Journal of Molecular and Cellular Cardiology 48 (2010) 220–229

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

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

Original article

5-HT4 and 5-HT2 receptors antagonistically influence gap junctional coupling between rat auricular myocytes

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ARTICLE INFO

Article history:
Received 24 April 2009
Received in revised form 15 June 2009
Accepted 3 July 2009
Available online 15 July 2009

Keywords:
Serotonin
5-HT2A
5-HT2B
5-HT4a
5-HT4b
Calcium current
cAMP
Connexin
Gap junction
Cx40
Cx45
Cx43
Cardiomyocytes
Syncytial properties

ABSTRACT

5-hydroxytryptamine-4 (5-HT_4) receptors have been proposed to contribute to the generation of atrial fibrillation in human atrial myocytes, but it is unclear if these receptors are present in the hearts of small laboratory animals (e.g. rat). In this study, we examined presence and functionality of 5-HT_4 receptors in auricular myocytes of newborn rats and their possible involvement in regulation of gap junctional intercellular communication (GJIC, responsible for the cell-to-cell propagation of the cardiac excitation). Western-blotting assays showed that 5-HT_4 receptors were present and real-time RT-PCR analysis revealed that 5-HT_{4b} was the predominant isoform. Serotonin (1 μM) significantly reduced cAMP concentration unless a selective 5-HT_4 inhibitor (GR113808 or ML10375, both 1 μM) was present. Serotonin also reduced the amplitude of L-type calcium currents and influenced the strength of GJIC without modifying the phosphorylation profiles of the different channel-forming proteins or connexins (Cxs), namely Cx40, Cx43 and Cx45. GJIC was markedly increased when serotonin exposure occurred in presence of a 5-HT_4 inhibitor but strongly reduced when 5-HT_{2A} and 5-HT_{2B} receptors were inhibited, showing that activation of these receptors antagonistically regulated GJIC. The serotoninergic response was completely abolished when 5-HT_4, 5-HT_{2A} and 5-HT_{2B} were simultaneously inhibited. A 24 h serotonin exposure strongly reduced Cx40 expression whereas Cx45 was less affected and Cx43 still less. In conclusion, this study revealed that 5-HT_4 (mainly 5-HT_{4b}), 5-HT_{2A} and 5-HT_{2B} receptors coexisted in auricular myocytes of newborn rat, that 5-HT_4 activation reduced cAMP concentration, I_{CaL} and intercellular coupling whereas 5-HT_{2A} or 5-HT_{2B} activation conversely enhanced GJIC.

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is an ubiquitous monoamine neurotransmitter and local hormone which exerts its diverse actions by binding to cell surface receptors which can be classified into seven major families of receptors (5-HT_1 to 5-HT_7) classified according to their pharmacological profile and mechanism of signal transduction [1]. The receptor signalling cascades frequently target membrane channels. In human atrial tissues for example, the stimulation of 5-HT_4 receptors affects, through the cAMP pathway, the L-type Ca^{2+} (I_{CaL}) [2] as well as the pacemaker "I(f)" [3] current, with effects on the cardiac rhythm and possible generation of arrhythmias. In contrast, in the adult rat atrium, the 5-HT-induced increase of contractile force (inotropic action) was ascribed to 5-HT_{2A} activation [4] and it is unclear if 5-HT_4 receptors are present in the heart of rat (and of other small laboratory animals as guinea pig or rabbit). Therefore the presence and functionality of 5-HT_4 receptors have at first been investigated in auricular myocytes of newborn rats, then the possible involvement of gap junctional intercellular communication in their signalling cascade was examined. Individual cardiac cells are indeed directly interconnected by intercellular channels clustered in gap junctions that allow the cell-to-cell propagation of action potentials, and alterations in the intercellular coupling affect the syncytial properties of the tissue. Being that 5-HT was shown to influence gap junctional intercellular communication (GJIC) in vascular smooth muscle [5], somatosensory cortex [6] or astrocytes [7], as did the ligands of some other G-protein coupled receptors, as endothelin or angiotensin II [8] receptors, we hypothesized that this important transmitter may underlie alterations in cell–cell coupling of the atrial myocytes as

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doi:10.1016/j.yjmcc.2009.07.005

well. We now report study that 5-HT working through both 5-HT_4 and 5-HT_2 receptors is involved in the regulation of GJIC in atrial myocytes.

Both 5-HT_4 and 5-HT_2 receptors are coded by complex genes, with multiple splice variants termed 5-HT_{4a} to 5-HT_{4i} and 5-HT_{4n} [9] for the first family, 5-HT_{2A} to 5-HT_{2C} (see [1]) for the second. Up to now, 5-HT_4 receptors have mainly been found in human and pig atria; in the rat heart, 5-HT_{4a} and 5-HT_{4b} mRNAs were detected in the atria of adult animals but 5-HT_4 receptors did not seem to be functionally active, whereas 5-HT_{2A} receptors were [4]. In contrast, though 5-HT_4 receptor agonists did not alter $[Ca^{2+}]_i$, they enhanced the stretch-induced secretion of atrial natriuretic peptide, suggesting that 5-HT_4 receptors would be present and functional [10]. As most 5-HT_4 receptors are positively coupled to adenylyl cyclase via Gs proteins and activate the cAMP-dependent pathway (see [1]), the influence of serotonin on the cAMP level and on the amplitude of I_{CaL} have been examined in native, freshly dissociated auricular myocytes.

5-HT_2 receptors, involved in multiple physiological functions, have been implicated in several disorders of the central nervous system but 5-HT_{2A} and 5-HT_{2B} isotypes are also present in the cardiovascular system. However, so far, consequences of 5-HT_2 activation or mutation mainly concerned vascular 5-HT_2, but might indirectly produce life-threatening arrhythmias and cardiodepression (for review, see [11]). Direct effects were however reported, for example in acute failing ventricles of adult rat, where appearing 5-HT_{2A} receptors were found to take part to the inotropic response [12]. Liang et al. [13] also observed that 5-HT_{2B} expression was up-regulated in newborn rat cardiomyocytes obtained from hypertensive cardiomyopathy models.

In human or rat hearts, cardiomyocytes most prominently express three isoforms of junctional channel-forming proteins (connexins, Cxs), namely Cx40, Cx43 and Cx45 (see [14,15]). In ventricular working myocardium, Cx43 is the major isoform whereas in atrial tissue Cx43 and Cx40 are largely expressed. In both auricle and ventricle, Cx45 is expressed in very low quantities [14,15]. Cx40, Cx43 and Cx45 are essential for both heart morphogenesis and cardiac impulse conduction and changes in cardiac Cx gene expression during development or in response to pathophysiological signals could play a major role in normal cardiac development and functions, as well as in several cardiac diseases. Alterations in the distribution and/or level of Cx expression have been associated with atrial fibrillation, hypertrophy and heart failure, but the underlying molecular mechanisms have not yet been elucidated [16]. In the same pathologies, 5-HT receptor involvement has also been postulated, as mentioned above. The present study revealed that 5-HT_4 (mainly 5-HT_{4b}), 5-HT_{2A} and 5-HT_{2B} receptors coexist in auricular myocytes of newborn rat, that 5-HT_4 activation reduces $[cAMP]_i$, I_{CaL} and intercellular coupling whereas 5-HT_{2A} or 5-HT_{2B} activation conversely enhances GJIC, and that prolonged 5-HT exposure alters the Cx expression pattern.

2. Materials and methods

2.1. Biological preparations

For membrane preparation, rat atrial tissue was removed from newborn Wistar rats and the membrane preparation procedure immediately performed at 4 °C. To identify 5-HT_4 receptor by Western-blotting, atrial tissue was homogenized in HEPES buffer supplemented with 5 mM glucose and protease inhibitor cocktail (Sigma, St Quentin Fallavier, France) and centrifuged at 500 *g* for 15 min. Supernatant was collected and membranes were pelleted by centrifugation at 28,000 *g* for 30 min. Final pellet containing plasma membranes was resuspended in an appropriate volume of PBS buffer with glycerol 10% (v/v) and protease inhibitor cocktail, then protein concentrations were determined (more details are given in the Online supplementary material).

Individual atrial myocytes were obtained with procedures derived from either the one previously described by Sallé et al. [17] for cAMP quantification and patch-clamp recordings or from Duthe et al. [18] for culture and cell-to-cell dye transfer assessments, with some modifications (see Online data supplement). For quantification of cAMP production and patch-clamp experiments, cells were used within 2–3 h after isolation whereas cell-to-cell dye transfer estimations were performed after culturing the cells for 2 days. The spontaneous synchronised mechanical activity was used as evidence to avoid confusion with non-muscle cells. The purity of the cell culture was controlled by striated actin immunolabelling.

2.2. Real-time reverse transcription-PCR

Specific primers presented in Table 1 were synthesized by Eurogentec (Angers, France). Real-time RT-PCR was carried out as recently described [19]. Two normalizing controls (PO and GAPDH) were included in each experiment and yielded similar results. Shown data were normalized to the GAPDH housekeeping genes (see Online data supplement).

2.3. Quantification of adenylyl cyclase activity

Intracellular cAMP measurements were carried out as previously described [2] on approximately $4.5.10^4$ myocytes and all values were obtained in triplicate (see Online data supplement for more details).

2.4. Patch clamp experiments

Whole-cell voltage-clamp experiments were conducted as previously described [17]. I_{Ca} amplitude was taken as the difference between peak inward current and the current at the end of a 1 s depolarising pulse.

2.5. Cell-to-cell dye diffusion

The kinetics of 6-carboxyfluorescein (6-CF) diffusion was assessed by the technique of fluorescence recovery after photobleaching (FRAP) as recently described by Derangeon et al. [20], presented in more details in the Online data supplement.

Table 1
Oligonucleotide sequences for forward and reverse primers.

cDNA	Primer	Sequence (5′_3′)	PCR product size (pd)	Accession Number in Genbank
5HT_{4a}	Forward	*GTGCTAAGGTATACAGTTTTGC*	81	U20906
	Reverse	*CCAGGGACTCTGGGTCATTG*		
5HT_{4b}	Forward	*GCTAAGGGATACAGTGGAATGT*	77	U20907
	Reverse	*GCAGCCACCAAAGGAGAAGTT*		
5HT_{2a}	Forward	*TTCACCACAGCCGCTTCAA*	109	NM-017254
	Reverse	*ATCCTGTAGTCCAAAGACTGGGATT*		
5HT_{2b}	Forward	*GGCTGATTTGCTGGTTGGATTG*	72	NM-017250
	Reverse	*GGGCCATGTAGCCTCAAACATG*		
Cx40	Forward	*GGAAAGAGGTGAACGGGAAG*	196	NM-019280
	Reverse	*GGGCCTCGAGACATAACAGTT*		
Cx43	Forward	*TCTGCCTTTCGCTGTAACACT*	116	NM-012567
	Reverse	*GGGCACAGACACGAATATGAT*		
Cx45	Forward	*GTGATGTACCTGGGATATGC*	144	AF536559
	Reverse	*CCTCTTCATGGTCCTCTTCC*		
PO	Forward	*ATGCCCAGGGAAGACAGGGC*	165	NM-022402
	Reverse	*CCATCAGCACCACAGCCTTC*		
GAPDH	Forward	*ATTCTACCCACGGCAAGTT*	152	M17701
	Reverse	*CGCCAGTAGACTCCACGACATA*		