Journal of Molecular and Cellular Cardiology 57 (2013) 129–136

Contents lists available at SciVerse ScienceDirect

Journal of Molecular and Cellular Cardiology

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

Original article

Abundance, distribution, mobility and oligomeric state of M_2 muscarinic acetylcholine receptors in live cardiac muscle

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ARTICLE INFO

Article history:
Received 26 November 2012
Received in revised form 21 December 2012
Accepted 11 January 2013
Available online 25 January 2013

Keywords:
Single molecule
TIRF
Acetylcholine receptor
Muscarinic
Cardiomyocyte

ABSTRACT

M_2 muscarinic acetylcholine receptors modulate cardiac rhythm via regulation of the inward potassium current. To increase our understanding of M_2 receptor physiology we used Total Internal Reflection Fluorescence Microscopy to visualize individual receptors at the plasma membrane of transformed CHO^{M2} cells, a cardiac cell line (HL-1), primary cardiomyocytes and tissue slices from pre- and post-natal mice. Receptor expression levels between individual cells in dissociated cardiomyocytes and heart slices were highly variable and only 10% of murine cardiomyocytes expressed muscarinic receptors. M_2 receptors were evenly distributed across individual cells and their density in freshly isolated embryonic cardiomyocytes was ~1 μm^{-2}, increasing at birth (to ~3 μm^{-2}) and decreasing back to ~1 μm^{-2} after birth. M_2 receptors were primarily monomeric but formed reversible dimers. They diffused freely at the plasma membrane, moving approximately 4-times faster in heart slices than in cultured cardiomyocytes. Knowledge of receptor density and mobility has allowed receptor collision rate to be modeled by Monte Carlo simulations. Our estimated encounter rate of 5–10 collisions per second, may explain the latency between acetylcholine application and GIRK channel opening.

1. Introduction

Basal heart rate is regulated by cardiac pacemaker cells under the influence of cholinergic, parasympathetic, vagal stimulation. Acetylcholine (ACh) acts via the M_2 subtype of muscarinic receptors, which are canonical G-protein coupled receptors (GPCRs) with 7-transmembrane spanning alpha-helices [1]. They bind a heterotrimeric ($\alpha\beta\gamma$) G-protein on their intracellular face and the ligand, ACh, at the extracellular side. ACh binding causes the $\beta\gamma$ G-protein subunits to activate inwardly rectifying, hetero-tetrameric [2] potassium GIRK channels [$(Kir3.1)_2 + (Kir3.4)_2$], increasing their potassium current and hyperpolarizing the cell membrane. This prolongs the electrical oscillation period of the pacemaker cell which slows the heartbeat. Patch clamp studies have shown that coupling between ACh binding to M_2 receptors and downstream GIRK channel opening works via an "in-membrane" mechanism i.e. via direct interaction between the membrane bound G-protein $\beta\gamma$ subunits and the integral-membrane GIRK channel [3–6]. The activation pathway therefore requires diffusion and collision of at least two membrane bound protein partners. Since the diffusional encounter rate contributes to the delay in GIRK channel conductance change following ACh stimulation [7,8] it is important to know the distribution, density, mobility and oligomeric state of the M_2 receptors at the plasma membrane.

Previously, the surface density and tissue distribution of M_2 receptors, the predominant ACh receptor subtype expressed in cardiac muscle [9], has been estimated using bulk methods such as quantitative antibody labeling [10–12]. A limitation of these methods is that they require high specificity of labeling [13] and assume homogenous receptor expression in all cells. The approach taken here has been to use a single molecule, fluorescence-based, method to directly observe and count M_2 receptors in individual cells and live tissue samples using light microscopy. Inter alia, this approach also enables receptor density, mobility, and oligomeric state to be measured by direct observation.

Total internal reflection fluorescence (TIRF) microscopy uses an obliquely-angled incident laser beam to produce an evanescent field that penetrates just 100 nm beyond the microscope coverslip surface into the biological specimen [14]. This means that if a receptor protein is labeled with a suitable fluorophore it appears as a discrete spot of light that can be pinpointed and tracked as it diffuses within the lipid membrane. Recently, we applied TIRFM to visualize individual M_1 receptors on the plasma membrane of mammalian cells, grown in culture [15]. By analyzing fluorescent spot intensities and spatial trajectories it was shown that M_1 receptors underwent transient dimerization. The goal of the present study has been to apply similar methods to live tissue sections obtained from mouse cardiac muscle in order to visualize individual M_2 receptors in their native context.

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http://dx.doi.org/10.1016/j.yjmcc.2013.01.009

Various cell lines were used to check the binding specificity of the fluorescent ligands used to investigate the properties of the M_2 receptors. A clonal CHO cell line, stably transfected with M_2 receptors, allowed comparison with previous data obtained for CHO cells expressing M_1 receptors [15]. An immortalized atrial cell line, HL-1 [16], which natively expresses M_2 receptors [17], was also studied to see if the receptor properties were dependent on the cellular background. The results from these cell lines were compared with those from the investigations of primary dissociated cardiomyocytes and freshly dissected tissue slices from mouse heart.

We report, for the first time, that TIRF microscopy has enabled individual molecules to be counted and tracked on the plasma membrane of a tissue slice. The biophysical properties of individual M_2 receptors in cardiac muscle slices have been quantitated at different stages of fetal and neonatal development. In addition, M_2 receptors were shown to form reversible dimers on the surface of cells. The new ability to study single molecules in freshly dissected heart slices opens the future possibility to directly visualize other molecules involved in the ACh signaling cascade in cardiac cells.

2. Materials and methods

2.1. Radioligand binding assays

CHO cell culture, membrane preparation and the [^{3}H]-NMS binding assays were as described previously [18] with minor modifications detailed in Supplementary information.

2.2. Fluorophore labeling procedure

The labeling conditions were designed to ensure rapid, maximal labeling of the M_2 receptors with minimum background fluorescence [15]. This was facilitated by the very high affinity of Cy3B–telenzepine which enabled >98% labeling of the receptors by the use of 1 to 10 nM ligand (50–500 times its K_d) (see SD for full details).

2.3. TIRF imaging and image analysis

The TIRF microscope system and image analysis procedures have been described in detail previously [15,19,20] (see SD for full details).

2.4. Confocal microscopy

Confocal microscopy was performed at 23 °C using a Leica SP5-II confocal microscope (Leica Microsystems, UK) equipped with GaAsP hybrid detector, 488 nm and 561 nm lasers and 20× NA 1.0 water-dipping objective lens giving a magnification of 200 nm per pixel in the x–y plane and using 1 μm z-steps to acquire volume sections.

3. Results

3.1. Characterization of fluorescent ligand binding to M_2 receptors

CHO cell lines transfected singly with each of the muscarinic receptor subtypes (M_1–M_5) have been used for many years for the characterization of the binding and functional properties of individual muscarinic receptor subtypes and have been shown to accurately reflect the binding properties of those receptors in whole tissue [21]. Radioligand binding studies on membranes from CHO^{M2} cells showed that M_2 muscarinic receptors bound Cy3B–telenzepine with a K_d of ~45 pM (log affinity 10.35 ± 0.02, n = 3, Fig. S1), comparable to the affinity for M_1 receptors in CHO^{M1} cells [15]. Alexa488–telenzepine bound to M_2 receptors with a 30-fold lower affinity than found for Cy3B–telenzepine (log affinity 8.83 ± 0.02, n = 2, Fig. S1), but still with nanomolar potency. This allowed >85% of the receptors to be labeled using 10 nM fluorescent ligand. In comparison, 10 nM Cy3B–telenzepine labels >99.5% of the M_2 receptors. The inhibition curves (Fig. S1), show no evidence of cooperative or allosteric behavior and are compatible with a 1:1 stoichiometry of M_2 receptor and ligand, as found for the same ligands with M_1 receptors [15].

3.2. Evidence that M_2 muscarinic receptors are specifically labeled by Cy3B–telenzepine

Wild type CHO cells do not natively express muscarinic receptors and we found no fluorescence signal when wild-type cells were incubated with Cy3B–telenzepine (using our standard labeling protocol). Furthermore, preincubation of the CHO^{M2} cell line with 10 μM of the potent muscarinic antagonist, atropine (which binds at all muscarinic receptor subtypes with ~1 nM K_d), was sufficient to block Cy3B–telenzepine binding. Following atropine treatment, there was no cell-specific labeling although a small number of non-specifically adhered fluorophores were readily identified because they were completely immobile. These control experiments confirmed that the overwhelming majority of mobile fluorescent spots observed in our later experiments (described below) were muscarinic receptors and the small number of static spots was due to nonspecific fluorophore binding (probably to the coverslip surface). The only muscarinic receptor subtype present in the CHO^{M2} cells [22] and the predominant muscarinic receptor subtype present in the HL-1 cell line [17] and rodent cardiac cells [9,23,24] are M_2 receptors, so we conclude that our data arises from specific labeling of the M_2 receptor subtype (see SD and Fig. S3 for additional control experiments).

When viewed by TIRF microscopy, CHO^{M2} cells labeled with Cy3B–telenzepine appeared similar to CHO^{M1} cells. M_2 receptors could be identified as individual fluorescent spots moving rapidly on the plasma membrane (Fig. 1A, Fig. S2 and Video SV1). The intensity profile of single spots had the expected, diffraction limited, spot size (~300 nm full width at half height) and had a sufficiently high signal-to-noise ratio to be reliably identified and tracked by an automated image analysis routine (See SD and Fig. S2A). The mean intensity of the majority of spots (Fig. S2B) was similar to that found for mono-dispersed, individual, Cy3B fluorophores viewed under identical conditions on a coverslip in vitro (Fig. S2B). Finally, the analysis of individual intensity trajectories over time demonstrated that the majority showed single step photobleaching (Fig. S2B). However, a significant population (40%) showed more complex behavior in which intensity alternated between two intensity levels.

3.3. M_2 receptors visualized in CHO^{M2}, HL-1 cells and isolated cardiomyocytes

The CHO^{M2} receptor density, determined from the number of fluorescent spots present on the cell membrane, was ~3 μm^{-2} similar to bulk estimates calculated from the radioligand binding assays (see Fig. 1, SD, and Fig. S4). Analysis of individual receptor spatial trajectories ("tracks") allowed the averaged mean squared displacement to be plotted as a function of time interval (Δt). The linearity of the MSD vs. Δt plots is consistent with receptors undergoing a Brownian random walk, characteristic of free diffusion within the lipid bilayer (Fig. 1A). The gradient of these plots gave an estimated D_{lat} ~0.1 $\mu m^2 \cdot s^{-1}$, close to the value measured for M_1 receptors in CHO^{M1} cells [15]. There was no evidence of receptor clustering or of cell regions deprived of moving fluorescent spots. Atrial derived HL-1 myocytes which natively express M_2 receptors were labeled with Cy3B–telenzepine and examined by TIRFM. All cells were labeled (Fig. 1B, and Video SV2) to approximately the same extent and the distribution of individual fluorescent spot intensities was similar to that found on CHO^{M2} cells, indicating that they resulted primarily from a single fluorophore. The average receptor