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G protein-coupled receptor mediated sensing of TMA

Anke Suska[a]*, Ana B. Ibáñez[a], Pakorn Preechaburana[a], Ingemar Lundström[a], and Anna Berghard[b]

[a] Division of Applied Physics, IFM, Linköping University, SE-58183 Linköping, Sweden.
[b] Department of Molecular Biology, Umeå University, SE-90187 Umeå, Sweden

Abstract

A new approach for the detection of trimethylamine (TMA) using recombinant *Xenopus laevis* melanophores was developed. The cells were genetically modified to express the mouse trace amine-associated receptor 5 (mTAAR5), a G protein-coupled receptor from the olfactory epithelium, which conferred high sensitivity to TMA. A focused chemical screen allowed the discovery of additional, previously unknown stimuli of mTAAR5. The cell-based sensor demonstrated no sensitivity to trimethylamine *N*-oxide (TMAO), making it suitable for a convenient evaluation of TMA levels in fish tissue extracts. The developed gas measurement platform was able to detect TMA from 1 to 100 ppm within thirty-five minutes.

Keywords: Cell-based sensor; trace amine-associated receptor; TAAR5; trimethylamine detection; *Xenopus laevis* melanophores.

1. Introduction

Trimethylamine is a volatile low molecular weight tertiary aliphatic amine with known toxicity and an unpleasant smell to rotten fish. The National Institute for Occupational Safety and Health (NIOSH) has established a recommended exposure limit (REL) for trimethylamine of 10 ppm (24 mg/m^3). However, only a few methods exist to control atmospheric TMA levels, and they are rarely field-applicable and simple for routine analysis [1,2].

TMA content in fish can be seen as an indicator of its freshness [2]. TMA is produced by decomposition of TMAO by microorganisms and its concentration rapidly increases in marine products after their death, which explains the characteristic smell of TMA. The sense of smell depends on available sensory receptors that respond to airborne chemicals. Recently, it was demonstrated that trace amine-associated receptors (TAARs) function as chemosensory receptors in the olfactory epithelium in mice [3]. TMA could be shown to specifically activate mTAAR5.

We report on the functional expression of mTAAR5 in *Xenopus* melanophores. In these cells GPCR activation leads to the dispersion or aggregation of intracellular pigment granules, i.e. melanin filled vesicles termed melanosomes. Both states (dispersion or aggregation) of intracellular melanosome distribution are easily detectable

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* Corresponding author. Tel.: +46 13 282625; fax: +46 13 137568.
E-mail address: anksu@ifm.liu.se

by either measuring the change in light transmittance through the cells or by imaging the cells as they are responding. This feature makes them an excellent system for cell based sensing.

2. Materials and methods

The coding sequence of mTAAR5 (NCBI accession no. NM_001009574) was amplified from genomic DNA using PCR and subcloned into pcDNA3.1/Hygro$^{(+)}$ (Life Technologies, Carlsbad, CA, USA). The *Xenopus* melanophores were electroporated according to a protocol described previously [4], with minor modifications. The cell suspension was immediately transferred to a tissue culture flask and propagated for two days before selection with 100 µg/ml hygromycin B for one month to obtain a cell line that stably expressed mTAAR5.

mTAAR5 activation assay was performed in flat bottom 96 well microtiter plates, modified from a protocol previously described [4]. As control measurements, non-transfected melanophores were analyzed. They did not respond to any of the ligands. Boltzmann four parameter sigmoid function was used for curve fitting.

For the gas measurement, melanophores were cultured in 35 mm culture dishes and covered with 1 ml of 0.2 % agarose. After the cells were preaggregated with melatomin, the medium was removed and the cells were placed into a 250 ml polystyrene container (petri dish) on top of the stage of an inverted microscope. The gaseous standards of TMA were generated by placing a drop of 2.3 µl standard solutions of 0.42 M, 0.042 M and 0.0042 M TMA, respectively, into the container (1ppm = 2.4 mg/m^3). Immediately upon addition of TMA, images of the cells were digitally recorded and melanosome distribution in the pictures was determined by counting the overall area covered by dark pigments in a defined portion of the image. A threshold of 160 (intensity level) was chosen to avoid artifact caused by subtle changes in overall light intensity. Data processing was performed using ImageJ software [5].

Cell viability after air exposure was assessed using erythrosine B in a vital dye exclusion test. The cells were exposed to air for 0, 1, 2, 4, 6 and 8 h at room temperature under subdued light conditions, stained with 30 µg/ ml erythrosine B, washed and imaged by both phase-contrast and fluorescence microscopy.

3. Results and discussion

We created a *Xenopus laevis* melanophore based sensor for TMA, by genetically engineering melanophores to express mTAAR5, a GPCR previously shown to mediate sensitivity to TMA [3]. Fig. 1a demonstrates the fractional change in absorbance of the obtained cell line in response to TMA. The dose-response curve obtained followed a characteristic sigmoidal shape and the observed half maximal effective concentration (EC$_{50}$) was 4 µM.
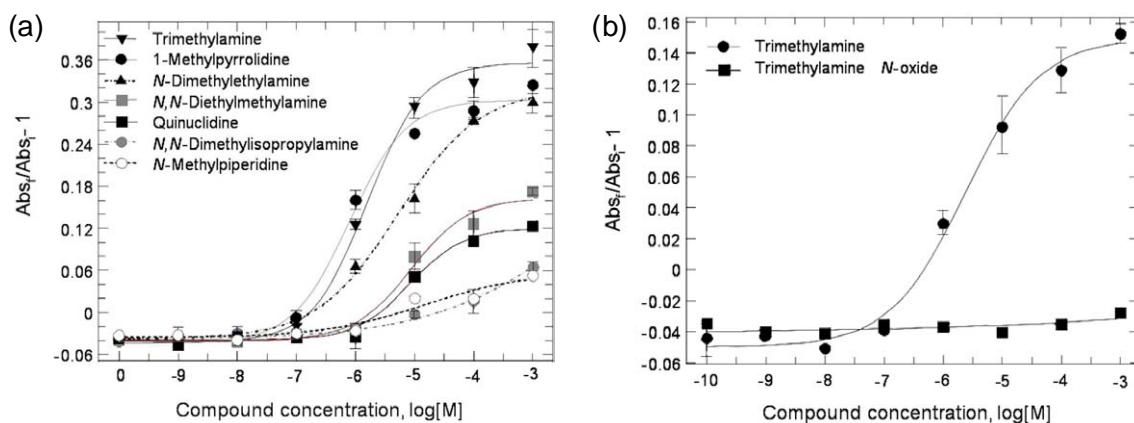

Fig. 1: (a) mTAAR5 transfected *Xenopus* melanophores respond to a selection of amines, including TMA. Melanosomes were aggregated in the presence of melatonin to obtain a baseline minimum absorbance. Varying concentrations of different amines were added and incubated with the cells for 30 min. Different amines exert varying potencies and maximal efficacies on the mTAAR5 receptor. Abs$_i$ is the initial absorbance immediately before the addition of compounds and Abs$_f$ is the final absorbance after 30 min incubation. (b) TMA but not TMAO induced pigment dispersion in mTAAR5 transfected Xenopus melanophores, indicating the suitability of this sensor system for the detection of fish freshness using simple extracts. . Measurements were performed in duplicates and expressed as means ± standard deviation (SD).

Besides TMA, there are two additional mTAAR5 ligands known, *N,N*-dimethylethylamine and *N*-methylpiperidine [3]. Both of these ligands showed lower potency (EC_{50}) and efficacy in the ability to disperse the melanosomes, than TMA (Fig. 1a). The maximum level of dispersion achieved by *N,N*-dimethylethylamine (at 1 mM) was only about 50 % of the maximum response that was elicited by TMA, and *N*-methylpiperidine only dispersed melanosomes to about 20%, suggesting that these are partial agonists for the receptor.

To further examine the mTAAR5 receptors selectivity, a total of 24 compounds which are structurally related to TMA or *N*-methylpiperidine were screened for melanosome dispersion. This allowed for the identification of four additional agonists for the mTAAR5 receptor (Fig. 1a). One compound, 1-methylpyrrolidine (EC_{50} = 0.9 µM) showed a slightly higher potency than TMA and its efficacy was comparable. *N*-dimethylethylamine (EC_{50} = 6.6 µM) exhibited similar potency, but showed slightly lower efficacy compared to TMA. Quinuclidine (EC_{50} = 9 µM) and *N,N*-dimethylisopropylamine (EC_{50} = 9.5 µM) were the weakest agonists found, exhibiting potencies and efficacies comparable to *N*-methylpiperidine. This focused screen adds to a previous general ligand screen, where a total of 94 amines where tested [3] and thus extended knowledge on activation of mTAAR5 by tertiary amines and confirmed its narrow selectivity.

One possible application of this cell line is its use in the determination of TMA emitted from fish products and/or TMA content in fish extracts for the determination of its freshness. Fresh marine products contain little TMA, but plenty of odorless trimethylamine *N*-oxide (TMAO). TMA is produced by metabolism of TMAO by microorganisms and its concentration rapidly increases in marine organisms following their death [2]. In certain fish, such as tuna, TMA levels showed a high correlation with levels of histamine, which has been associated to scombroid poisoning [6]. In order to utilize the mTAAR5 expressing melanophores for determinations of TMA in crude fish extracts, without previous extraction of volatile basic nitrogen, it is fundamental that there is no cross sensitivity to its precursor TMAO. Fig. 1b shows that TMAO did not elicit a response in the mTAAR5 expressing melanophores, facilitating a possible application of this cell based sensor for the straightforward measurement of TMA in fish extracts.
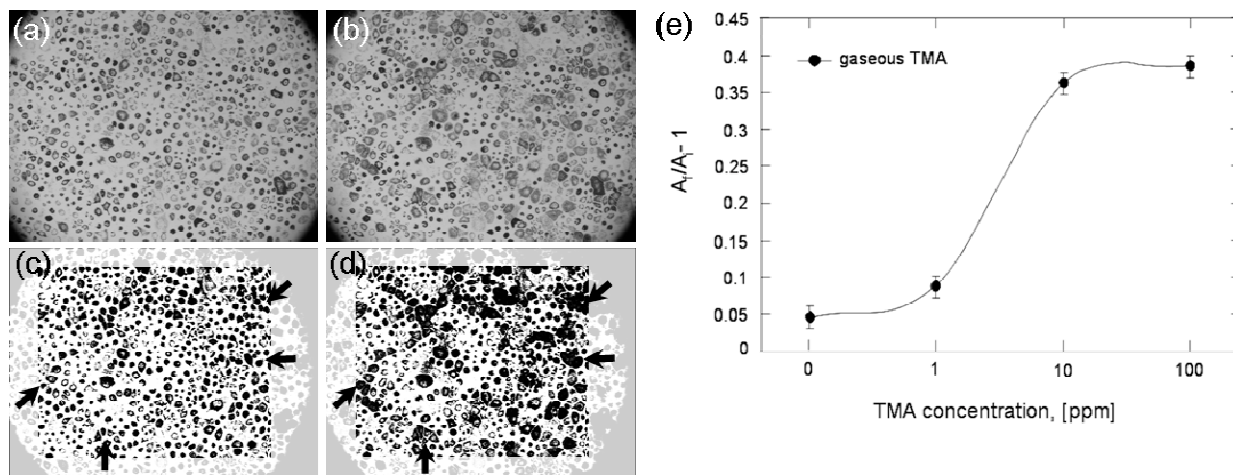

Fig. 2: (a) Imaging of agarose covered *Xenopus* melanophores. First frame immediately after exposure to 100 ppm TMA and (b) last frame after 35 min. (c) and (d) When applying a threshold of 160 (intensity level), differences between the two shown frames become accentuated. (c) First frame immediately after exposure to 100 ppm TMA and (d) last frame after 35 min. For further processing a defined area was chosen which is kept constant in each frame (indicated as clear inset in (c) and (d)). Arrows indicate different groups of cells, to exemplify how pigment dispersion caused an increase of total area covered by pigments. *(e)* Dose-response curve of *Xenopus* melanophore-based TMA gas sensing. Melanosomes were preaggregated in the presence of melatonin and subsequently exposed to standard concentrations of gaseous TMA. Cellular responses were imaged and a conventional image processing protocol evaluated the fractional change of area covered by pigments (A_i is the initial area covered by pigments and A_f is the final area after 35 min exposure).

To the best of our knowledge, *Xenopus* melanophores have never been used in gas measurements before. In order to facilitate the measurement of TMA in air, it was necessary to develop a platform that ensured the viability of the air exposed cells for at least the time a measurement takes, which is around 30 min. For this purpose, the cells were

covered with a 0.2 % agarose gel. A viability test based on the vital exclusion stain erythrosine B showed that cell survival was $97.8 \pm 0.86\%$ (mean $\pm$ SD) up to 6h of air exposure. The agarose covered cells that were exposed to air for 8 h, started to detach from the plate in patches (due to dehydration of the agarose sheet), resulting in an about 50% loss of cells, but remaining cells did not stain positive with erythrosine B and continued to grow normally after the experiment. This data corroborates the usefulness of this melanophore-based biosensor design for the measurement of gaseous substances like TMA, since the normal duration of a measurement does not exceed 35 minutes.

The agarose covered mTAAR5-melanophores were pre-aggregated by 1nM melatonin, the medium was removed and the cells were exposed to defined concentrations of TMA vapor in a polystyrene container. Cell culture images were digitally recorded at 5 min intervals for 35 min immediately upon addition of 1 ppm, 10 ppm, and 100 ppm TMA, respectively. As control, the cellular responses in the absence of TMA were monitored.

Fig. 2 a shows the first frame immediately after exposure to 100 ppm TMA and Fig. 2 (b) shows the same cells 35 min later. For the chosen illuminating band (510–560 nm), most of the acquired information is contained in the (8-bits) green camera channel, which was used. Differences between the two frames were visible, i.e. after 35 min a larger area of the plate was covered by pigments due to pigment dispersion within the cells.

When applying a threshold of 160 (intensity level), avoiding artifacts caused by subtle changes in overall light intensity or camera exposure, these differences became accentuated (Fig. 2 (c) and (d)). For further processing a defined area was chosen which is kept constant in each frame. Pigment dispersion was assessed by evaluating the fractional change in area covered by pigments, $A_f/A_i - 1$.

Fig. 2 (e) shows the obtained dose response to TMA vapor. The obtained threshold concentration for TMA using these conditions was as low as 1 ppm.

4. Conclusions

We have demonstrated a novel cell based sensor for the determination of TMA in liquid and air. The sensor is based on mTAAR5 expressing *Xenopus* melanophores, and shows high sensitivity and specificity to a very limited number of tertiary amines, including TMA. As the system does not show sensitivity to the TMA precursor TMAO, it offers the possible application for fast and convenient determination of TMA in fish containing products. Moreover, a platform for the detection of gaseous substances using this sensor system has been developed. It consists of a cell monolayer covered with a thin sheet of agarose gel. Cellular responses to TMA are imaged on an inverted microscope and the evaluation of cell responses are performed with conventional image processing tools, which facilitated the detection of TMA with a threshold concentration of 1 ppm.

Acknowledgements

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