Insulin activates epithelial sodium channel (ENaC) via phosphoinositide 3-kinase in mammalian taste receptor cells

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Baquero AF, Gilbertson TA. Insulin activates epithelial sodium channel (ENaC) via phosphoinositide 3-kinase in mammalian taste receptor cells. Am J Physiol Cell Physiol 300: C860–C871, 2011. First published November 24, 2010; doi:10.1152/ajpcell.00318.2010.—Diabetes is a profound disease that results in a severe lack of regulation of systemic salt and water balance. From our earlier work on the endocrine regulation of salt taste at the level of the epithelial sodium channel (ENaC), we have begun to investigate the ability of insulin to alter ENaC function with patch-clamp recording on isolated mouse taste receptor cells (TRCs). In fungiform and vallate TRCs that exhibit functional ENaC currents (e.g., amiloride-sensitive Na⁺ influx), insulin (5–20 nM) caused a significant increase in Na⁺ influx at ~80 mV (EC₅₀ = 7.53 nM). The insulin-enhanced currents were inhibited by amiloride (30 μM). Similarly, in ratimetric Na⁺ imaging using SBFI, insulin treatment (20 nM) enhanced Na⁺ movement in TRCs, consistent with its action in electrophysiological assays. The ability of insulin to regulate ENaC function is dependent on the enzyme phosphoinositide 3-kinase since treatment with the inhibitor LY294002 (10 μM) abolished insulin-induced changes in ENaC. To test the role of insulin in the regulation of salt taste, we have characterized behavioral responses to NaCl using a mouse model of acute hyperinsulinemia. Insulin-treated mice show significant avoidance of NaCl at lower concentrations than the control group. Interestingly, these differences between groups were abolished when amiloride (100 μM) was added into NaCl solutions, suggesting that insulin was regulating ENaC. Our results are consistent with a role for insulin in maintaining functional expression of ENaC in mouse TRCs.

TASTE RECEPTOR CELLS (TRCs) must recognize a vast array of different chemical structures, ranging from those that are small and ionic to compounds with complex tertiary structures. This chemo sensory ability allows animals to distinguish compounds that may be either harmful to the organism or, alternatively, necessary for nutritional needs. In mammals, it is well established that salt taste transduction is mediated by sodium (Na⁺) ions through the apical amiloride-sensitive epithelial sodium channel (ENaC). The influx of Na⁺ ions directly depolarizes the taste cell, eventually leading to the release of neurotransmitter onto the afferent nerve fibers (10, 12). ENaC has been characterized both in TRCs and in other transporting epithelia, and it shares a number of common features across tissue types. Similarities include small conductance (~5 pS), Na⁺ > Li⁺ >> K⁺ ion selectivity, regulation by extracellular Na⁺ (self-inhibition) and intracellular Na⁺ (feedback inhibition), and regulation by hormones. These functional similarities between ENaC in the taste system and other transporting epithelia extend to the molecular level. Experiments in different rodent species have shown that ENaC channels expressed in TRCs have a high sequence homology with those channels expressed in other organs. Thus, in many regards, ENaC appears similar across organ types (11, 15, 23, 27, 39).

The regulation of ENaC is essential for salt and water balance in various Na⁺-transporting epithelia. Current evidence in taste cells and other transporting epithelia has demonstrated that ENaC expression and/or function may be altered by a number of hormones (e.g., aldosterone, vasopressin, atrial natriuretic peptide). Aldosterone (Aldo) and arginine-vasopressin (AVP) appear to increase ENaC-mediated currents, while atrial natriuretic peptide and, perhaps, oxytocin reduce these Na⁺ currents. Hormones like Aldo and AVP can increase Na⁺ influx through ENaC by increasing the channel’s open probability and/or decreasing the turnover of ENaC in the cell membrane, thus increasing functional ENaC expression (13, 19, 26). Additionally, the importance of ENaC regulation is well understood for sodium balance, blood pressure, and extracellular fluid volume (46).

Recently, research in kidney epithelia has suggested that insulin contributes to Na⁺ movement via ENaC. In general, both ENaC open probability and membrane expression of ENaC in the cell membrane can be increased by insulin stimulation acting through insulin/IGF-I receptors. Insulin-mediated Na⁺ reabsorption is believed to occur through the phosphoinositide 3-kinase OH (PI3-kinase) signaling pathway (38, 45). Activation of PI3-kinase by insulin leads to phosphorylation of two downstream signaling cascades: 1) a PI3-kinase signaling pathway leading to the phosphorylation of ENaC and/or 2) the activation of phosphoinositide-dependent kinase 1 (PDK1). Thus, synthesis of phosphatidylinositol 4,5-bisphosphate [PtdIns(3,4)P₂ or PIP₂]/phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P₃ or PIP₃] by PI3-kinase directly phosphorylates PDK1 and then activates serum- and glucocorticoid-regulated kinase 1 (SGK1). SGK1 is considered a key regulator of Na⁺ reabsorption via ENaC in kidney epithelia since several hormones such as insulin, Aldo, and AVP regulate ENaC through this pathway (8, 47). SGK1 function may increase either the open probability of apical ENaCs or the number of active channels in the membrane, although the mechanism of ENaC trafficking by insulin-SGK1-mediated responses is not well understood (21, 25, 28).

Little is known about the importance of insulin in the peripheral gustatory system. In the present study, we use a combination of whole cell patch-clamp recording and ratiometric functional Na⁺ imaging to determine whether insulin influences Na⁺ transport via ENaC in taste cells. Our results showed that insulin stimulates Na⁺ movement through...
amiloride-sensitive channels (i.e., ENaC) in these cell-based assays. In agreement with earlier reports in other transporting epithelia, insulin/ENaC-mediated responses in the peripheral gustatory system occurred via PI3-kinase-mediated pathways and its phospholipid products (40, 48). Following our functional and molecular studies, we attempted to investigate the role of insulin in the regulation of salt taste. Our results showed that insulin modulation of ENaC activity is extended to the animals’ ability to detect NaCl. These results reveal a novel insulin pathway that modulates ENaC function and salt taste in mouse TRCs.

METHODS

All experiments were performed on adult (2–4 mo old) male C57BL/6 mice that were maintained on a 12:12-h light-dark cycle with normal chow and water provided ad libitum. Mice lacking sgk (sgk–/– mice) were provided by Dr. A. Náray-Fejes-Tóth (9). All procedures involving animals were approved by the Institutional Animal Care and Use Committee of Utah State University and were performed in accordance with American Veterinary Medical Association guidelines.

Isolation of Taste Receptor Cells

Individual taste buds were isolated from mouse tongues following protocols used in earlier reports (2, 6). Briefly, tongues were removed and immediately immersed in Tyrode’s solution containing (in mM) 140 NaCl, 5 KCl, 1 CaCl2, 1 MgCl2, 10 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), 10 glucose, and 10 Na+ pyruvate (osmolality, 310 mosM). The pH was adjusted to 7.4 with NaOH. The anterior portion of the tongue containing the fungiform papilla was injected between the muscle layer and the lingual epithelium with ~0.15 ml of physiological saline (Tyrode’s) containing a mixture of collagenase A (1.1 mg/ml; Roche Applied Science, Indianapolis, IN), dispase II (2.4 mg/ml; Roche Applied Science), and trypsin inhibitor (1 mg/ml; type I-S; Sigma Chemical, St. Louis, MO). Approximately 0.1 ml of the same enzyme solution was also used to inject the area surrounding the circumvallate papilla. The injected tongue was incubated in Tyrode’s solution and bubbled with O2 for 45 min. The lingual epithelium was then peeled from the underlying tissue and pinned out in Tyrode’s in a Sylgard-lined petri dish with the mucosal side facing down. The epithelium was then incubated for 7 min with the same enzyme cocktail. Following the incubation, the epithelium was incubated in Ca2+-free Tyrode’s solution containing 2 mM BAPTA (Invitrogen, Eugene, OR) for 5 min. Amiloride (10 μM; Sigma Chemical) was added to all solutions to help protect against enzymatic degradation of ENaCs (11). Taste buds were removed from the epithelium using a large bire (150–250 μm) pipette and plated either onto a charged microscope slide in a Tyrode’s-containing Sylgard ring for patch-clamp recording or onto a Cell-Tak-coated coverslip in a laminar flow perfusion chamber for functional imaging.

For isolation of single taste cells, this procedure was slightly modified and set the chloride equilibrium potential (ECl) and potassium equilibrium potential (EK) at ~80 mV. In this experiment, recording pipettes were filled with a solution containing (in mM) 140 K-gluconate, 1 CaCl2, 2 MgCl2, 10 HEPES, 11 EGTA, and 3 ATP. The pH and osmolality were adjusted to 7.2 and ~310 mosM, respectively, with KOH. This low intracellular Cl− (10 mM) helped to eliminate most of the inward Cl− current, which facilitates the analysis of insulin-activated ENaC current and set the ECl near ~80 mV

Individual fungiform TRCs were recorded using conventional whole cell patch clamp. Patch pipettes were pulled to a resistance of 5–8 MΩ when filled with intracellular solution. Series resistance and capacitance were compensated optimally before recording. Currents were recorded in the presence and absence of test solutions in a continuous (gap-free) recording mode. The holding potential in all experiments was ~80 mV. Current data were recorded and command potentials were delivered using pClamp software (version 10). This software was interfaced with an AxoPatch 200B amplifier and DigiData 1322A data acquisition system (Molecular Devices, Sunnyvale, CA). Current-time relationships (I−T curve) were used to determine whether test solutions significantly altered amiloride-sensitive currents in TRCs.

Functional Sodium (Na+) Imaging

Functional imaging of taste receptor cells was carried out on cells loaded with a Na+–sensitive dye, sodium-binding benzofuran isothi严密的olactone ester (SBFI-AM; Invitrogen). Single taste cells were isolated as described above and plated onto charged coverslips attached to a laminar flow perfusion chamber (RC-25F Warner Instruments, Hamden, CT). TRCs were loaded with ~4 μM SBFI-AM in Hanks’ buffer salt solution with HEPES, sodium pyruvate, 1% pluronic acid F-127 (Invitrogen), and 2% fetal bovine serum for 60 min. The cells were perfused with Na+-free Tyrode’s solution (in mM) 140 NMDG, 5 KCl, 1 MgCl2, 1 CaCl2, 10 HEPES, 10 glucose, and 10 Na+ pyruvate, adjusted to pH 7.4 with HCl. Increases in intracellular Na+ were recorded in Tyrode’s solution, and with and without insulin (20 nM) and/or amiloride (30 μM). The PI3-kinase inhibitor LY294002 (10 μM) and its inactive analog LY303511 (10 μM) were prepared from a stock solution of 30 mM (CalBiochem, San Diego, CA). Wortmannin (0.05 and 1 μM) was prepared from a 2 mM stock solution (Sigma). All inhibitors were diluted in Tyrode’s solution and made fresh daily before use. Data collection and analyses were recorded by InCyT High Speed I/M imaging system (Intracellular Imaging, Cincinnati, OH). Briefly, images were acquired with a monochrome integrating charge-coupled device camera through a ×40 oil immersion objective lens of an inverted Nikon TE-2000s microscope. Excitation wavelengths of 340 nm and 380 nm were emitted by a Benthos FGS 150 fast changing monochromator (Intracellular Imaging) with an emission wavelength ~510 nm. Images obtained were captured every 3 s by InCyT Im2 software (Intracellular Imaging). The SBFI ratio (340/380) was used to determine whether test solutions significantly altered Na+ influx on TRCs. Data analyses were carried out by measuring the area under the curve of the SBFI ratio in the presence and/or absence of both amiloride and/or insulin using Origin software (version 7; Northampton, MA).

RT-PCR

First-strand cDNA was synthesized using the iScript RT Kit (Bio-Rad, Hercules, CA). The maximum volume of taste RNA or 50 ng of kidney RNA was used for the reaction with the total volume being 20 μl. Reactions were also set up in which the reverse transcriptase enzyme was omitted as a control to detect genomic DNA contamination. After first-strand synthesis, 1 μl of cDNA was added to a PCR reaction mixture containing final concentrations of 50 mM KCl, 10 mM MgCl2, 10 mM TRIS pH 8.3, 0.2 mM dNTPs, 0.5 U TiRNaseHII (Promega), and 1 U Taq polymerase (Promega). The PCR reaction was set up in a 25 μl reaction mixture containing 100 ng of cDNA, 200 ng of each primer, and reaction buffer with 1.5 mM MgCl2 (Invitrogen). The specific PCR primers used for each of the genes were designed to correspond to the expected length of the amplicon. The PCR products were visualized using a UV transilluminator and excised for sequencing using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems). Reverse transcription primer (5′-CCCTAAGCGCTCTCTGTCG-3′) was used. The following PCR settings were used for each gene: 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C for 30 cycles. The PCR products were sequenced and aligned using SeqMan software (DNASTAR).
mM Tris-HCl (pH 8.3), 2.5 mM Mg\(^{2+}\), 200 μM dNTPs, ~500 nM forward and reverse primers, and 1.25 units Taq polymerase. PCR products were amplified using an initial 5-min denaturation step followed by 40 cycles of a 3-step PCR (30-s denaturation at 95°C, 30 s annealing at optimal temperature, and 45 s extension at 72°C), and concluding with a 7-min final extension step. Amplified sequences were visualized by electrophoresis in 2% agarose gels using 1× TAE buffer (40 mM Tris-acetate and 1 mM EDTA). Primer sequences, accession numbers, expected product sizes, and corresponding nucleotide sequences are shown in Table 1. Purification of PCR products for sequencing was performed using the QIAquick PCR purification kit (Qiagen, Valencia, CA). Sequences were determined by the dye-terminator method using an ABI Model 3100 Automated Sequencer (Foster City, CA). Partial sequences for each product were examined for sequencing was performed using the BLAST search engine (National Center for Biotechnology Information; http://blast.ncbi.nlm.nih.gov/BLAST.cgi).

**Animal Model of Acute Hyperinsulinemia**

Male, 2- to 4-mo-old C57BL/6 mice from Charles River Laboratories (Wilmington, MA) were administered either vehicle or insulin (Novolin; Novo Nordisk, Princeton, NJ) at 0.75 U/kg body wt in sterile 0.9% NaCl as a single intraperitoneal injection. Both insulin-treated \( (n=40) \) or vehicle-treated \( (n=40) \) mice received an injection 15 min before the start of behavioral testing. At the end of the behavioral assay, blood glucose (mg/dl) was measured with a glucometer (BD Biosciences, San Diego, CA) using blood obtained from the saphenous vein using techniques previously described (18).

**Behavioral Experiments**

**Lickometer training.** All behavioral experiments were performed using a computer-controlled stimulus delivery and a lick monitoring station (“Davis Rig”, model MS-180, Dilog Instruments, Tallahassee, FL). Mice were trained and tested under 22.5 h of water access restriction. To prevent excessive body mass losses, all animals received 1 h of water in their home cage after each section. Under this regimen, the mice were able to maintain a body mass between 85% and 90% of their initial body weight. Training consisted of 2 days: First, the mice were allowed one presentation of distilled water for 15 min and a 15-min time limit to first lick. The second day of training consisted of thirty 10- to 15-s presentations of distilled water with a 12-s intertrial interval and a 150-s time limit to first lick.

**Testing procedure.** Following the training phase, mice were subjected to behavioral testing for three consecutive days. Each day, all of the mice received either insulin or vehicle injection 15 min before the trial, and any animal that exhibited signs of distress was excluded from the study. In addition, we performed insulin tolerance tests to decide the best time frame to perform our behavioral assays based on the animal’s alertness and motivation to complete the task. All experiments began at the same time every morning, and each mouse from either insulin-treated or vehicle-injected group was presented with two 5-s presentations of each concentration in an ascending order from the lowest to the highest concentration followed by a 2-s rinse with distilled water. The intertrial interval was 10 s with a 150-s time limit for the first lick. Seven NaCl concentrations (30, 150, 270, 330, 450, 600, and 1,000 mM) were tested to determine relative preference for NaCl solutions using lick rates as the dependent variable. For experiments using orally administered amiloride, a 10 mM amiloride stock was prepared in ddH\(_2\)O, and 100 μM amiloride was made by dilution in each of the seven NaCl concentrations including the ddH\(_2\)O used for the water stimulus and rinses. All NaCl solutions were prepared fresh every day before testing. At the end of the behavioral experiments, data were analyzed by the average of the number of licks for each NaCl concentration divided by the average number of licks to water per trial. This lick ratio normalizes for individual differences and motivational state of the animal (16). The mean ± SE values of the tasteant/water lick ratio were plotted to obtain concentration-response curves for each group.

**RESULTS**

**Insulin Increases Amiloride-Sensitive Na\(^{+}\) Currents in Mouse Taste Cells**

To determine whether insulin stimulates sodium (Na\(^{+}\)) transport in the gustatory system, we recorded insulin effects in Na\(^{+}\) transport in taste cells using conventional whole cell patch-clamp recording. In all experiments, cells were held at −80 mV in a continuous (gap-free) recording mode with the pipette containing 140 mM K-gluconate and Tyrode’s as the extracellular solution. This intracellular solution helped to eliminate any inward Cl\(^{-}\) or K\(^{+}\) current since the \( E_{Cl} \) and \( E_{K} \) were approximately −80 mV. In most TRCs, insulin increased inward Na\(^{+}\) currents, consistent with previous studies in other transporting epithelia that showed that the ability of insulin to enhance Na\(^{+}\) movement is through the activation of ENaC (38, 44). To determine whether the insulin-enhanced responses we recorded were attributable to ENaC, we recorded insulin-induced responses in the presence or absence of amiloride (30 μM) and/or benzamil (10 μM). Insulin dramatically increased inward Na\(^{+}\) current in taste cells, and the enhancing effects of insulin were sensitive to amiloride (Fig. 1A) or benzamil (Fig. S1; Supplemental Material for this article is available online at the Journal website), suggesting that insulin activates ENaC in the taste system. In contrast, insulin had no effect on taste cell conductance when Na\(^{+}\) was replaced with NMDG.

To investigate whether insulin stimulation of inward Na\(^{+}\) currents occurred in a concentration-dependent manner, we characterized insulin responses in TRCs using whole cell patch clamp. In all fungiform cells, the enhancement of inward Na\(^{+}\) currents correlated with insulin concentration (Fig. 1B). The concentration-dependent changes in Na\(^{+}\) transport illustrated that the magnitude of Na\(^{+}\) current depends on insulin and that these responses occur

### Table 1. Nucleotide sequences for primers in the RT-PCR assays

<table>
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<tr>
<th>Target</th>
<th>GenBank Accession No.</th>
<th>Sense Primer/Antisense Primer</th>
<th>Corresponding Nucleotide Sequence</th>
</tr>
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<tbody>
<tr>
<td>IR</td>
<td>NM_010568</td>
<td>5′-CTT TGG GAA ATC ACT AGC TGG-3′</td>
<td>3694–3715</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5′-GT TGT TCT CCT CGG TGT AGG-3′</td>
<td>3929–3949</td>
</tr>
<tr>
<td>IRS-1</td>
<td>NM_010570</td>
<td>5′-CCG AAG TGC GGA AGA TTC-3′</td>
<td>3311–3329</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5′-CTT ATT CTG CCC AAC TCA ACT-3′</td>
<td>3781–3802</td>
</tr>
<tr>
<td>IRS-2</td>
<td>NM_001081212</td>
<td>5′-GGG GAG AGA ATG ACC ATG T-3′</td>
<td>2423–2445</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5′-GCT TGG GCT CTG TGG GTA GA-3′</td>
<td>2699–2719</td>
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<tr>
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<td>833–849</td>
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<tr>
<td></td>
<td></td>
<td>5′-GGC GTA GAG CAT ATG ATA CA-3′</td>
<td>928–947</td>
</tr>
</tbody>
</table>

IR, insulin receptor; IRS-1, insulin receptor substrate 1; IRS-2, insulin receptor substrate 2; SGK-1, serum- and glucocorticoid-regulated kinase-1.
The magnitude of inward Na\(^+\)/H\(^+\) concentrations of insulin was applied by bath perfusion at 1, 3, 5, 7.5, 10, and 20 nM. Data points are the statistically weighted best-fit logistic relation with EC\(_{50}\) was obtained by obtaining the area under the curve (AUC) resulting from changes in the 340/380 ratio for each condition in a manner analogous to the patch-clamp experiments. Conservatively, we classified as amiloride-sensitive cells only those cells that showed a reversible decrease in SBFI ratio. In all cases, fungiform or circumvallate cells were perfused with Na\(^+\)-free solution, and 140 mM NaCl was used as the prototypical salt stimulus. As shown in Fig. 2A, 140 mM NaCl evoked Na\(^+\) responses (AUC: 30.5 ± 2.9) in fungiform taste cells and these NaCl-induced responses were inhibited by amiloride (30 µM; AUC: 23.3 ± 3.4). Repeated-measures ANOVA showed statistically significant differences between NaCl and amiloride treatments (P < 0.01; Fig. 2B). Following from our electrophysiological results, we investigated whether insulin could stimulate Na\(^+\) movement into taste cells. Na\(^+\) movement in fungiform taste cells was dramatically increased from an AUC of 30.5 ± 2.9 to an AUC of 85.1 ± 13.6 when insulin (20 nM) was added into the solution (Fig. 2A). Insulin-induced Na\(^+\) transport in TRCs is mostly through ENaC since Na\(^+\) responses were dramatically decreased by amiloride (30 µM; AUC: 45.2 ± 7). Statistical analysis with repeated-measures ANOVA revealed a significant difference between Na\(^+\) responses with and without insulin (P < 0.01); i.e., amiloride + insulin-mediated responses were significantly different from those with insulin treatment alone (P < 0.01).

The ability of insulin to regulate Na\(^+\) movement via ENaC is extended to other areas of the tongue. Circumvallate cells loaded with SBFI evoked similar Na\(^+\) responses (AUC: 27.3 ± 4.3; Fig. 2C). These responses were inhibited by amiloride (30 µM; AUC: 16.9 ± 3). Similar to the anterior taste buds, the posterior part of the mouse tongue exhibited amiloride sensitivity. Insulin treatment in circumvallate cells stimulated Na\(^+\) movement approximately twofold (AUC: 27.3 ± 4.3 to 54.3 ± 5.1). Amiloride (30 µM) diminished the insulin-mediated Na\(^+\) enhancement back to near control levels (AUC: 24.0 ± 2) in the posterior tongue, consistent with the action of insulin on ENaC-mediated Na\(^+\) movement. Repeated-measures ANOVA analysis revealed significant differences between NaCl and amiloride (30 µM; P < 0.01). Insulin treatment showed significant differences compared with NaCl (P < 0.01), and Na\(^+\) responses between insulin and insulin + amiloride-treated cells were significantly different (P < 0.01). Importantly, the insulin-mediated enhancement of Na\(^+\) influx in taste cells was rapid and reversible. However, we observed a difference in the amount of Na\(^+\) movement mediated by insulin between fungiform and circumvallate taste cells (Fig. 2, C and D).

**Insulin-Mediated Na\(^+\) Transport Occurs via PI3-Kinase**

Recent studies in kidney epithelia have suggested that PI3-kinase is involved in the activation of ENaC by insulin (38, 40, 45). However, the role of PI3-kinase in the signaling pathway in taste transduction is unknown. Therefore, we tested the sensitivity of insulin-induced Na\(^+\) transport to applications of PI3-kinase inhibitors. LY294002 (10 µM), a specific pharmacological blocker of PI3-kinase, can decrease insulin’s effects on Na\(^+\) responses in SBFI-loaded mouse taste cells (Fig. 3A).
Insulin enhancement of Na\(^+\) movement dramatically decreases with LY294002 treatment from (0.89 ± 0.09) to (0.1 ± 0.09). Paired Student’s t-test statistical analysis revealed differences between insulin (20 nM) and insulin (20 nM) + LY294002 (10 \(\mu\)M) \((P < 0.01)\).

To determine whether PI3-kinase was involved in insulin-mediated responses in the posterior tongue, SBFI-loaded circumvallate taste cells were treated with insulin (20 nM) and LY294002 (10 \(\mu\)M). As shown in Fig. 3B, LY294002 blocked insulin’s effects on relative Na\(^+\) influx from 1.36 ± 0.2 to 0.22 ± 0.18, and the difference in Na\(^+\) response between insulin and insulin + LY294002 was statistically significant \((P < 0.01)\). In contrast, LY303511, an inactive analog of LY294002, had no effect on insulin stimulation of relative Na\(^+\) transport (from 2.87 ± 0.44 to 3.37 ± 0.45; Fig. 3C). Therefore, we observed reversible effects of LY294002 on insulin-stimulated Na\(^+\) movement within 10 min following the initial presence of the inhibitor (data not shown). Together, our observations showed that PI3-kinase is critical for insulin-enhanced Na\(^+\) transport in the taste system.

The Ability of Insulin to Enhance ENaC Function Is Mediated by Phosphatidylinositides

A number of phosphatidylinositides have been suggested to participate in ENaC activation. Several of these phosphatidylinositides are products of the PI4-kinase signaling pathway such as PtdIns(4,5)P\(_2\). Other phosphatidylinositides like PtdIns(3,4)P\(_2\)/PtdIns(3,4,5)P\(_3\) are synthesized by PI3-kinase, which is directly activated by insulin (29, 31, 34, 36, 37, 45). On the basis of our imaging results in which PI3-kinase blockers abolished acute insulin-enhanced Na\(^+\) responses, we
next wanted to investigate the role of phosphatidylinositides in insulin/ENaC-mediated responses by inhibiting the PI4-kinase signaling pathway. Using wortmannin (1 μM), which is an inhibitor of type III PI4-kinase and PI-related kinases at higher micromolar concentrations (1), we performed functional Na⁺ imaging in TRCs. The AUC obtained from SBFI ratio in the presence and absence of the test solution was analyzed by the relative amount of insulin enhancement (using the 140 mM Na⁺ response as baseline). Insulin-induced effects on amiloride-sensitive cells were abolished by wortmannin (1 μM) treatment (Fig. 4A). Inhibition of PI4-kinase decreased insulin-mediated Na⁺ responses from 0.96 ± 0.19 to −0.5 ± 0.07. Consistent with this response, amiloride sensitivity is absent in these cells, which suggests that PI4-kinase phospholipid products PtdIns(4)P/PtdIns(4,5)P₂ are vital to maintain Na⁺ influx via ENaC.

Since PI3-kinase is a downstream signaling effector of PI4-kinase, we wanted to determine the significance of PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃ on insulin/ENaC-mediated responses in TRCs. In these experiments, we used two pharmacological PI3-kinase blockers, wortmannin (50 nM) and LY294002 (10 μM). Insulin-evoked changes in taste cells were inhibited by both wortmannin, from 1.37 ± 0.23 to −0.11 ± 0.07, and LY294002, from 0.89 ± 0.09 to −0.01 ± 0.05 (Fig. 4, B and C). In contrast to what we found for PI4-kinase, inhibition of PI3-kinase does not affect 140 mM Na⁺ responses in the absence of insulin. Amiloride (30 μM) only reduces 140 mM Na⁺ responses, which suggests that there are functional ENaC channels in the plasma membrane but that PIP₂ and PIP₃ are necessary to increase insulin-mediated Na⁺ movement through ENaC. Clearly, our findings strongly suggest that ENaC function is regulated by phosphatidylinositol synthesis in the gustatory system and that both PI3-kinase and PI4-kinases are capable of enhancing and maintaining ENaC channel activity, respectively.

**Physiological Role of SGK in Insulin/ENaC-Mediated Responses**

Having established that PI3-kinase is crucial for ENaC activation by insulin in TRCs, we performed experiments to explore other mechanisms of insulin action. PtdIns(3,4,5)P₃, which is a product of PI3-kinase activation, is capable of interacting with the pleckstrin homology (PH) domain of PDK1 and PKB/Akt and then activating SGK (28, 31, 33). To discover whether insulin could enhance Na⁺ movement via SGK in the gustatory system, we first identified SGK expression in the taste system (Fig. S2). We performed functional Na⁺ imaging assays in the presence and absence of insulin (20 nM) in taste cells from transgenic SGK−/− mice (9). Insulin

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**Fig. 3.** Insulin enhancement of ENaC-mediated Na⁺ transport occurs via phosphoinositide 3-kinase (PI3-K) signaling pathway. A: effects of PI3-K inhibitor LY294002 (10 μM) on insulin-induced Na⁺ responses in mouse fungiform taste cells using functional Na⁺ imaging with SBFI. B: functional Na⁺ imaging from circumvallate taste receptor cells showing changes in insulin enhancement of Na⁺ influx in the absence and presence of LY294002 (10 μM). C: summary graph of insulin effects in Na⁺ influx before and after LY303511 treatment. Data are shown as means ± SE. *Significant reduction in insulin-mediated Na⁺ influx by the PI3-K inhibitor (P < 0.01, Student’s t-test).
had no effect on Na\(^+\) transport in taste cells from SGK\(^{-/-}\) mice (Fig. 5, B and D). In contrast, taste cells from SGK\(^{+/+}\) evoked greater Na\(^+\) influx compared with SGK\(^{-/-}\) and SGK\(^{+/+}\) mice. Student’s t-test analysis of the AUC showed a significant difference (P < 0.01) between 140 mM Na\(^+\) and amiloride (30 \(\mu\)M) treatment for both SGK\(^{-/-}\) and SGK\(^{+/+}\) TRCs (Fig. 5, C and D). Interestingly, insulin-induced Na\(^+\) influx was severely impaired in SGK\(^{-/-}\)-expressed Na\(^+\) compared with SGK\(^{+/+}\) littermates. The magnitude of the insulin-stimulated Na\(^+\) transport was found to be less in SGK\(^{-/-}\) (26.2 \(\pm\) 3) than SGK\(^{+/+}\) (48.9 \(\pm\) 11.7). Our observations imply that both SGK\(^{-/-}\) and SGK\(^{+/+}\) taste cells have functional ENaC channels. However, functional ENaC activity seems dramatically reduced in SGK\(^{-/-}\) taste cells. Consequently, our findings suggest that SGK is essential to maintain normal ENaC function and that the absence of SGK protein in the null mice severely inhibits insulin’s effects in the gustatory system.

Expression of Insulin Receptor, Insulin Receptor Substrate 1, and Insulin Receptor Substrate 2 in Taste Cells

In most transporting epithelia, insulin reabsorption of NaCl is initiated when insulin binds to the basolateral insulin receptor (IR). Autophosphorylation of IR elicits a number of downstream events including phosphorylation of IRS and PI3- kinase (3, 44). To verify the expression of IR, insulin receptor substrate 1 (IRS-1), and insulin receptor substrate 2 (IRS-2) in mouse taste buds, a series of RT-PCR assays were performed using total RNA isolated from fungiform, foliate, and circumvallate taste buds. Expression of IR, IRS-1, and IRS-2 was found in all three lingual taste bud types in a minimum of three independent experiments (Fig. 6). All PCR products were sequenced using a PE Biosystem 377 automated DNA sequencer. The sequences for mouse taste bud IR, IRS-1, and IRS-2 were at least 99% homologous with sequences from GenBank.

Insulin Regulates Salt Intake in Mice

We next performed a series of brief access behavioral tests to determine whether insulin enhancement of Na\(^+\) movement via ENaC in mouse taste cells was correlated with any differences in behavioral responsiveness to NaCl. Since we hypothesized that insulin could act primarily as a regulator of salt appetite in the taste system, we tested the ability of insulin to alter NaCl preference in a hyperinsulinemic animal model. For all experiments, 24 C57BL/6 mice were trained for 2 days and then divided into two groups. Each group received either intraperitoneal injections of insulin (0.75 U/kg) or vehicle. Both insulin-treated and vehicle-treated groups were injected 15 min before the start of the behavioral test, and preference for seven different NaCl concentrations was tested. Insulin-treated mice exhibited significant avoidance of NaCl at lower concentrations than the control group (Fig. 7A). These changes in NaCl preference in hyperinsulinemic mice were more evident at concentrations between 150 mM to 600 mM NaCl. Simple effects ANOVA analysis revealed highly significant differences (P < 0.01) between insulin-treated and control groups at the following NaCl concentrations: 150, 270, 330, 450, and 600 mM. Blood glucose levels (mg/dl) were lower in
insulin-treated mice (47.7 ± 5.6) compared with the vehicle-injected group (155.6 ± 3.8; \( P < 0.01 \) paired Student’s \( t \)-test; Fig. 7B).

To elucidate the functional role of ENaC in alterations of NaCl sensitivity by insulin treatment, we added amiloride (100 \( \mu \)M), a diuretic by means of its antagonism of ENaC, to all NaCl solutions (7). Additionally, amiloride was also added into the ddH2O used for the water stimulus and rinses to eliminate any possible taste cue from amiloride during the short-term taste assays. In contrast, behavioral effects of insulin in salt preference were abolished by amiloride (Fig. 7C). Thus, NaCl taste is diminished in both insulin-treated and control groups in the presence of amiloride (100 \( \mu \)M). The pharmacological effect of amiloride on ENaC channels attenuated any difference in NaCl sensitivity between hyperinsulinemic and control groups at concentrations below 330 mM NaCl. However, we observed differences between insulin-treated and control mice in the presence of amiloride (100 \( \mu \)M) at 600 mM NaCl (\( P = 0.01 \)). Blood glucose levels differed between the control group (160.8 ± 5.6) and the insulin-treated group (55.3 ± 4.1; \( P < 0.01 \), paired Student’s \( t \)-test; Fig. 7D).

**DISCUSSION**

The importance of ENaC in the transduction of sodium salts in taste cells is well established (4, 13, 14, 20, 39). Studies have shown ENaC in other tissues to be regulated by hormones, such as aldosterone, vasopressin, and insulin (13, 19, 26, 38, 39). The importance of serum- and glucocorticoid-regulated kinase (SGK) in insulin/ENaC mediated responses in taste cells. A: functional Na\(^+\) imaging with SBFI from SGK\(^{+/+}\) wild-type mice in the presence of insulin (20 nM) and, subsequently, amiloride (30 \( \mu \)M). B: insulin effects on Na\(^+\) influx in SGK\(^{+/+}\) mice in the absence and presence of amiloride (30 \( \mu \)M) treatment. C: summary graph showing insulin-mediated effects in Na\(^+\) transport in SGK\(^{+/+}\) wild-type animals. D: area under the curve graph from SGK\(^{-/-}\) mice showing a dramatic reduction in the magnitude of insulin-mediated Na\(^+\) responses. Data are shown as means ± SE. *Significant reduction in insulin-mediated Na\(^+\) influx by amiloride (30 \( \mu \)M; \( P < 0.01 \), Student’s \( t \)-test).
Recent studies indicate that ENaC activity can be increased directly by phosphatidylinositides (35, 37). In the present study, we revealed the importance of PI4-kinases in the taste system. Using wortmannin, which is a pharmacological blocker of PI4-kinases at high concentrations (i.e., 1 μM), we inhibited the synthesis of phosphatidylinositol 4-phosphate [PI(4)P]. In general, our results suggested that PtdIns(4)P/PtdIns(4,5)P2 are likely involved in Na+ transport (Fig. 4A). Though our results showed a reduction in 140 mM NaCl responses, we could not draw any meaningful conclusions about the role of PI4-kinase products since wortmannin (1 μM) also blocked PI3-kinases and PI-related kinases (1). To overcome the issue, we blocked PI3-kinase with a specific blocker, LY294002 (10 μM), then compared the effects with wortmannin (50 nM), which is a pharmacological blocker of PI3-kinase. As shown in Fig. 4, B and C, both LY294002 and wortmannin (50 nM) inhibited insulin’s effect on Na+ influx, yet did not affect 140 mM NaCl responses. In addition, there was evidence of amiloride-sensitive ENaC activity. In agreement with our results, it has been reported in other transporting epithelia that phosphatidylinositides bind ENaC and increase its open probability (31, 34, 37, 45). Although we have provided the first evidence of the role of PI4-kinase and PI3-kinase in taste cells, further experiments are needed to elucidate the characteristics of PtdIns(3,4)P2/PtdIns(3,4,5)P3 interaction with ENaC.

Insulin stimulation of ENaC-mediated Na+ transport occurs via PI3-kinase signaling pathway cascade by one of two mechanisms: 1) increasing the open probability of apical ENaCs and/or 2) increasing the number of active channels in the membrane. Insulin-induced PI3-kinase signaling cascade leads to phosphorylation of PDK1 and SGK1 activation. In kidney epithelia, stimulation of SGK1 by insulin increases the number of apical ENaC. The interaction between SGK and ENaC occurs via phosphorylation of the ubiquitin ligase NEDD4–2. Thus, NEDD4–2 removes and degrades ENaC protein from the cell membrane (5, 8, 41, 50). In the present study we were interested in demonstrating the physiological...
role of SGK in mammalian taste cells. Our results show that TRCs from SGK−/− mice evoked alterations in the magnitude of insulin-mediated Na⁺ movement (Fig. 5). These findings illustrate the inability of insulin to increase Na⁺ influx in SGK−/− taste cells. Moreover, the present observations are also consistent with a reduction in functional ENaC expression in SGK−/− taste cells. It could be possible that absence of SGK1 protein allows a greater degradation of ENaC in the peripheral taste system. Similar results have been shown in kidney epithelia in which insulin-mediated Na⁺ retention was abolished in SGK−/− mice (22). More studies are needed to investigate the link between SGK and insulin-mediated salt appetite.

Our results showed insulin-mediated enhancement of Na⁺ influx through ENaC in taste cells, and we hypothesize that insulin plays a physiological role in the regulation of salt taste. To test this, we characterized insulin’s effects on behavioral responses to NaCl using a mouse model of acute hyperinsulinemia. NaCl concentrations were presented in ascending order to both groups to maintain the behavioral momentum of the animal. We showed that insulin-treated mice displayed a strong avoidance of NaCl solutions (Fig. 7, A and B). Given this, we performed a separate experiment with five vehicle-injected mice. NaCl concentrations were presented in random order, and no differences in NaCl lick ratio were found between the two experiments (data not shown). We considered the possibility that low glucose levels may create distress in the animals during behavior assays. However, this is unlikely because there were no differences in latency to the first lick for all NaCl concentrations between control or insulin-treated mice (Fig. S3).

Our results show evidence that insulin-mediated effects on Na⁺ movement extend to the animal’s behavior. Using amiloride to block ENaC function in taste cells, we attempted

**Fig. 7.** Behavioral effects of insulin on salt preference appear to be via ENaC channels. A: NaCl/water lick ratio (means ± SE) measured in short-term taste assays using the Davis Rig in two groups of 22 mice. Insulin-treated mice significantly avoided NaCl solutions between 150 mM and 600 mM. *Significant difference in NaCl preference compared with control mice (P < 0.01, simple effects ANOVA). B: blood glucose levels (mg/dl) in each group. Values are means ± SE. C: NaCl/water lick ratio. Each point represents 12 mice for each group. Avoidance of NaCl solutions was reduced in both control and insulin-treated mice due to the blocking of ENaC channel by amiloride (100 μM), which was present in all solutions. Values are means ± SE. *Significant difference in NaCl preference compared with control mice (P < 0.01, simple effects ANOVA). D: graph of blood glucose levels (mg/dl) in each group. Values are mean ± SE. *Significant reduction in blood glucose levels by insulin treatment compared with control (P < 0.01, Student’s t-test).
to determine the specificity of insulin’s effect on ENaC. If insulin was targeting Na\(^+\) movement through ENaC, it would be expected that NaCl preference would be similar in both hyperinsulinemic and control groups. Clearly, the presence of amiloride in all solutions was very effective at suppressing insulin’s effects on NaCl responses (Fig. 7, C and D). These data suggest that insulin activates ENaC channels expressed in the apical membrane of taste cells, causing these animals to experience a change in their NaCl taste preference. However, more studies are needed in this area to understand in greater detail the effects of insulin in the animal’s taste behavior.

In conclusion, the present study has demonstrated a new signaling pathway for maintaining functional ENaC expression in the taste system. Thus, insulin leads to PI3-kinase activation which either increases ENaC activity with its phospholipid products or increases the number of apical ENaCs by the activation of SGK. Our results are consistent with the emerging idea that the gustatory system is capable of responding to activation of SGK. Our results are consistent with the emerging idea that the gustatory system is capable of responding to the gustatory epithelium from the hamster and rat. Chem Sens 23: 283–293, 1998.


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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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