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Transcellular and Paracellular Elements of Salt Chemosensation in Toad Skin

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Abstract

Dehydrated toads absorb water by pressing a specialized (seat patch) area of the skin to moist surfaces. This behavior, the water absorption response (WR), is preceded by periods of more limited skin contact (seat patch down, SPD) in which the suitability of the rehydration source is evaluated. WR and SPD behaviors were suppressed on 250 mM NaCl and 200 mM KCl solutions. Ten micromolar amiloride partially restored SPD and WR on NaCl solutions. The addition of 5 mM La^{3+} also partially restored the initiation of WR and this effect was additive to the effect of amiloride, suggesting transcellular and paracellular pathways exist in parallel. Similarly, 5 mM La^{3+} partially restored the initiation of WR on KCl solutions, to levels comparable to those with K^+gluconate, suggesting a paracellular pathway for detection of K^+. Hyperosmotic (250 mM) NaCl solutions bathing the mucosal surface rapidly and reversibly increased the paracellular conductance of isolated skin and this increase was partially inhibited by 5 mM La^{3+}. These results suggest that the regulation of tight junctions has a chemosensory role in toad skin.

Key words: amphibian skin, chemosensory epithelium, epithelial sodium channels, paracellular pathway

Introduction

Dehydrated toads, *Bufo punctatus*, reject hyperosmotic (250 mM NaCl and KCl) solutions offered as hydration sources. Amiloride, a specific inhibitor of epithelial Na^+ channels (ENaCs) partially restores tolerance of NaCl but not KCl solutions in contact with the skin (Hoff and Hillyard, 1993a,b). Since anuran amphibians, including toads in the genus *Bufo*, obtain water almost exclusively by osmosis across their skin, these results suggest that the skin serves a chemosensory function similar to that of the mammalian tongue where Na^+ taste transduction is mediated by ENaCs (DeSimone *et al.*, 1984). However, amiloride only partially restores tolerance to NaCl, and the amiloride-insensitive component of aversion to NaCl and KCl remains unresolved.

A possible paracellular pathway for chemosensory transduction in amphibian skin, similar to that described for the mammalian tongue (Ye *et al.*, 1991), was suggested by Sullivan *et al.* (2000) who showed that the tolerance by toads of 250 mM Na^+ salt solutions increased linearly with the mol. wt of the anion.

The studies cited above used an assay of hydration behavior for quantitative analysis of aversion to, and tolerance of, salt solutions by toads. Dehydrated toads take up water by pressing a specialized area of the ventral skin (the seat patch) to moist surfaces using a fixed sequence of body postures. The behavioral sequence, from contact with feet only to contact with the seat patch (seat patch down, SPD) and to abduction of the rear legs so that the entire ventral skin is pressed to the surface (water absorption response, WR; Stille, 1958), follows a predictable time course that is dependent on the constituents of the presented substrate (reviewed by Hillyard *et al.*, 1998). SPD behavior corresponds to the perceptual correlate of tasting salts by the tongue of vertebrates that drink orally (Maleek *et al.*, 1999) and WR is the behavioral correlate of drinking. The initiation and duration of these postures provide quantitative measures of the animals' ability to evaluate and then accept or reject a hydration source.

The first objective of the present study was to evaluate the relative contributions of transcellular and paracellular transduction pathways to aversion and tolerance of NaCl solutions by *B. punctatus*. We used our behavioral assay on dehydrated toads presented with 250 mM NaCl solutions and combinations of amiloride and lanthanum ions (La^{3+}) to separate the two transduction pathways. La^{3+} has been shown to decrease tight junction conductance in lingual epithelia (Simon *et al.*, 1993; Gilbertson and Zhang, 1998a,b). Therefore, we examined its effects on isolated toad

skin bathed with 250 mM NaCl, in Ussing chamber preparations, to assess its efficacy in blocking tight junctions in this tissue.

Our second objective was to characterize the transduction pathway for K^+. Since the aversion to 250 mM KCl solutions is insensitive to amiloride, mechanisms for detecting K^+ salts could include: (i) apical K^+ channels that have been suggested for urodele (*Necturus*) tongue (Kinnamon and Cummings, 1992) and (ii) a paracellular pathway suggested for rat tongue (Ye *et al.*, 1991). We evaluated these hypotheses by comparing the duration of SPD + WR behaviors and the frequency of WR initiation on hyperosmotic KCl solutions alone and after the addition of Ba^{2+} or La^{3+}, with Cl^- and gluconate as the anions. We hypothesize that if the transduction pathway for K^+ is transcellular Ba^{2+} will partially restore the expression of SPD or WR behavior, while restoration of these behaviors by La^{3+} or gluconate would support the alternate hypothesis that K^+ is detected via a paracellular pathway.

Materials and methods

Maintenance of animals and dehydration protocol

Red-spotted toads (*Bufo punctatus*) were collected from a single drainage in the Spring Mountains, Clark County, NV after the breeding season (IACUC protocol R701-089-043). Toads were housed in a terrarium that contained moist soil, rocks and standing tap water so they could select from among a range of substrate textures and water potentials similar to their natural habitiat. Toads were kept on a 12:12 light/dark cycle and were fed crickets two or three times each week. Toads were acclimated to laboratory conditions for at least 2 weeks before experimentation and only toads with stable or increasing weight were used.

We assumed that with water available *ad libitum* the toads would maintain a hydrated state (Jorgensen, 1994). Toads from the home terrarium had their urinary bladders emptied by inserting a polyethylene cannula into the cloaca and applying gentle abdominal pressure. The toads were then placed in a dry glass terrarium for 2–4 h, until dehydrated by ~10% of their standard weight (weight of the hydrated toads with an empty bladder; Ruibal, 1962). This level of dehydration consistently results in the initiation of the WR in this species (Brekke *et al.*, 1991). Only trials that met the following conditions were used in the analysis: dehydration by >8 and <20%, relative humidity <35% and barometric pressure steady or rising. Previous experiments in our laboratory have shown these factors to affect hydration behaviors (e.g. Hoff and Hillyard, 1993b). Trials that did not meet these conditions were excluded from the analysis. The exclusions are reflected in the variation in sample size.

Behavioral assay

Dehydrated toads were placed on a 10 × 10 cm piece of laboratory tissue saturated with 4 ml of a treatment solution, on the bottom of a 20 × 20 × 20 cm terrarium. The terrarium was elevated so the contact of the skin with the moist tissue could be viewed from below. The initiation and duration of SPD and WR behaviors were recorded to the nearest second during each observation period. The criterion for the transition from SPD to WR was the abduction of the hindlimbs so that the hind feet extended outside of the region of the seat patch pressed to the moist surface (Hillyard *et al.*, 1998).

Three sets of experiments were conducted. Each set used a separate group of animals. Each animal was used only once for each treatment within that set of experiments. Experiment 1 evaluated the dose effect of amiloride on the initiation of WR behavior on 250 mM NaCl solutions. Five minute (300 s) trials were sufficient to evaluate the initial reaction of the toads including SPD duration and initiation of WR. Experimental treatments included NaCl alone and with 1 or 10 μM amiloride added to the salt solution. Controls used deionized water, also in the presence and absence of amiloride.

Experiment 2 evaluated the separate and combined effects of amiloride and La^{3+}. In these trials, toads were presented with 250 mM NaCl alone and with either 10 μM amiloride, 5 mM La^{3+}, or both amiloride and La^{3+}. Fifteen minute trials (900 s) were used because open-ended control studies showed that 15 min was sufficient to capture behavioral complexities including observations that toads walk back on the tissue and initiate SPD or even WR behavior after an initial aversive reaction.

Experiment 3 examined the response to 200 mM KCl or K gluconate solutions. Initially we used 250 mM KCl solutions, but we found that toads rapidly stepped away from the solutions and exhibited no hydration behaviors. When we decreased the concentration to 200 mM KCl, toads consistently showed SPD and occasionally WR behavior. Thus, skin contact time (SPD + WR) could be used to quantify the behavior of toads presented with 200 mM KCl, or to compare the effect of a larger anion. Behavior was evaluated on both salts separately and with 5 mM Ba^{2+} or 5 mM La^{3+}.

Isolated skin experiments

For these experiments, skin was obtained from commercially available *Bufo marinus*. Preliminary experiments with isolated skin from a small number of *B. punctatus* showed electrophysiological responses that were similar to those of *B. marinus*.

Toads were killed by double pithing and the skin from the pelvic seat patch region was dissected and mounted in a modified Ussing chamber that allowed continuous perfusion of the chamber halves so that a continuous voltage or current clamp could be maintained while solutions were changed (De Wolf and Van Driessche, 1988). The skin was initially maintained under short-circuit current conditions (I_{sc}; Ussing and Zerhan, 1951) with identical Ringer's bathing both sides of the tissue (115 mM NaCl, 2.5 mM

$KHCO_3$, 1 mM $CaCl_2$). After stable I_{sc} values were achieved, the tissue conductance was measured as the slope of a plot of current versus voltage, measured at clamp voltages between –80 and + 80 mV (serosal relative to the mucosal side of the chamber). The mucosal solution was then changed to 250 mM NaCl, under either voltage or current clamped conditions. Current clamp evaluates the time course for the change in transepithelial potential following the solution change. The current–voltage relationship was again measured and the mucosal bath returned to the Ringer's solution. In some preparations, the mucosal solution was again changed to 250 mM NaCl with 5 mM $LaCl_3$, to duplicate the conditions of the behavioral experiments while in others 250 mM NaCl was added alone. As before, conductance was calculated from the current voltage relationship. It should be noted that the Cl^- conductance of toad skin increases markedly when the mucosa is negative relative to the serosa due to activation of a voltage-sensitive transport mechanism in mitochondria-rich cells (Larsen, 1991). We used brief voltage pulses (~5 s) to prevent the activation of this chloride conductance and obtain linear current–voltage plots.

Analysis

Statistical analysis used STATVIEW (AbacusConcepts) software. Comparisons of frequencies of initiation of WR used χ^2. Unless otherwise stated in the text, comparisons of the duration of behavior used two-way ANOVA (substrate × treatment) with Fisher's PLSD for *post hoc* tests. Significance levels are given for *post hoc* comparisons, with $P < 0.05$ considered significant. Comparisons of conductance were made with Student's *t*-test for paired data.

Results

In all experiments, dehydrated toads placed on deionized water consistently initiated the WR and maintained this posture for most of the observation period. Neither amiloride, Ba^{2+} nor La^{3+}, at the doses provided, significantly affected behavior on deionized water (data not shown).

In experiment 1, toads briefly stayed on 250 mM NaCl but did not show either SPD or WR behavior (n = 11). With 1 μM amiloride, the time to first exit from the tissue increased and WR was initiated in a few trials (n = 10). However, these changes were not significantly different from the controls. With 10 μM amiloride (n = 12), toads remained longer on the tissue (P = 0.022 versus NaCl alone), showed a significant increase in SPD time (P = 0.005 versus NaCl) and the WR was initiated in about half of the trials (P = 0.023 versus NaCl; Figure 1A,B).

In experiment 2, toads placed on 250 mM NaCl returned to the wetted surface, showed SPD behavior and remained on the tissue (Figure 2A). However, WR was not initiated in any trial (n = 13). In contrast, the addition of either amiloride (n = 13) or La^{3+} (n = 15) resulted in the initiation of WR in ~25% of the trials which indicates a trend but was not significant (P = 0.065). With the combination of amiloride and La^{3+}, WR (n = 15) was significantly initiated, in about 50% of the trials (P = 0.005 when compared to NaCl alone; Figure 2B).

In isolated skin preparations, the substitution of the mucosal Ringer's with 250 mM NaCl caused a decrease in transepithelial voltage that was not sensitive to La^{3+} during the first 12 s (Figure 3A, n = 5). However, during the

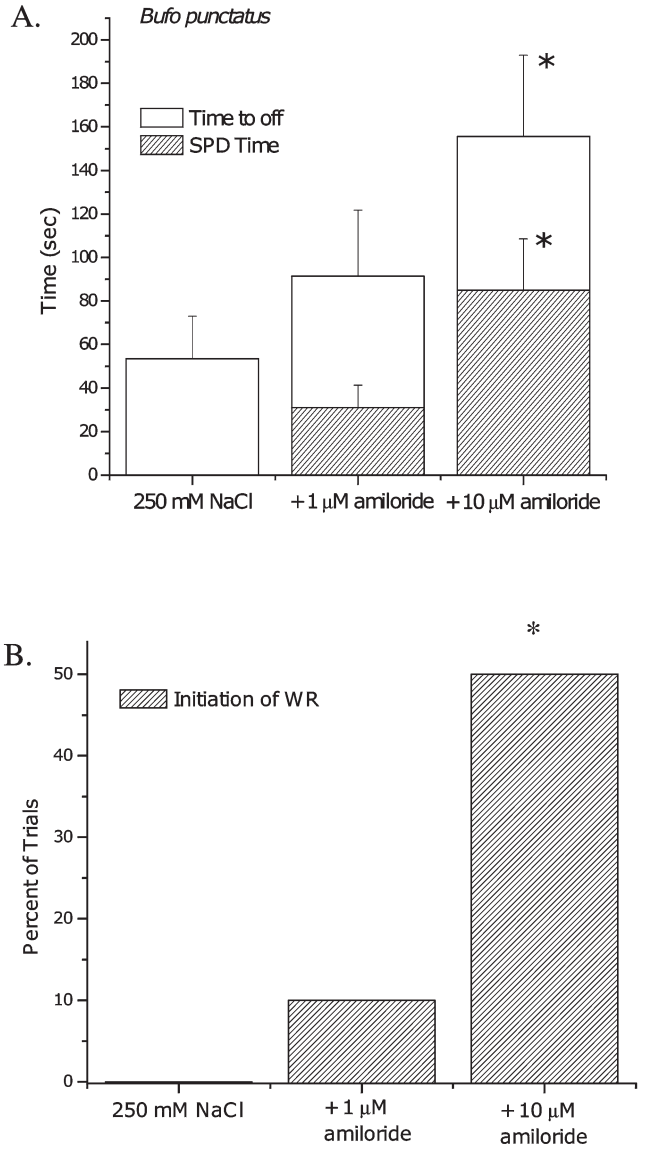
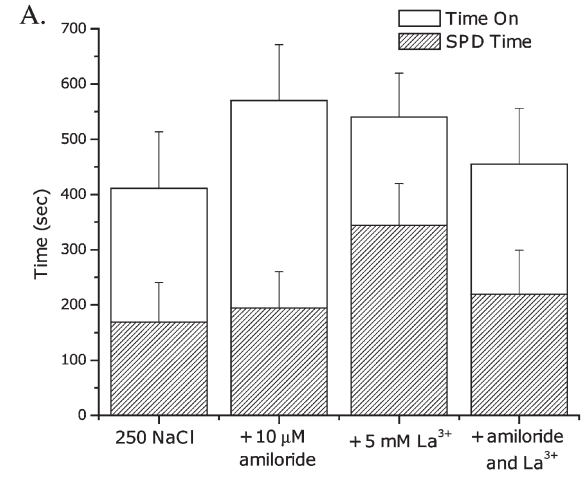


Figure 1 **(A)** Ten micromolar but not 1 μM amiloride significantly increased the time that toads remained on 250 mM NaCl before initially walking off the surface (time to first off), during 5 min (300 s) observation trials. The same dose effect was seen for the duration of time that toads allowed their seat patch to contact the surface (seat patch down = SPD). SPD behavior was not observed in the absence of amiloride. **(B)** Ten micromolar amiloride in the 250 mM NaCl solution also significantly increased the percent of trials where the WR posture was initiated. Asterisk (*) indicates a significant difference. Sample size and significance levels for specific comparisons are given in the text for all presented data.



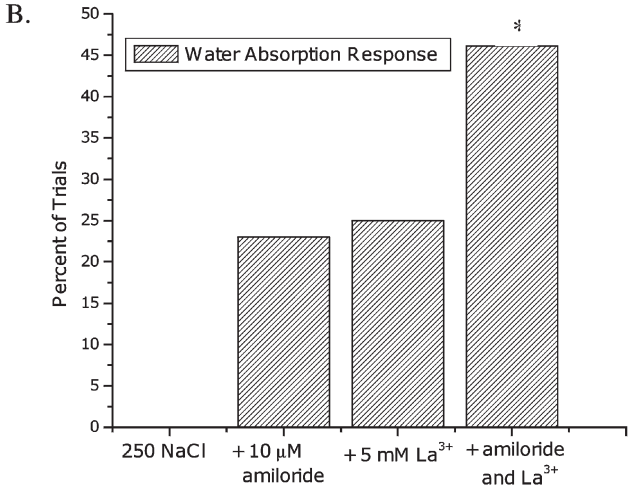
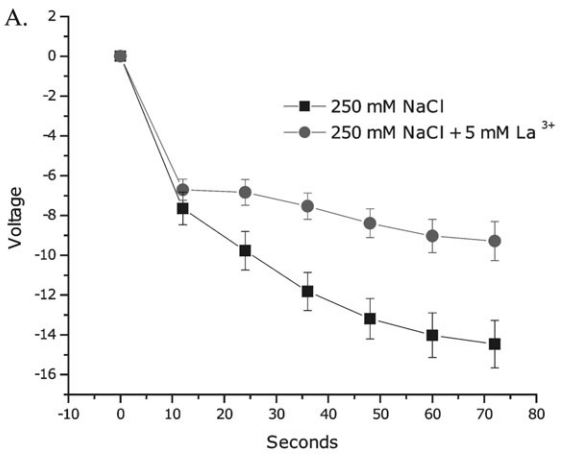


Figure 2 **(A)** In 15 min (900 s) observation periods, toads returned to the 250 mM NaCl surface and allowed contact of the seat patch (SPD). Neither the inhibition of the transcellular (amiloride-sensitive) or paracellular (La^{3+}-sensitive) pathways significantly affected this behavior. **(B)** Amiloride and La^{3+} individually increased initiation of the WR posture but the differences are not significant. However, in combination the increase in WR initiation was significant (*).



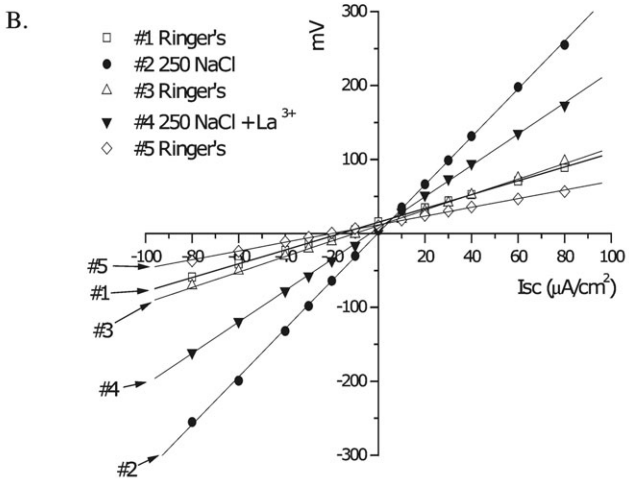


Figure 3 **(A)** Under current clamp conditions, exchange of Ringer's solution in the mucosal bath with 250 mM NaCl resulted in a negative shift in transepithelial potential. In the presence of 5 mM La^{3+} the response to 250 mM NaCl was not affected during the first 12 s but became significantly reduced over the following 60 s. **(B)** A representative current voltage plot showing the sequential substitution of: 1, Ringer's; 2, 250 mM NaCl; 3, Ringer's; 4, 250 mM NaCl + 5 mM La^{3+}; and 5, Ringer's. The conductances, calculated from the slopes of the linear regressions are, respectively, 1.87, 6.49, 2.10, 4.24 and 1.18 mS.

remainder of a 72 s observation period, the transepithelial potential stabilized at a less negative value in the presence of La^{3+} ($P < 0.01$). A representative current–voltage plot is shown in Figure 3B for an experiment where conductance was measured with 250 mM NaCl in the presence and absence of La^{3+}, with a Ringer's control initially and between the two solution changes. The sequential effects of Ringer's and 250 mM NaCl substitution, in the presence and absence of La^{3+}, are presented in Figure 4A,B ($n = 26$), respectively. Tissue conductance increased significantly with 250 mM NaCl and could be reversibly decreased by subsequent replacement with Ringer's as the mucosal bathing solution. A second substitution with 250 mM NaCl resulted in a similar increase in conductance. If the second 250 mM NaCl substitution also contained La^{3+}, the increase in conductance was significantly lower than with the salt solution alone ($P < 0.011$).

In experiment 3, toads placed on 200 mM KCl consistently showed SPD behavior for brief periods and initiated the WR in a small percentage of the trials. The combined time showing SPD and WR (SPD + WR) is shown in Figure 5A, while the percentage of trials in which WR was initiated is shown in Figure 5B. The addition of Ba^{2+} to the 200 mM KCl solution had no effect on the SPD + WR time and WR

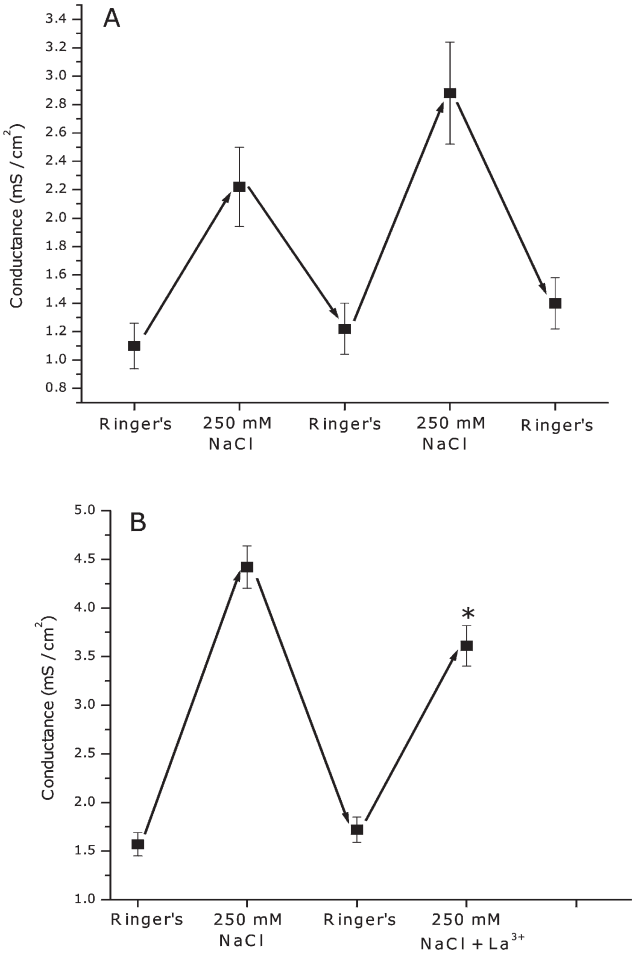


Figure 4 **(A)** The increase in conductance produced by 250 mM NaCl is reversible and can be repeated with subsequent exposure to the salt solution. The conductance following the first and second exposures to 250 mM NaCl is not different. **(B)** When the second salt substitution contains 5 mM La^{3+}, the increase in conductance is significantly reduced relative to the first (*).

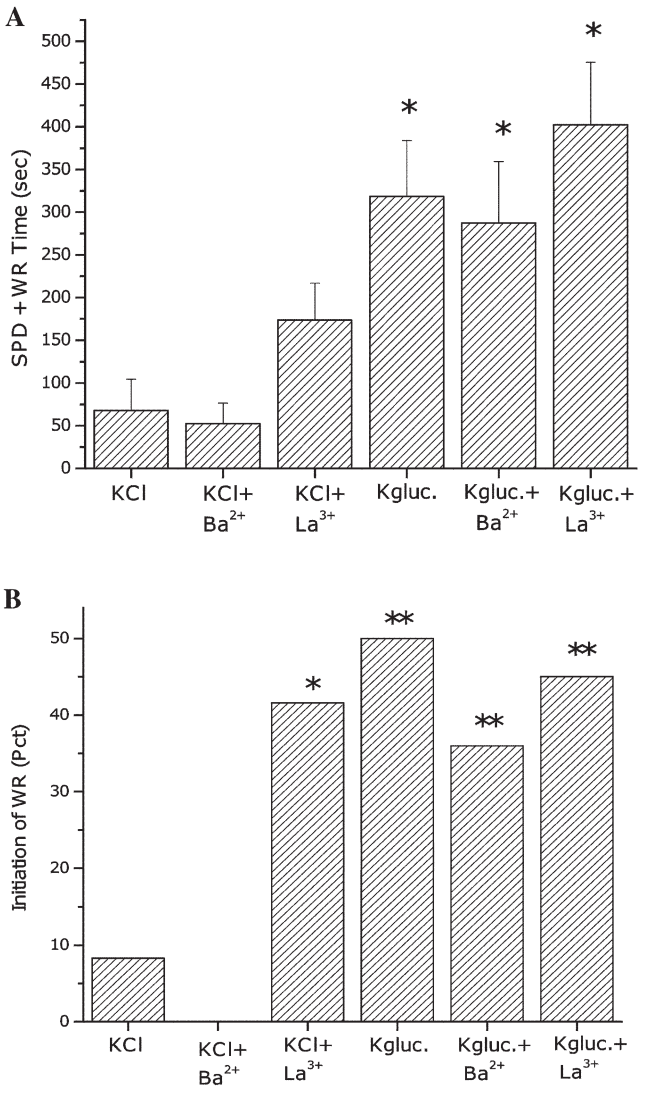


Figure 5 **(A)** When placed on 200 mM KCl solutions, toads consistently initiated SPD behavior and in a few trials WR was initiated for brief periods. Ba^{2+} had no significant effect on the combined time showing SPD and WR. La^{3+}, when compared with either Ba^{2+} or KCl alone, did not significantly increase SPD + WR time. With gluconate as the anion, the duration of combined SPD and WR behavior was significantly increased relative to the Cl^- trials for each treatment (*) but was not further affected by either Ba^{2+} or La^{3+}. **(B)** The percentage of trials where WR was initiated on 200 mM KCl was significantly greater with La^{3+} than with Ba^{2+} in the solution (*). A similar increase was seen with gluconate as the anion and the anion effect was not further stimulated by either Ba^{2+} or La^{3+} (**).

was not observed in any trial (n = 11). With La^{3+} in the KCl solution (n = 12) the increase in SPD + WR time was not significantly different from that with Ba^{2+} or KCl alone. However, the initiation of WR, in ~40% of the trials, was significantly greater than that with Ba^{2+} (P = 0.016). With gluconate as the anion (n = 12), both SPD time and WR initiation were significantly increased relative to trials with Cl^- as the anion (P = 0.0017). Neither Ba^{2+} nor La^{3+} increased SPD time or WR initiation significantly above that of gluconate alone.

Discussion

One micromolar amiloride did not have a significant effect on SPD and WR, despite the fact that the Ki of amiloride for ENaCs in toad skin is ~200–300 nM (Smith and Benos, 1991). In Ussing chamber preparations complete inhibition of Na^+ current by 100 μM amiloride takes ~2 min after addition to an apical solution of amphibian Ringer's (Fischer and Lockard, 1988). With 250 mM NaCl bathing the outer surface of the skin and a similar time lag for inhibition it is likely that the unsuitable hydration source will be detected before the chemosensory pathway becomes completely blocked. In about half of the trials with 10 μM amiloride concentration the blockage of ENaCs appears to be rapid enough to suppress the sensory transduction mechanism to the point that toads will initiate the WR. The increased

frequency of initiating WR observed in Figure 1B was similar to the results of Hoff and Hillyard (1993a), who found that 10 μM amiloride increased WR initiation from 0 to 44%. However, over the same time frame the hyperosmotic solution causes cell junctions to open and activates a paracellular transduction pathway that is amiloride-insensitive. Hypertonic bathing solutions on the apical surface of amphibian skin have long been known to activate a paracellular conductance pathway (Ussing and Windhager, 1964; Erlij and Martinez-Palomo, 1972).

With 15 min trials on 250 mM NaCl, toads returned to the surface and displayed SPD behavior but not the WR (Figure 2A,B). Unlike the previous experiments, amiloride did not further increase the time spent in contact with the salt solution or SPD time, but it did stimulate WR initiation in about one-quarter of the trials. It is noteworthy that the experiments of Figure 2 were conducted in a different group of toads at a different time of the year (summer) versus winter for the experiments of Figure 1. Seasonal variation plus differences in the molting cycle could affect the paracellular versus the transcellular conductance (Larsen, 1991). A greater paracellular conductance could reduce the amiloride sensitivity of the skin so that an amiloride effect is only seen when the paracellular pathway is blocked. An alternative explanation for amiloride-insensitive SPD behavior could be self-inhibition of ENaCs in the presence of increased Na^+ concentration in the apical bathing solution. This phenomenon was first described in amphibian skin (Fuchs *et al.*, 1977). More recently, Gilbertson and Zhang (1998a) suggested that self inhibition may explain taste receptor cell (TRC) adaptation in response to elevated Na^+ exposure in rat tongue.

La^{3+} restored WR initiation in ~25% of the trials with 250 mM NaCl and the amiloride and La^{3+} effects were additive. La^{3+} is believed to block cation transport through tight junctions (Simon *et al.*, 1993; Gilbertson and Zhang, 1998b). The latter study found that 6 mM La^{3+} produced a 40% reduction in transepithelial current across isolated rat and hamster tongue epithelia that was bathed with 500 mM NaCl in the mucosal solution. In the present study, 5 mM La^{3+} reduced tissue conductance from 4.4 to 3.6 mS (18.3%) when 250 mM NaCl bathed the mucosal surface of the skin. An emerging body of evidence indicates that epithelial tight junctions are regulated by signaling pathways that include claudins, occludins and the ZO family of membrane associated gualylate kinases (Mitic and Anderson, 1998; Rothen-Rutishauser *et al.*, 2002). Similar mechanisms in chemosensory epithelia may be involved in the activation of paracellular conductance and amiloride insensitivity.

In the isolated skins, substitution of Ringer's with 250 mM NaCl produced a large increase in conductance and a serosa-negative potential, suggesting anion conductance for the paracellular pathway, as proposed by Ussing and Windhager (1964) for frog skin exposed to a double-strength Ringer's in the mucosal solution. This will depolarize the basolateral membrane of the epithelial cells and, as proposed by Nagai *et al.* (1999), may be the source for the stimulation of branches of spinal nerves that form close associations with cells in the stratum germanitivum of toad skin. The demonstration that La^{3+} only partially offsets both the negative shift in transepithelial potential and the increase in conductance produced by 250 mM NaCl is consistent with the behavioral experiments where the small increase in WR behavior produced by La^{3+} is only significant when the transcellular pathway is also blocked. For rat tongue, Ye *et al.* (1994) have suggested that the paracellular mechanism for K^+ and also the amiloride-insensitive component of Na^+ taste is mediated by a 'subtight junctional transducer . . . with access limited by anion mobility'. Sullivan *et al.* (2000) came to a similar conclusion, observing that the time *B. punctatus* remained on tissues moistened with hypertonic Na^+ salt solutions increased with the mol. wt of the anion; gluconate > phosphate > acetate > Cl^-. Feldman *et al.* (2003) observed that the lingual surface potential in humans becomes significantly hyperpolarized when a rinse NaCl solution is raised from 100 to 600 mM, but that the inhibition by amiloride is variable among individuals. They conclude that the role of ENaCs appear to 'vary among individuals'. The activation of a paracellular pathway might account for this variability.

In the initial trials of experiment 3, using 250 mM KCl as a hydration source resulted in very brief contact with the salt solution and no sensitivity to either Ba^{2+} or La^{3+}. Reducing the concentration to 200 mM resulted in consistent display of SPD behavior that was increased with gluconate as the anion but not by the addition of Ba^{2+}. The ability of La^{3+} but not Ba^{2+} to stimulate the initiation of WR behavior indicates that the paracellular pathway is the primary mechanism for K^+ detection. Because La^{3+} in 5 mM concentration is unable to overcome the higher K^+ concentration gradient and its effect on conductance with NaCl solutions is incomplete, it is likely that it does not completely block paracellular K^+ conductance at 200 mM concentration and thus La^{3+} only partially restored the initiation of the WR. With gluconate as the anion, SPD times were significantly increased but the initiation of WR was not further stimulated by La^{3+}.

In all of the experiments described above, blocking of transcellular (amiloride) and paracellular (La^{3+} and gluconate) pathways restores the initiation of the WR in only about half of the trials and, when initiated, the duration of the WR posture is short. Thus, the initiation of the WR posture and the continued application of the seat patch skin to a hydration surface involves separate and sequentially applied sensory capabilities of the skin. Among these, the ability to detect an osmotic gradient favoring water absorption is certainly important. In support of this hypothesis, toads presented with hydration sources made hyperosmotic with a non-electrolyte (urea) similarly reduce time spent on that source and do not initiate the WR (Brekke *et al.*, 1991).

Water absorption involves water movement across the skin epithelial cells via aquaporins and may be as rapid as 30% of the body weight per hour in dehydrated toads (Hoff and Hillyard, 1993b). Recent RT–PCR studies demonstrate the presence of aquaporins 2 and 3 in the posterioventral skin of *Hyla japonica* (Tanii *et al.*, 2001; Hasegawa *et al.*, 2003) and *Bufo bufo* (Willumsen *et al.*, 2003). Kim *et al.* (1999) demonstrated that aquaporins exist in taste cells and suggested a role for water channels in osmotic sensing. Changes in cell volume that result from water loss to hyperosmotic media could be a mechanism for activation of mechanosensory cation channels in the skin that activate sensory neurons associated with the epithelium. Gilbertson (2002) observed that 'the osmotic status of the taste stimulus has received comparatively little attention'. The amphibian skin, as a chemosensory and absorptive epithelium, is an excellent model to investigate this phenomenon.

Acknowledgements

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