's as the apical solution  $G_t$  remained low,  $V_b$  transiently hyperpolarized to values near the equilibrium potential for  $K^+$  and corresponded with the reduced neural response. These results support the hypothesis that chemosensory function of the skin is analogous to that of mammalian taste cells but utilizes paracellular ion transport to a greater degree.

**Key words:** behavior, chemosensation, NaCl, toad skin

## Introduction

In contrast with other vertebrate classes, amphibians obtain water by absorption across their skin and rely on chemosensory mechanisms in the skin to assess the ionic and osmotic properties of hydration sources (Brekke et al. 1991; Hoff and Hillyard 1993). Terrestrial species such as toads in the genus *Bufo* have a region of the ventral skin termed the seat patch that is specialized for water absorption, and they display a behavior termed the water absorption response (WR) in which the hindlimbs are abducted to maximize the area of the seat patch in contact with hydration sources (Hillyard et al. 1998). Dehydrated toads will consistently initiate the WR with NaCl at concentrations as high as 100 mM and can rehydrate from dilute solutions at rates equal to or greater than from deionized water (DI; Sullivan et al. 2000; Hillyard and Larsen 2001; Hillyard et al. 2007). On the other hand, toads reject hyperosmotic NaCl (250 mM) and KCl (200 mM) solutions (Hoff and Hillyard 1993; Hillyard et al. 2004). Amiloride, in concentrations as low as 10  $\mu$ M, partially restored the WR on NaCl but not KCl solutions, suggesting that epithelial  $Na^+$  channels in the apical membrane of epidermal cells in the skin serve a chemosensory function analogous

to those in taste cells of the mammalian tongue (Hoff and Hillyard 1993; Nagai et al. 1999).

Amiloride sensitivity of salt taste in the mammalian tongue has been extensively studied in rats and varies according to a variety of factors that include postnatal salt exposure, conditioned responses, and salt depletion (Formaker and Hill 1990; Hill et al. 1990; Roitman and Bernstein 1999; Brot et al. 2000). Brot et al. (2000) found that control, water-deprived, and salt-depleted rats readily accepted 100 mM NaCl and that amiloride reduced the acceptance of this solution in salt-depleted animals. Aversion to NaCl solutions was seen above 200 mM for control and water-deprived rats and 300 mM for salt-depleted rats (all salt solutions also contained 150 mM sucrose). Neurophysiological recordings from the chorda tympani nerve (CT) showed a graded increase in the amiloride-sensitive response that DeSimone and Ferrell (1985) calculated to originate at 25 mM and reach a plateau at 250 mM. Thus, detection of NaCl solutions by rats and the associated neural response can be observed at concentrations where salt appetite favors consumption and where salt is rejected. Dehydrated toads used

in our experiments show aversion at hyperosmotic salt concentrations that are similar to that of rats.

Mammalian studies have also identified amiloride-insensitive salt taste that was initially ascribed to paracellular transport (Ye et al. 1991, 1993, 1994). In this model, impermeant anions such as gluconate are unable to cross tight junctions between taste cells and an excess of cationic charge in the intercellular space will hyperpolarize the basolateral membrane potential ( $V_b$ ) leading to a reduced neural response and behavioral sensitivity to the salt, a phenomenon that was termed the “anion paradox.” More recent studies with rat taste cells and the CT response indicate that cell junctions become tighter in the presence of hyperosmotic solutions, and the amiloride-insensitive CT response is the result of  $\text{Na}^+$  entry via a vanilloid receptor variant in the apical membrane of the taste cells (Lyll et al. 2004; 2005a, 2005b).

It has long been known that hyperosmotic solutions bathing the apical surface of amphibian skin will open tight junctions (Ussing and Windhager 1964), so the earlier hypothesis regarding paracellular transport may still apply for the chemosensory response of toad skin that is amiloride insensitive. Behaviorally, we have shown that the suppression of the WR by 250 mM  $\text{Na}^+$  salts decreased in a linear manner with increasing molecular weight of the anion (Sullivan et al. 2000). In the present study, we evaluated the expression of the WR at NaCl concentrations between 100 mM, where behavior is consistently initiated, and 250 mM, where it is consistently suppressed. This allowed us to better characterize the point at which NaCl solutions are rejected and compare the effect of the anion on expression of the WR at these higher concentrations. Water gain and loss were also measured as they relate to the efficacy of the behavior. Next, we measured the response of spinal nerve #6 that innervates the ventral skin to determine whether the increased tolerance for Na-gluconate is associated with a reduced neural response. If the neural response in toad skin is transduced by depolarization of the epithelial cells, as proposed for the mammalian tongue (Stewart et al. 1997), a larger neural response to 250 mM NaCl versus Na-gluconate should result from a greater depolarization of  $V_b$ . We tested this hypothesis with isolated epidermis from the toad skin in an Ussing-type chamber that allowed changes in  $V_b$ , measured with a microelectrode, to be evaluated concurrently with transepithelial potential ( $V_t$ ) and tissue conductance ( $G_t$ ) that provided an estimate of paracellular permeability to chloride and gluconate salts.

## Methods

### Behavior

We have found similar avoidance of WR behavior on 250 mM NaCl with all species of *Bufo* that have been examined (*Bufo punctatus*, Hoff and Hillyard 1993; *Bufo alvarius*,

Nagai et al. 1999; *Bufo marinus*, Maleek et al. 1999). However, most of our behavioral studies have been conducted with *B. punctatus* which were selected for the present study that was designed to show the tolerance limits to NaCl and comparison with Na-gluconate.

Toads were captured from the Spring Mountains, Clark County, Nevada, under permit from the Nevada Department of Wildlife. They were maintained in terraria with sand, rocks, and water to duplicate conditions in their natural habitat. Crickets were provided as food twice per week, and only toads maintaining or increasing in body weight were used for study. Toads with water available ad lib were assumed to be hydrated.

At the beginning of an experiment, toads were weighed before and after the urinary bladder was emptied. Because toads are able to store 30–50% of their body weight as dilute bladder water, the weight of a hydrated toad with an empty bladder, the standard weight (SW; Ruibal 1962), serves as a basis for evaluating the level of dehydration and subsequent water absorption or loss. Brekke et al. (1991) found *B. punctatus* frequently initiated WR behavior when dehydrated by as little as 0.6% of the SW. However, we used dehydration levels of 10–15% of the SW to insure a consistent and sustained level of rehydration behavior in dilute solutions that served as a control for behavior at higher salt concentrations.

Dehydrated toads were placed on a 10 × 10 cm piece of laboratory tissue that was saturated with 4 ml of DI or NaCl solutions between 100 and 250 mM. Each trial lasted 20 min, and exposure to specific salt concentrations was done at random. One group of 12 toads was used for comparing DI and NaCl concentrations between 100 and 200 mM. A second group of 9 toads from the same laboratory population was used to compare DI with 250 NaCl and the Na-gluconate (200 and 250 mM) solutions. The initiation of WR behavior was easily observed as a rapid abduction of the hindlimbs and pressing of the seat patch to the tissue. The frequency of trials in which WR was initiated and the changes in body mass were calculated for each salt concentration as percentage of the values observed on DI.

### Neural recording

We selected *B. marinus* for these experiments because they are commercially available in sufficient numbers for the study, whereas *B. punctatus* occur in small isolated populations that limit the number of animals that can ethically be collected. Spinal nerves of *B. marinus* are also larger and more easily dissected and recorded from by undergraduates trained in the laboratory. We have previously obtained similar results with *B. marinus* (Maleek et al. 1999), *B. alvarius* (Nagai et al. 1999), and a limited number of *B. punctatus* (Hillyard SD, Nagai T, unpublished observations). Toads were doubly pithed to eliminate reflexive muscle twitches that can interfere with recordings. An incision was made

on the lateral side to expose spinal nerve #6 in a subcutaneous lymph sac. The nerve was cut to insure that only afferent signals were recorded and placed over one lead of a silver–silver chloride electrode. The other lead was inserted into soft tissue near the nerve. A ground electrode was also inserted into soft tissue of the toad and connected to a Faraday cage surrounding the animal. After the nerve was placed on the electrode, the sensory field was outlined by gentle pressure applied to the skin. A gravity-based perfusion system was used to produce a flow of approximately 0.1 ml/s from a series of reservoirs containing the control and experimental solutions. The control solution was 0.5 mM NaCl. We have found that 0.5–1 mM NaCl solutions produce a stable baseline against which the response to 250 mM salt solutions can be evaluated (Maleek et al. 1999; Nagai et al. 1999).

Neural activity was filtered with a bandwidth of 10–100 Hz and amplified by a Grass P 511 amplifier. The signal was recorded on digital audiotape (Bio-Logic DTR 1205) and passed through an integrator with a time constant set at 1 s. Both records were converted to a digital file with an iWorks/214 computer interface (iWorks/CB Sciences, Dover, NH) and exported to Origin 7.5 for presentation. The integrated response was measured with the integration function of iWorks LabScribe software using a sampling period of 5 s. This period encompassed the rapid attenuation of the rinse responses and allowed comparison of the peak perfusion responses over the same timescale. In a given experiment, the skin was alternately perfused with 250 mM Na-gluconate and NaCl solutions followed by 250 and 500 mM KCl solutions. In some cases, the NaCl and Na-gluconate solutions were perfused a second time before the KCl solutions. The responses to 250 mM NaCl and Na-gluconate are presented as a fraction of the response to 500 mM KCl.

### Basolateral membrane potential

*Bufo bufo* were used because of their availability to the laboratory in Copenhagen where microelectrode recordings were made. *Bufo bufo* exhibits a pattern of WR behavior and water absorption that is similar to other bufonid species including *B. punctatus* and *B. marinus* (Viborg and Rosenkilde 2004; Viborg and Hillyard 2005; Viborg et al. 2006). Toads were maintained in a large room with water and food (mealworms) available ad lib. A piece of whole skin was dissected and the serosal surface gently scraped to allow collagenase to penetrate to the epidermis. The skin was then stretched over a piece of polyvinyl chloride tubing with the external surface facing outward and bathed with amphibian Ringer's (112 mM NaCl, 2.4 mM  $\text{KHCO}_3$ , 1 mM  $\text{CaCl}_2$ ). The inner surface was bathed with the same Ringer's solution containing 2 mg/ml collagenase (Worthington, NJ). After 2 h, the epidermis was separated and placed horizontally between halves of a modified Ussing chamber (Willumsen et al. 1992) mounted on the stage of an upright

Optiphot-2 UD microscope (Nikon, Tokyo, Japan). The chamber allowed for gravity perfusion of both the mucosal (apical) and the serosal (basolateral) surfaces of the skin, with the basolateral surface facing upward and accessible to a microelectrode.

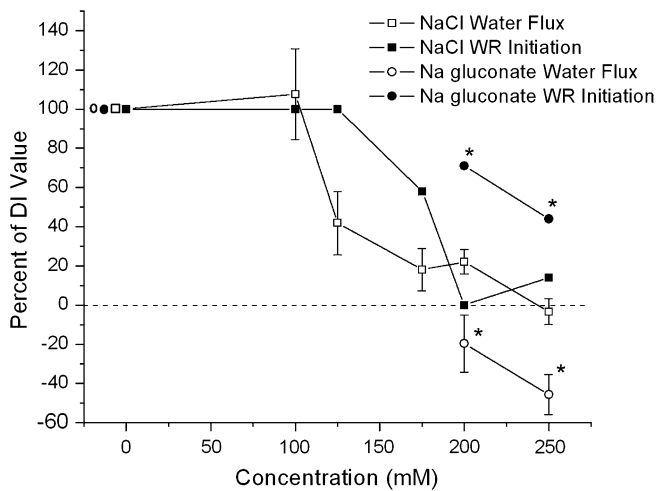
Microelectrodes made according to Willumsen et al. (1992) were filled with 2 M KCl and positioned in a principal cell from the serosal side under visual inspection with a WR60 micromanipulator (Narashige, Tokyo, Japan). The basolateral membrane potential ( $V_b$ ) was measured with a DUO 773 WPI amplifier (World Precision Instruments, Sarasota, FL). The transepithelial potential ( $V_t$ ) was monitored by a VCC600 current and voltage clamp amplifier (Physiological Instruments, San Diego, CA) operating in the current clamp mode. Transepithelial and cell membrane potentials were measured relative to a common reference electrode in the basolateral solution. With this convention,  $V_t$  resulting from inward  $\text{Na}^+$  transport and  $V_b$  both had a negative sign. Transepithelial conductance was calculated from changes in  $V_t$  produced by either 5 or 50  $\mu\text{A}$  pulses applied in current clamp mode.  $V_b$ ,  $V_t$ , and the transepithelial clamping current were digitized by a 1401plus A/D converter with Spike2 data acquisition and analysis software (version 5.16, Cambridge Electronic Design, Cambridge, UK).

In a typical experiment, control values for  $V_b$ ,  $V_t$ , and  $G_t$  were recorded with Ringer's bathing both sides of the epidermis via a gravity flow system. The flow rates varied between preparations because of variability in the stability of microelectrode impalements. In general, slower perfusion rates gave more stable measurements of  $V_b$ . Once stable values for  $V_b$  were obtained, either 250 mM NaCl or Na-gluconate was switched as the apical bathing solution and  $V_b$  was recorded until a new stable value was attained. At this point, perfusion of the Ringer's solution resumed and  $V_b$  was again recorded until a stable value was obtained.

## Results

### Behavior

WR behavior was consistently initiated on DI and with NaCl concentrations up to 125 mM and declined to 58% and 0% of trials as the concentration increased to 175 and 200 mM, respectively (Figure 1). Water absorption at 100 mM was not different from that on DI but declined as the concentration increased to 200 mM. With 250 mM NaCl, one toad briefly initiated the WR but, like the group as a whole, experienced water loss. For the Na-gluconate experiments, WR behavior was initiated in all the control trials with DI water and declined, respectively, to 71% and 44% of trials with concentrations of 200 and 250 mM. Chi-square analysis showed WR initiation and water loss to be greater on the gluconate versus the chloride solutions at 200 and 250 mM concentrations ( $P < 0.01$ ).



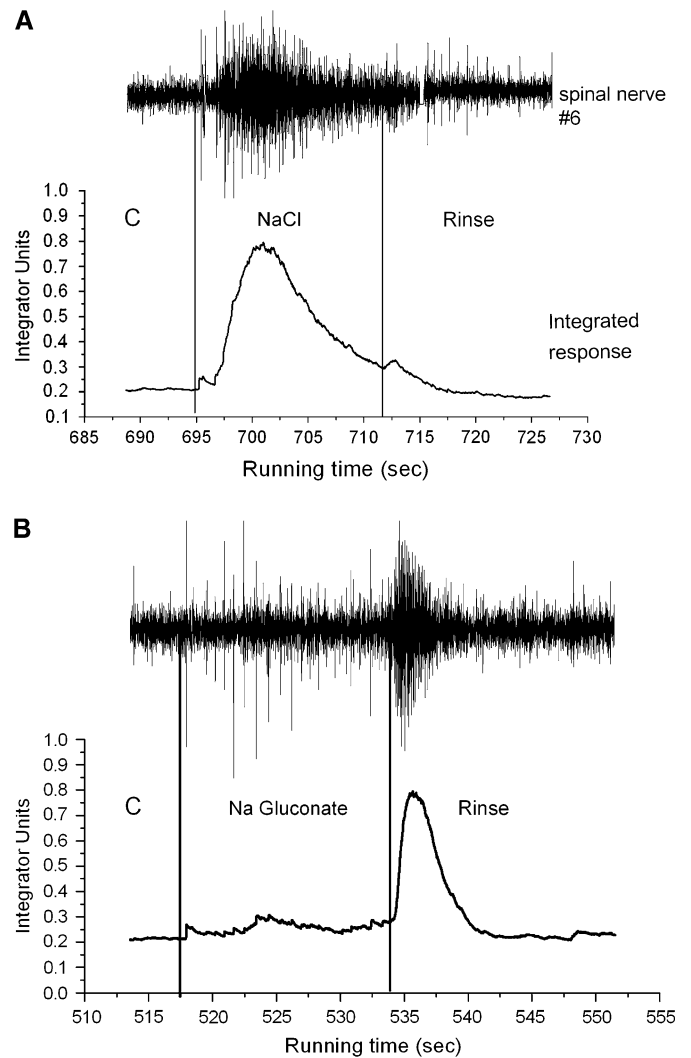
**Figure 1** The frequency of trials in which the WR was initiated and changes in body mass, both expressed as a percentage of values observed on DI. Water weight gain from 100 mM NaCl was not different from DI but declined as the NaCl concentration was increased to 125 mM and above. WR was consistently initiated on 125 mM NaCl but declined as water absorption fell below 40% of the DI value. At 200 and 250 mM concentrations, water loss and the initiation of WR behavior were both significantly greater in toads attempting to rehydrate from Na-gluconate compared with NaCl (\*  $P < 0.01$ ).

### Neural response

When 250 mM NaCl perfused the skin, a rapid burst of action potentials was recorded prior to replacement with the control solution (Figure 2A). The degree to which the neural response declined varied between preparations; in some cases, the response continued until the rinse solution was applied. A small rinse response was observed when perfusion of the control solution resumed. In contrast, perfusion with Na-gluconate produced a smaller response but a much larger rinse response (Figure 2B). The difference between the integrated neural and rinse responses for NaCl versus Na-gluconate is presented in Figure 3 for 14 trials with 9 preparations. Comparison of trials with Student's  $t$ -test (13 degrees of freedom [df]) showed that the response to perfusion was significantly larger with NaCl ( $t = 3.55$ ,  $P = 0.004$ ), whereas the rinse response was greater with Na-gluconate ( $t = 4.70$ ,  $P = 0.007$ ). Similar levels of significance were obtained with analysis of single trials from each preparation (8 df,  $T = 3.94$ ,  $P = 0.004$  for perfusion;  $T = 3.13$ ,  $P = 0.007$  for rinse).

### Basolateral membrane potential

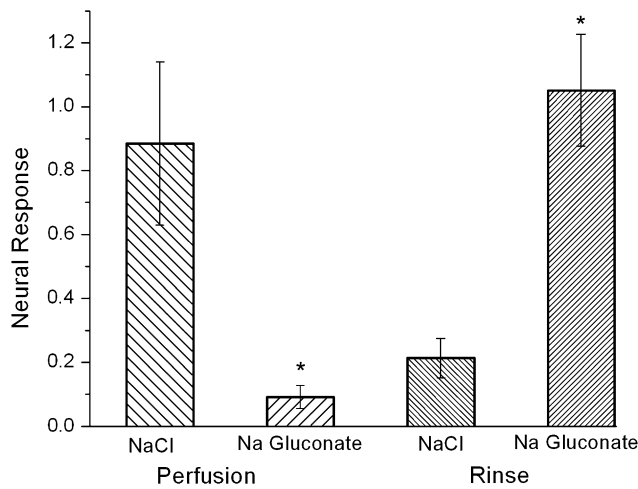
Replacing Ringer's solution with 250 mM NaCl as the apical bathing solution resulted in depolarization of both  $V_b$  and  $V_t$  (Figure 4A). Note also that as  $V_t$  declined, the 5- $\mu$ A current pulses became smaller indicating an increase in  $G_t$  that presumably reflected the opening of tight junctions. The apical membrane potential ( $V_a$ ) calculated from the relationship  $V_t = V_b - V_a$  is also plotted in Figure 4A. Note that both  $V_a$  and  $V_b$  began to depolarize before  $V_t$  depolarized. This



**Figure 2** (A) Upper trace: representative experiment showing the response of spinal nerve #6 to perfusion of 250 mM NaCl over the skin in the receptive area of the nerve. The control solution (C) was 0.5 mM NaCl. Lower trace: the integrated response of the neural activity obtained with a 1-s time constant over a 5-s interval covering the peak response. (B) The same procedure with 250 mM Na-gluconate produced a much smaller integrated response but a much larger rinse response.

was variable among preparations. When Ringer's replaced 250 mM NaCl,  $V_t$ ,  $V_a$ , and  $V_b$  all repolarized to values near that of the control and  $G_t$  decreased to near-control values. For comparison, the integrated response for the neural recording from Figure 2A has been superimposed over the epithelial voltage measurements with the addition and removal of 250 mM NaCl aligned between the traces. A scale bar has been added to illustrate the more rapid time course for the neural recording experiments. Five distinct intervals have been identified to correlate the neural and epithelial events: 1) the control period prior to addition of the concentrated salt solution, 2) the initial level of depolarization of  $V_t$  and  $V_b$  that approximate the peak neural stimulation, 3) the values for  $V_t$  and  $V_b$  just prior to washout of the





**Figure 3** Na-gluconate produced a significantly smaller neural response than 250 mM NaCl, but a larger rinse response arose when the apical perfusate returned to 0.5 mM NaCl (\*  $P < 0.01$ ; specific values for  $t$  and  $P$  are given in Results). The response of both solutions is expressed as the fraction of the integrated response to 500 mM KCl. The integrated responses for all solutions were obtained by integrating the neural responses for 5 s covering the peak response, with a time constant of 1 s. Each bar represents the mean  $\pm$  1 standard error for 14 trials with 9 preparations.

concentrated salt solution, 4) the values for  $V_t$  and  $V_b$  during the washout period, and 5) the stable values of  $V_t$  and  $V_b$  following washout.

When Ringer's was replaced with 250 mM Na-gluconate as the apical bathing solution (Figure 4B),  $V_t$  depolarized slowly but to a much smaller extent than with NaCl and  $G_t$  remained constant (1–2). At the same time,  $V_b$  and by calculation  $V_a$  transiently hyperpolarized and then returned to values that were depolarized relative to the control (2–3). Returning to Ringer's produced a transient depolarization of  $V_b$  and  $V_t$  (3–4) that gradually returned to values near that of the control (4–5). The transient depolarization was accompanied by a marked increase in  $G_t$  that returned to near-control values as  $V_t$  and  $V_b$  stabilized. Superimposing the integrated response from Figure 2B showed that the smaller neural response to Na-gluconate corresponded to the hyperpolarization and recovery of  $V_b$  and the larger rinse response corresponded with the transient increase in  $G_t$  and the depolarization of  $V_b$  and  $V_t$ . As with Figure 4A, the time course for the neural response experiments was much more rapid than for measurement of  $V_t$ ,  $V_b$ , and  $G_t$  because of the need to maintain the microelectrode impalement during solution changes.

The results from 11 records for experiments with 250 mM Na-gluconate and 12 for 250 mM NaCl are presented in Figure 5. The sequential changes in  $V_t$ ,  $V_b$ , and  $G_t$  were analyzed between the 5 measuring intervals with a repeated-measures analysis of variance using a Bonferroni post hoc adjustment for multiple comparisons (SPSS, Chicago, IL). Results for 250 mM NaCl experiments, with 4 df, indicate significant overall differences for all 3 parameters ( $V_t$ :  $F =$

7.67,  $P = 0.008$ ;  $V_b$ :  $F = 33.50$ ,  $P < 0.001$ ;  $G_t$ :  $F = 21.98$ ,  $P < 0.001$ ). For 250 mM NaCl substitution (1–2),  $V_t$  and  $V_b$  depolarized significantly ( $P = 0.003$  and  $P < 0.001$ , respectively) in conjunction with an increase in  $G_t$  ( $P < 0.001$ ), and the differences continued to be significant during exposure to the concentrated solution. All 3 parameters returned to control values when the concentrated salt solution was washed out (3–4, 4–5).

Results for 250 mM gluconate experiments, also with 4 df, indicate significant overall differences for all 3 parameters ( $V_t$ :  $F = 32.85$ ,  $P < 0.001$ ;  $V_b$ :  $F = 62.17$ ,  $P < 0.001$ ;  $G_t$ :  $F = 11.50$ ,  $P = 0.003$ ). Individual comparisons showed that substitution with 250 mM Na-gluconate produced a small but significant depolarization of  $V_t$  ( $P = 0.045$  for 1–2) that continued with exposure to the concentrated solution ( $P = 0.007$  for 2–3). During the same intervals,  $V_b$  transiently hyperpolarized ( $P < 0.001$  for 1–2) and then depolarized significantly ( $P < 0.001$  for 2–3) to a level that was not different from the control ( $P = 0.185$  for 1–3).  $G_t$  did not change during periods 1–3 ( $P = 1.0$ ). However, return to Ringer's solution (3–4) produced a transient depolarization of  $V_t$  ( $P < 0.006$ ) and  $V_b$  ( $P < 0.001$ ) accompanied by a significant increase in  $G_t$  ( $P = 0.001$ ). Continued exposure to the rinse solution (4–5) produced a recovery of  $V_t$ ,  $V_b$ , and  $G_t$  to control values.

## Discussion

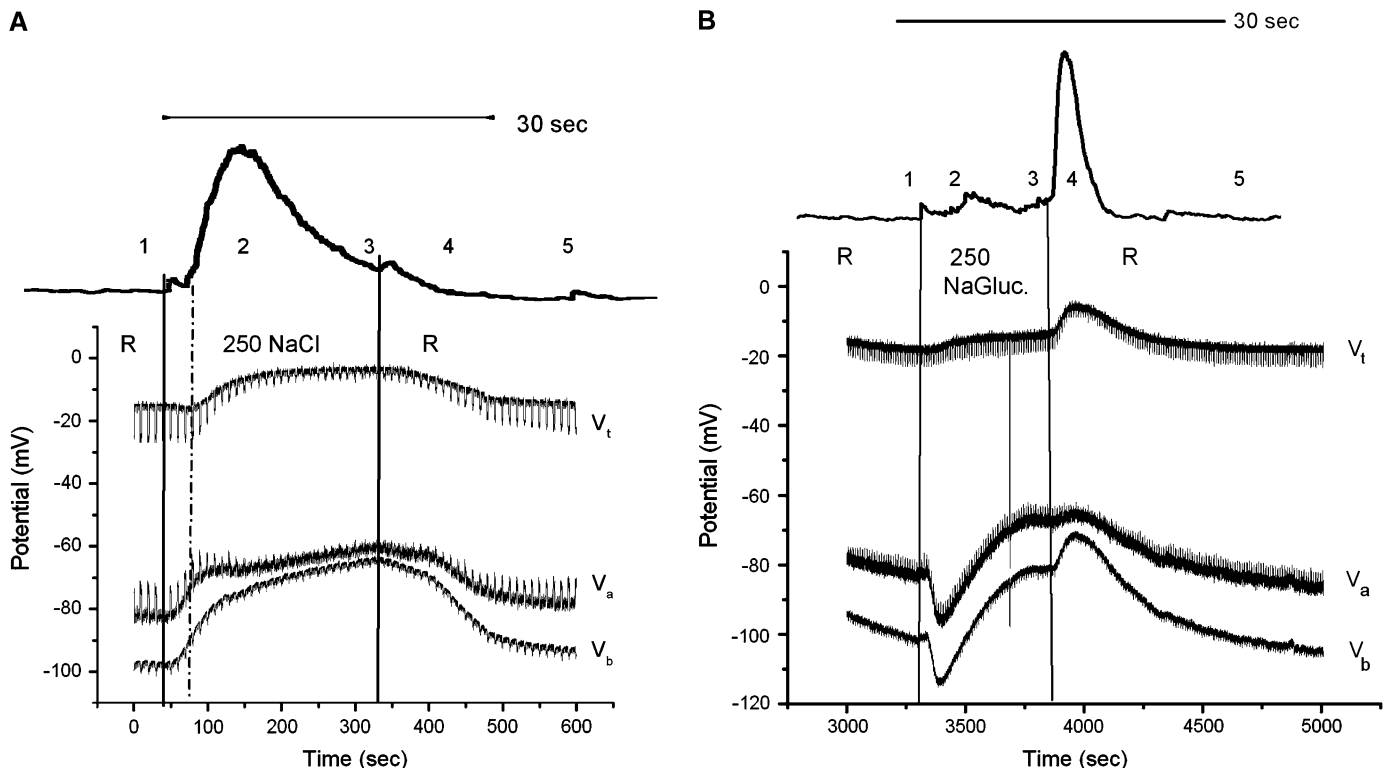
The ability of toads to rehydrate from 50 to 100 mM NaCl solutions at rates near that of DI water, despite the reduced osmotic gradient, has been noted by ourselves (Sullivan et al. 2000; Hillyard et al. 2007) and others (Ferriera and Jesus 1973). The mechanism for this has been variously speculated to result from solute coupling of water transport (Guo et al. 2003), increased blood flow to the seat patch (Hillyard and Larsen 2001), and increased insertion of intramembranous particles (e.g., aquaporins; Katz 1987). All these possibilities require sensory input to coordinate the physiological and behavioral responses. For example, skin contact with a hydration solution rather than dehydration, per se, is required to stimulate blood flow in conjunction with WR behavior in all the species used in the present study (Viborg and Rosenkilde 2004; Viborg and Hillyard 2005; Viborg et al. 2006). It is of interest that toads continued to initiate the WR on 125 mM NaCl where water absorption was reduced to 40% of DI water but reduced WR initiation to near zero as water absorption fell to less than 20% of DI values, that is, toads are able to detect NaCl concentrations at which they become less able to physiologically compensate for the reduced osmotic gradient.

Initiation of the WR on 200 and 250 mM Na-gluconate solutions was significantly greater than on NaCl solutions even though water loss was significantly greater on Na-gluconate. This suggests that the sensory input required for toads to avoid unfavorable solutions is not as effective

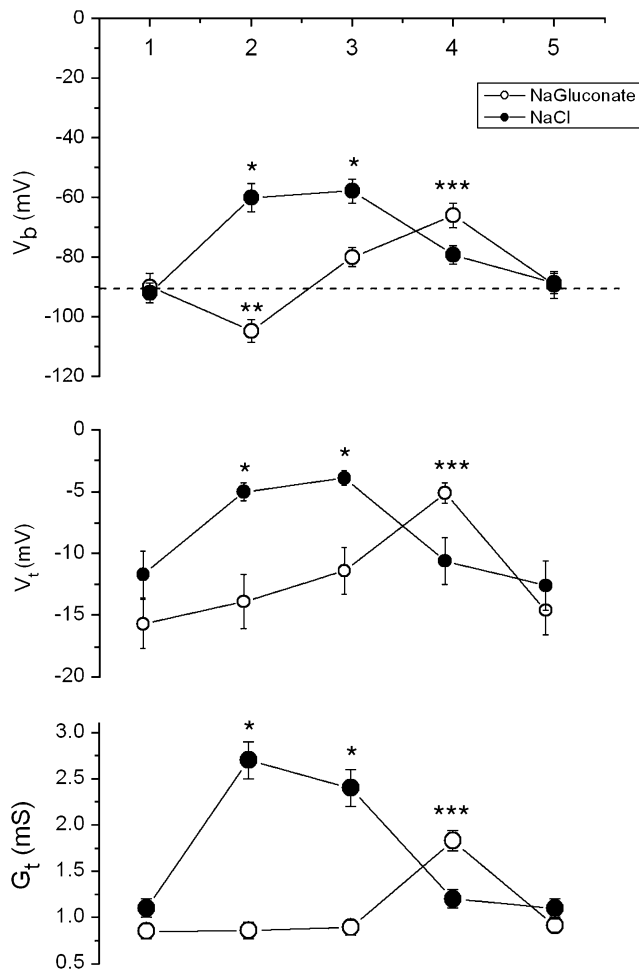
with the impermeant anion. We have previously shown that dehydrated *B. punctatus* spend more time attempting to rehydrate on 250 mM Na-gluconate solutions relative to 250 mM NaCl, but water loss during immersion in these solutions was not different (Sullivan et al. 2000). In the present study, the continued attempts to rehydrate from an open surface may have resulted in increased evaporative water loss in addition to osmotic water loss.

The reduced neural response to Na-gluconate in conjunction with hyperpolarization of  $V_b$  and no change in  $G_t$  indicates that gluconate was unable to cross the cell junctions and its anionic charge limited  $\text{Na}^+$  diffusion as well. Because the apical membrane of the principal cells of the skin have a very low  $\text{Cl}^-$  conductance (Willumsen and Larsen 1986; Willumsen et al. 1992), a reduction in the paracellular shunt conductance that is anion selective would reduce  $\text{Na}^+$  entry via the transcellular pathway to insure electroneutrality. In this case,  $V_b$  would approach the equilibrium potential for  $\text{K}^+$ , which was estimated to be  $-106 \pm 2$  mV using double barreled  $\text{K}^+$ -sensitive microelectrodes inserted into the basal cell layer of isolated *B. bufo* epidermis (Larsen et al. 1992).

This is remarkably similar to the value for  $V_b$  ( $-105.2 \pm 3.4$  mV) observed during maximal hyperpolarization in the present study (Figure 5). The depolarization of  $V_t$  (from  $-15.7 \pm 2.0$  to  $-11.4 \pm 1.9$  V, Figure 5) although significant can be largely explained from the liquid junction potential of 2.4 mV for the mucosal agar bridge solution. With continued perfusion of 250 mM Na-gluconate,  $V_b$  depolarized to a level not different from the control. However, when Ringer's was reintroduced as the apical bathing solution there was a transient depolarization of  $V_b$  and  $V_t$ . These changes are expected if the shunt current carried by  $\text{Cl}^-$  and the transcellular current carried by  $\text{Na}^+$  are increased. This explanation is supported by the near doubling of  $G_t$ . These results suggest that the cell junctions were opened by the initial exposure to 250 mM Na-gluconate and the replacement with  $\text{Cl}^-$  allowed transient diffusion of anionic charge into the paracellular pathway and depolarization of  $V_b$ . The transient depolarization of  $V_b$  corresponds with the large transient rinse response observed when 250 mM Na-gluconate was replaced with 0.5 mM NaCl in the intact animal. Ye et al. (1994) reported a similar rinse response when K-gluconate



**Figure 4** (A) Perfusion of the apical surface of isolated epidermis with 250 mM NaCl resulted in depolarization of  $V_a$ ,  $V_b$ , and  $V_t$  as the tissue conductance increased, as indicated by the smaller deflection of  $V_t$  when 5  $\mu\text{A}$  pulses were delivered in current clamp mode. The dashed line indicates that depolarization of  $V_a$  and  $V_b$  begins before the decline in resistance. The upper trace is the integrated response from Figure 2A aligned, so the application and removal of 250 mM NaCl in the neural recording experiments correspond with those of the experiments with the isolated epithelium (solid vertical lines). (B) The same procedure with 250 mM Na-gluconate shows an initial hyperpolarization followed by a slow depolarization. Return to Ringer's perfusion produced a transient depolarization of  $V_b$  and  $V_t$  that corresponds with the rinse response observed when the trace from Figure 2B is aligned with the measurements of  $V_t$  and  $V_b$  with the isolated epidermis. The slower time course for the isolated skin preparation makes the current pulses less distinct. In both Figures, a horizontal bar indicates the time span for the neural response. The control (Ringer's) perfusion value is indicated by #1. Data points at 2 indicate the initial response to 250 mM NaCl and Na-gluconate solutions, and data points at 3 are the values recorded immediately before returning to Ringer's. Data points at 4 and 5 show the initial and stabilized values after return to Ringer's perfusion.



**Figure 5** Exposure of the mucosal surface of the skin to 250 mM NaCl significantly depolarized  $V_b$  and  $V_t$  as  $G_t$  significantly rose (\*). All 3 parameters returned to control values when the mucosal solution was replaced with Ringer's. Exposure of the mucosal surface to 250 mM Na-gluconate significantly hyperpolarized  $V_b$  (\*\*) with no change in  $G_t$ . With continued perfusion,  $V_t$  depolarized to a value not different than control. Replacement of the mucosal bath with Ringer's produced a significant but transient depolarization of  $V_b$  and  $V_t$  in conjunction with a significant increase in  $G_t$  (\*\*\*). Significance levels are provided in the text.

was replaced with KCl bathing the apical surface of rat taste cells. They proposed, "Rinsing rapidly collapses the hyperpolarizing transepithelial potential. This could result in a transient depolarization of the receptor cells and a transient neural response." As the paracellular pathway closed,  $V_b$ ,  $V_t$ , and  $G_t$  returned to control values showing the reversibility of tight junction opening.

With 250 mM NaCl, depolarization of  $V_b$  proceeded as  $G_t$  increased, presumably due to opening of the shunt pathway in response to raised osmolality. At the same time,  $V_t$  depolarized suggests a greater mobility of  $\text{Cl}^-$  and thus an increased current through the cell junctions which tend to short circuit the preparation. The junction potential for the higher NaCl concentration was calculated to be only 0.9 mV.

The increase in shunt current is associated with a similar increase in transcellular current, which can account for the depolarization of  $V_b$ . In some cases, the depolarization of  $V_a$  preceded the opening of the shunt pathway (Figure 4A). This may reflect increased apical entry of  $\text{Na}^+$ , which represents the amiloride-sensitive component of the neural response. Variability in the degree and time course of opening of the shunt pathway can explain the variability we have observed in the amiloride sensitivity of the neural and behavioral responses (Hoff and Hillyard 1993; Nagai et al. 1999; Hillyard et al. 2004).

The demonstration of a vanilloid receptor (TRPV-1) variant in the apical membrane of rat and mouse taste cells and the absence of an amiloride-insensitive CT response to NaCl in TRPV-1 knockout mice has led to the hypothesis that paracellular transduction is not as important in mammalian salt taste as previously suggested (Lyall et al. 2004). As noted in the Introduction,  $G_t$  in mammalian tongue decreased in response to hyperosmotic solutions (Lyall et al. 2005a), whereas paracellular conductance of toad skin increased markedly as the mucosal NaCl concentration was raised to 250 mM (Hillyard et al. 2004 and present study). Associated with the increase in  $G_t$ , the amiloride-insensitive neural response increased as NaCl concentration was increased from 200 to 300 mM (Nagai et al. 1999). Interestingly, acute exposure to NaCl at or above 300 mM results in hyponatremia and death in many bufonid species within a few days (Gordon 1962; Liggins and Grigg 1985; Sinsch et al. 1992).

It should be noted that TRPV-1 knockout mice retain a reduced and phasic CT response to NaCl, and the phasic response is more prevalent when the perfusion rates are rapid, whereas a more tonic response is seen with slower perfusion rates (Lyall et al. 2005a; DeSimone and Lyall 2006). The phasic neural response of toad skin to 250 mM NaCl was obtained with rapid flow over the skin, whereas the more tonic depolarization of  $V_b$  and  $V_t$  was obtained with slower flow rates and the need to exchange the Ringer's solution in the mucosal chamber. Also, the control solution was 0.5 mM NaCl for the neural responses, whereas Ringer's was the control for the isolated epithelium to eliminate the need for series resistance compensation under current clamp conditions. Nonetheless, the stimulation of neural activity correlated with depolarization of  $V_b$ , as predicted if the epithelial cells serve a chemosensory function as proposed by Nagai et al. (1999) and Koyama et al. (2001), who showed that the carbocyanine dye DiI applied to spinal nerves labeled basal cells of the epithelium.

The correspondence between  $V_b$  depolarization and the magnitude of the neural and rinse responses with Na-gluconate provide additional support to our hypothesis that the toad skin epithelium serves a sensory function like that of the lingual epithelium of animals that take in fluids by mouth. Both systems have an amiloride-sensitive, transcellular component to the neural and behavioral responses

to hyperosmotic NaCl solutions (DeSimone and Ferrell 1985; Hoff and Hillyard 1993; Nagai et al. 1999; Brot et al. 2000). Unlike the tongue, the paracellular transduction pathway in toad skin appears to be more important at salinities that are harmful to the animals. The role of vanilloid receptors in amphibian skin remains to be determined. However, we have recently identified a protein in isolated skin from both toads (*B. marinus*) and frogs (*Rana catesbeiana*) that gives a positive reaction to mammalian TRPV-1 antisera (Santa Cruz Laboratories) in Western blot analysis (Hillyard SD, Marrero M, unpublished observations).

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