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The Multitarget Ligand 3-Iodothyronamine Modulates β-Adrenergic Receptor 2 Signaling

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Key Words

β-Adrenergic receptors · Thyronamine · Signaling · Calcium homeostasis · Calcium channel · Human conjunctiva

Abstract

Background: 3-Iodothyronamine (3-T_1AM), a signaling molecule with structural similarities to thyroid hormones, induces numerous physiological responses including reversible body temperature decline. One target of 3-T_1AM is the trace amine-associated receptor 1 (TAAR1), which is a member of the rhodopsin-like family of G protein-coupled receptors (GPCRs). Interestingly, the effects of 3-T_1AM remain detectable in TAAR1 knockout mice, suggesting further targets for 3-T_1AM such as adrenergic receptors. Therefore, we evaluated whether β-adrenergic receptor 1 (ADRB1) and 2 (ADRB2) signaling is affected by 3-T_1AM in HEK293 cells and in human conjunctival epithelial cells (IOBA-NHC), where these receptors are highly expressed endogenously. ***Methods:*** A label-free EPIC system for prescreening the 3-T_1AM-induced effects on ADRB1 and ADRB2 in transfected HEK293 cells was used. In addition, ADRB1 and ADRB2 activation was analyzed using a cyclic AMP assay and a MAPK reporter gene assay. Finally, fluorescence Ca^{2+} imaging was utilized to delineate 3-T_1AM-induced Ca^{2+} signaling. ***Results:*** 3-T_1AM (10^{-5}–10^{-10} M) enhanced isoprenaline-induced ADRB2-mediated G_s signaling but not that of ADRB1-mediated signaling. MAPK signaling remained unaffected for both receptors. In IOBA-NHC cells, norepinephrine-induced Ca^{2+} influxes were blocked by the nonselective ADRB blocker timolol (10 μM), indicating that ADRBs are most likely linked with Ca^{2+} channels. Notably, timolol was also found to block 3-T_1AM (10^{-5} M)-induced Ca^{2+} influx. ***Conclusions:*** The presented data support that 3-T_1AM directly modulates β-adrenergic receptor signaling. The relationship between 3-T_1AM and β-adrenergic signaling also reveals a potential therapeutic value for suppressing Ca^{2+} channel-mediated inflammation processes, occurring in eye diseases such as conjunctivitis.

Introduction

3-Iodothyronamine (3-T_1AM) is an amine with structural similarities to thyroid hormones [1]. 3-T_1AM modifies several important physiological parameters in rodents (for reviews see Zucchi et al. [2] and Piehl et al. [3]).

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In vitro, the receptor target of 3-T_1AM was assigned to the trace amine-associated receptor 1 (TAAR1) [4]. Interaction of 3-T_1AM with TAAR1 activates the G_s/adenylyl cyclase signaling pathways [4]. Paradoxically, several 3-T_1AM-induced physiological effects still persist in *mTaar1* knockout mice, suggesting further receptor targets in vivo. In this context, previous studies using turkey erythrocytes indirectly suggested β-adrenergic receptors as potential targets for thyronamines [3, 5]. Furthermore, several findings indicate that 3-T_1AM binds with high affinity to the locus coeruleus [6].

3-T_1AM has been proposed to be a multitarget ligand (reviewed by Zucchi et al. [2]), interacting also with a member of nonselective Ca^{2+} entry channels such as the transient receptor potential melastatin channel 8 (TRPM8), which is also known as menthol receptor [7]. In the eye, functional expression of TRPM8 and other thermosensitive TRP isoforms such as the TRP vanilloid 1 channel (capsaicin receptor) have been identified in corneal neurons as well as in corneal endothelial cells [8, 9]. Both are temperature-sensitive TRP channels occurring in corneal tissue layers and cells [10]. Interestingly, TRPV1, in connection with a putative modulation by thyronamines via G protein-coupled receptors (GPCRs), may be a potential drug target in reducing inflammatory symptoms in eye and conjunctiva diseases. It is known that TRPV1 can be stimulated during exposure to the same hypertonic conditions identified in tear samples obtained from dry eye patients or patients with conjunctivitis [11, 12].

The present study was undertaken to elucidate a potential role of β-adrenergic receptor 1 (ADRB1) and 2 (ADRB2) as new targets for 3-T_1AM. We also explored a putative link between 3-T_1AM actions on Ca^{2+} channels and these adrenergic receptors, which are able to specifically modulate the aforementioned TRP channels. The human conjunctival epithelial cell line (IOBA-NHC) was utilized due to the increased expression of β-adrenergic receptors, which has also been observed in the conjunctiva and eye [13–15].

Materials and Methods

Cloning of Adrenergic Receptors

All full-length β-adrenergic receptors were cloned from genomic human DNA into the eukaryotic expression vector pcDps. The β_1- (ADRB1, NM_000684.2) and β_2-adrenergic receptors (ADRB2, NM_000024.5) were N-terminally tagged with a hemagglutinin (YPYDVPDYA) epitope. Plasmids were sequenced and verified with BigDye-terminator sequencing (PerkinElmer Inc., Waltham, Mass., USA) using an automatic sequencer (ABI 3710xl; Applied Biosystems, Foster City, Calif., USA).

Cell Culture and Transient Transfection

For the cyclic AMP (cAMP) assay and MAPK characterization, HEK293 cells were cultured in MEM Earle's media containing L-glutamine supplemented with 5% FBS and nonessential amino acids (Biochrom AG, Berlin, Germany) in a humidified 5% CO_2 incubator at 37°C. For functional assays, transfections were performed as described previously [16]. The IOBA-NHC cell line was used as a cell model for human conjunctival epithelial cells. Cells were grown in DMEM/HAMs F12 1:1 supplemented with 10% FBS, 1 μg/ml insulin, 5 μg/ml hydrocortisone and antibiotics in a humidified 5% CO_2 incubator at 37°C [17].

Dynamic Mass Redistribution

HEK293 cells (500,000 cells/well) were seeded in poly-L-lysine-coated 50-ml cell culture flasks. Twenty-four hours after seeding, cells were transiently transfected using Lipofectamine™ 2000 (Life Technologies). On the next day, cells were detached using Versene solution (Life Technologies, Darmstadt, Germany) and were transferred into fibronectin-coated 384-well plates (15,000 cells/well) and incubated for 24 h at 37°C with 5% CO_2. For receptor activation, a high-throughput dynamic mass redistribution (DMR) technology (Corning® Epic® system) was used [18] to determine direct effects of 3-T_1AM (Santa Cruz Biotechnology Inc., Dallas, Tex., USA; final concentration of DMSO 0.1%) on ADRB1 and ADRB2 following costimulation with isoprenaline (ISOP, Sigma Aldrich, St. Louis, Mo., USA, dissolved in H_2O).

cAMP Assay

G_s signaling was determined by measuring cAMP in transiently transfected HEK293 cells. For control purposes, we transfected cells with empty vector. All experiments were performed in comparison to ADRB1 and ADRB2 transfection. 3-T_1AM was dissolved in DMSO and used in a concentration of 0.1% for 10^{-5} M 3-T_1AM, which did not affect cAMP accumulation. Stimulation was performed 48 h after transfection as described previously [19]. Cells were incubated for 40 min without ligand or with 3-T_1AM (final concentration of DMSO 0.1%), ISOP or norepinephrine (NorEpi; Sigma Aldrich, St. Louis, Mo., USA; both dissolved in H_2O), or were costimulated with ISOP, or NorEpi and 3-T_1AM, or NorEpi with 3,3′,5-triiodothyronine (T_3; Sigma Aldrich; dissolved in HCl final concentration of 0.01%). Intracellular cAMP accumulation was determined as previously described [19].

MAPK Activation Assay

MAPK activation was measured using a luciferase reporter gene assay (SRE-luc) (Promega, Fitchburg, Wis., USA). HEK293 cells were cotransfected with serum response element (SRE) plasmid DNA (pGL4.33), a reporter construct containing an SRE and the firefly luciferase reporter gene, and either receptor or empty vector plasmid DNA (mock). Two days after transfection, cells were incubated for 6 h with the respective substances (ISOP, NorEpi or 3-T_1AM) in supplement-free MEM at 37°C with 5% CO_2. Reactions were terminated by aspirating the media. Cells were lysed using 1× passive lysis buffer (Promega). Measurement was conducted with automatic luciferase substrate injection of 40 μl in a black 96-well plate using a Berthold Microplate Reader (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany).

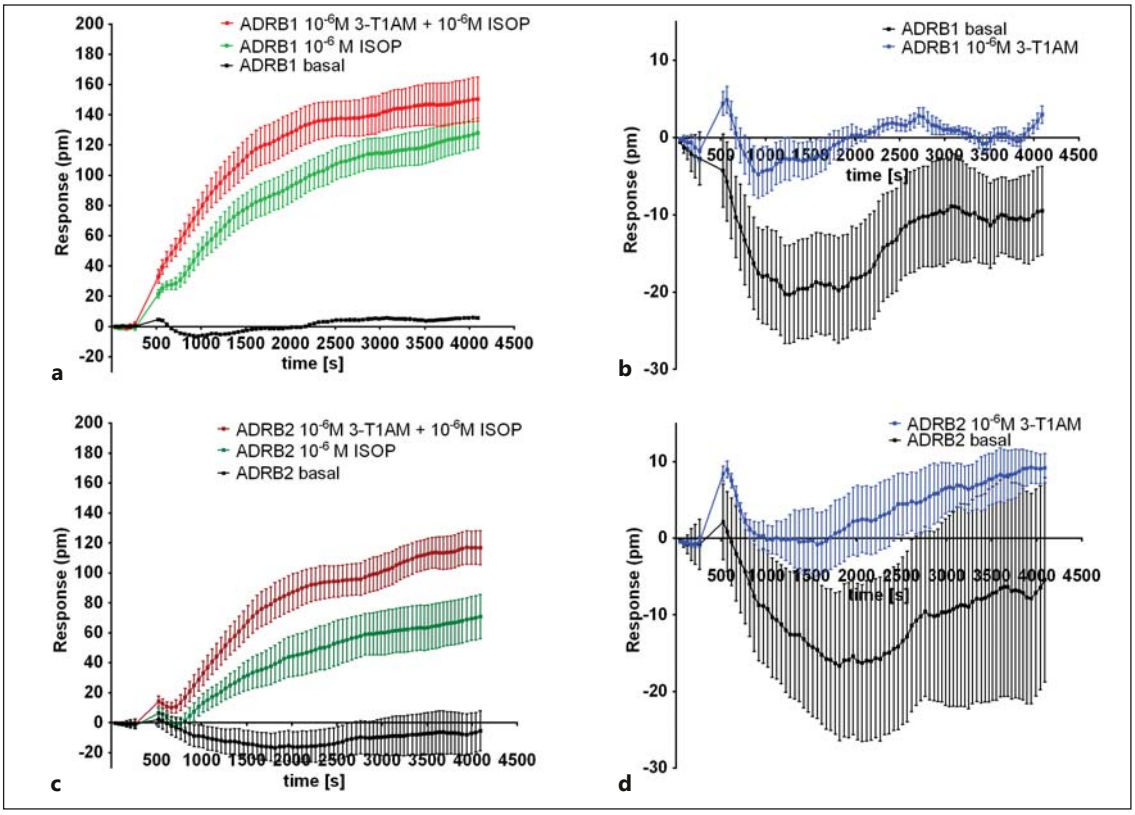

Fig. 1. EPIC technology to identify 3-T_1AM effects on ADRB1 and ADRB2 signaling. HEK293 cells were transiently transfected with ADRB1 or ADRB2, and their response to ISOP and 3-T_1AM alone or in costimulation was measured using the label-free dynamic mass distribution assay (EPIC technology). **a** Signaling of ADRB1 followed by application of 10^{-6} M ISOP with coincubation of 10^{-6} M ISOP and 10^{-6} M 3-T_1AM in comparison to untreated cells (basal) was measured. **b** 3-T_1AM stimulation in comparison to basal level of ADRB1 was measured. **c** The basal level of ADRB2 compared to 10^{-6} M ISOP or in coincubation with 10^{-6} M ISOP and 10^{-6} M 3-T_1AM is shown. **d** Signaling was measured in cells expressing ADRB2 following incubation with 10^{-6} M 3-T_1AM in comparison to untreated cells. Data are represented as means ± SEM of mock-corrected response over time. Original data are shown as one representative experiment of four independent experiments performed in three technical replicates.

Fluorescence Calcium Measurements

The intracellular Ca^{2+} concentration in IOBA-NHC cells was measured as previously reported [17, 19]. In brief, IOBA-NHC cells were loaded with 1 μM fura-2/AM for 30–40 min at 37°C. Fluorescence measurements were performed with a microscope (Olympus BW50WI) at room temperature using a Ringer-like solution containing 150 mM NaCl, 6 mM CsCl, 1.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES and 10 mM glucose (pH 7.4) (~300 mOsM). In addition, a digital-imaging system (TILL-Photonics, Munich, Germany) and TIDA software were used (HEKA-Electronics, Lamprecht, Germany). Fura-2 fluorescence was alternately excited at wavelengths of 340 and 380 nm and emission was measured at 510 nm. The fluorescence ratio ($f_{340\,nm}/f_{380\,nm}$) is a relative index of changes in $[Ca^{2+}]_i$ [20]. Results are presented as mean traces of f_{340}/f_{380} ± SEM. Measurements lasted 10 min.

Statistics

Data are shown as means ± SEM of independent experiments as indicated in the respective figure legends. For statistical analysis, one-way ANOVA was performed with Dunnett's multiple comparisons test with a threshold of statistical significance of $p \leq 0.05$. Student's t test was used if the values were normally distributed in accordance with the Gaussian distribution (normality test). GraphPad Prism 6.0 (GraphPad software, San Diego, Calif., USA) was chosen for data analysis. SigmaPlot software version 12.5 (Systat, San Jose, Calif., USA) was used for the creation of particular diagrams.

Results

ADRB1 and ADRB2 Are Targets of 3-T_1AM

Using DMR technology, we prescreened the potential effects of 3-T_1AM alone and in combination with ISOP on ADRB1 and ADRB2 signaling. A robust change in DMR following stimulation with 10^{-6} M ISOP for ADRB1

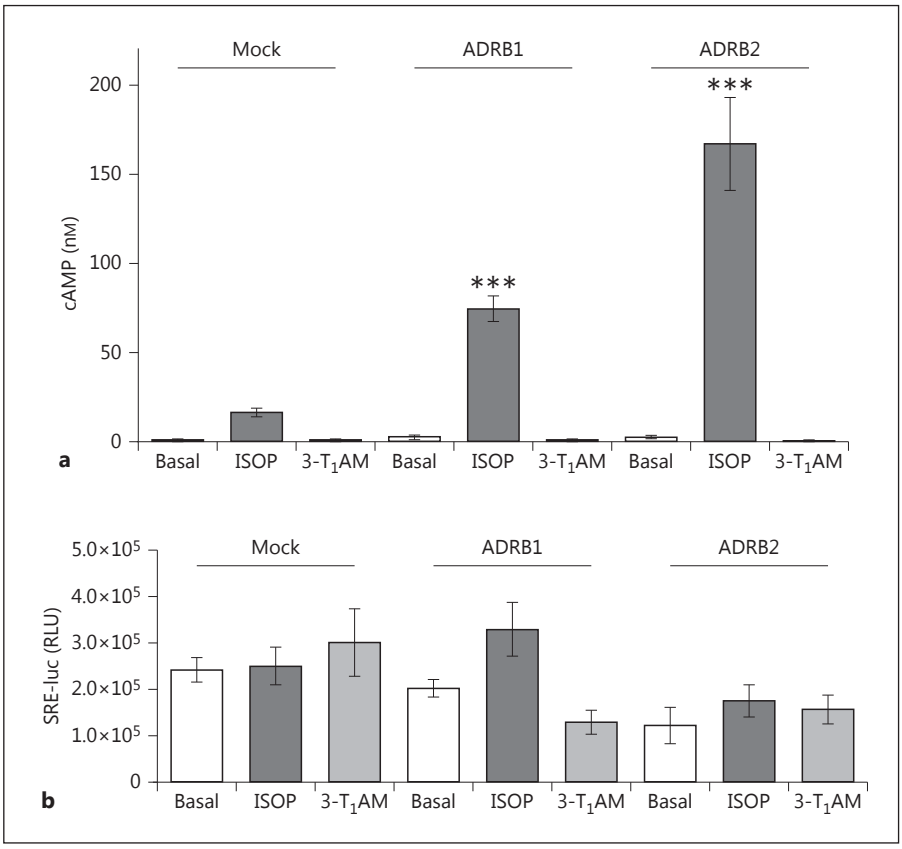

Fig. 2. 3-T_1AM supplementation shows no classic signaling effects on ADRB1 and ADRB2. **a** HEK293 cells transfected with mock, ADRB1 or ADRB2 were left unstimulated or treated with 10^{-6} M ISOP or 10^{-5} M 3-T_1AM, followed by measurement of cAMP levels. **b** Cells were cotransfected with mock, ADRB1 or ADRB2, and with SRE-luc, a reporter construct for MAPK activation. Relative light units (RLU) were then measured. Data were obtained from 3–4 independent experiments measured in at least triplicate and are means ± SEM. A one-way ANOVA against ISOP-stimulated mock was used for statistical analysis. *** $p \leq 0.001$.

(fig. 1a) and ADRB2 (fig. 1b) in comparison to nonstimulated cells was registered. We also investigated the direct effect of 10^{-5} M 3-T_1AM on ADRB1 (fig. 1c) and ADRB2 (fig. 1d). The effect of 3-T_1AM on both receptors was small but statistically significant at several time points in comparison to nonstimulated cells (one-way ANOVA with a Dunnett's multiple comparisons test, $p < 0.05$). In addition, the modulatory effect of 3-T_1AM in the presence of 10^{-6} M ISOP was examined ($p < 0.05$; fig. 1a, b), with a shift in DMR for both receptors demonstrated in comparison to ISOP stimulation alone.

3-T_1AM Alone Does Not Activate G_s and MAPK at ADRB1 and ADRB2

Stimulation of ADRB1 and ADRB2 with 10^{-6} M ISOP resulted in a robust increase of ADRB1- and ADRB2-mediated G_s signaling (fig. 2a). However, a high 3-T_1AM concentration of 10^{-5} M was not sufficient to activate these receptors (fig. 2a). Therefore, the observed effect in DMR was not compatible with G_s activation.

To test whether the observed signal in DMR was due to MAPK activation, stimulation of 10^{-6} M ISOP or 10^{-5} M 3-T_1AM was performed. A slight increase in MAPK signaling for ADRB1 with ISOP was observed, which was not found to be statistically significant (fig. 2b). In addition, no increase of MAPK signaling following 3-T_1AM stimulation was observed in comparison to basal levels (fig. 2b).

3-T_1AM Modulates ISOP-Induced cAMP Signaling of ADRB2

To determine a modulatory effect of 3-T_1AM on ADRB1 and ADRB2 on ISOP-induced signaling, we performed costimulation studies with a constant concentration of ISOP (10^{-6} M) and increasing concentrations of 3-T_1AM (10^{-10}–10^{-5} M) in G_s signaling (fig. 3).

Coincubation of ISOP and increasing concentrations of 3-T_1AM resulted in a modest effect for ADRB1, particularly at a concentration of 10^{-7} M 3-T_1AM (fig. 3a). The formation of cAMP at an ISOP concentration of 10^{-6} M was normalized to 1 and the fold change in cAMP formation induced by 3-T_1AM was indicated. A significant modulatory effect for ADRB2 signaling with a maximal enhancement of G_s signaling of twofold over mock-

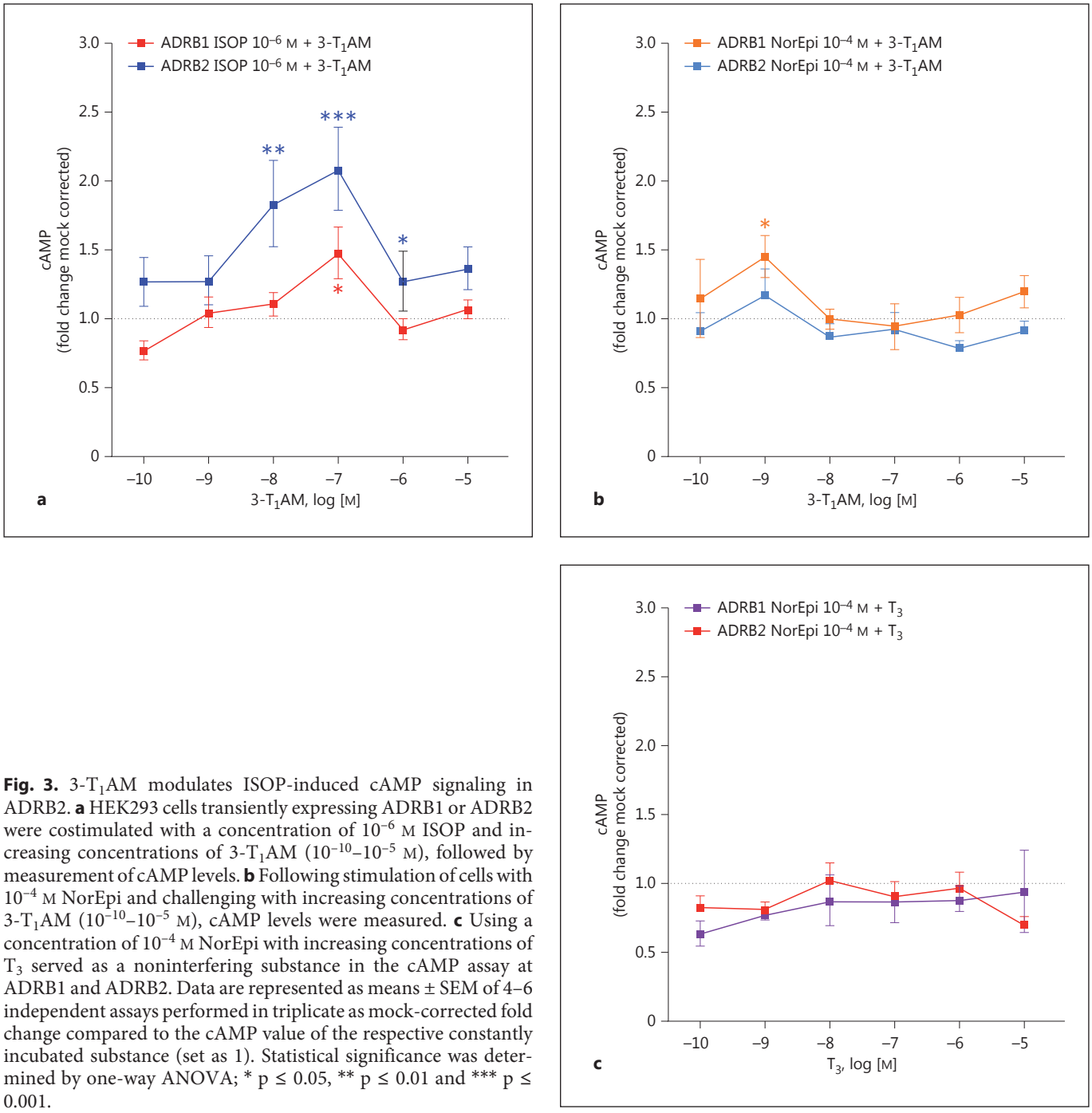

Fig. 3. 3-T_1AM modulates ISOP-induced cAMP signaling in ADRB2. **a** HEK293 cells transiently expressing ADRB1 or ADRB2 were costimulated with a concentration of 10^{-6} M ISOP and increasing concentrations of 3-T_1AM (10^{-10}–10^{-5} M), followed by measurement of cAMP levels. **b** Following stimulation of cells with 10^{-4} M NorEpi and challenging with increasing concentrations of 3-T_1AM (10^{-10}–10^{-5} M), cAMP levels were measured. **c** Using a concentration of 10^{-4} M NorEpi with increasing concentrations of T_3 served as a noninterfering substance in the cAMP assay at ADRB1 and ADRB2. Data are represented as means ± SEM of 4–6 independent assays performed in triplicate as mock-corrected fold change compared to the cAMP value of the respective constantly incubated substance (set as 1). Statistical significance was determined by one-way ANOVA; * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.

corrected 10^{-6} M ISOP signaling (fig. 3a) was demonstrated. Incubation of 3-T_1AM on mock-transfected cells did not result in cAMP formation (data not shown).

To further analyze whether this effect is reproducible with the endogenous ligand NorEpi, we performed costimulation studies with a concentration of 10^{-4} M NorEpi and increasing concentrations of 3-T_1AM (10^{-10}–10^{-5} M; fig. 3b). For the endogenous ligand, which has a lower affinity at ADRB1 and ADRB2 in comparison to ISOP [21], the modulatory effects were much smaller (fig. 3b). Only a stimulatory effect for costimulation with 10^{-9} M 3-T_1AM was observed.

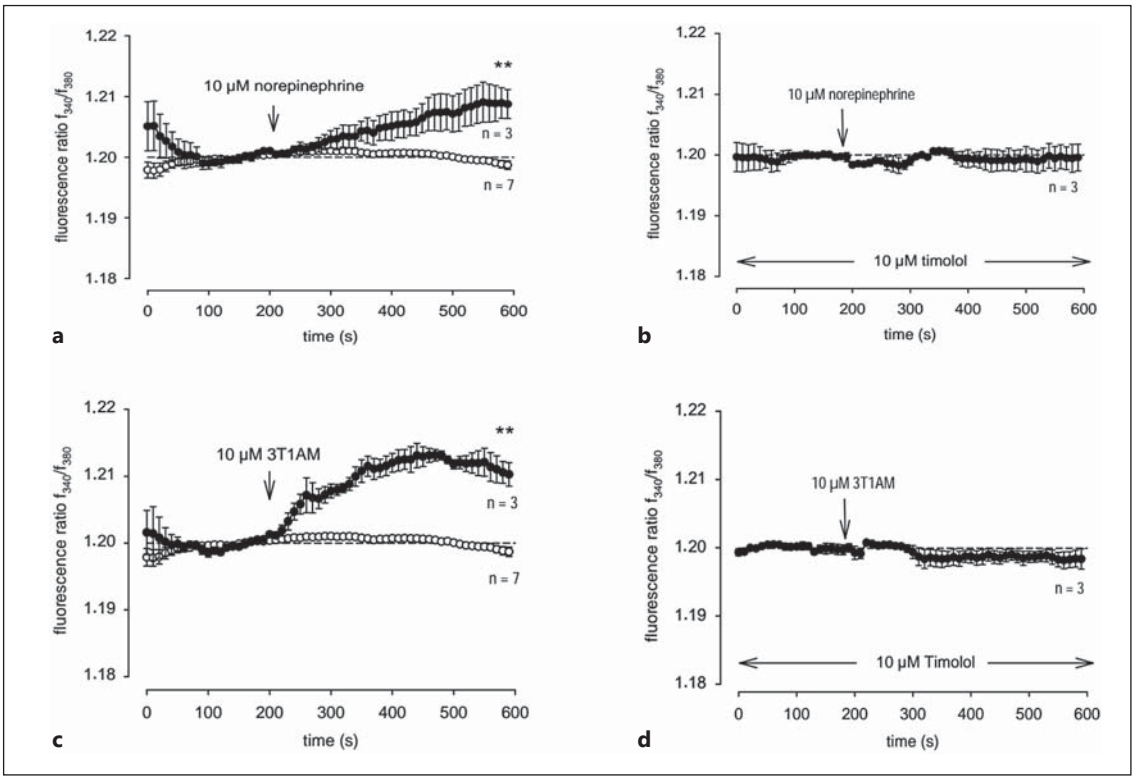

Fig. 4. NorEpi and 3-T_1AM-induced Ca^{2+} entry suppressed by the nonselective ADRB blocker timolol. Changes in cytosolic free Ca^{2+} are depicted as the ratio of the fluorescence induced by the excitation wavelength at 340 and 380 nm. Reagents were added to nontransfected IOBA-NHC cells at the time points indicated by the arrows. **a** Cells were stimulated with 10^{-6} M NorEpi (filled circles) and Ca^{2+} influxes were measured (n = 3) in comparison to basal levels (n = 7, open circles). **b** Cells were incubated with 10^{-6} M NorEpi in the presence of 10^{-6} M timolol (n = 3). **c** 10^{-6} M 3-T_1AM (filled circles) were added to the cells, and Ca^{2+} levels were measured (n = 3). **d** Cells were coincubated with 10^{-6} M 3-T_1AM and 10^{-6} M timolol (n = 3). Data are represented as means ± SEM of 3–7 experiments. Ca^{2+} baselines were recorded as controls (n = 7).

To rule out that the modulation of signaling is an artificial result of costimulation, we repeated the experiments by costimulation of 10^{-4} M NorEpi and increasing concentrations of T_3 (10^{-10}–10^{-5} M). Only for stimulation of ADRB1 with 3-T_1AM (at a concentration of 1 nM) was a significant difference compared to stimulation with NorEpi alone observed (fig. 3c).

3-T_1AM-Induces Ca^{2+} Influx in Human Conjunctival Epithelial Cells

To ascertain whether endogenously expressed adrenergic receptors respond to 3-T_1AM, we investigated IOBA-NHC cells [22] since β-adrenergic receptors are highly expressed in eye layers and cells [14, 23, 24]. It is also known that there is an association between voltage-dependent Ca^{2+} channels and adrenergic receptors [25]. In addition, there is also an association between adrenergic receptors and nonvoltage-dependent Ca^{2+} channels, such as the aforementioned TRP channels which are expressed in IOBA-NHC cells [17]. As shown in figure 4a and b, a NorEpi-induced Ca^{2+} influx was blocked by the nonselective β-adrenergic receptor antagonist timolol. More specifically, stimulation with 10^{-6} M NorEpi increased the fluorescence ratio $f_{340\,nm}/f_{380\,nm}$ from 1.200 ± 0.001 to 1.209 ± 0.002 ($p \leq 0.05$, n = 3) at 600 s (fig. 4a), whereas the NorEpi-induced Ca^{2+} increase was clearly suppressed in the presence of 10^{-6} M timolol to 1.200 ± 0.02 ($p \leq 0.05$; n = 3; at 600 s; fig. 4b). Similarly, extracellular application of 10^{-6} M 3-T_1AM increased the fluorescence ratio from 1.199 ± 0.001 to 1.210 ± 0.002 ($p < 0.01$; n = 3; at 600 s) at 600 s (fig. 4c), which was also blocked by 10^{-6} M timolol (1.199 ± 0.002; $p \leq 0.01$; n = 3; at 600 s; fig. 4d). The effect of 3-T_1AM in DMR might therefore be due to an effect of endogenously expressed TRP channels and ADRB1 or ADRB2.

Discussion

3-T_1AM Targets ADRB2 and Is Involved in Regulation of Calcium Influx

3-T_1AM is an endogenous thyroid hormone derivative that activates TAAR1 (reviewed by Piehl et al. [3]). It has been suggested that 3-T_1AM is a multitarget ligand and affects further GPCRs or interacts with non-GPCR proteins [2]. In this study, DMR, cAMP assay and a MAPK activation assay were used to ascertain whether 3-T_1AM-induced cellular signaling is linked with ADRB1 and ADRB2. Furthermore, modulation of Ca^{2+} signaling by 3-T_1AM was evaluated using an IOBA-NHC cell line which has been described as a cell model for the investigation of human conjunctiva [22], also in an electrophysiological context [12, 17].

Our studies revealed that 3-T_1AM enhances G_s-mediated signaling of ISOP-stimulated ADRB2 as a modulator. This finding might be reasoned by positive allosteric effects, which means that binding of 3-T_1AM enhances binding and/or agonistic signaling properties (maximum of capacity) of ISOP. This may be explained by two different scenarios. In the case of so-called off-target effects, dimers constituting two interacting receptor protomers would mutually influence one another, and binding of 3-T_1AM at one protomer increases the capacity for ISOP-induced signaling at the second protomer. Several examples for such a signaling (or binding) modification in GPCR dimers have been reported (for review see Smith and Milligan [26]) and ADRB2 is known to constitute dimeric arrangements [27]. In the second potential scenario of 'on-target' allosterism, 3-T_1AM would bind at an allosteric receptor site and improve, by spatial rearrangements of the monomeric receptor conformation, the signaling capacity for ISOP bound in the orthosteric binding site. Such a principle scenario of two different ligand-binding sites at a GPCR monomer has been previously suggested, for example at the lutropin receptor [28].

Interestingly, the observed effect of signaling inhibition at higher 3-T_1AM concentrations compared to lower concentrations (fig. 3) would be compatible with a two-binding site model for 3-T_1AM (low and high affinity), whereby one site would have a stimulating effect and the second an inhibitory influence. Such mechanisms have been described for the M_3 muscarinic acetylcholine receptor [29] or the β_1-adrenergic receptor [30].

It can be also hypothesized that the effect of a decreased G_s-mediated cAMP accumulation at increasing 3-T_1AM concentrations in costimulation experiments with ISOP or NorEpi (constant concentration) should be related to the activation of G_i. This would inhibit G_s-mediated signaling. It is known that β-adrenergic receptors are promiscuous for different G protein subtypes and that their differentiated activation can be dependent on the ligand variant or concentration [31].

Furthermore, NorEpi as well as 3-T_1AM increased intracellular Ca^{2+} levels in IOBA-NHC cells, which could be blocked by the nonselective adrenergic receptor blocker timolol (fig. 4). This prompts the suggestion that 3-T_1AM is connected with Ca^{2+}-permeable channels or intracellular store depletion processes in connection with adrenergic receptors, which was also reported in cardiomyocytes [32].

Since regulation of body temperature is more or less conducted by temperature-sensitive receptors, we postulate that 3-T_1AM somehow activates cold 'receptors' such as the aforementioned menthol receptor TRPM8. In previous studies using the same cell line, we could delineate that the 3-T_1AM-induced Ca^{2+} effect was due to TRPM8 activation since the specific TRPM8 blocker BCTC abolished the 3-T_1AM effect [7]. Interestingly, timolol was observed to have the same effect, suggesting that 3-T_1AM binds to ADRBs, in particular ADRB2, which in turn activates Ca^{2+} channels such as TRPM8. A first assumption might be that 3-T_1AM effects are directly mediated via protein interactions between the GPCR and TRP channel (reviewed by Veldhuis et al. [33]), or indirectly via the β/γ-subunits of $G_{i/o}$ [34], which can also be activated by the β-adrenergic receptors [31]. This hypothesis is supported by our observations in costimulation experiments (fig. 3), which might show activation of G_i at higher 3-T_1AM concentrations combined with constant ISOP or NorEpi levels (and therefore inhibition of G_s signaling). In consequence, the direct 3-T_1AM-mediated G_i-induced signaling should be investigated in future studies.

Potential Role of 3-T_1AM in Conjunctival Epithelial Cells

The established human conjunctival IOBA-NHC cell line was used as a relevant model of ocular surface cell biology as well as for investigation of electrophysiological investigation of TRP channels [10, 17]. Notably, an association between direct interaction of 3-T_1AM with TRPM8 and suppression of TRPV1 activity could be demonstrated in this cell line [7]. The results of the present study further indicate that the multitarget 3-T_1AM is a modulator of ADRB2 and mediates cellular and Ca^{2+} signaling. At this point, 3-T_1AM appears to play a potential role in both TRPM8- and TRPV1-mediated Ca^{2+} regulation and linked biological processes.

Conclusions

We demonstrate that 3-T_1AM-enhances ISOP-induced ADRB2 signaling via the G_s/adenylyl cyclase pathway. This suggests that ADRB2 is a GPCR target for 3-T_1AM and that 3-T_1AM might also have implications for tissues expressing ADRB2 such as the lung, skeletal muscles and skin [24]. In addition, there is evidence that ADRB is linked with Ca^{2+} channels in human conjunctival epithelial cells. This conclusion is warranted since the nonselective ADRB blocker timolol had an inhibitory effect on both NorEpi- and 3-T_1AM-induced Ca^{2+} increases. In summary, 3-T_1AM is a multitarget ligand and modulates ADRB-mediated signaling in human conjunctival epithelial cells. Therefore, thyronamines, in connection with ADRB, may provide novel compounds for suppressing Ca^{2+} channels such as TRPV1 since its activation is associated with inflammation processes occurring in conjunctivitis or dry eye syndrome. Our findings shed new light on the previously proposed complex spectrum of 3-T_1AM action [35] and point to a diversified functional profile of 3-T_1AM.

Acknowledgements

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Disclosure Statement

The authors have nothing to disclose.

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