Effects of bitter receptor antagonists on behavioral lick responses of mice

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Running title: Effects of bitter antagonists

Abstract

Bitter taste receptors TAS2Rs detect noxious compounds in the oral cavity. Recent heterologous expression studies reported that some compounds function as antagonists for human TAS2Rs. For examples, amino acid derivatives such as γ-aminobutyric acid (GABA) and Nα,Nα-bis(carboxymethyl)-L-Lysine (BCML) blocked responses to quinine mediated by human TAS2R4. Probenecid inhibited responses to phenylthiocarbamide mediated by human TAS2R38. In this study, we investigated the effects of these human bitter receptor antagonists on behavioral lick responses of mice to elucidate whether these compounds also function as bitter taste blockers. In short-term (10 s) lick tests, concentration-dependent lick responses to bitter compounds (quinine-HCl, denatonium and phenylthiourea) were not affected by the addition of GABA or BCML. Probenecid reduced aversive lick responses to denatonium and phenylthiourea but not to quinine-HCl. In addition, taste cell responses to phenylthiourea were inhibited by probenecid. These results suggest some bitter antagonists of human TAS2Rs can work for bitter sense of mouse.

Key words: bitter inhibitor, gustatory response, taste perception, bitter coding, species difference

Abbreviations

BCML, Nα,Nα-bis(carboxymethyl)-L-Lysine; CV, circumvallate papillae; Den, denatonium benzoate; DW, distilled water; FP, fungiform papillae; GABA, γ-aminobutyric acid; GIV 3727, 4-(2,2,3-trimethylcyclopentyl) butanoic acid; HEK, human embryonic kidney; IP_3R3, inositol-1,4,5-triophosphate receptor type 3; KO, knockout; PLCβ2, phospholipase Cβ2; PROP, 6-n-propyl-2-thiouracil; PTC, phenylthiocarbamide; PTU, phenylthiourea; QHCl, quinine-HCl; MPG, monopotassium glutamate; SE, standard error; Tas2Rs, type 2 taste receptors; TRPM5, transient receptor potential channel M5;

Introduction

Among the basic taste modalities, bitter taste is an aversive taste elicited by numerous chemically different compounds [1]. Many noxious and poisonous compounds have a bitter taste, therefore the bitter taste is thought to play an important role in protecting from ingestion of these noxious compounds. Although more than 1,000 compounds have a bitter taste, only a limited number of receptors detect these compounds. The bitter receptors, type 2 taste receptors (TAS2Rs), comprise a G-protein-coupled receptor family, which includes 25 and 35 functional receptors in humans and mice, respectively [2-6]. Binding of bitter compounds to a TAS2R elicits activation of intracellular signaling cascades in taste cells, including α-gustducin [7], phospholipase Cβ2 (PLCβ2) [8], inositol-1,4,5-triophosphate receptor type 3 (IP_3R3) [9] and transient receptor potential channel M5 (TRPM5) [8], leading to depolarization and generation of action potentials in taste cells [10, 11].

The agonists of each bitter receptor have been determined by functional expression assays. The first report demonstrated that human embryonic kidney (HEK) cells expressing mouse Tas2r5 (Tas2r105) responded to cycloheximide [3]. The same report also showed that human TAS2R4 and mouse Tas2r8 (Tas2r108) responded to denatonium and high concentrations of 6-n-propyl-2-thiouracil (PROP). Functional expression assays for bitter receptors also revealed the molecular basis for the differences in bitterness recognition of phenylthiocarbamide [PTC, also known as phenylthiourea (PTU)], which was explained by single nucleotide polymorphisms (SNPs) in the human *TAS2R38* gene [12, 13]. More recent high throughput assays have determined the molecular receptive ranges of human and mouse bitter receptors [14, 15]. Thus, our knowledge of cognate agonists for bitter receptors is gradually increasing, but further studies are required to fully elucidate the ligands of each of the bitter receptors.

Antagonists of bitter taste receptors may be useful for suppressing the bitter taste and may help in ingesting bitter tasting drugs. Similar to bitter agonists, some bitter antagonists have been identified by functional expression assays. The first reported antagonist was the small molecule, 4-(2,2,3-trimethylcyclopentyl) butanoic acid (GIV3727), which suppresses the activation of human TAS2R31 [16]. Subsequently, probenecid [17], sesquiterpene lactones [18], 6-methoxyflavanones [19], γ-aminobutyric acid (GABA) and Nα,Nα-bis(carboxymethyl)-L-Lysine (BCML) [20] have been identified as antagonists of human TAS2Rs. Although some bitter antagonists have been shown to inhibit cat and chicken bitter receptors [21, 22], it is unclear whether bitter antagonists also function and affect bitter sensitivity in non-human species.

In this study, we investigated the effects of the bitter antagonists, GABA, BCML and probenecid on behavioral lick responses of mice to multiple tastants, to reveal whether these bitter antagonists block perception of bitterness in mice.

Material and Methods

Animals

All experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the committee for Laboratory Animal Care and Use at Kyushu University and Okayama University, Japan. Subjects were adult C57BL6/J (>8 weeks old) male mice. All mice were housed under a 12:12-h light-dark cycle (lights on 0800-2000h) and had *ad libitum* access to tap water and food pellets (CE-2, CLEA Japan, Tokyo, Japan). Mice were divided into two groups: one for testing the effect of GABA and BCML (n = 9), and one for testing the effect of probenecid (n = 10). Gustducin-GFP mice [23] were used for recording of taste cell responses.

Solutions

Taste stimuli used were as follows (mM): 10–1000 NaCl, 10–1000 sucrose, 1–30 HCl, 10–300 monopotassium glutamate (MPG), 0.01–3 quinine-HCl (QHCl), 0.1–3 denatonium benzoate (Den), and 0.3–10 phenylthiourea (PTU). Tastants were dissolved in distilled water (DW) and used at room temperature (25°C). These concentrations of tastants were used in previous studies [24, 25]. To test the effect of GABA and BCML, 1/1000 volume of 100 mM GABA (final concentration: 100 μM) or 10 mM BCML (final concentration: 10 μM) was added to these solutions [20]. To test the effect of probenecid, 1/1000 volume of 1 M probenecid dissolved in 1 N NaOH (final concentration: 1 mM probenecid) or 1/1000 volume of 1 N NaOH (for control) was added to these solutions [16]. In the probenecid group, HCl was not assessed because of the precipitation of probenecid by acids. All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA) or Wako Pure Chemical Industries (Osaka, Japan).

Short term lick test

Taste behavior was assessed using a short term (10 s) lick test [24]. On the first day of training, each animal was water deprived for 23 h, then placed in the test cage and given free access to DW during the 1 h session. Day 2-5 were training sessions. During this period, the animal was trained to drink DW on an interval schedule, consisting of 10-s periods of DW presentation alternating with 20-s inter-trial intervals. From day 6, the number of licks for each taste solutions and DW was counted during the first 10 s after the animal's first lick using a lick meter (Yutaka Electronics Co. Gifu, Japan). On each test day, the first test stimulus given to the animal was DW. Then, test stimuli were tested in a randomized order. After bitter tastants (QHCl, Den and PTU) were tested, other tastants or DW were tested to avoid successive application of bitter solutions. The

schedule for testing the effect of GABA and BCML was as follows: days 6, 11 and 13 for control taste solutions; days 7, 9 and 14 for taste solutions + GABA; days 8, 10 and 12 for taste solutions + BCML. The schedule for testing the effect of probenecid was as follows: days 6, 9 and 10 for control taste solutions with NaOH; days 7, 8 and 11 for taste solutions + probenecid. The mean number of licks across three days by each animal was used for statistical analysis.

Taste cell recording

Recording procedures were similar to those used previously [26]. Briefly, the tongue were removed and treated with 0.5 mg/ml elastase (Elastin Products, Owensville, MO, USA). Then, the lingual epithelium was peeled and individual taste buds with a piece of surrounding epithelium were excised and set to the stimulating pipette. Taste stimuli were applied to the apical membrane via stimulating pipette. Tyrode solution (140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 5 $NaHCO_3$, 10 HEPES, 10 Glucose, 10 sodium pyruvate in mM; pH = 7.4 with NaOH) was continuously flowed into the recording chamber. GFP taste cells were identified by confocal laser scanning microscopy (FV-300; Olympus, Tokyo, Japan) and were approached by a recording electrode. Electrical signals were recorded by a high-impedance patch-clamp amplifier (Axopatch 200B; Axon Instruments, Foster City, CA, USA) interfaced to a computer (Windows 7) by an analog-to-digital board (Digidata 1440A; Axon Instruments).

Data analysis

Two-way or one-way repeated ANOVA with post hoc Holm test was used to statistically evaluate the effect of GABA, BCML and probenecid. All statistical calculations were performed using the EZR program on Windows 10 [27]. All summarized data are presented as means ± standard error (SE).

Results

The effect of GABA and BCML on lick responses to tastants

GABA and BCML have been reported to inhibit responses to quinine in HEK cells expressing human TAS2R4 [20]. Therefore, we tested whether the addition of GABA or BCML to bitter solutions disturbs bitter perception in mice. Mice showed a concentration-dependent avoidance from the bitter compounds, QHCl, Den and PTU in the short-term lick tests (Fig. 1). This concentration-dependent avoidance from bitter compounds was not affected by the addition of 100 μM GABA or 10 μM BCML (Fig. 1). The mean lick rates for QHCl, Den and PTU were not significantly different between the control, GABA and BCML treatments (Supplemental table 1). We also tested whether GABA or BCML affects the lick responses to other tastants. The mean lick rates for sucrose (sweet), NaCl (salt), MPG (umami) and HCl (sour) were not significantly different between the control, GABA and BCML treatments (Fig. 2, Supplemental table 1). Taken together, addition of GABA or BCML to taste solutions did not appear to affect taste perception in mice.

The effect of probenecid on lick responses to tastants

Probenecid is another bitter antagonist reported to inhibit human TAS2R16, 38 and 43 [17]. Next, we tested whether the addition of probenecid to bitter solutions affects lick responses to bitter solutions in mice. In contrast to GABA and BCML, probenecid reduced avoidance from Den and PTU in the short-term lick tests (Fig. 3). The mean lick rates for Den and PTU were significantly different between the control and probenecid treatments (Fig. 3B, C, Supplemental table 2). However, the lick responses

to QHCl were not affected by the addition of probenecid (Fig. 3A, Supplemental table 2). The mean lick rates for sucrose, NaCl, and MPG were not significantly different between the control and probenecid treatments (Fig. 4, Supplemental table 2). These results suggest that probenecid inhibits some mouse bitter receptors contributing to the detection of Den and PTU.

Taste cell responses

We tested whether probenecid inhibits taste cell responses to PTU. Responses of Gustducin-GFP taste cells to 10 mM PTU were inhibited by the addition of 1 mM probenecid (Fig. 5). These results indicate that probenecid affects taste cell responses to PTU.

Discussion

More than 1,000 chemical compounds have bitter tastes in humans, but only a small number of compounds have been reported to function as bitter antagonists. The effects of bitter antagonists have been analyzed by heterologous expression studies of human TAS2Rs [16-20]. In this study, we investigated mainly by behavioral tests whether some of bitter antagonists have a similar effect on mouse bitter receptors. We used the effective concentrations of bitter antagonists that have been reported in previous heterologous expression studies [17, 20]. At the tested concentrations, these antagonists did not elicit a taste or change preferences for lick responses of mice, as the addition of each antagonist did not affect both the preferable (sucrose) and aversive (HCl, QHCl) responses (Figs. 1–4). We found that the human TAS2R4 antagonists, GABA and BCML, had no effect on taste perception in mice (Fig. 1, 2). These results are consistent with previous studies of taste cell responses [26]. Thus, similarly to bitter agonists [15], some bitter antagonists (GABA and BCML) may have different efficiencies among

species. Differences in the effects of taste receptor antagonists among species have also been reported for sweet taste: gymnemic acids inhibit human, but not mouse, sweet receptors [28-30]. The species-specific effect of gymnemic acids depends on the amino acid changes in the sweet receptor component, Tas1R3, between humans and mice [31]. Therefore, amino acid changes between human TAS2R4 and its ortholog mouse Tas2r108 may account for the different effect of GABA and BCML on human and mouse bitter receptors. The important residues for the effect of GABA and BCML have been reported to be Ala-82 and Lys-262 in human TAS2R4 [20]. The corresponding residues in mouse Tas2r108 are substituted by Thr and Gln, respectively. These amino acid changes may cause less or no binding of GABA and BCML to mouse Tas2r108. Additionally, methodological differences may contribute to the different results. In the heterologous expression experiments, the analyzed cells expressed a specific bitter receptor, such as human TAS2R4, and the effect of antagonists on the specific receptor was analyzed. However, in the behavioral tests, tested mice possess multiple types of bitter receptors including mouse Tas2r108. If the effect of GABA and BCML is specific to mouse Tas2r108, activation of other bitter receptors by bitter agonists [15] may neutralize the effect of bitter antagonists at the behavioral level. To our knowledge, the effects of GABA and BCML on bitter receptors other than human TAS2R4 have not been tested.

We also found that probenecid significantly reduced aversive lick responses to Den and PTU, but not to QHCl in mice. Probenecid blocked activation of human TAS2R16, 38 and 43 [17]. These human bitter receptors have been reported to respond to Den (human TAS2R43), PTU (human TAS2R38) and QHCl (human TAS2R43) [14]. The mouse orthologs of human TAS2R16 and 38 are mouse Tas2r118 and 138, respectively. However, there is no mouse ortholog of human TAS2R43. Mouse Tas2r138 responded to 5-propyl-2-thiouracil and Yohimbin, whereas agonists of mouse Tas2r118 were not

determined, although it is quite abundantly expressed in taste tissues [15]. In a heterologous expression study, a bitter receptor for PTC was not determined when it was used at 0.1 mM [15]. Our results showed that aversion to PTU was observed at higher concentration (3–10 mM) and taste cells were activated by 10 mM PTU, namely, mouse Tas2r138 was activated by high concentrations of PTC (PTU). Taken together, the blockade of mouse Tas2r138 by probenecid may lead to reduced aversive responses to PTU. Conversely, reduction in aversive responses to Den may not be explained by blockade of mouse Tas2r138 because this receptor was not activated by Den in heterologous expression experiments [15]. Probenecid may inhibit mouse Tas2r118 or other Tas2rs [15], leading to reduction in aversive responses to Den. Probenecid has been shown to inhibit human and cat Tas2R38 and 43 [21]. Taken together, probenecid may be used as a common antagonist of PTU bitter receptors in humans, cats and mice. Probenecid is an allosteric antagonist that targets the intracellular part of several human TAS2Rs [17], whereas the other antagonists are assumed to target the orthosteric TAS2R binding site, which has been demonstrated for GIV3727 [16, 18]. Because the intracellular part of TAS2Rs is more conserved than the extracellular part [32], it is reasonable to assume that probenecid shows cross-species activity compared with the other antagonists.

Conclusion

In this study, we revealed that the efficacy of some bitter antagonists (GABA and BCML) may be different among species. Additionally, we demonstrated the consistent effect of the bitter antagonist, probenecid, on human and mouse PTC bitter taste receptors. Bitter antagonists are powerful tools for investigating the function of bitter taste receptors. However, such differences should be considered when these antagonists are used in both *in vivo* and *in vitro* studies. Further studies are required to reveal the

molecular mechanisms that underlie the similarities and differences in the effects of bitter antagonists among species.

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Conflicts of interest

The authors declare no competing financial interests.

Author contributions

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Figure legends

Fig. 1. The effect of addition of GABA and BCML on lick responses to bitter compounds. Concentration-dependent lick responses to quinine (A), denatonium (B) and PTU (C) with or without 100 μM GABA or 10 μM BCML in mice (n = 9). All data are presented as the mean ± SE.

Fig. 2. The effect of addition of GABA and BCML on lick responses to sweet, salty, umami and sour compounds. Concentration-dependent lick responses to sucrose (A), NaCl (B), MPG (C) and HCl (D) with or without 100 μM GABA or 10 μM BCML in mice (n = 9). All data are presented as the mean ± SE.

Fig. 3. The effect of addition of probenecid on lick responses to bitter compounds. Concentration-dependent lick responses to quinine (A), denatonium (B) and PTU (C) with or without 1 mM probenecid in mice (n = 10). All data are presented as the mean ± SE. +: $P<0.05$, +++: $P<0.001$, two-way ANOVA. *: $P<0.05$, post hoc Holm test.

Fig. 4. The effect of addition of probenecid on lick responses to sweet, salty and umami compounds. Concentration-dependent lick responses to sucrose (A), NaCl (B), MPG (C) and HCl (D) with or without 1 mM probenecid in mice (n = 10). All data are presented as the mean ± SE.

Fig. 5. The effect of probenecid on taste cell responses to PTU. Sample recordings (A) and individual data (B) showing the effect of 1 mM probenecid (+proben) on PTU responses of gustducin-GFP taste cells (n=5, $F=9.0$, $P<0.01$, One-way repeated ANOVA).

Supplemental table 1. Two-way ANOVA results for the effect of GABA and BCML

compounds	factor	degree of freedom	F Value
QHCl	Additive	2, 120	0.079
	Concentration	4, 120	163.2***
	Interaction	8, 120	0.05
Den	Additive	2, 96	0.111
	Concentration	3, 96	164.9***
	Interaction	6, 96	0.497
PTU	Additive	2, 96	0.634
	Concentration	3, 96	81.3***
	Interaction	6, 96	0.266
Sucrose	Additive	2, 120	2.126
	Concentration	4, 120	8.125***
	Interaction	8, 120	1.2
NaCl	Additive	2, 120	0.771
	Concentration	4, 120	766.4***
	Interaction	8, 120	0.419
MPG	Additive	2, 96	1.984
	Concentration	3, 96	4.744**
	Interaction	6, 96	0.228
HCl	Additive	2, 96	0.607
	Concentration	3, 96	369.6***
	Interaction	6, 96	0.472

**: $P<0.01$, ***: $P<0.001$

Supplemental table 2. Two-way ANOVA results for the effect of probenecid

compounds	factor	degree of freedom	F value
QHCl	Additive	1, 90	0.638
	Concentration	4, 90	109.5***
	Interaction	4, 90	0.49
Den	Additive	1, 72	86.5***
	Concentration	3, 72	4.874*
	Interaction	3, 72	1.597
PTU	Additive	1, 72	12.8***
	Concentration	3, 72	31.4***
	Interaction	3, 72	4.415**
Sucrose	Additive	1, 90	0.408
	Concentration	4, 90	1.091
	Interaction	4, 90	0.162
NaCl	Additive	1, 90	1.432
	Concentration	4, 90	292.9***
	Interaction	4, 90	0.206
MPG	Additive	1, 72	0.326
	Concentration	3, 72	1.076
	Interaction	3, 72	0.122

*: $P<0.05$, **: $P<0.01$, ***: $P<0.001$

Fig.1

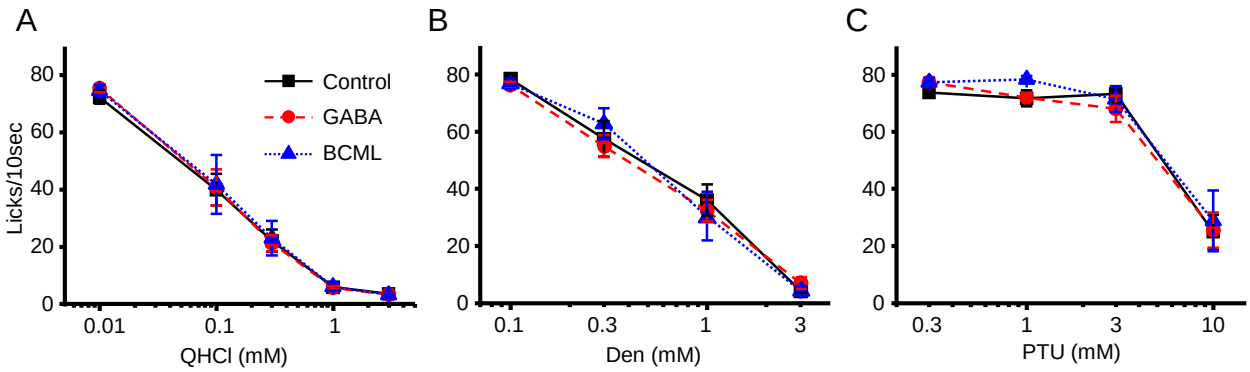

A
B
C
Licks/10sec
80
60
40
20
0
Control
GABA
BCML
0.01
0.1
1
QHCl (mM)
0.1
0.3
1
3
Den (mM)
0.3
1
3
10
PTU (mM)

Fig.2

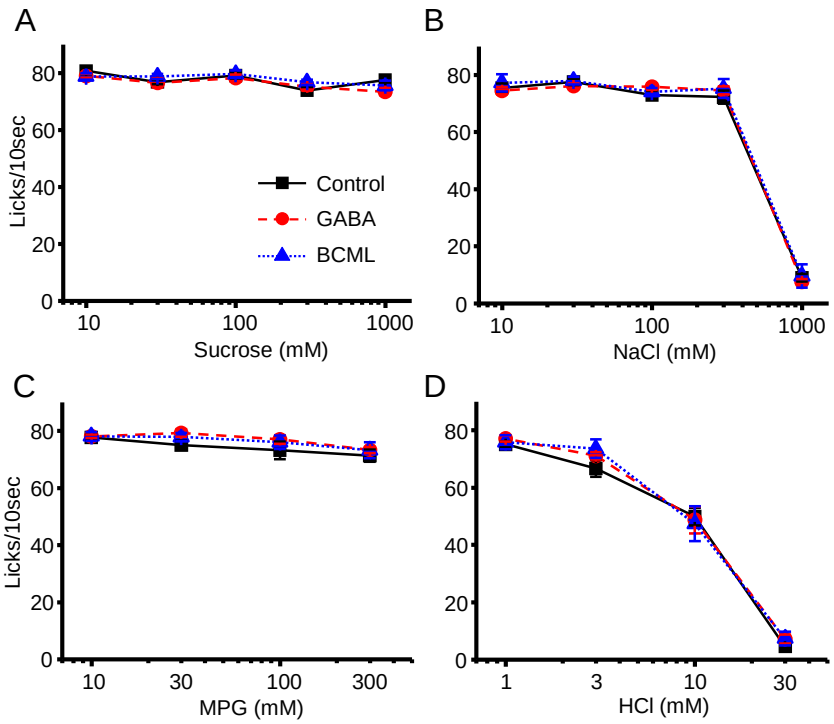

A
B
C
D
Licks/10sec
Control
GABA
BCML
Sucrose (mM)
NaCl (mM)
MPG (mM)
HCl (mM)
0
20
40
60
80
10
30
100
300
1000
1
3

Fig.3

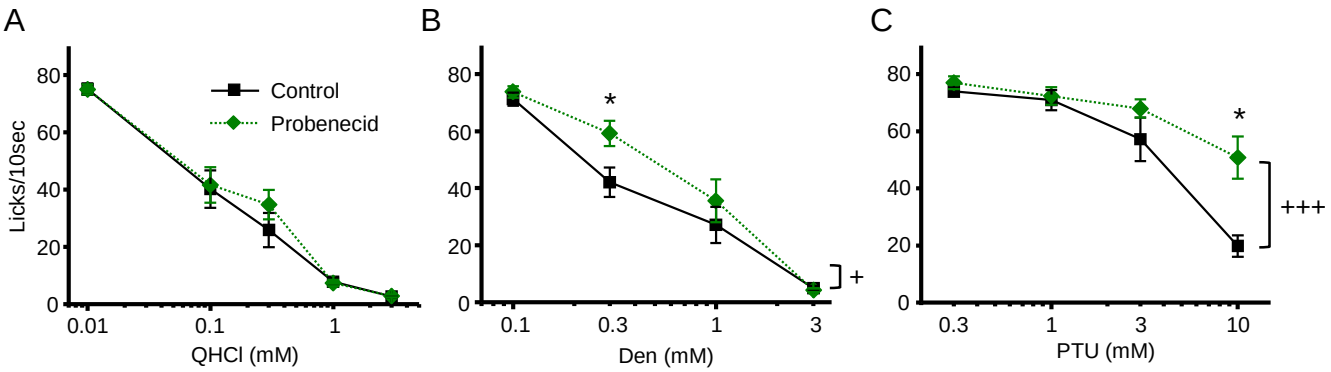

A
B
C
Licks/10sec
Control
Probenecid
QHCl (mM)
Den (mM)
PTU (mM)
*
*
+
+++
0
20
40
60
80
0.01
0.1
1
3
10
0.3

Fig.4

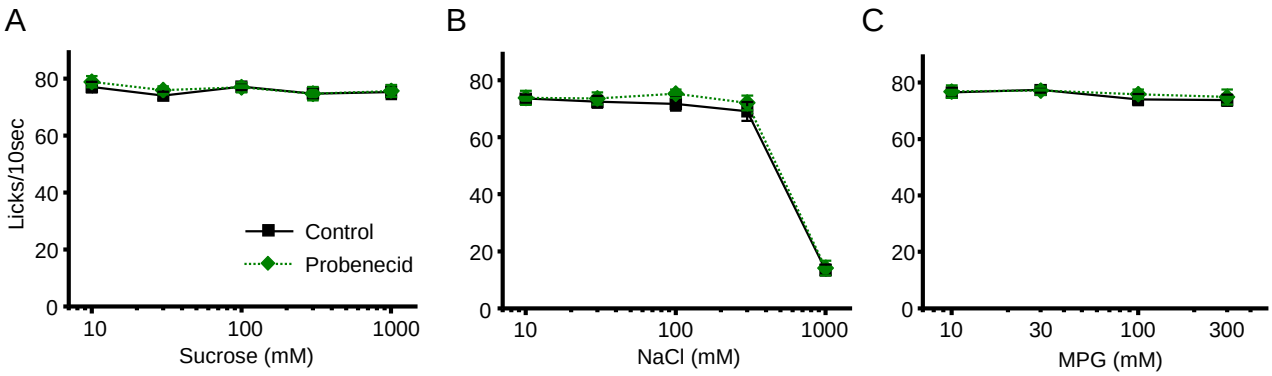
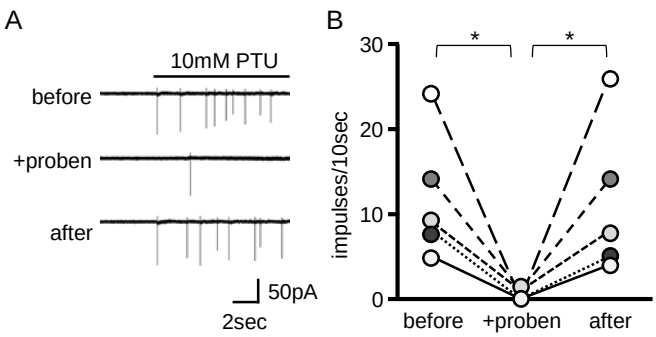

A
B
C
Licks/10sec
0
20
40
60
80
10
100
1000
Sucrose (mM)
Control
Probenecid
NaCl (mM)
30
300
MPG (mM)

Fig.5


A
10mM PTU
before
+proben
after
50pA
2sec
B
*
*
30
20
10
0
impulses/10sec
before
+proben
after