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Membrane-permeable tastants amplify β2-adrenergic receptor signaling and delay receptor desensitization via intracellular inhibition of GRK2's kinase activity ☆

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ABSTRACT

Background: Amphipathic sweet and bitter tastants inhibit purified forms of the protein kinases GRK2, GRK5 and PKA activities. Here we tested whether membrane-permeable tastants may intracellularly interfere with GPCR desensitization at the whole cell context.
Methods: β_2AR-transfected cells and cells containing endogenous β_2AR were preincubated with membrane-permeable or impermeable tastants and then stimulated with isoproterenol (ISO). cAMP formation, β_2AR phosphorylation and β_2AR internalization were monitored in response to ISO stimulation. IBMX and H89 inhibitors and GRK2 silencing were used to explore possible roles of PDE, PKA, and GRK2 in the tastants-mediated amplification of cAMP formation and the tastant delay of β_2AR phosphorylation and internalization.
Results: Membrane-permeable but not impermeable tastants amplified the ISO-stimulated cAMP formation in a concentration- and time-dependent manner. Without ISO stimulation, amphipathic tastants, except caffeine, had no effect on cAMP formation. The amplification of ISO-stimulated cAMP formation by the amphipathic tastants was not affected by PDE and PKA activities, but was completely abolished by GRK2 silencing. Amphipathic tastants delayed the ISO-induced GRK-mediated phosphorylation of β_2ARs and GRK2 silencing abolished it. Further, tastants also delayed the ISO-stimulated β_2AR internalization.
Conclusion: Amphipathic tastants significantly amplify β_2AR signaling and delay its desensitization via their intracellular inhibition of GRK2.
General Significance: Commonly used amphipathic tastants may potentially affect similar GPCR pathways whose desensitization depends on GRK2's kinase activity. Because GRK2 also modulates phosphorylation of non-receptor components in multiple cellular pathways, these gut-absorbable tastants may permeate into various cells, and potentially affect GRK2-dependent phosphorylation processes in these cells as well.

1. Introduction

Sweet, bitter and umami substances which act on taste G-protein-coupled receptors (GPCRs), T1Rs/T2Rs, are expressed not only in the oral cavity, but also along the gastrointestinal tract and in other organs such as pancreas, airway smooth muscle, testis and the heart [1–5]. By their interaction with T1Rs/T2Rs along the gastrointestinal tract, these tastants may induce post-oral physiological effect such as nutrient absorption (e.g., glucose) [3] and satiety signals [6]. In addition, many non-sugar sweeteners and bitter tastants (e.g., saccharin, acesulfame K, cyclamate, sucralose, naringin, caffeine and quinine) are absorbable through the gut into the circulation after oral feeding [7–10] and thus may potentially act on similar receptors located in other extra-oral tissues [4]. Except of the long-term safety tests conducted by the FDA

Abbreviations: GPCR, G protein-coupled receptor; GRK, GPCR kinase; PKA, protein kinase A; β_2AR, β_2-adrenergic receptor; β_2-HA, HA-tagged β_2AR; EGFR, epidermal growth factor receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ISO, isoproterenol; IBMX, 3-isobutyl 1-methylxanthine; DMEM, Dulbecco's Modified Eagle's Medium; NHD, neohesperidin dihydrochalcone; SACC, saccharin; D-TRP, D-tryptophan; NAR, naringin; QUIN, quinine; CAFF, caffeine; MELI, melibiose; MALT, maltose; SUC, sucrose

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and other public authorities, little information is available on the nature of physiological responses that such absorbable tastants may induce *in vivo*. The sweeteners aspartame and thaumatin are metabolized to their amino acids in the gastrointestinal tract, whereas saccharin (SACC), acesulfame K, cyclamate and sucralose are absorbed through the gut and then secreted in the urine with little metabolism [7,9]. In addition, SACC can stimulate or inhibit adenylyl cyclase activity in muscle and liver membranes, respectively [11], and the sweeteners acesulfame K and SACC, were found to stimulate adipogenesis and suppress lipolysis independently of the T1R2/T1R3 sweet taste receptors [12]. Also, sweeteners such as SACC, sucralose and aspartame were found to induce glucose intolerance by altering the gut microbiota [13]. In fact, our past study showed that feeding SACC-containing diets to rats increased proteolytic activity *in vivo* in the cecum, most probably due to SACC bacteriostatic effect rather than direct effect on the exocrine pancreas [14].

Many bitter and non-sugar sweeteners are amphipathic (containing both hydrophobic and hydrophilic domains), which allow them to permeate *in vitro* and *in vivo* into taste bud cells [15,16] as well as *ex vivo* into other epithelial cells unrelated to taste [17,18]. Depending on the tissue and cell type, such tastant permeation has been shown to be rapid, reaching millimolar intracellular concentrations in less than one minute. This phenomenon raises the hypothesis that amphipathic tastants may, in addition to stimulation of T1Rs/T2Rs, produce intracellular post-receptor cellular effects. For example, several amphipathic tastants were found to stimulate the GTPase activity of some purified G-proteins directly (e.g., G_i/G_o and transducin) [19], as well as inhibit purified protein kinase A (PKA) and GPCR kinase (GRK) activity (e.g., phosphorylation of rhodopsin) [15]. The latter molecular results have led us to hypothesize that such membrane-permeable compounds may interact intracellularly with GPCR signal-termination kinases, and thus delay the desensitization of certain GPCRs including the taste T1R/T2Rs, whose pathways of desensitization are yet to be characterized.

Desensitization of GPCRs is an important physiological feedback mechanism that protects against acute and chronic receptor overstimulation [20,21]. GPCR responsiveness occurs shortly after exposure to the agonist, followed by receptor phosphorylation as an initial step of desensitization, and receptor internalization as the subsequent step. GPCR phosphorylation can be mediated by two families of protein kinases: one is the second-messenger-dependent kinases, such as PKA and protein kinase C (PKC), which carry out heterologous desensitization; the other is Ser/Thr kinases, also known as GRKs, which perform homologous desensitization by phosphorylating Ser/Thr residues in the intracellular domains, C-terminal tail or third intracellular loop of agonist-occupied GPCRs [20]. The desensitization pathway of GPCRs usually involves the recruitment of cytosolic β-arrestin proteins to the cytoplasmic surface of the receptor, a process enhanced by GRK phosphorylation. The binding of β-arrestins to the receptors uncouples the receptors from their G proteins, thereby terminating G-protein signaling [20]. This β-arrestin binding further directs the internalization of the desensitized GPCRs via clathrin-coated vesicles [22], where the receptors are either degraded or recycled back to the plasma membrane.

Inhibition of GRK-mediated receptor phosphorylation can delay GPCR-signal termination, as shown in the visual [23] and other transduction systems [24]. In certain cases, e.g., in the metabotropic glutamate receptor 5 (mGluR5), the RH domain of GRK2 can sequester $G\alpha_q$ and interfere with $G\alpha_q$-coupled receptor signaling by targeting it for internalization via a phosphorylation-independent mechanism [25,26]. Although kinase-inactive mutant of GRK2 attenuated some $G\alpha_s$-coupled receptor signaling, GRK2's kinase activity is primarily responsible for β_2-adrenergic receptor (β_2AR) desensitization during the first 30 min of stimulation [27–29].

In view of previous phosphorylation assays indicating inhibition of purified GRK2/5 and PKA kinase activities by amphipathic tastants [15], the main objective of the present study was to investigate whether such tastants, via intracellular inhibition of GRK2, modify β_2AR downstream signaling, its phosphorylation and consequently β_2AR internalization. Using either heterologously or endogenously expressing β_2AR and its downstream signaling components allowed us to investigate the effect of amphipathic tastants on β_2AR function in a controlled cellular context.

2. Materials and methods

2.1. Chemicals and reagents

Dulbecco's Modified Eagle's Medium (DMEM), ISO, sugars and non-sugar sweeteners: SUC, MELI, MALT, NHD, SACC, D-TRP and bitter tastants: NAR, QUIN and CAFF, were purchased from Sigma-Aldrich. Primary antibodies, polyclonal anti-β_2AR, monoclonal anti-HA, and polyclonal anti-phosphoSer(355–356) β_2AR were purchased from Santa Cruz Biotechnology. FITC- or HRP-conjugated secondary antibodies were purchased from Jackson ImmunoResearch. Buffer A contained 50 mM β-glycerophosphate, 1.5 mM EGTA, 0.1 mM sodium orthovanadate, 1 mM EDTA, and 1 mM DTT, pH 7.3. Buffer H was the same as Buffer A but also contained 1 mM benzamidine, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 2 μg/ml pepstatin A. RIPA buffer contained 20 mM Tris, 137 mM NaCl, 10% (v/v) glycerol, 0.1% (w/v) SDS, 0.5% (w/v) deoxycholate, 1% (v/v) Triton X-100, 2 mM EDTA, 1 mM PMSF, and 20 μM leupeptin. The β_2AR plasmid was kindly provided by Dr. R. J. Lefkowitz, Howard Hughes Medical Institute, Duke University, Durham, NC, USA. HA-tagged β_2AR (β_2HA) was purchased from the Missouri S&T cDNA Resource Center, USA (www.cdna.org).

2.2. Cell culture

HeLa, HEK293T and HCT116 cells were obtained from American Type Culture Collection (Manassas, VA, USA). Cells were maintained in DMEM containing 10% (v/v) fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine at 37 °C in a humidified 5% CO_2 incubator. HeLa cells were transfected at about 50–70% confluence using MaxFect reagent (Molecular Research Laboratories, Columbia, MD, USA) according to manufacturer's instructions. HEK293T cells were transfected at about 90% confluence using Lipofectamine 2000 according to manufacturer's specification (Invitrogen, Carlsbad, CA, USA). Transfection was applied for 5 to 7 h in serum-free DMEM, and stopped by adding 20% (v/v) FBS in DMEM to cells. The day after transfection, cells were quickly trypsinized and split to approximately 60% confluence, and grown for the next 24 h. Cells were starved with 0.1% (v/v) FBS in DMEM overnight before experiments.

2.3. Transfection of small interfering RNA (siRNA)

siRNAs were chemically synthesized by Dharmacon (Lafayette, CO, USA). HeLa and HEK293T cells were plated in antibiotic-free medium, at 30–40% confluence in 100-mm dishes and were transfected simultaneously with 100 nM siRNA and 9 μg of plasmid encoding β_2AR, by using DharmaFECT Duo transfection reagent (Dharmacon, Lafayette, CO, USA) according to manufacturer's instructions. After 24 h cells were split into 24-well plates at approximately 60% confluence, cultured for 2 days, and then starved with 0.1% (v/v) FBS in DMEM overnight for further cAMP assay. For phosphorylation experiments HEK293T cells were plated in antibiotic-free medium, at 30–40% confluence in 6-well plates and transfected simultaneously for 48 h with 50 nM siRNA and 2 μg of plasmid encoding β_2AR, using DharmaFECT Duo transfection reagent (Dharmacon, Lafayette, CO, USA) according to manufacturer's instructions.

2.4. HPLC determination of tastant permeation into HeLa and HCT116 cells

Serum-starved HeLa or HCT116 cells at 70% confluence were incubated for 10 min with 10 mM SACC, 10 mM D-TRP, 0.6 mM (1.25 mM

for HCT116) NHD, 5 mM (10 mM for HCT116) CAFF, 0.5 mM NAR or 0.03 mM QUIN (3 min). They were then washed four times with cold 1× PBS and scraped in water. The cells were centrifuged at 4 °C, 17,000*g* for 20 min. The supernatant was collected, frozen and thawed twice. After the last freeze, the cell lysates were lyophilized and stored at −20 °C until analysis. The intracellular levels of SACC, QUIN, D-TRP and CAFF were determined by HPLC as previously described [17,18]. NHD and NAR were determined by HPLC similar to D-TRP and CAFF with the following modifications: the mobile phase for NHD was composed of acetonitrile and 0.5% (v/v) acetic acid at an isocratic ratio of 65:35, a flow rate of 0.75 ml/min, and detection at 282 nm. NAR was detected using an isocratic mobile phase composed of water and acetonitrile (20:80), a flow rate of 1 ml/min, and detection at 280 nm.

2.5. cAMP assay

β_2AR-transfected HeLa or HEK293T cells, and non-transfected HCT116 cells (expressing endogenous β_2AR, GRK2, but not GRK5, data not shown) were split into 24-well plates at approximately 70% confluence, cultured for one day, and then starved with 0.1% (v/v) FBS in DMEM overnight. The cAMP concentration was monitored essentially as previously described [17]. Briefly, cells were preincubated with tastants at a series of concentrations for different time periods. These and all subsequent experiments were conducted at 37 °C. In some experiments, cells were also incubated with the PDE inhibitor IBMX (150 μM) or the PKA inhibitor H89 (20 μM) for 20 min prior to 10-min preincubation with the tastants. To dissolve NAR, IBMX, H89 or QUIN, DMSO or ethanol was used but the final concentration of these solvents was no more than 0.1% (v/v). Following preincubation, β_2AR-transfected cells were stimulated with ISO for different times. The reaction was stopped with 5% trichloroacetic acid (TCA) and samples were prepared for RIA [17] to determine intracellular cAMP levels, using ^{125}I-labeled cAMP and anti-cAMP BSA serum [30]. After TCA treatment, cells were disrupted by adding 0.1 M NaOH for 30 min at room temperature and protein concentration was determined according to Bradford [31].

2.6. β_2AR phosphorylation

β_2HA-transfected HeLa cells were starved overnight, preincubated in the presence or absence of a tastant for 10 min and then stimulated with 10 μM ISO for 0 to 20 min. The reaction was stopped by aspiration of the medium, three washes with cold 1× PBS on ice and addition of RIPA buffer containing 25 mM NaF. Cells were scraped, collected in cold centrifuge tubes and centrifuged (17,000*g*, 4 °C, 20 min). For immunoprecipitation of β_2HA on Protein G-Plus agarose beads, the supernatants were transferred into Eppendorf tubes containing monoclonal anti-HA antibody previously conjugated to Protein G-agarose beads and rotated end-to-end for 2 h at 4 °C. The beads were washed three times with cold 0.5 M LiCl and centrifuged for 1 min (10,000*g*, 4 °C). Sample buffer was added and the samples were heated at 95 °C for 5 min, then subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane for Western blotting. Anti-phosphoSer(355–356) antibody at 1:700 dilution was used to recognize GRK2/5-phosphorylated sites on β_2HA [32] whereas the total amount of β_2HA was determined with anti-HA antibody at 1:1000 dilution. Horseradish peroxidase-conjugated secondary antibodies were used for ECL detection. Similar phosphorylation experiments were conducted with siRNA- and β_2AR-transfected HEK293T cells. However, in HEK293T cells, no immunoprecipitation procedure was used to identify the anti-β_2AR and anti-phosphoSer(355–356) antibodies. siRNA- and β_2AR-transfected HEK293T cells were plated in 6-well plates at approximately 60% confluence, grown for 24 h and starved overnight. Cells were preincubated for 10 min in the presence or absence of a tastant and stimulated with 1 μM of ISO for 0 to 25 min. Stimulation was stopped by aspiration of the medium and three washes with cold 1× PBS, after which cells were scraped in the presence of RIPA buffer and the cell lysates were centrifuged (4 °C, 17,000*g*, 20 min). Supernatants were collected and heated at 95 °C for 5 min in the presence of sample buffer. Samples were then subjected to 12% SDS-PAGE and transferred to a nitrocellulose membrane for Western blotting. Anti-β_2AR (gβ_2AR) and anti-phosphoSer(355–356) β_2AR (pβ_2AR) antibodies at 1:500 dilutions were used to detect total and phosphorylated β_2AR, respectively. Horseradish peroxidase-conjugated secondary antibody was used for ECL detection. Bands were quantified using ImageJ (NIH) software.

2.7. β_2AR internalization

(a) Confocal imaging: β_2AR-transfected HeLa cells were seeded at approximately 70% confluence onto glass coverslips placed in 12-well plates and cultured for 24 h. Following serum starvation overnight, cells were preincubated in the presence or absence of a tastant (3 min for QUIN, 10 min for the others) and then stimulated with 10 μM ISO from 0 to 20 min. After treatment, cells were washed twice with 1× PBS, fixed with 3% (w/v) paraformaldehyde in 0.1 M phosphate buffer for 15 min, then washed three more times with 1× PBS. Cells were then permeabilized and blocked for 2 h with a blocking solution containing 3% (w/v) BSA and 0.3% (v/v) Triton X-100 in 0.1 M PBS at RT. The primary antibody used specifically to detect β_2AR was diluted at 1:100 in the blocking solution and added to the cells overnight at 4 °C in a wet chamber. After washing with 1× PBS, cells were incubated with an FITC-conjugated anti-rabbit secondary antibody diluted at 1:200 in 1× PBS for 1 h at RT. Cells were then washed three times with 1× PBS and mounted on microscope slides. Fluorescent images were taken with a confocal microscope (BioRad) equipped with 60× immersion oil objective and LaserSharp 2000 software (BioRad). Images were slightly processed using Adobe Photoshop 7.0 software.

(b) Western blot analysis: β_2AR-transfected HeLa cells were cultured and stimulated as in (a). At the end of the reaction, cells were washed twice with cold 1× PBS and once with Buffer A. Buffer H containing 0.25 M sucrose was added and cells were scraped and transferred to cold centrifuge tubes. The samples were subjected to three centrifugation steps: first at 3000*g*, 4 °C, 10 min, then once at 10,000*g*, 4 °C, 10 min and finally once at 100,000*g* (4 °C, 45 min). At the end of the last centrifugation, the supernatant (cytosolic fraction) was transferred to centrifuge tubes whereas the pellet (containing the plasma membrane) was resuspended in RIPA buffer. Sample buffer was added and all the samples were heated at 95 °C for 5 min. The samples were subject to 12% SDS-PAGE and transferred to nitrocellulose membranes for Western blotting. Anti-HA antibody at 1:1000 dilution was used to detect the total amount of β_2HA in both fractions. Horseradish peroxidase-conjugated secondary antibody was used for ECL detection. EGF receptor (EGFR) and GAPDH, which are known to appear specifically in the membrane and the cytosol fractions, respectively, were used as control proteins.

3. Results

3.1. Certain amphipathic tastants permeate into HeLa, and HCT116 cells

In line with previous *in vitro* and *in vivo* data [15–18], three non-sugar sweeteners (NHD, SACC and D-TRP) and three bitter tastants (NAR, QUIN and CAFF) permeated rapidly into HeLa cells when cells were exposed to extracellular tastants at concentrations comparable with those present in various food items [33,34]. Following a 10-min incubation of HeLa cells with extracellular concentrations (in mM) of SACC (10), NHD (0.6), D-TRP (10), CAFF (5), NAR (0.5) and QUIN

(0.03, 3 min incubation), the intracellular concentrations of these compounds were (in mM) 51 ± 3, 3.5 ± 0.5, 51 ± 8.5, 20 ± 2, 3.7 ± 0.2 and 2.2 ± 0.1, respectively. Thus, the intracellular concentrations of these compounds were increased by four- to six-fold compared to their concentrations applied outside of the cells (note: that of QUIN was extremely high). Following a 10-min incubation of HCT116 cells with extracellular concentrations of 10 mM of D-TRP or 10 mM of caffeine, the intracellular concentrations were 12 ± 1 and 86 ± 4 mM, respectively, whereas 5-min incubation of HCT116 with extracellular concentration of 1.25 mM NHD resulted in intracellular concentration of 19 ± 6 mM. According to Fridman (Fridman, T., M.Sc. thesis, The Hebrew University of Jerusalem, Rehovot, 2009, pp. 67, published in Hebrew), who used the same procedure (confluency 50%) for tastants permeation, it was found that HEK293T cells were less permeable. The magnitude of permeation, however, is still significant with intracellular concentrations (in mM) of 4.5 ± 0.1, 0.14 ± 0.003, 0.3 ± 0.02, and 2.8 ± 0.07 for SACC, NHD, NAR and D-TRP, respectively.

3.2. Preincubation of β_2AR-transfected HeLa cells with amphipathic tastants amplifies ISO-stimulated cAMP formation

ISO is a specific ligand of β_2AR that activates adenylyl cyclase via Gs_α to form cAMP. Following ISO stimulation of the β_2AR-transfected HeLa cells, a rapid elevation in cAMP formation was observed, reaching (except CAFF) a peak at 30 s and then gradually decreasing for the rest of the 5-min incubation (Fig. 1A). On the other hand, a 10-min preincubation of the same β_2AR-transfected cells with each of the six amphipathic tastants prior to the 30 s ISO stimulation, significantly amplified the ISO-stimulated cAMP formation over the level obtained in samples that had been preincubated without these tastants (Fig. 1A). CAFF is a known inhibitor of PDE which elevates cellular cAMP. Therefore, to eliminate the elevation in cAMP level due to PDE inhibition by CAFF, IBMX was added to the CAFF-treated cells and their controls. Note that except CAFF the preincubation with the amphipathic tastants did not change the cAMP formation peak time point at 30 s (Fig. 1A) and 1 min (Fig. 1C) post-ISO stimulation. In CAFF-preincubated samples, cAMP formation peaked at 2 min (Fig. 1A) and at 5 min (Fig. 1C) post-ISO stimulation. In all cases, cAMP levels remained higher in samples preincubated with the tastants than in control samples during 2–3 min (Fig. 1A) or even during longer than 5 min (Fig. 1C) post-stimulation with ISO. This increased cAMP formation by each tastant was concentration-dependent (Supplementary material, Fig. S1A), resulting in about two-fold amplification in cells preincubated with a high concentration of tastants.

Amplification of ISO-stimulated cAMP formation by tastants also depended on the duration of tastant preincubation (from 1 to 10 min) (Supplementary material, Fig. S1B). For most tastants, a preincubation of at least 5 min was required to significantly amplify cAMP formation following ISO stimulation. Except for CAFF-treated cells, a 10-min preincubation of β_2AR-transfected HeLa cells with each of the amphipathic tastants alone, without subsequent stimulation by ISO, did not increase cAMP formation above basal (Supplementary material, Fig. S1C). Therefore, these amphipathic tastants per se do not act as β_2AR ligands, and their permeation into the cells was a prerequisite for the tastant amplification of cAMP formation.

To further verify the significance of tastant permeability for the tastant amplification of ISO-stimulated cAMP formation, we compared the effect of three membrane-permeable non-sugar sweeteners (D-TRP, SACC and NHD) with that of three sugar sweeteners (MELI, MALT and SUC) known to be membrane impermeable in mammalian cells (Fig. 1B). As shown in HeLa cells (Supplementary material, Fig. S1C), incubation of β_2AR-transfected HEK293T cells with either membrane-permeable or impermeable sweeteners, without subsequent stimulation by ISO, did not elevate the basal level of cellular cAMP. Furthermore, preincubation with the three membrane impermeable sweeteners prior to stimulation with ISO, did not enhance the subsequent ISO-stimulated cAMP formation compared with the stimulation by ISO alone. On the other hand, as expected, preincubation with the three membrane-permeable sweeteners prior to the stimulation by ISO, amplified the ISO-stimulated cAMP formation by almost 2-fold. It is therefore evident that the tastant amplification of ISO stimulation of cAMP formation is of intracellular source.

HCT116 cells contain endogenous β_2AR and GRK2 but not GRK5 (data not shown). These cells were permeable to NHD, D-TRP and CAFF tastants, and therefore, were used as a control model to test whether the phenomenon of amphipathic tastants-induced ISO-stimulated cAMP formation could be observed in cells containing endogenous β_2AR. As shown in Fig. 1C, preincubation of these cells with these three membrane-permeable tastants significantly amplified the ISO-stimulated cAMP formation similar to that found with the β2AR-transfected HeLa cells (Fig. 1 A and B). In the HCT116 experiments, preliminary trials indicated that the kinetics of cAMP formation was slightly slower, and hence, the time course in subsequent experiments was tested during 10 min.

3.3. The amplification of ISO-stimulated cAMP formation by the amphipathic tastants is likely to be independent of phosphodiesterase (PDE) or PKA inhibition

We next investigated whether these membrane-permeable tastants exert their cAMP-amplification effect via inhibition of intracellular cAMP breakdown by phosphodiesterases (PDE). Since the amphipathic compounds, except of the bitter and PDE inhibitor CAFF, did not increase the basal level of cAMP (Supplementary material, Fig. S1C), inhibition of PDE by the membrane-permeable tastants is of low probability. To further explore this possibility, β_2AR-transfected cells were preincubated with the same amphipathic tastants but in the presence or absence of the non-specific PDE inhibitor IBMX applied at a concentration known to effectively inhibit PDE activity [35], prior to the stimulation by ISO (Fig. 2A). As expected, in the presence of IBMX, the basal level of cellular cAMP was elevated compared with that observed in the absence of IBMX and ISO stimulation in the presence of IBMX resulted in an approximately 10-fold increase in cAMP formation above basal, compared to 3–5 fold increase in cAMP in response to ISO stimulation of samples lacking IBMX. Importantly, in the presence of IBMX, preincubation of the β_2AR-transfected cells with the amphipathic tastants further enhanced the ISO-stimulated cAMP formation, but the relative magnitude of this amplification induced by the tastants was similar in both the presence and absence of IBMX. These results are in agreement with the tastants' inability to elevate the cellular level of cAMP (Supplementary material, Fig. S1C) and supports our hypothesis that the putative contribution of PDE inhibition by the amphipathic tastants to tastants' amplification of ISO-stimulated cAMP formation is unlikely.

Given the above results, we hypothesized that these tastants, after permeating into the cells, may inhibit signal-termination pathways such as PKA (Fig. 2B). PKA can phosphorylate β_2AR [36], and amphipathic tastants inhibited the activity of purified PKA [15]. Results showed that preincubation of β_2AR-transfected cells with the membrane-permeable PKA kinase inhibitor, H89, significantly amplified the subsequent stimulation of cAMP formation by ISO (Fig. 2B), perhaps via inhibition of the PKA-β_2AR-G_i route [37]. We then measured the effect of tastants' amplification of ISO-stimulated cAMP formation in the presence or absence of H89 at a concentration known to result in about maximal inhibition of PKA activity [38]. Preincubation of β_2AR-transfected cells with amphipathic tastants produced additional amplification of ISO-stimulated cAMP formation in H89-treated cells (Fig. 2B). Nevertheless, the relative additional amplification of cAMP formation by amphipathic tastants in H89 treated cells was proportionally similar to tastants' amplification of cAMP formation in cells lacking H89. It thus appears that under the present experimental conditions, the tastants' amplification effect was essentially PKA-independent.

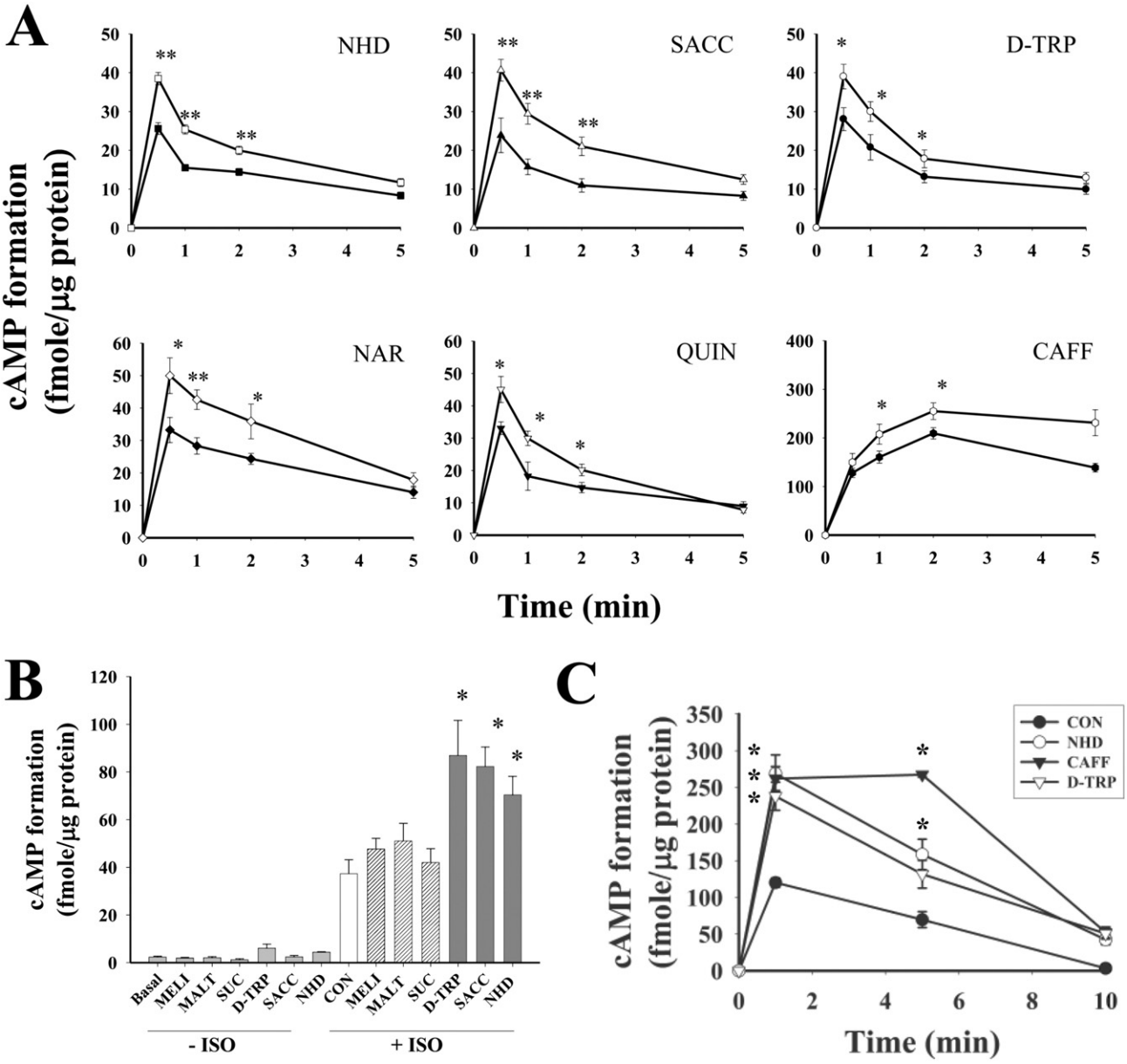


Fig. 1. Preincubation of cells expressing transfected or non-transfected β_2AR with amphipathic tastants amplifies ISO-stimulated cAMP formation. (A) Effect of tastant preincubation on time-dependent cAMP formation is shown. β_2AR-transfected HeLa cells were preincubated with either NHD (1.25 mM), SACC (10 mM), D-TRP (10 mM), NAR (0.5 mM), QUIN (0.03 mM) and CAFF (5 mM) (open symbols) or without (solid symbols) for 10 min. Samples containing CAFF (and their corresponding controls) were additionally preincubated with 150 µM IBMX for 20 min. Cells were then stimulated with ISO (10 µM) for different periods of time, up to 5 min and the intracellular concentration of cAMP was determined by RIA. Results are the means ± SEM for six measurements of an experiment repeated two to three times. * indicates significant ($P < 0.05$, two-tailed paired *t*-test) difference between the control and the tastant-containing samples. ** indicates significant difference ($P < 0.01$). (B) Only membrane-permeable tastants amplify the ISO stimulation of cAMP formation. β_2AR-transfected HEK293T cells were preincubated for 10 min with 100 mM of membrane impermeable disaccharide sweeteners of either MELI, MALT or SUC, and with membrane-permeable sweeteners of either D-TRP (10 mM), SACC (10 mM) or NHD (1.25 mM), without or with subsequent stimulation with ISO (10 µM) for 30 s. CON cells were preincubated without tastants and then stimulated by ISO for 30 s. Intracellular concentration of cAMP was determined by RIA. Results are the means and SEM for six measurements of an independent experiment repeated two times. * indicates significant (at least $P < 0.05$, two-tailed paired *t*-test) difference between the control and the tastant-containing samples. (C) Membrane-permeable tastants amplify the time-dependent ISO stimulation of cAMP formation in a cell model containing endogenous β_2AR and signaling components. HCT116 cells which contain endogenous β_2ARs and GRK2 were preincubated with either NHD (1.25 mM), CAFF (10 mM) and D-TRP (10 mM) for 10 min. Samples containing CAFF (and their corresponding controls) were additionally preincubated with 150 µM IBMX for 20 min. Cells were then stimulated with ISO (1 µM) for different periods of time, up to 10 min and the intracellular concentration of cAMP was determined by RIA. Results are the means ± SEM for six measurements of an experiment repeated two times. One- and two-way ANOVA (at least $P < 0.001$) and Tukey-Kramer HSD post-hoc comparisons were performed. * indicates significant (at least $P < 0.05$) between CON and the tastants containing samples in each time period.

3.4. Preincubation of β_2AR-transfected HeLa cells with amphipathic tastants delays the ISO-stimulated β_2AR phosphorylation

We then tested whether preincubation of the β_2AR-transfected cells with amphipathic tastants exerts the amplification of ISO-stimulated cAMP formation via inhibition of GRK-mediated phosphorylation of β_2AR (Fig. 3). GRK2 and GRK5 are known for their ability to phosphorylate β_2AR [32] and are endogenously present in the tested HeLa cells (data are not shown). The effect of ISO stimulation on β_2AR phosphorylation at various time points was monitored with an anti-phosphoSer(355–356) antibody, which recognizes GRK2/5 phosphorylation sites in β_2AR [28,32,39] (Fig. 3A, B). The rate of β_2AR phosphorylation in control cells (see CON, Fig. 3A, B) peaked at about 5 min post-ISO stimulation, and then gradually returned to basal level over the next 20 min. On the other hand, a 10-min preincubation of the β_2AR-transfected cells with each of the six tastants delayed the

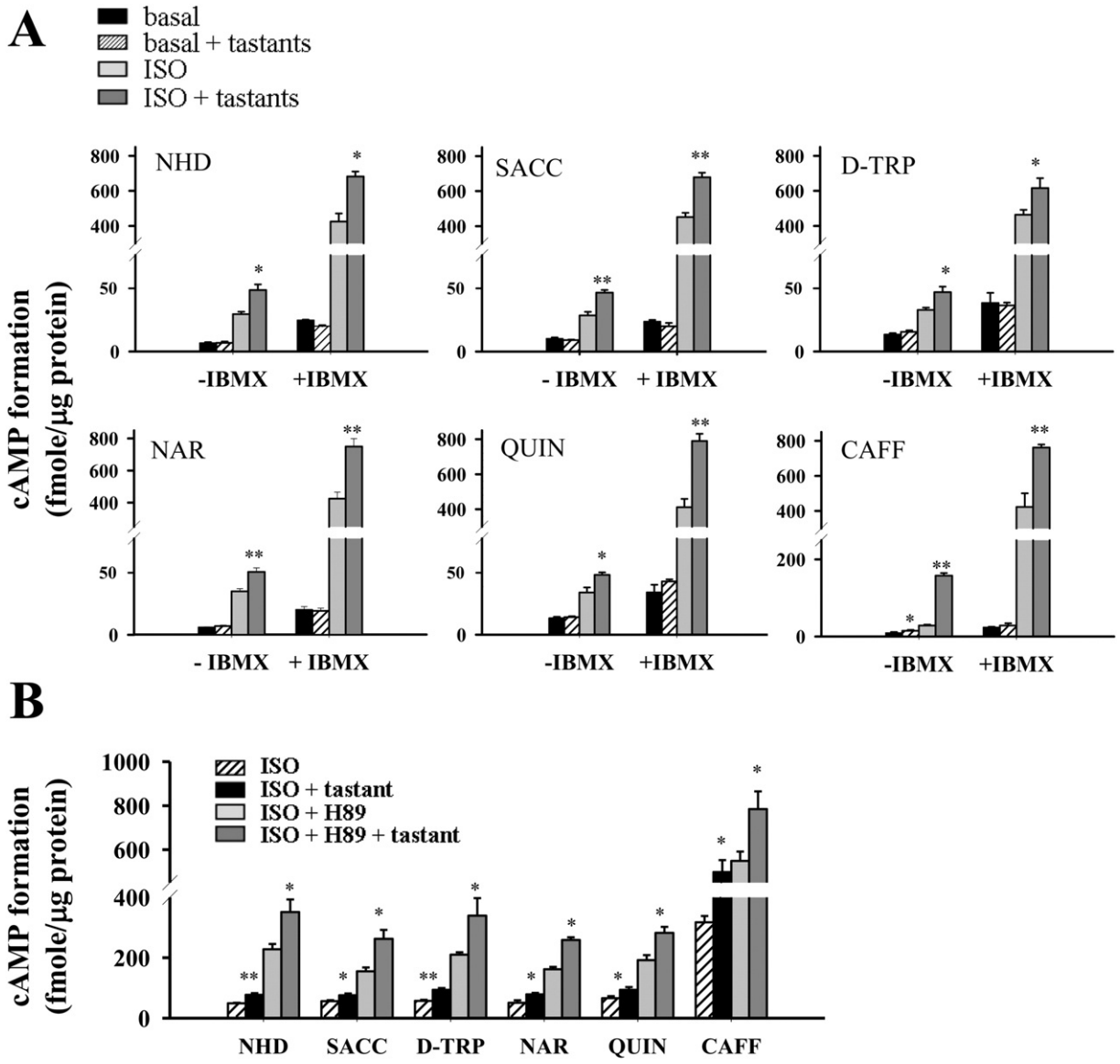


Fig. 2. Tastant amplification of ISO-stimulated cAMP formation is independent of PDE and PKA activities. (A) Effect of tastant preincubation on PDE activity. β_2AR-transfected HeLa cells were preincubated for 10 min with each tastant at the concentrations indicated in Fig. 1A, with or without preincubation with 150 μM IBMX for 20 min. Cells were then stimulated with ISO (10 μM) for 1 min and the intracellular concentration of cAMP was determined as in Fig. 1. Results are the means and SEM of six measurements for each data point of an experiment repeated two to three times. * indicates significant ($P < 0.05$, two-tailed paired *t*-test) difference between the corresponding control (either basal or post-ISO stimulation) and the tastant-containing samples. ** indicates significant difference at $P < 0.01$. (B) Effect of tastant preincubation on PKA activity. β_2AR-transfected HeLa cells were preincubated for 10 min with or without each tastant at the concentrations indicated in Fig. 1A, and with or without preincubation for 20 min with 20 μM H89. Cells were then stimulated with ISO (10 μM) for 1 min and the intracellular concentration of cAMP was determined as in Fig. 1. Results are the means and SEM of six measurements of an experiment repeated two to three times. * indicates significant ($P < 0.05$, two-tailed paired *t*-test) difference between the control (either post-ISO stimulation or after preincubation with H89 and post-ISO stimulation) and the tastant-containing samples. ** indicates significant difference ($P < 0.01$).

appearance of the β_2AR-phosphorylation peak in response to stimulation with ISO. Preincubation with NHD, SACC, QUIN, and CAFF resulted in β_2AR phosphorylation peak at 15 to 20 min post-ISO stimulation, whereas preincubation with NAR and D-TRP resulted in even a longer delay of the β_2AR phosphorylation peak. Therefore, the membrane-permeable tastants slowed the phosphorylation rate of β_2AR at Ser355/Ser356 sites after the ISO stimulation. Statistical analyses indicated significant (at least $P < 0.05$) differences in peak phosphorylation time point between CON and each of the tastants-containing samples.

3.5. Silencing of GRK2 activity abolishes the tastants' amplification of ISO-stimulated cAMP formation and β_2AR phosphorylation

To test whether GRK 2/5 are the targets for the membrane-permeable tastants to act on and amplify the cAMP formation following the ISO stimulation of β_2AR, GRK2/5 RNA silencing was performed. We tested several siRNA constructs for GRK 2/5 silencing in both HeLa and HEK293T cells. However, the silencing with HeLa cells was ineffective. In contrast, Western blotting data showed that silencing of GRK2 in HEK293T was successful, reduced by 70%, but the endogenous GRK5 could not be traced in these cells (Fig. 4A). To further evaluate GRK2's role in the tastant amplification of β_2AR activity in response to ISO, experiments were conducted with GRK2 silenced in β_2AR-transfected HEK293T cells. The concentration-dependent curve for ISO stimulation of cAMP formation in HEK293T cells was determined in order to identify an ISO concentration that stimulates β_2AR activity to a level below saturation. A range of concentrations of ISO that lead to sub-maximal stimulation of β_2AR activity was obtained (Supplementary material Fig. S2), with maximal stimulation at about 10 μM ISO, similar to that previously published [40]. Subsequently, it was evident that SACC

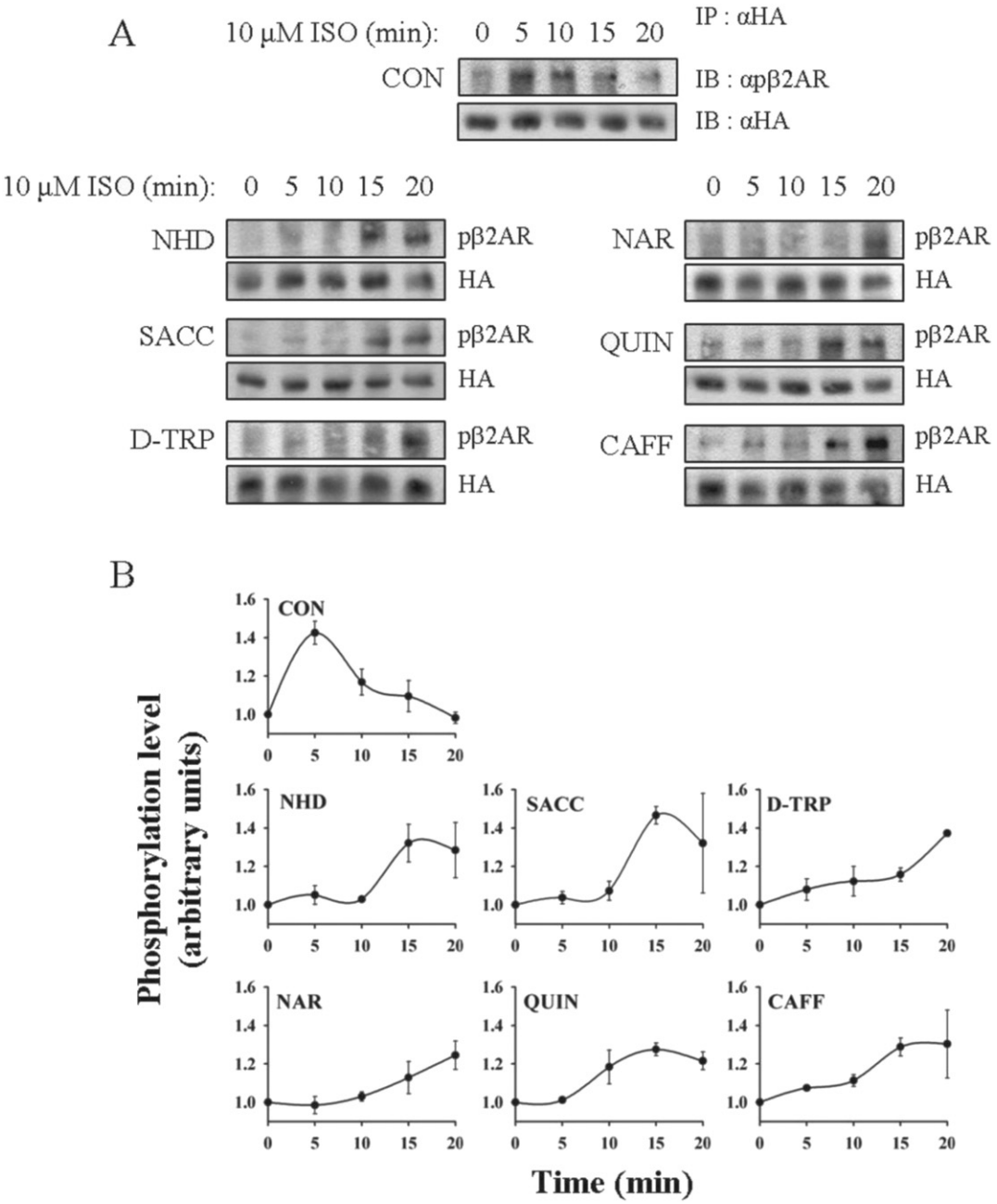


Fig. 3. Preincubation of β_2AR-transfected HeLa cells with amphipathic tastants delays the β_2AR-phosphorylation peak after ISO stimulation. (A) HA-tagged β_2AR-transfected HeLa cells were preincubated in the presence (at concentrations as in Fig. 1A) or absence of tastants for 10 min and then stimulated with ISO (10 μM) for different times. Cells were scraped in RIPA buffer, cold-centrifuged, and the supernatant was used for immunoprecipitation of β_2HA using monoclonal anti-HA antibody bound to agarose beads and subjected to SDS-PAGE and Western blot analysis (see Materials and methods). Anti-phosphoSer(355–356) antibody was used to recognize GRK2/5 phosphorylation sites on β_2HA. Shown is a representative experiment repeated at least three times. (B) Quantification of phosphorylation-band intensities (derived from (A)). The results are the means ± SEM of three independent experiments. One-way ANOVA ($P < 0.001$) and Dunnett's test was performed to compare the obtained maximal phosphorylation level between CON and each of the tastant-containing samples. Significant (at least $P < 0.05$) differences in peak phosphorylation time point were found between CON and each of the tastants-containing samples.

amplification effect of ISO-stimulated cAMP formation following preincubation of β_2AR-transfected HEK293T was absent when GRK2 was silenced under three experimental conditions, using 0.03, 0.1, and 1 μM of ISO (Fig. 4B). Accordingly, the sub-maximal concentration of 0.1 μM ISO was selected to evaluate the significance of GRK2 silencing for SACC, D-TRP and NAR amplification effect of ISO-stimulated cAMP formation (Fig. 4C). Preincubation with these membrane-permeable tastants amplified ISO-stimulated cAMP formation by about 2 folds. As expected, under GRK2 silencing, stimulation of β_2AR-transfected cells by ISO significantly elevated cAMP formation (about 40 folds over basal). Concomitantly, GRK2 silencing completely abolished the tastant-amplifying effect of ISO-stimulated cAMP formation. These results demonstrate that the ability of the tested amphipathic tastants to amplify the ISO-stimulated cAMP formation depends on their ability to intracellularly inhibit GRK2.

The next experiment was designed to monitor the effects of amphipathic tastants on β_2AR phosphorylation in HEK293T and to determine how GRK2 knock down affects this phosphorylation. As shown in Fig. 5, the kinetics for ISO-stimulated β_2AR phosphorylation in the HEK293T-treated cells were similar to those obtained for the HeLa-treated cells (Fig. 3). In CON samples, β2AR phosphorylation peaked at about 5 min after stimulation with ISO, whereas in the tastants-treated samples, a significant shift of β2AR phosphorylation peak to longer time periods (20 min and more) was observed. Most important, GRK2 knock down

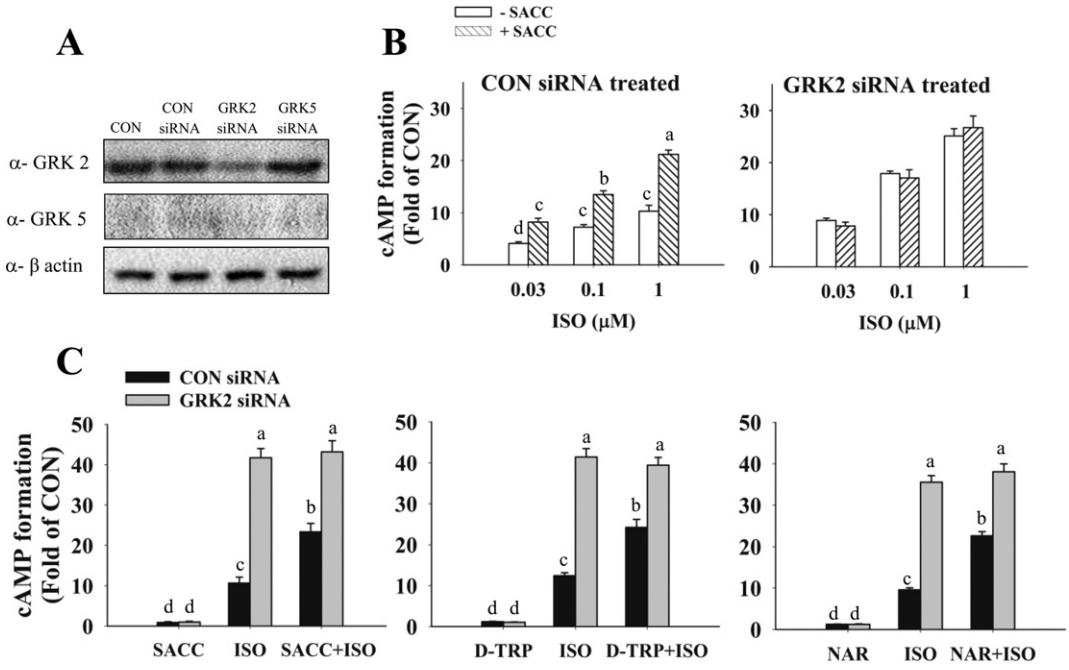


Fig. 4. Silencing of GRK2 abolishes the tastant-mediated amplification of ISO-stimulated cAMP formation. (A) HEK293T cells were transfected with either control siRNA (CON siRNA) or the indicated GRK-specific siRNAs. Three days after transfection, cells were lysed and analyzed by immunoblotting using GRK2- and GRK5-specific antibodies. β-Actin was used as a loading control. (B) Effect of preincubation with SACC (10 mM) on cAMP formation in the presence (left panel) or absence (right panel) of GRK2. HEK293T cells, were simultaneously transfected with the β_2AR plasmid and with either CON siRNA or GRK2 siRNAs, and then preincubated with or without SACC for 10 min. Cells were then stimulated with three sub-maximal concentrations of ISO (0.03, 0.1 and 1 μM) for 30 s and the intracellular concentration of cAMP was determined by RIA. Results are the means and SEM for six measurements of an independent experiment repeated twice. Basal cAMP levels in the CON siRNA and GRK2 siRNA were: 84.9 and 100.9 fmole/μg protein, respectively. Two-way ANOVA ($P < 0.001$) and Tukey-Kramer HSD post-hoc comparisons were performed on the data of each panel. Superscripts not sharing the same letter within each panel indicate significant (at least $P < 0.05$) differences. (C) Effect of tastants preincubation on cAMP formation in the presence or absence of GRK2. HEK293T cells were simultaneously transfected with the β_2AR plasmid and with either CON siRNA or GRK2 siRNAs. Cells were then preincubated for 10 min without or with SACC (10 mM), D-TRP (10 mM) or NAR (0.5 mM). Cells were then stimulated with 0.1 μM ISO for 30 s, and the intracellular concentration of cAMP was determined by RIA. Results are the means and SEM for six measurements of an independent experiment repeated twice. Basal cAMP levels for SACC control siRNA and GRK2 siRNA were: 42.1 and 38.5 fmole/μg protein, respectively. For D-TRP, basal cAMP levels in CON siRNA and GRK2 siRNA were: 37.2 and 42.9 fmole/μg protein, respectively. For NAR, basal cAMP levels in CON siRNA and basal GRK2 siRNA were: 42.7 and 40.6 fmole/μg protein, respectively. One-way ANOVA (at least $P < 0.001$) and Tukey-Kramer HSD post-hoc comparisons were performed on the data of each panel. Superscripts not sharing the same letter within each panel indicate significant (at least $P < 0.05$) differences.

in CON-treated samples and in tastants-treated samples completely abolished β_2AR phosphorylation following stimulation with ISO. This suggests that β_2AR phosphorylation in the Ser355–356 sites was very likely produced solely by GRK2, and since amphipathic tastants completely inhibit GRK2 (Fig. 4), no shift of β2AR phosphorylation peaks could be seen when GRK2 was knocked down in the HEK293T cells.

3.6. Amphipathic tastants delay the ISO-induced β_2AR internalization

Here we tested the putative effect of the above-mentioned membrane-permeable tastants on β_2AR internalization using two different procedures. First, the location of β_2ARs in membrane vs. cytosol was visualized and counted via fluorescence microscopy before and after stimulation by ISO (Fig. 6A, B). Pictures showing the effect of CAFF and SACC are first presented (Fig. 6A). At time 0, most of the β_2ARs were present on the plasma membrane. In the control cells (CON), at 5 and 10 min post-ISO stimulation, β_2ARs began to move into the cytosol, and after 15 and 20 min most of the β_2ARs were located in the cytosol. On the other hand, ISO stimulation of cells preincubated with either CAFF or SACC delayed the β_2ARs movement into the cytosol: even 20 min since ISO stimulation began, a significant fraction of cells still had receptors on their membrane surface. Preincubation with CAFF or SACC alone, without ISO stimulation, did not induce any internalization of β_2ARs (data are not shown). Quantitative determination of the effect of the six amphipathic tastants (Fig. 6B) indicated that the delay in β_2ARs internalization correlated well with tastant concentration during preincubation of the β_2AR-transfected cells prior to stimulation by ISO. The number of β_2ARs in the membrane of cells preincubated with the amphipathic tastants for 15 min was 2.0- to 2.5-fold higher than that found in CON cells preincubated with no tastants. Regression analyses resulted in significant correlation (at least $R^2 = 0.83$, $P < 0.001$) between tastants concentration during preincubation and the number of cells containing membranal β_2AR.

The delay in β_2AR internalization due to preincubation of cells with all six tastants was also quantified by Western blotting (Fig. 7A, B). The signal intensities of β_2AR proteins in Western blots of the membranal versus the cytosolic fractions, suggest complementary distribution of β_2AR protein in these two portions. The membrane EGFR control protein solely appeared in the plasma membrane and the cytosolic GAPDH control protein solely appeared in the cytosolic fraction (Fig. 7A), both proteins were not affected by the treatments. Following stimulation of β_2AR-transfected CON cells by ISO, β_2AR content in the membrane decreased gradually (Fig. 7A) with only traces remaining after 20 min. On the other hand, in β_2AR-transfected cells which had been preincubated with amphipathic tastants, ISO stimulation had a relatively minor effect on the β_2AR content of the membranal fraction during the 20 min of ISO stimulation. Co-variance linear regression analysis to compare the slope differences among tastants (membranal fraction, Fig. 7B left panel) resulted in negative slope values of −0.03, −0.01, −0.008, −0.015, −0.015, −0.004, and 0.007, for CON, CAFF, NAR, NHD, QUIN, SAC and D-TRP, respectively. The negative slope value for each tastant was significantly lower (at least $P < 0.005$) than that for CON. As expected, there was a concomitant increase in the amount of β_2ARs in the cytosolic fraction of the CON cells. In the cytosolic fraction of cells that had been preincubated with tastants, in most cases, there was a low content of β_2ARs, especially during the initial time periods with some increase at the latter times. A similar

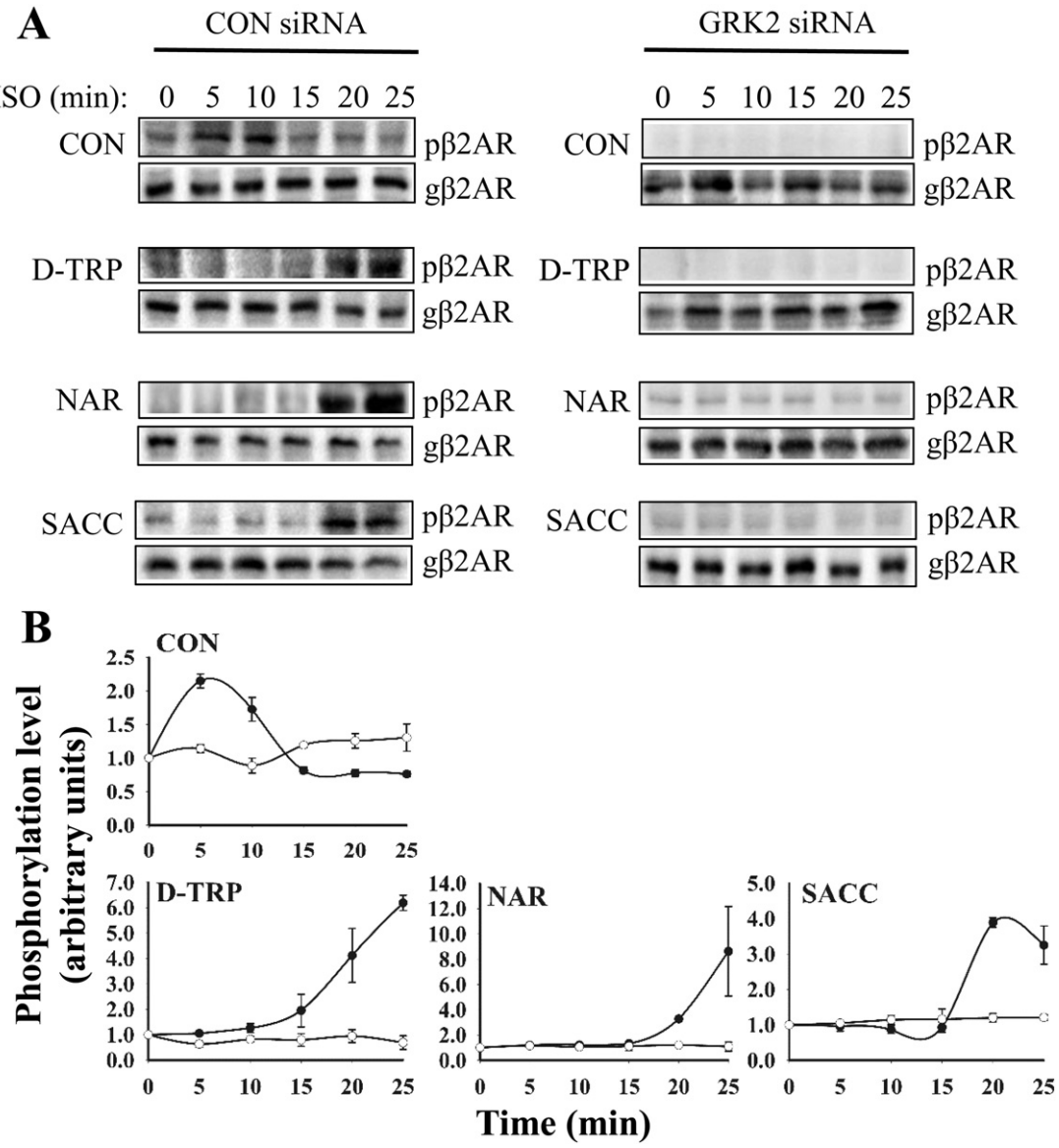


Fig. 5. GRK2 silencing abolishes β_2AR phosphorylation and tastants-induced delay in β_2AR phosphorylation. (A) CON siRNA and siGRK2 β_2AR-transfected HEK293T cells were preincubated in the presence (at concentrations as in Fig. 4C) or absence of tastants for 10 min and then stimulated with ISO (1 μM) for different times. Cells were scraped in RIPA buffer, cold-centrifuged, and the supernatant was subjected to SDS-PAGE and Western blot analysis (see Materials and methods). Anti-phosphoSer(355–356) antibody was used to recognize GRK2 phosphorylation sites on β_2AR. Shown is a representative experiment repeated two times. (B) Quantification of phosphorylation-band intensities (derived from (A)). Results are the means ± SEM of two independent experiments. Shown are CON siRNA (filled circles) and GRK2 siRNA (open circles) β_2AR phosphorylation levels. One-way ANOVA ($P < 0.001$) and Dunnett's test were performed to compare the obtained maximal phosphorylation level between CON and each of the tastant-containing samples. Significant (at least $P < 0.005$) differences in peak phosphorylation time point were found between CON and each of the tastant-containing samples. Only traces of phosphorylation were found for the GRK2 siRNA samples.

co-variance analysis (Fig. 7B, right panel) resulted in positive slope values of 0.05, 0.003, 0.018, 0.019, 0.022, 0.016 and 0.009, for CON, CAFF, NAR, NHD, QUIN, SAC and D-TRP respectively. The positive slope value for each tastant was significantly lower (at least $P < 0.01$) than that for CON.

4. Discussion

The ability of amphipathic tastants to rapidly permeate the tested cells was an essential prerequisite for their amplification of β_2AR signaling in both heterologous systems and in cells containing endogenous β_2AR and signaling components. Consequently, this amplification of β_2AR activity led to the delay in β_2AR desensitization. These tastants, when applied at extracellular concentrations comparable with those present in various food items [33,34] permeated rapidly into cells, and could even exceed the extracellular concentrations. Since such tastants can translocate through multilamellar lipid vesicles (MLV) [18] and permeate into taste-bud cells without using metabolic energy [16], a mechanism of passive and/or facilitated diffusion has been proposed [16,18,41].

The minimal 5 min preincubation time required for the membrane-permeable tastants to amplify the ISO-stimulated cAMP formation, and the inability of membrane impermeable tastants to mimic such effect, strongly suggest that amphipathic tastants exert their amplification of β_2AR activity via acting at intracellular site(s). It should be noted that although we focus here on β_2AR rather than the taste T1R or T2R signaling that should be initiated within the ms time range, the lingering extinction (taste persistence) of certain non-sugar sweeteners has been found to occur in the 5 min (and even longer) time range (e.g., Refs. [42,43]). The inability of the tested amphipathic compounds to elevate the cellular basal level of cAMP in the β_2AR-transfected cells without subsequent stimulation by ISO indicates that these tastants do not act as β_2AR

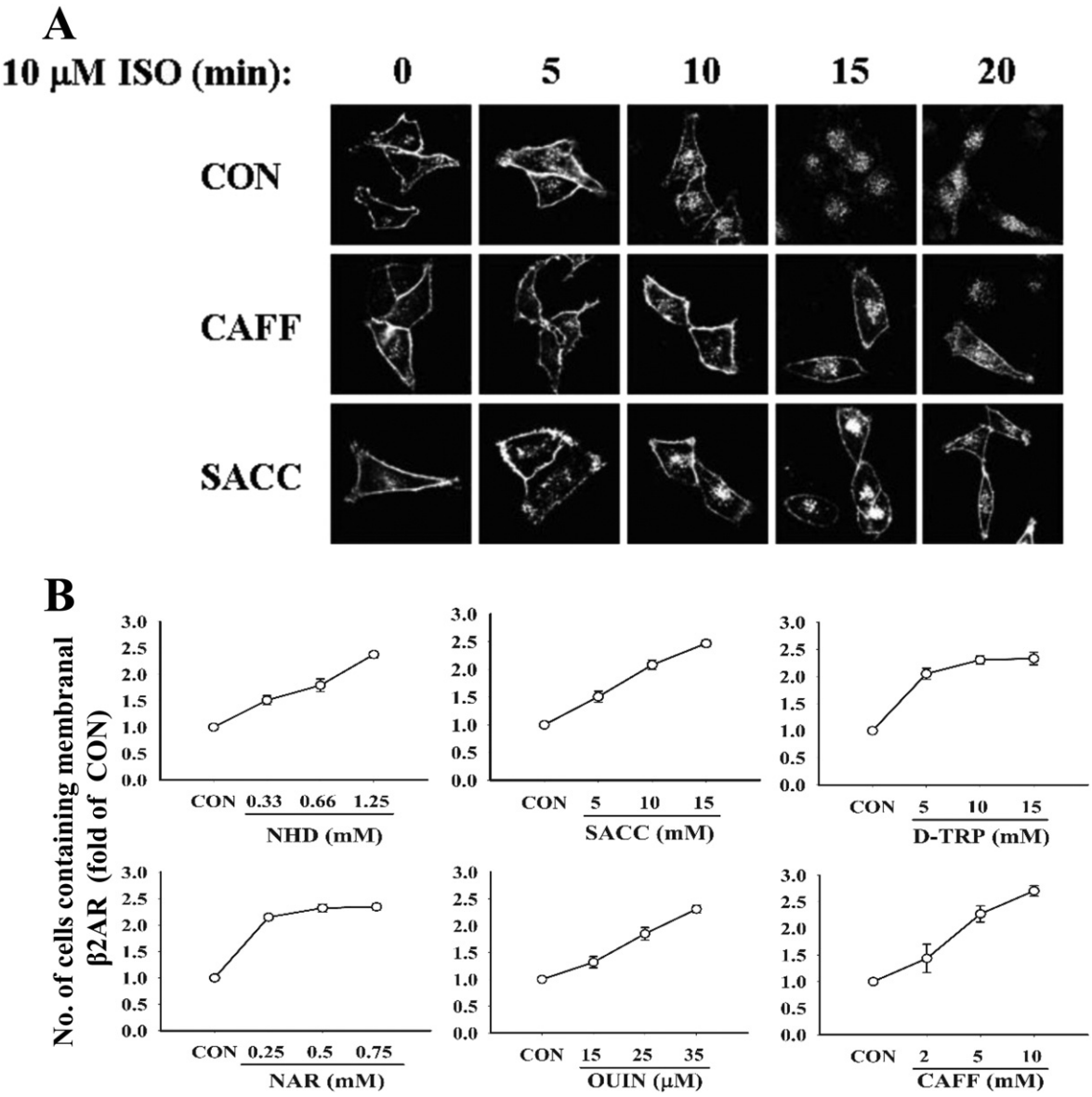


Fig. 6. Amphipathic tastants delay ISO-stimulated β_2AR internalization — Direct observation under confocal imaging. (A) Pictures showing the effects of CAFF and SACC. β_2AR-transfected HeLa cells were preincubated with or without 5 mM CAFF or 10 mM SACC for 10 min and then stimulated with 10 μM ISO for different times. Cells were then washed, fixed and blocked. To detect β_2AR, the primary antibody was added, and an anti-rabbit FITC-conjugated secondary antibody was used to visualize the receptors. Shown is the direct observation of an experiment repeated at least three times. (B) The effect of amphipathic tastants on the delay of ISO-stimulated β_2AR internalization is concentration-dependent. β_2AR-transfected cells were preincubated for 10 min without or with three different sweeteners or three different bitter stimuli for 10 min (3 min for QUIN), and then stimulated with ISO (10 μM) for 15 min. Cells were fixed as in A. Values in B are derived from three wells, each containing about 300 cells. The number of β_2AR-transfected cells containing β_2ARs on their surface (plasma membrane) in cells preincubated with the amphipathic tastants was counted, and expressed (means ± SEM) relative to that of the control (CON) cells preincubated without tastants. Note: at some data points, SEM values were too small to be visible. Linear (for NHD, SACC, QUIN, and CAF) and semi-log (for D-TRP and NAR) regression analyses resulted in significant correlation (at least $R^2 = 0.83$, $P < 0.001$) between tastants concentration during preincubation and the number of cells containing membranal β_2AR.

ligands. Furthermore, results support the notion that after their permeation into the cells, these tastants (unlike the bitter and the PDE inhibitor CAFF) did not inhibit PDE, and under the experimental conditions, did not activate $G\alpha_s$ proteins directly [19,44].

Further investigation suggested that putative intracellular sites such as inhibition of PDE or PKA by the amphipathic tastants did not appear to play a significant role in the tastant amplification of the ISO-stimulated cAMP formation. Rather, additive (or slight synergistic) amplification effects by PDE or PKA inhibitors (IBMX or H89, respectively) on one side, and the amphipathic tastants on the other were produced, suggesting the action of two separated independent mechanisms of kinase inhibition.

The hypothesis is further supported by the present data showing that membrane-permeable tastants intracellularly inhibit GRK2 activity. Phosphorylation of β_2AR by GRK is a major step of its desensitization [20,21], and reduced GRK activity is associated with increased cAMP production and increased sensitivity to β_2AR activation [45]. Our previous *in vitro* data [15] indicated that such tastants inhibit the phosphorylation of rhodopsin by pure forms of GRK2 and GRK5 via non-competitive inhibition, as well as the phosphorylation of casein by PKA, suggesting that direct inhibition of GRK2/5 by these tastants was the cause for a delay in β_2AR phosphorylation peak at Ser355/356, GRK2/5 sites in the β_2AR. However, it does not rule out the possibility that inhibition of GRK6 could also be involved [29], a pathway which was not tested here. It should be noted that there is significant variance among amphipathic tastants in their ability to inhibit signal termination-related kinases. For example, our previous *in vitro* experiments indicated that the sweeteners NHD, cyclamate and D-TRP and

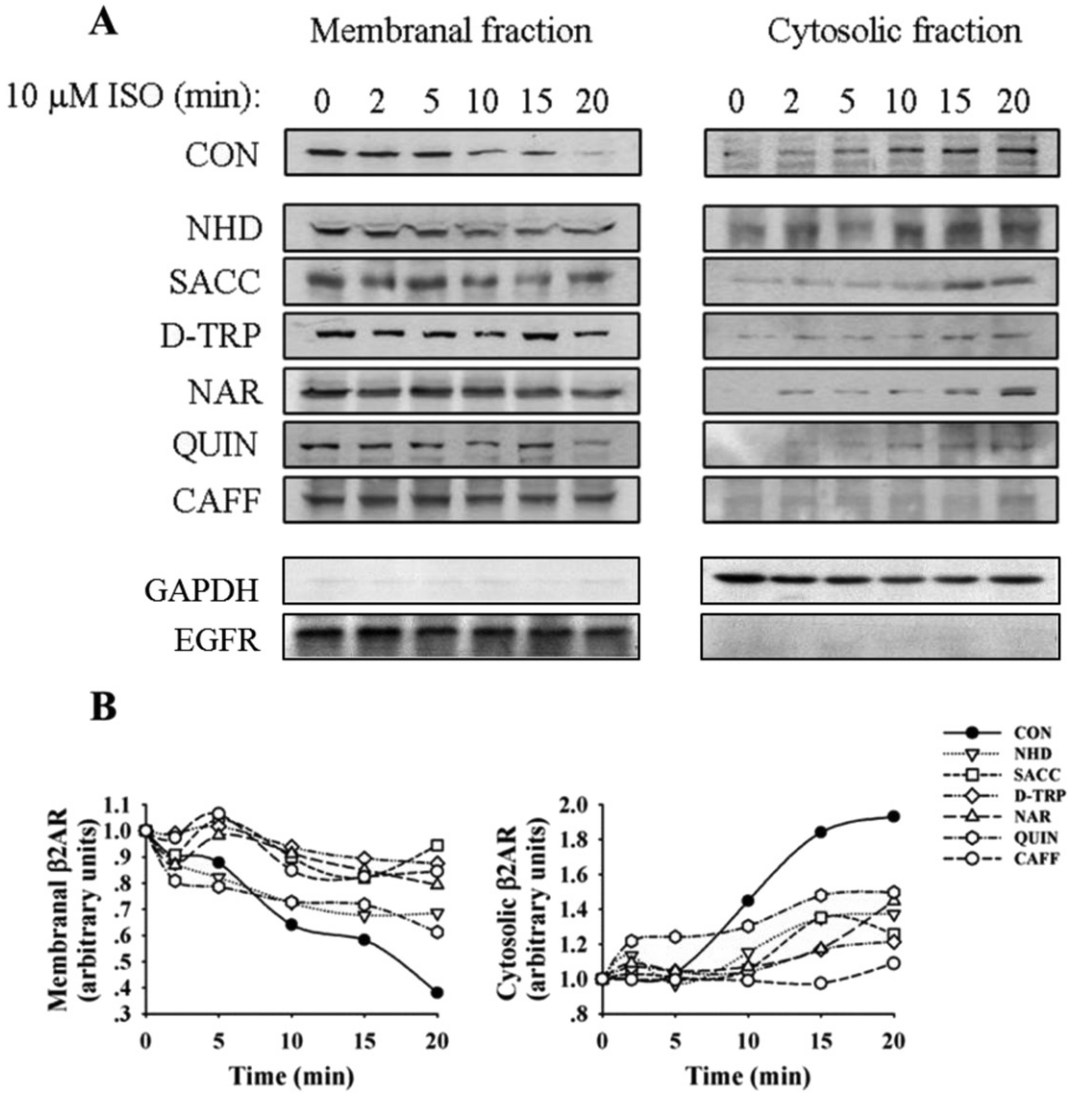


Fig. 7. Preincubation of β_2AR-transfected cells with the amphipathic tastants prior to stimulation with ISO subsequently reduces the amount of the cytosolic β_2ARs. (A) Cells were preincubated without (CON) or with each of the six tastants at concentrations indicated in Fig. 1A, for 10 min and then stimulated with 10 μM ISO for different time periods. Loading control proteins for the plasma membrane and the cytosolic fraction were EGFR and GAPDH, respectively. Cells were then washed, scraped and subjected to three centrifugation steps as indicated in Materials and methods. At the end of the last centrifugation, two fractions were separated. The pellet (containing the plasma membrane) was resuspended in RIPA buffer, sample buffer was added to both fractions, all samples were then heated at 95 °C for 5 min, resolved by 12% SDS-PAGE and transferred to nitrocellulose membrane for Western blotting. Anti-HA antibody diluted to 1:1000 was used to specify the total amount of β_2HA. Horseradish peroxidase-conjugated secondary antibody was used for ECL detection. Shown is a representative experiment repeated at least twice. (B) Quantification of band intensities (derived from A). Co-variance regression analyses to compare the slope differences among tastants and CON curves were performed. The negative (membranal fraction, Fig. 7B, left panel) and positive slope values (Fig. 7B, right panel) for each tastant was significantly different (at least $P < 0.001$) from that for CON.

the bitter tastants CAFF and L-TRP inhibited the kinase activity of GRK2, GRK5 and PKA whereas bitter ligands such as limonin, NAR, quinine or cyclo(Leu-Trp) and the sweet ligands SACC and acesulfame K inhibited the activity of only one or two of these kinases. Therefore, specificity is not obvious and probably depends, as other kinase inhibitors [46], on the binding (apparently allosteric) of the amphipathic ligands to the appropriate domains in each kinase.

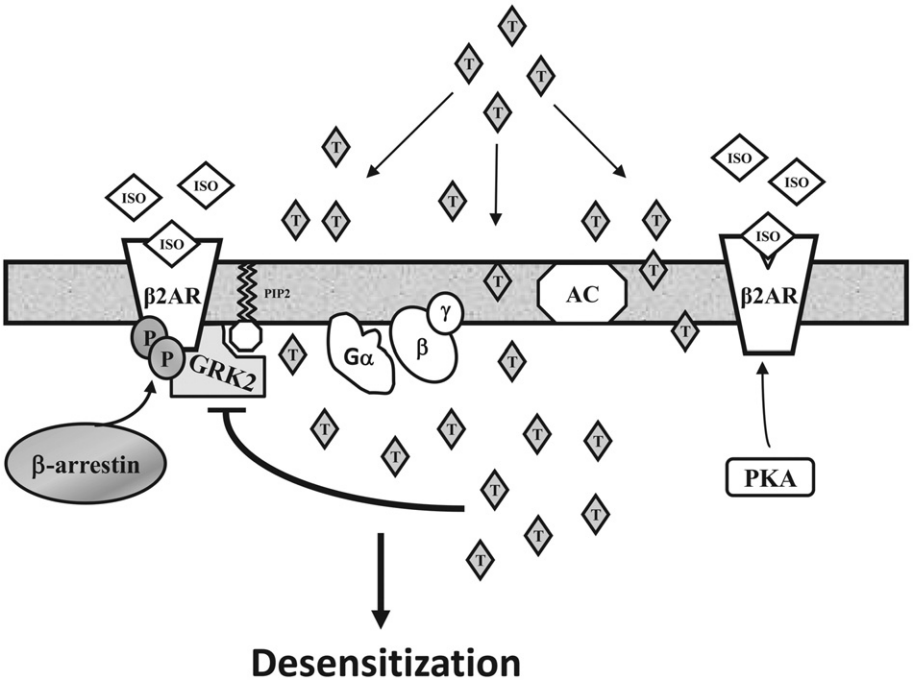
A significant shift was observed in the kinetic of amphipathic tastants-induced delay in β_2AR phosphorylation following stimulation by ISO compared with CON samples stimulated without preincubation with these tastants (Figs. 3 and 5). On the other hand, the effect of tastants on ISO-stimulated cAMP production resulted in different kinetic (Fig. 1A and C). It appears that the kinetics for cAMP production were similar in the absence and presence of tastants, even though the levels of cAMP were higher when the tastants present. This difference may be related to the fact that except CAFF, the amphipathic tastants were not PDE inhibitors but effectively inhibited GRK2-phosphorylated β_2AR. Indeed, this assumption is supported by the results of CAFF-treated samples (Fig. 1C). Compared with CON samples, the peak for CAFF-amplified ISO stimulation of cAMP formation was delayed from 30 s to 2 min (HeLa cells, Fig. 1A) and from 1 min to about 5 min (HCT116 cells, Fig. 1C), and so were the slower declines in cAMP levels in the caffeine-treated samples. Additional putative reason for the difference in the kinetics of cAMP degradation and β_2AR phosphorylation could be some inhibition of PKA-phosphorylated β_2AR in sites that were not determined here and that differ from those of GRK2-phosphorylated β_2AR. Both, PKA- and GRK2-phosphorylated β_2AR are very rapid, but as proposed for ERK (extracellular signal-regulated kinase) activation, the PKA-dependent phosphorylation of β_2AR could potentially precede that induced by the GRK [47,48]. Nevertheless, the lack of the tastants' effects on both cAMP production and β_2AR phosphorylation when GRK2 was knocked down (Fig. 4A and 4B, Fig. 5) indicates that the delayed β_2AR desensitization induced by these tastants was mainly due to their inhibition of GRK2 rather than PKA.

In line with the tastants' delaying effect of ISO-induced β_2AR phosphorylation, β_2AR internalization was also delayed. First, under confocal imaging, tastant preincubation prior to ISO stimulation produced significantly slower movement of β_2ARs into the cytosol compared to CON samples, and this delay in β_2AR internalization correlated well with tastant concentration. Western blotting data showed that in CON samples β_2AR content in the membrane gradually decreased over time after ISO stimulation with concomitant increase in the cytosol. On the other hand, only minor changes in β_2AR content occurred in the membrane of cells that had been preincubated with the amphipathic tastants.

Direct identification of the intracellular site by which the amphipathic tastants exerted their effect on β_2AR signaling was imperative. Successful silencing of GRK2 in HEK293T cells was a useful cell model. Because GRK2's kinase activity is considered to play a primary role for the desensitization of β_2AR signal transduction during the first 30 min of β_2AR signaling [27–29,32], we considered GRK2 as the likely target on which the amphipathic compounds exerted their amplification of β_2AR activity. If this hypothesis is correct, then the ISO-stimulated β_2AR activity should be tested at level below saturation (sub-maximal level of ISO) when GRK2 activity is knocked down. This should allow additional interactions of the tastants e.g., acting upstream and blocking activation of GRK2 rather than inhibit GRK2 activity directly. Most notable, under experimental conditions in which GRK2 silencing and sub-maximal concentrations of ISO (Fig. 4B) were applied, SACC failed to amplify the ISO-stimulated cAMP formation independently of ISO concentrations. Hence, this phenomenon was also true for D-TRP and NAR (Fig. 4C). Similarly, when GRK2 activity was silenced, the ISO-stimulated phosphorylation of β_2AR was abolished and so was the delayed β_2AR phosphorylation induced by the amphipathic tastants (Fig. 5). Overall, these results demonstrate that the tastants' amplification effect on cAMP formation and the tastant delay of β_2AR phosphorylation depended on their ability to intracellularly inhibit GRK2 (Scheme 1).

Further research is needed to elucidate mechanism(s) by which amphipathic tastants inhibit GRK2's kinase activity. Evidently, the chemical structure of the tested tastants is diverse and includes flavonoids, sulfamate, xanthine, and a D-amino acid. A diverse chemical structure has also been reported for various potent and less potent kinase inhibitors. The ATP-binding site is highly conserved among protein kinases and this binding site is involved in the mode of action of many reported kinase inhibitors [45,46,49,50]. Heparin (and perhaps other polyanions), a known GRK inhibitor, was found to be a competitive inhibitor of the substrate (e.g., rhodopsin) but mixed type inhibitor with respect to ATP [51]. Peptide inhibitors of GRKs were found to be non-competitive for the receptor and for the ATP [24]. We previously found that certain amphipathic tastants inhibit GRK2 and GRK5 phosphorylation of rhodopsin via non-competitive inhibition for rhodopsin and for ATP [15]. Additional inhibitory mechanisms have been proposed for certain GRK2 inhibitors. Thal et al., [46] using structural analysis and homology modeling determined the crystal structures of GRK2-$G_{\beta\gamma}$ complex in the presence of three heterocyclic small molecules of GRK2 inhibitors (Balanol, CMPD103A, CMPD101) considered to be highly potent. They proposed that these compounds bind to the kinase active site and induce a slight closure of the kinase domain which relates to their inhibition potency whereas selectivity of these GRK2 inhibitors is achieved by their ability to stabilize an inactive conformation of the GRK2's kinase domain.

The significant delay in β_2AR desensitization caused by the tastants' inhibition of GRK2 calls for studies to evaluate potential implications of the present results for the desensitization of other GPCRs whose mechanism of desensitization is coupled with GRK2's kinase activity. These should include GPCR pathways along the gastro-intestinal tract whose physiological role has not been previously recognized [52,53]. We previously proposed a hypothesis [15] that inhibition of signal-termination kinases such as GRKs by the membrane-permeable tastants, may be related to the lingering aftertaste (taste persistence) that they produce in humans [54]. GRK2 is present in taste-bud cells [15] but the desensitization pathway(s) of the taste T1Rs/T2Rs needs to be elucidated before subsequent investigation of this phenomenon can proceed. Potential implications of these results to post-receptor signaling pathways such as the MAPK (e.g., Ref. [55]) and, consequently to additional



Scheme 1. Proposed pathway for amphipathic tastants-inhibited β_2AR phosphorylation-dependent desensitization. Isoproterenol (ISO) stimulates β_2AR from the extracellular side to activate $G\alpha_s$ proteins which, in turn, activates adenylyl cyclase (AC). β_2AR-mediated signaling may be desensitized by GRK2 which recruits to the membrane, binds to PIP_2 and phosphorylates β_2AR. This phosphorylation promotes the binding of arrestin proteins to β_2AR, uncoupling β_2AR from G-proteins and induce its internalization. Direct inhibition of GRK2's kinase activity by membrane-permeable tastants (T) amplifies β_2AR activity and delays its desensitization.

downstream pathways [56] should also be explored. Preliminary results in our laboratory (unpublished, using a similar methodology to that described here) indicated that preincubation of β_2AR-transfected cells with amphipathic tastants prior to stimulation by ISO resulted in a delay of the time course of ERK1/2 phosphorylation. Importantly, GRK2 modulates additional multiple non-receptor cellular responses of various physiological contexts (see Ref. [56] for updated review). For example, GRK2 phosphorylates tubulin following β_2AR stimulation [20] and IkBα to mediate TNFα-induced NF-kB signaling [57]. Because inhibition of GRK2 is one option for treatment heart failure [58], many studies are designed to explore membrane-permeable, potent and selective GRK2 inhibitors. The effective concentrations of the amphipathic tastants found in the present study to inhibit GRK2 activity were high compared with drugs currently considered as GRK2 inhibitors [46]. Nevertheless, as mentioned in the Introduction, a variety of membrane-permeable sweeteners and bitter tastants are absorbed through the gut. Based on their ability to accumulated inside various cells after membrane permeation [17,18], once absorbed through the gut, their intracellular concentrations in certain tissues might reach those found in this study to inhibit GRK2.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

Transparency Document

The transparency document associated with this article can be found, in the online version.

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