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Activation of histamine H₃ receptor decreased cytoplasmic Ca²⁺ imaging during electrical stimulation in the skeletal myotubes

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A B S T R A C T

Histamine plays an important role as an inflammatory mediator and neurotransmitter and exerts its effects by binding to G protein-coupled histamine receptors H₁–H₄ receptor with different affinities. H₁ receptor (pKᵢ for histamine 4.2) and H₂ receptor (pKᵢ for histamine 4.3) have long been established as histamine receptors that are expressed in various tissues (Walter and Stark, 2012), but in the last decades, H₃ receptor and H₄ receptor, which have a higher affinity for histamine (pKᵢ 8.0 and 8.2, respectively), have been characterized mostly in non-professional histamine-producing cells, such as dendritic cells and lymphocytes (Szabó and Nagy, 2001 and Kubo and Nakano, 1999). The data obtained using HR agonists and/or antagonists have suggested that H₄ receptor is mainly involved in immunity and is expressed in immune cells, while H₃ receptor has been reported to be

1. Introduction

Histamine is a well-known biogenic cationic amine that is synthesized, stored, and released by professional histamine-synthesizing cells, such as mast cells, basophils, and enterochromaffin cells. These cells express histidine decarboxylase, which effectively converts L-histidine to histamine (Ichikawa et al., 2010). Histamine plays an important role as an inflammatory mediator and neurotransmitter and exerts its effects by binding to G protein-coupled histamine receptors H₁–H₄ receptor with different affinities. H₁ receptor (pKᵢ for histamine 4.2) and H₂ receptor (pKᵢ for histamine 4.3) have long been established as histamine receptors that are expressed in various tissues (Walter and Stark, 2012), but in the last decades, H₃ receptor and H₄ receptor, which have a higher affinity for histamine (pKᵢ 8.0 and 8.2, respectively), have been characterized mostly in non-professional histamine-producing cells, such as dendritic cells and lymphocytes (Szabó and Nagy, 2001 and Kubo and Nakano, 1999). The data obtained using HR agonists and/or antagonists have suggested that H₄ receptor is mainly involved in immunity and is expressed in immune cells, while H₃ receptor has been reported to be...
expressed in the brain and presynaptic membranes of myoneural junctions (Zampeli and Tilgada, 2009 and Leurs et al., 2005). H3 receptor activation with a selective agonist in the cardiac sympathetic nerve endings reduced the rise in intracellular Ca2+ concentration in response to membrane depolarization, while a selective H3 receptor antagonist inhibited this effect (Silver et al., 2002). Some studies have also found functional H3 receptor expressed in smooth muscle cells, such as bronchial smooth muscle cells (Cardell and Edvinsson, 1994) and bladder detrusor cells (Neuhaus et al., 2006). However, there has been no information on H3 receptor expression in muscle cells led us to hypothesize that H3 receptor may also be expressed in skeletal muscle cells, myoblasts, or myotubes, and, consequently, on the role of H3 receptor in skeletal myogenesis.

The presence and functional role of H3 receptor in smooth muscle cells led us to hypothesize that H3 receptor may also be expressed in skeletal muscles, where it may play a role in cytoplasm and calcium regulation of the myotubes.

2. Materials and methods

2.1. Cell culture

Mouse C2C12 myoblasts were obtained from the Turku Center for Biotechnology, University of Turku, Finland (Pallari et al., 2011), and were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM, Lonza/BioWhittaker, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (FBS; HyClone, Boston, MA, USA) and penicillin/streptomycin (100 U/ml in xylene before being mounted in Vectashield.

For Biotechnology, University of Turku, Finland (Pallari et al., 2011), rabbit anti-human H3 receptor polyclonal antibodies (LS-A476, MBL International, Woburn, MA, USA), or non-immune rabbit IgG (R&D Systems, Minneapolis, MN, USA) was used as negative control. Cells were washed three times for 5 min in PBS and incubated with Alexa Fluor® 488-conjugated donkey anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA) diluted 1:400 in 0.1% BSA–PBS for 1 h, washed three times for 5 min in PBS each time, and counterstained with 4', 6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:2000 in distilled water, for 5 min. The coverslips were washed quickly twice in PBS and distilled water for 10 min before mounting with Vectashield (Vector Laboratories Inc., Burlingame, CA, USA). Labeled slides were photographed using a Leica DM 6000 B/M fluorescence microscope equipped with a motorized Leica XY-stage and connected to a Leica DFC 420 digital camera (Leica Microsystems, Wetzlar, Germany) and analyzed using the Leica Application Suite Advanced Fluorescence 2.5.6.6735 software.

2.4. Immunohistochemistry

The study protocol was approved by the institutional Medical Ethics Committee and was in accord with the 1983 Declaration of Helsinki. Informed consent was obtained from all volunteers.

The mid-urethral striated muscle samples were obtained from three healthy male volunteers by biopsy. The samples were fixed in 40% formaldehyde solution for 24 h and embedded in paraffin. Paraffin blocks were sliced into 3–5-μm sections, which were placed on the slides and incubated at 37 °C overnight. Samples were deparaffinized by using xylene and antigens were retrieved by heating samples in a microwave in 10 mM citrate buffer, and were then incubated in 1% hydrogen peroxide to quench endogenous peroxidase activity. The samples were stained using a VECTASTAIN Elite ABC Kit (Rabbit IgG) (PK-6101, Vector Laboratories, Burlingame, CA, USA). The slides were blocked in 10% normal donkey serum for 1 h and incubated at 4 °C overnight with 1 μg/ml of primary antibodies, viz., polyclonal affinity-purified rabbit anti-human H3 receptor, rabbit anti-human H3 receptor polyclonal antibodies, or non-immune rabbit IgG used as negative control. The slides were washed three times for 5 min in PBS and were then incubated with the secondary donkey anti-rabbit IgG diluted 1:400 in 0.1% BSA–PBS for 1 h. After washing three times for 5 min in PBS, the slides were incubated with the ABC complex for 1 h, washed three times for 5 min in PBS, and were then incubated in the peroxidase substrate solution until the desired staining intensity appeared (maximum time: 10 min). The slides were then washed in distilled water, counterstained in Mayer’s hematoxylin, washed in running water, dehydrated in graded ethanol, and cleared in xylene before being mounted in Vectashield.

2.2. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

To investigate the expression of H3 receptor during C2C12 myogenesis, 5 × 10^4 cells/well were seeded in 12-well plates (CellStar, Sigma-Aldrich, St Louis, MO, USA) and allowed to grow in DMEM with 10% FCS for 2 days to reach 80% confluence; then, the cells were switched to differentiation medium to induce myogenesis. The cells from differentiation days 0 and 6 were fixed in 4% paraformaldehyde in 10 mM phosphate-buffered 140 mM saline (PBS, pH 7.4) for 15–20 min, washed three times for 5 min in PBS and permeabilized in 0.5% Triton X-100 in PBS for 15 min. Cells were blocked with 10% normal donkey serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 1 h and incubated at 4 °C overnight with 1 μg/ml of primary antibodies: polyclonal affinity-purified rabbit anti-human MHC IgG (a gift of Dr. John E. Erikson, University of Turku, Turku, Finland) (Pallari et al., 2011), rabbit anti-human H3 receptor polyclonal antibodies (LS-A476, MBL International, Woburn, MA, USA), or non-immune rabbit IgG (R&D Systems, Minneapolis, MN, USA) was used as negative control. Cells were washed three times for 5 min in PBS and incubated with Alexa Fluor® 488-conjugated donkey anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA) diluted 1:400 in 0.1% BSA–PBS for 1 h, washed three times for 5 min in PBS each time, and counterstained with 4', 6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:2000 in distilled water, for 5 min. The coverslips were washed quickly twice in PBS and distilled water for 10 min before mounting with Vectashield (Vector Laboratories Inc., Burlingame, CA, USA). Labeled slides were photographed using a Leica DM 6000 B/M fluorescence microscope equipped with a motorized Leica XY-stage and connected to a Leica DFC 420 digital camera (Leica Microsystems, Wetzlar, Germany) and analyzed using the Leica Application Suite Advanced Fluorescence 2.5.6.6735 software.

2.3. Immunofluorescence

C2C12 cells were seeded at a density of 2 × 10^4 cells/well on coverslips in 24-well plates (Cell Star, Sigma-Aldrich, St Louis, MO, USA) and allowed to grow in DMEM with 10% FCS for 2 days to reach 80% confluence; then, the cells were switched to differentiation medium to induce myogenesis. The cells from differentiation days 0 and 6 were fixed in 4% paraformaldehyde in 10 mM phosphate-buffered 140 mM saline (PBS, pH 7.4) for 15–20 min, washed three times for 5 min in PBS and permeabilized in 0.5% Triton X-100 in PBS for 15 min. Cells were blocked with 10% normal donkey serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 1 h and incubated at 4 °C overnight with 1 μg/ml of primary antibodies: polyclonal affinity-purified rabbit anti-human MHC IgG (a gift of Dr. John E. Erikson, University of Turku, Turku, Finland) (Pallari et al., 2011), rabbit anti-human H3 receptor polyclonal antibodies (LS-A476, MBL International, Woburn, MA, USA), or non-immune rabbit IgG (R&D Systems, Minneapolis, MN, USA) was used as negative control. Cells were washed three times for 5 min in PBS and incubated with Alexa Fluor® 488-conjugated donkey anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA) diluted 1:400 in 0.1% BSA–PBS for 1 h, washed three times for 5 min in PBS each time, and counterstained with 4', 6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:2000 in distilled water, for 5 min. The coverslips were washed quickly twice in PBS and distilled water for 10 min before mounting with Vectashield (Vector Laboratories Inc., Burlingame, CA, USA). Labeled slides were photographed using a Leica DM 6000 B/M fluorescence microscope equipped with a motorized Leica XY-stage and connected to a Leica DFC 420 digital camera (Leica Microsystems, Wetzlar, Germany) and analyzed using the Leica Application Suite Advanced Fluorescence 2.5.6.6735 software.
2.5. Cytoplasmic Ca\(^{2+}\) imaging

C2C12 cells were differentiated for 7 days into myotubes on 18-mm coverslips. The cells were then loaded with 4 \(\mu\)mol/L Fluo-4 AM Ca\(^{2+}\) indicator dye (F-14201, Invitrogen, Carlsbad, CA, USA) for 30 min at room temperature in Elliot medium, containing (mM): 137 NaCl, 5 KCl, 0.44 KH\(_2\)PO\(_4\), 20 HEPES, 4.2 NaHCO\(_3\), 5 d-glucose, 2 CaCl\(_2\), 1.2 MgCl\(_2\), and 1 Na-pyruvate (pH adjusted to 7.4 with NaOH). Then, a 30-min de-esterification was performed at 37 °C and the coverslips were transferred to an RC-49MFS recording chamber equipped with electrodes for field stimulation (Warner Instruments Inc., Hamden, CT, USA) and maintained at 37 °C during imaging.

H\(_3\) receptor agonist R-(\(\alpha\))-methylhistamine ((R)-\(\alpha\)-MeHA), at different concentrations (1 nM, 10 nM, 100 nM, 1 \(\mu\)M, 10 \(\mu\)M, and 100 \(\mu\)M) in Elliot medium, and a blank control (Elliot medium) were added into parallel coverslip chambers for 5, 10, 20 min, and 30 min before electrical pacing. Ca\(^{2+}\) imaging in the cytoplasm was recorded during stimulation. The cells were electrically paced at a frequency of 0.5 Hz with 10-ms bipolar pulses (200–360 mA) using a SIU-102 Stimulus Isolation Unit (Warner Instruments, Hamden, CT, USA) connected to a Master-8 stimulator (A.M.P.I., Jerusalem, Israel). Ca\(^{2+}\) imaging was observed under an inverted IX70 microscope (Olympus Corporation, Hamburg, Germany) equipped with transparent housing, a heater, and a temperature control unit (Solent Scientific, Ltd, Segensworth, UK) using a UPlanSAPo 20 \(\times\) air objective (Olympus Corporation, Hamburg, Germany). Polychrome IV (TILL Photonics, Graefelfing, Germany) was used to illuminate the sample at a wavelength of 488 nm; emitted light was filtered through a Chroma filter set (EM HQ535/50 m, BS Q505LP, Chroma Technology Corporation, Bellows Falls, VT, USA). Images were recorded with a Hamamatsu ORCA-Flash 4.0 sCMOS camera (Hamamatsu Photonics K.K., Hamamatsu, Japan) at 50 frames/s. Data acquisition was controlled and the recordings were analyzed using HC Image software (Hamamatsu Photonics K.K., Hamamatsu, Japan). The cells for analysis were manually selected in the regions of interest and the background was subtracted before quantifying the fluorescence relative to baseline fluorescence \((F/F_0)\).

2.6. Statistical analysis

All values are given as mean ± standard error of the mean (S.E.M.). Statistical differences were evaluated using the Mann–Whitney test (Matlab, MathWorks Inc., Natick, MA, USA).

3. Results

3.1. C2C12 cell morphology during myogenesis

Before differentiation, C2C12 cells presented as mononuclear myoblasts around 10-μm in diameter, round or spindle-like; they were well spread and showed many contacts with the substrate (Fig. 1A). When the cells were induced to differentiate, they started proliferating and migrating and then arranged themselves along a single direction to fuse into myotubes. After 6 days of differentiation, the cells fused into long, multinucleated myotubes of over 150-μm in diameter (Fig. 1B).

3.2. H\(_3\) receptor expression during myogenesis and in human midurethral striated muscles

The expression of H\(_3\) receptor and the late myogenesis marker MHC during C2C12 differentiation was assessed using qRT-PCR. MHC increased over the course of differentiation by 7168-, 89487-
and 279897-fold at days 2, 4, and 6, respectively, compared to day 0 (Fig. 1D). The expression of H3 receptor, which was initially low in C2C12 myoblasts, also strongly increased with differentiation when compared to day 0; by 26-, 91-, and 182-fold at days 2, 4, and 6, respectively (Fig. 1C).

The immunofluorescence staining of the MHC protein was negative before differentiation (Fig. 2), but was strongly positive at day 6 (Fig. 2). Similarly, immunostaining for the H3 receptor protein was negative at day 0 (Fig. 2), but was strongly positive at day 6 (Fig. 2).

Human mid-urethral striated muscles showed positive immunostaining for both MHC and H3 receptor proteins in the filiform-like muscle cells (Fig. 2).

3.3. The H3 receptor agonist (R)-α-MeHA decreased cytoplasmic Ca\(^{2+}\) imaging evoked by electrical stimulation

(R)-α-MeHA of different concentrations was added to stimulate 7 day-differentiated myotubes for 5, 10, and 20 min prior to electrical pacing, and the Ca\(^{2+}\) imaging in the cytoplasm was assessed during stimulation (Fig. 3). Incubation with 1 μM (R)-α-MeHA for 10 or 20 min significantly (55%) decreased the increase of cytoplasmic Ca\(^{2+}\) imaging upon electrical pacing. Shorter (5 min) incubation with 100 nM (R)-α-MeHA decreased Ca\(^{2+}\) imaging by 45%; longer treatments further increased the decrease-effect when (R)-α-MeHA was over 100 nM. However, (R)-α-MeHA activity in C2C12 cells displayed bell-shaped dynamics: the concentration increase over 1 μM significantly reduced the decrease-effect of intracellular Ca\(^{2+}\) imaging upon electrical stimulation (Fig. 3). The result of (R)-α-MeHA treatment at 30 min is not shown, because obvious decay of the Ca\(^{2+}\)-labeled Fluo-4 was detected at this time.

4. Discussion

To our knowledge, this is the first investigation of the role of H3 receptor in skeletal muscle cells. When we first observed H3 receptor immunostaining in adult skeletal muscle, we hypothesized that H3 receptor may play a role in skeletal myogenesis or mature skeletal muscle cells. To test this proposal, we assessed H3 receptor expression kinetics during differentiation of mouse C2C12 myoblast cells. H3 receptor expression was almost absent in
myoblasts, but increased markedly with myogenesis, suggesting that H3 receptor mostly functions in differentiated myoblasts that were fused into myotubes. H3 receptor functional analysis, using an H3 receptor agonist (R)-α-MeHA, revealed that in fully differentiated 7-day myotubes, H3 receptor activation decreased Ca\(^{2+}\) imaging evoked by electrical stimulation. The lowest (R)-α-MeHA concentration used (1 mM) was below the agonist affinity for H3 receptor (pK\(_{i}\) = 8.4) in the brain (Chen et al., 2003 and Li et al., 2014) and had no effect on the cytoplasmic Ca\(^{2+}\) imaging under electrical stimulation in our study. On the other hand, the highest (R)-α-MeHA concentration used (100 μM) was also less active, probably because of combinatorial effects, due to nonspecific activation of other receptors (H1 receptor or H2 receptor) on the cell membrane (Chen et al., 2015), which counteracted the response (Leurs et al., 1998), or probably as a self-protective mechanism. The highest inhibitory effect of 1 μM (R)-α-MeHA, exerted after 10- min or 20 min treatment, was in agreement with a previous pharmacological study on histamine receptor ligands, where (R)-α-MeHA also showed the highest efficacy at 1 μM (Kotte et al., 2011).

The histamine receptors, including H3 receptor, are a class of G-protein-coupled receptors. H3 receptor is coupled to the G\(_{i/o}\) proteins, which have prominent effects on Ca\(^{2+}\) influx. H3 receptor-mediated activation of G\(_{i/o}\) proteins may cause the activation of phospholipase A2 (PLA2), which induces the release of arachidonic acid and the inhibition of the Na\(^+\)/H\(^+\) exchanger, leading to a decrease in intracellular Ca\(^{2+}\) levels involving the impaired entrance of Ca\(^{2+}\) through voltage-gated ion channels (Leurs et al., 2005). In cardiac sympathetic nerves, H3 receptor-mediated inhibition of norepinephrine exocytosis was caused by H3 receptor-G\(_{i/o}\) coupling, and inhibition of adenyl cyclase activity and cAMP formation, leading to diminished protein kinase A (PKA) activity, and decreased Ca\(^{2+}\) influx through voltage-operated Ca\(^{2+}\) channels (Leurs et al., 2005). In our experiments, H3 receptor activation in the differentiated C2C12 myotubes decreased intracellular Ca\(^{2+}\) imaging in response to electrical pacing, which is similar to the effect observed in cardiac sympathetic nerve endings.

In mature skeletal muscle cells, the major source of Ca\(^{2+}\) is the intracellular Ca\(^{2+}\) store in the sarcoplasmic reticulum (SR), which accumulates Ca\(^{2+}\) from the cytoplasm in an ATP-dependent manner by the action of Ca\(^{2+}\)-ATPase (Endo, 2009). Most of this calcium moves back and forth across the SR membrane in cycles of contraction and relaxation. The channel responsible for release from the SR is the ryanodine receptor (RyR). The RyR that mediates the efflux of Ca\(^{2+}\) from the SR has a central role in excitation–contraction coupling between sarcocemal depolarization and SR Ca\(^{2+}\) release (Stokes and Wagenknecht, 2000).

In early studies of isolated frog skeletal muscles, catecholamines induced sarcocemal-depolarized contraction through elevation of cAMP, which also stimulates RyR on the SR, and increased Ca\(^{2+}\) flux out from the SR into the cytoplasm (Gonzalez-Serratos et al., 1981). Regulation of Ca\(^{2+}\) concentration in the cytoplasm is important in coupling excitation and contraction in skeletal muscle (Emrick et al., 2010). Evidence has suggested that the level of cyclic AMP may be involved in the increase of Ca\(^{2+}\) in the cytoplasm (Ong and Steiner, 1977 and Lanner et al., 2010) and is associated with contraction of both cardiac and skeletal muscles (Gonzalez-Serratos et al., 1981). In cultured skeletal myotubes, the activated calcitonin gene-related peptide (CGRP) receptor can positively couple to Gs proteins and activation of adenylate cyclase, increasing levels of cAMP generation and subsequent protein kinase A (PKA) activation to increase contraction force. During this process, CGRP increases voltage-gated SR Ca\(^{2+}\) release within hours, resulting from a quantitatively similar increase in releasable SR Ca\(^{2+}\) content (Avila et al., 2007). When chicken and rat skeletal muscle cells were cultured for 7 days and were then subjected to electrical stimulation for additional 2 days, the ability of these cells to synthesize cAMP was increased by almost two-fold (Young et al., 2000). In our study, electrical stimulation of differentiated C2C12 myotubes may have caused upregulation of cAMP biosynthesis; in this case, H3 receptor activation may inhibit cAMP elevation and prevent overcontraction. Other studies found histidine decarboxylase activity was very low in normal non exercised muscle (Endo et al., 1998) and mice treated with a H3 receptor antagonist display no significant effects on prolonged walking endurance (Niijima-Yaota et al., 2012), suggesting that histamine and H3 receptor signaling have a limited role on normal muscle physiology. It is the possibility that H3 receptor is activated only in exercised and/or excited muscle. In addition, we also tried ciprofloxin (histamine H3 receptor antagonist, pK\(_{i}\) = 9.1–9.4) 1 μM instead of (R)-α-MeHA to do the experiment, and found there was no cytoplasmic Ca\(^{2+}\) imaging arise compare to the control. So H3 receptor blockage might not enhance cell contraction.

In conclusion, we found that the histamine H3 receptor agonist (R)-α-MeHA can attenuate cytoplasmic Ca\(^{2+}\) imaging during electrical stimulation in differentiated C2C12 myotubes, which may facilitate relaxations of cells and prevent over-contraction in electrically stimulated C2C12 myotubes. Attenuation of the cytoplasmic Ca\(^{2+}\) imaging may occur via activation of H3 receptor to induce inhibition of cAMP formation, or activation of PLA2, release of arachidonic acid, and the subsequent decrease in intracellular Ca\(^{2+}\) levels. These effects are most prominent early during myotube maturation. Whether these effects also occur in muscle regeneration remains to be elucidated.

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