European Journal of Pharmacology 793 (2016) 35–42

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Molecular and cellular pharmacology

3-iodothyronamine (T1AM), a novel antagonist of muscarinic receptors

Annunziatina Laurino[a], Rosanna Matucci[a], Giulio Vistoli[b], Laura Raimondi[a,*]

[a] Dept. of NEUROFARBA, Section of Pharmacology, University of Florence, 50139 Florence, Italy
[b] Dept. of Pharmaceutical Science, University of Milan, 20100 Milan, Italy

ARTICLE INFO

Keywords:
3-iodothyrothyronamine
3-iodothyroacetic acid
Thyroid
Muscarinic receptors

ABSTRACT

3-iodothyronamine (T1AM) is a trace amine suspected to derive from thyroid hormone metabolism. T1AM was described as a ligand of G-protein coupled monoaminergic receptors, including trace amine associated receptors, suggesting the amine may exert a modulatory role on the monoaminergic transmission. Nothing is known on the possibility that T1AM could also modulate the cholinergic transmission interacting with muscarinic receptors.

We evaluated whether T1AM (10 nM–100 μM) was able to i) displace [^{3}H]-NMS (0.20 nM) binding to membrane preparations from CHO cells stably transfected with human muscarinic receptor subtypes (M1-M5); ii) modify basal or acetylcholine induced $pERK_{1/2}$ levels in CHO expressing the human muscarinic type 3 receptor subtype by Western blot iii) modify basal and carbachol-induced contraction of isolated rat urinary bladder. T1AM fitting within rat muscarinic type 3 receptor was simulated by Docking studies.

T1AM recognized all muscarinic receptor subtypes (pKi values in the micromolar range). Interacting at type 3, T1AM reduced acetylcholine-increased $pERK_{1/2}$ levels. T1AM reduced carbachol-induced contraction of the rat urinary bladder. The fenoxyl residue and the iodide ion were found essential for establishing contacts with the active site of the rat muscarinic type 3 receptor subtype.

Our results indicate that T1AM binds at muscarinic receptors behaving as a weak, not selective, antagonist. This finding adds knowledge on the pharmacodynamics features of T1AM and it may prompt investigation on novel pharmacological effects of T1AM at conditions of hyper-activation of the muscarinic tone including the overactive urinary bladder.

1. Introduction

3-iodothyronamine (T1AM) is an iodinated primary amine circulating in trace in human and rodents (Galli et al., 2012; Saba et al., 2010). T1AM is produced in the thyroid as well as in extra-thyroid tissues (Hoefig et al., 2011, 2015) and is rapidly metabolized by monoamine oxidases (MAO) and deiodinase activities to 3-iodothyroacetic acid (TA1) and iodothyronamine, respectively (Hackenmueller et al., 2012). The physiological meaning of T1AM tissue levels is currently unknown. Much is instead known on the pharmacological effects of T1AM. In this respect, we described that T1AM acutely modifies mice behavior and metabolism (Manni et al., 2012, 2013; Laurino et al., 2015) with a mechanism which remains to be defined but that depends, at least in part, on the activation of the histaminergic system and on T1AM biotransformation into TA1 (Manni et al., 2012, 2013; Laurino et al., 2015). In 2004, Scanlan et al. described the hibernating effect of T1AM while recently James et al. (2013) reported a pro-awaking effect of T1AM in rats. Notwithstanding the increasing numbers of hypotheses, the mechanisms responsible for such effects remain elusive yet.

However, T1AM has the potential to interact at several targets as documented by different experimental approaches. Scanlan et al. (2004) described T1AM as an high affinity ligand (in the nanomolar range of affinity) for the trace amine-associated receptor type 1 (TAAR1). Later, by virtue of its β-phenylethyl skeleton, in-vitro studies indicated T1AM recognized, in the micromolar range of concentrations, monoaminergic targets including beta 2 and alpha 2 receptors (Regard et al., 2007) (Dinter et al., 2015c), working as an agonist and as an inverse agonist respectively (Dinter et al., 2015a, 2015b). More recently, Dinter et al. (2015c) reported T1AM as an inverse agonist of TAAR type 5 (TAAR5) (Dinter et al., 2015cc). Further, recent electrophysiological evidence indicated T1AM (in the range of 0.1–10 μM) as an activator of the TRPM8 cold channel (Lucius et al., 2016). Except for alpha2 adrenoceptors, whether any of these targets are involved in pharmacological effects of T1AM are currently unknown. This uncertainty also includes the involvement of TAAR1. In fact, Chiellini et al. (2012), reported findings which argued against the involvement of

* Corresponding author.
E-mail address: laura.raimondi@unifi.it (L. Raimondi).

http://dx.doi.org/10.1016/j.ejphar.2016.10.027
Received 2 August 2016; Received in revised form 17 October 2016; Accepted 20 October 2016
Available online 02 November 2016

TAAR1 in T1AM-induced reduction of body temperature. However, the relationship between TAAR1 and T1AM would merit to be further explored in light of the findings from Szumska et al. (2015). These authors reported TAAR1 expression on thyroid follicles, a localization supporting their role, and of T1AM, its endogenous ligand, in thyroid regulation. Taken together all these evidence indicate that T1AM, as other trace amines, may be considered a modulator of the monoaminergic transmission (Borowsky et al., 2001). Nothing is known instead about the possibility that T1AM could also modulate the cholinergic transmission interacting with muscarinic receptors.

Muscarinic receptors are a family of five different G-coupled receptors (M1-M5) linked to two main intracellular cascades governed by Gi or Gq proteins. Muscarinic receptors are expressed at the central nervous system, where they have a main role in memory and pain circuits, and in the periphery were they are involved in smooth muscle contraction, in exocrine and endocrine gland secretion, including the thyroid (Szumska et al., 2015) and the pancreas (Nakajima et al., 2013). The recognition of muscarinic receptors by T1AM might rationally sustain some pharmacological effects of the amine and open new perspectives for investigating unexplored T1AM functions governed by muscarinic signaling, including the control of visceral smooth muscle tone.

To explore this possibility we performed binding experiments with T1AM and with TA1 on human muscarinic receptor subtypes (M1-M5) stably expressed in CHO cells. Then, to investigate on the agonistic/antagonistic nature of T1AM towards muscarinic receptors, we evaluated whether T1AM modified i) basal or acetylcholine-induced pERK1/2 levels in CHO stably transfected with human muscarinic type 3 receptor and ii) basal or carbachol-induced contraction of the rat urinary bladder.

2. Materials and methods

2.1. Animals

Male Sprague Dawley rats (275–300 g) from ENVIGO (San Pietro al Natisone, Udine, Italy) were used. Animals were kept at 23 ± 1 °C with a 12 h light–dark cycle (light on at 07:00 h) and were fed a standard laboratory diet with water ad libitum.

Experiments and animal use procedures were in compliance with ARRIVE guidelines (McGrath et al., 2010). All the experimental procedures were in accordance with EU Directive 2010/63/EU for animal experiments.

2.2. Membrane preparation

CHO cells stably expressing cDNA encoding the human muscarinic receptors (M1-M5) were generously provided by Prof. R. Maggio (Department of Experimental Medicine, University of L'Aquila, Italy).

Confluent CHO cell lines were trypsinized, washed with buffer (25 mM sodium phosphate-containing 5 mM $MgCl_2$ at pH 7.4) and homogenized for 30 s using an Ultra-Turrax (16,000 rpm). The pellet was centrifuged (17,000×*g* for 15 min at 4 °C) and the membranes were resuspended in the same buffer, re-homogenized with Ultra-Turrax and stored at −80 °C until use.

2.2.1. Binding assay in CHO cells

The radioligand binding assay was run in polypropylene 96-well plates (Sarstedt, Verona, Italy) and performed for 120 min at room temperature in a final volume of 0.25 mL of 25 mM sodium phosphate buffer, pH 7.4, containing 5 mM $MgCl_2$. Final membrane protein concentrations were: 30 μg/mL for human muscarinic type 1, 70 μg/mL for human muscarinic type 2, 25 μg/mL for human muscarinic type 3, 50 μg/mL for human muscarinic type 4 and 25 μg/mL for human muscarinic type 5. In heterologous competition curves, fixed concentrations of the tracer (0.2 nM [^{3}H]-NMS, ca. K_D) were displaced by increasing concentrations of T1AM and TA1 (Dei et al., 2005). All measurements were performed in duplicate. At the end of the binding reaction, free radioligand was separated from bound ligand by rapid filtration through UniFilter GF/C plates (Perkin–Elmer Life and Analytical Science, Boston, MA, USA) using a FilterMate Cell Harvester (Perkin–Elmer Life and Analytical Science, Boston, MA); after filtration, the filters were washed several times with ice cold water, allowed to dry overnight at room temperature under air flow. 25 μl of scintillation liquid (Microscint-20, Perkin–Elmer Life and Analytical Science, Boston, MA) were then added and the filter-bound radioactivity counted by TopCount NXT Microplate Scintillation Counter (Perkin–Elmer Life and Analytical Science, Boston, MA).

Off-Rate assays were performed to estimate the affinity of T1AM for the [^{3}H]NMS occupied receptor. In these experiments, designed to investigate the effects of a range of modulator concentrations on the [^{3}H]NMS dissociation rate, a “one-point kinetic assay” approach was used, where the effect on radioligand dissociation of the test ligand was determined at 0 min and at one time point, which was chosen to be ca 2.5 dissociation half-lives of [^{3}H]NMS alone (Lazareno et al., 1995). A high concentration of human M1-M5 CHO membranes (50–75 μg/ml) was incubated with a high concentration of [^{3}H]NMS (2 nM) for about 60 min at room temperature. Then, 100 μl aliquots were distributed into tubes which contained 10 μl of 100 μM atropine alone, or in the presence of a different concentrations of test compounds, and diluted in 1 ml total volume of buffer. The time zero data point was obtained using only 100 μl of the mixture containing membranes plus [^{3}H]NMS. Incubation (100 min for hM_3) was terminated by rapid filtration through Whatman GF/C filters (Brandel, USA), that had been pre-soaked in a 0.05% polyethylenimine (PEI) solution for at least 1 h, using a Brandell cell harvester (Biomedical Research and Development Laboratory, Inc Atlas Drive, Gaithersburg, MD, USA). Filters were washed three times with 3 ml aliquots of ice-cold milliQ water and dried before the addition of 4.5 mL of scintillation cocktail (Filter Count, Perkin-Elmer Life and Analytical Science, Monza, Italy). The radioactivity was determined using scintillation counting (TRI-CARB 1100, Perkin-Elmer Life and Analytical Science, Monza, Italy).

2.3. Docking studies

All derivatives were simulated in their protonated state, that one involved in ligand recognition by rat muscarinic type 3 receptor. The ligand's conformational profile was analyzed by MonteCarlo simulations which produced 1000 conformers by randomly rotating the rotatable bonds. In detail, the study was focused on the X-ray structure of the rM3R in complex with tiotropium (PDB Id: 4DAJ). The protein structure was prepared by removing the water molecules and all crystallization additives and then underwent energy minimation by keeping the backbone atoms fixed to preserve the resolved folding. After deleting the bound ligand, the so optimized protein structure was utilized by docking simulations as performed by using Plants which generates reliable ligand poses by ant colony optimization (ACO) algorithms (Korb et al., 2009).

In detail, the search was focused on a 10 Å radius sphere around the key aspartate residue (Asp147 in rat muscarinic type 3 receptor) thus including the entire binding site; 10 poses were generated and scored by the ChemPlp function and the speed was equal to 1. The so obtained lowest score complex was minimized by keeping fixed all atoms outside a 10 Å radius sphere around the bound ligand and finally underwent a targeted 1 ns simulations with constant temperature at 300 K and the above mentioned constraints to assess the relative stability of the computed complex. All cited calculations were performed by using the conjugate gradient algorithm until a rms equal to 0.001. Namd2.9 was used with the CHARMM force field and the Gasteiger's atomic charges (Phillips et al., 2005).