



Cardiac 5-HT₄ Serotonergic Receptors, 52 kD SSA/Ro and Autoimmune-Associated Congenital Heart Block

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It was recently reported that sera from patients with systemic lupus erythematosus contain antibodies reactive with the second extracellular loop of the serotonergic 5-HT₄ receptor expressed in the human heart. This antibody response was associated with antibodies to 52 kD SSA/Ro, a reactivity prevalent in mothers of children with congenital heart block (CHB). The current study was undertaken to determine whether the 5-HT₄ receptor is a target of the immune response in these mothers. Initial experiments demonstrated mRNA expression of the 5-HT₄ receptor in the human foetal atrium. Electrophysiologic studies established that human foetal atrial cells express functional 5-HT₄ receptors. Sera from 116 mothers enrolled in the Research Registry for Neonatal Lupus, whose children have CHB, were evaluated. Ninety-nine (85%) of these maternal sera contained antibodies to SSA/Ro, 84% of which were reactive with the 52 kD SSA/Ro component by immunoblot. None of the 116 sera were reactive with the peptide spanning aa165–185 of the serotonergic receptor. Rabbit antisera which recognized this peptide did not react with 52 kD SSA/Ro or peptide aa365–382 in the C terminus. Although 5-HT₄ receptors are present and functional in the human foetal heart, maternal antibodies to the 5-HT₄ receptor are not associated with the development of CHB.

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Introduction

It is almost predictable that when a foetus is identified to have heart block in the absence of structural abnormalities, the maternal serum contains antibodies reactive with one or more components of the SSA/Ro-SSB/La ribonucleoprotein complex [1]. The association of congenital heart block (CHB) with maternal autoantibodies is independent of whether the mother is asymptomatic or has a defined rheumatologic disease such as systemic lupus erythematosus (SLE) or Sjögren's syndrome (SS). Clinical and experimental data support a pathogenic role of SSA/Ro and SSB/La antibodies in the development of CHB. There is a temporal relationship between transplacental transport of antibodies and the subsequent detection of bradyarrhythmia. Maternal IgG anti-SSB/La idiotypes have been identified on the surface of foetal myo-

cardial fibres [2] and anti-SSA/Ro antibodies have been eluted from an affected foetal heart [3]. Third degree block is induced in the rabbit heart [4] as well as the human foetal heart [5] after perfusion of the aorta with sera containing anti-SSA/Ro antibodies and affinity-purified anti-52 kD SSA/Ro antibodies. At the cellular level it has been demonstrated that the candidate antibodies inhibit whole cell and single channel L-type calcium currents (I_{CaL}) channels in human foetal ventriculocytes [5]. Mice immunized with the recombinant SSA/Ro antigens have given birth to pups with CHB [6].

Despite evidence highly suggestive of pathogenicity, accessibility of intracellular target antigens to circulating maternal antibodies has been perplexing. A molecular explanation has been provided by the observation that in cultured human foetal cardiocytes rendered apoptotic, the SSA/Ro-SSB/La antigens translocate from the nucleus through the cytoplasm to the membrane surface and are bound by the cognate antibodies [7, 8]. However, an alternative hypothesis which continues to attract attention is the consideration of crossreactivity between one or any of

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the SSA/Ro-SSB/La components and a cardiac receptor. Indeed, support for this second hypothesis is the recent report by Eftekhari *et al.* that antibodies reactive with the serotonergic 5-hydroxytryptamine (5-HT)_{4A} receptor, cloned from human adult atrium, also bind 52 kD SSA/Ro [9]. Moreover, affinity-purified 5-HT₄ antibodies antagonized the serotonin-induced L-type Ca channel activation in human atrial cells. Two peptides in the C terminus of 52 kD SSA/Ro, aa365–382 and aa380–396, were identified that shared some similarity with the 5-HT₄ receptor. The former was recognized by sera from mothers of children with neonatal lupus and it was this 52 kD SSA/Ro peptide that was reported to be crossreactive with antibodies to peptide aa165–185, derived from the second extracellular loop of the 5-HT₄ receptor [9]. These findings are of particular importance, since over 75% of serum from mothers whose children have CHB contain antibodies to 52 kD SSA/Ro as detected by ELISA, immunoblot and immunoprecipitation [10, 11].

Given the intriguing possibility that antibodies to the 5-HT₄ receptor might represent the hitherto elusive reactivity which could directly contribute to atrioventricular (AV) block, the current study was initiated to determine the prevalence of anti-5-HT₄ antibodies in mothers whose children have CHB. RT-PCR was employed to first establish mRNA expression of the 5-HT₄ receptor in the human foetal heart. Additional experiments addressed the functional status of the serotonergic 5-HT₄ receptor in the human foetal heart. One hundred and sixteen sera, obtained from the Research Registry for Neonatal Lupus [12], and 31 control sera were evaluated for anti-5-HT₄ reactivity.

Materials and Methods

Research Registry for Neonatal Lupus and maternal antisera

The Research Registry for Neonatal Lupus was established by the National Institute for Arthritis, Musculoskeletal and Skin Diseases (NIAMS) in September, 1994 and has been extensively described [12]. In contrast to SLE, a disease for which criteria have been defined by the American College of Rheumatology (ACR) [13], no such formal criteria yet exist for classification of neonatal lupus. However, for the purposes of the Registry, a foetus, neonate or infant was considered to have definite neonatal lupus if the following two criteria were met: (1) heart block or characteristic skin rash, and (2) maternal antibodies to the 52 kD SSA/Ro, 60 kD SSA/Ro, or 48 kD SSB/La ribonucleoproteins.

The health status of the mother was determined by review of available medical records. Mothers were classified as having SLE if they met at least 4 of the ACR criteria [13]. Mothers were categorized as having SS if they had: (1) both dry eyes and dry mouth, or (2) either dry eyes or dry mouth along with objective documentation of salivary or lacrimal gland hypofunction or lymphocytic infiltration of these glands.

Undifferentiated autoimmune syndromes (UAS) were diagnosed in those patients with features of a rheumatic disease who did not have prominent sicca complaints and did not meet criteria for SLE. Asymptomatic mothers were those without any clinical evidence of a rheumatic illness.

Sera used in the present study were obtained from 99 mothers with previously documented antibodies to SSA/Ro and/or SSB/La and whose children had CHB (in the presence or absence of cutaneous involvement) and 17 mothers without the candidate antibodies but whose children had CHB. Additionally, sera from 31 healthy volunteers who did not have children with CHB were used as controls.

Peptides

A peptide, GIIDLIEKRKFNQNSNSTYCV, corresponding to the second extra cellular loop of the human 5-HT₄ receptor [9], was synthesized by BACHEM, Bioscience, Inc. (King of Prussia, PA, USA). A second peptide, RKGHFLSSKSGFWTIWL, corresponding to amino acids 365–382 of the 52 kD SSA/Ro protein, was synthesized by Bio-Synthesis, Inc. (Lewisville, TX, USA). The homogeneity of each peptide was evaluated by analytical HPLC and the peptide identity checked by mass spectrometry.

Rabbit antibodies

For immunization, the 5-HT₄ peptide was conjugated to BSA (Sigma St. Louis, MO, USA) using the MBS (m-Maleimidobenzoyl-N-hydroxysuccinimide ester, Pierce, Rockford, IL, USA) crosslinker [14]. In brief, 10 mg BSA was dialysed overnight in phosphate-buffered saline (PBS), pH 7.2, and was allowed to react with 1.5 mg MBS for 30 min at 22°C. The reaction was then passed through a Sephadex G25 column (1×25 cm) equilibrated with 50 mM phosphate buffer, pH 6.0. Fractions corresponding to the void volume were pooled and mixed with 5 mg of peptide. The reaction was adjusted to pH 7.5 with 1.0 N NaOH and stirred 3 h at 22°C. A 2.5 kg New Zealand White rabbit was immunized subcutaneously with 1 mg peptide-BSA conjugate in 0.5 ml mixed with an equal volume of complete Freund's adjuvant. Two booster injections at 1-month intervals were given in incomplete Freund's adjuvant. Serum was collected prior to immunization and 10 days after the last booster injection.

ELISA and immunoblot

These assays were performed as previously described [10, 15], with some modifications. Anti-SSA/Ro antibodies were evaluated with a commercial ELISA kit (Diamedix Corp, Miami, FL, USA) which used calf thymus SSA/Ro and did not contain 52 kD SSA/Ro. For evaluation of anti-peptide responses, peptides were prepared in accordance with the supplier. One milligram of peptide aa365–382 of 52 kD SSA/Ro

was diluted in 1 ml of double distilled H₂O, and subsequently diluted to a final concentration of 1 µg/ml in carbonate buffer. One milligram of the 5-HT₄ peptide was initially diluted in 0.3 ml 20% acetic acid. Subsequent dilution to 1 µg/ml was done in PBS with the addition of DTT (to ensure thiols of peptide remained in a reduced state) and adjusted to pH 7.4. Briefly, for ELISA, wells were coated overnight with 0.2 µg of either peptide, washed with PBS containing 0.05% Tween 20 (PBS-Tween), blocked with 3% BSA/PBS-Tween or soy milk (to eliminate potential background reactivity with BSA), washed with PBS-Tween and incubated with human antibody in PBS-Tween (1:600) for 1 h at 22°C. Goat anti-human IgG [F(ab')₂ fragment] or anti-rabbit IgG [F(ab')₂ fragment] conjugated to alkaline phosphatase at a dilution of 1:5,000 or 1:30,000 respectively (Sigma) was added for 1 h at 22°C. After washing with PBS-Tween, the substrate p-nitrophenyl phosphate (Sigma) was added for 10 min at 37°C, and OD read at 405 nm. Control and experimental sera were tested in duplicate and results confirmed on different runs. Results were considered positive if they exceeded 2 SD greater than the mean obtained with 31 normal human sera.

SDS-immunoblots to optimally identify antibody reactivity to 52 kD SSA/Ro were performed using MOLT4 as previously described [15] with minor modification of the detection system. In place of Protein A-I¹²⁵ to detect human antibodies, membranes were incubated for 1 h in blocking buffer containing 1/5,000 dilution of HRP-linked anti-human IgG or 1/3,000 dilution of HRP-linked anti-rabbit IgG (New England Bio-Labs, Beverly, MA, USA). Filters were washed again in PBS-Tween and detection was accomplished using Phototope-HRP Western Blot detection kit (New England Bio-Labs) as per manufacturer. Membranes were wrapped in Saran Wrap and exposed to X-ray film.

RT-PCR analysis for human 5-HT₄ and 52 kD SSA/Ro mRNA transcripts

First strand synthesis was accomplished using 5 µg of total RNA containing 40 U RNasin (Promega, Madison, WI, USA) obtained from atrial or ventricular tissue of a 24-week human foetal heart as previously described [16]. PCR was performed using a TC9600 Cycler (Perkin-Elmer, Foster City, CA, USA). Briefly, the 50 µl reaction in 1×Taq buffer contained 2 U Taq polymerase (Gibco BRL, Gaithersburg, MD, USA), 2.5 mM MgCl₂, 200 µM of each dNTP, approximately 10 ng of cDNA and 20 pmol of the following primers: for detection of full length 52 kD SSA/Ro (52α); a sense primer spanning bases 423–443 (5'-TATGTGCCAGTCTCGGAAAC-3') located upstream of exon 4 and an antisense primer covering bases 1377–1396 (5'-GGCACATTCAGAGAA GGAGT-3') downstream of exon 4 [16]; for amplification of the 5-HT₄ receptor, a sense primer spanning bases 3–20 (5'-ATGGACAACTTGATGCT-3') and antisense primer spanning bases 1127–1145 (5'-TCAG AAGCATGATTCCAG-3'); and for amplification of the

housekeeping gene coding for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a sense primer (5'-GTGAGGAGGGGAGATTCAG-3') and antisense primer (5'-GCATCCTGGGCTACTACTG-3'). Oligonucleotide primers were synthesized by Gibco-BRL.

Amplification was started by heating for 2 min to 94°C. Thirty cycles followed for 52 kD SSA/Ro and the 5-HT₄ receptor, or 20 cycles for GAPDH, each consisting of 1 min at 94°C, 2 min at 55°C, and 1.5 min at 72°C. The temperature was then held for 10 min at 72°C and cooled down to 4°C. Six microlitres of the respective product were mixed with 1.5 µl of sample buffer (50% glycerol, 0.25% bromophenol blue in H₂O) and applied to a 1.5–1.8% agarose gel in TBE buffer for analysis. For determination of the molecular weights of the PCR products, 250 ng of øX174 DNA *Hae* III digest and 250 ng λ/*Hind*III fragments (Gibco BRL) were run in parallel. The gels were subsequently stained with ethidium bromide and photographs were taken with Polaroid film 667.

Isolation of human atrial myocytes and calcium current (I_{CaL}) recordings

Foetal atrial myocytes were obtained as previously described [5, 7, 17]. Briefly, human foetal hearts were aseptically obtained after elective termination of normal pregnancy by dilatation and evacuation. This was done in accordance with the guidelines of the Institutional Review Board and after obtaining consent from the mothers. Cardiac myocytes were obtained from Langendorff-perfused [17] human foetal hearts. Hearts (of gestational age 18–23 weeks) were perfused at 37°C with a HEPES buffered solution containing (in mM): NaCl 117, KCl 5.7, NaHCO₃ 4.4, NaH₂PO₄ 1.5, MgCl₂ 1.7, HEPES 20, glucose 11, creatine 10, taurine 20, and 21 milliunits/ml insulin and gassed with 100% O₂, at pH 7.4. After 5 min of washing, the heart was then perfused with fresh buffer mixed with 1 mg/ml collagenase type A or B (Boehringer Mannheim Corp., Indianapolis, IN, USA) and 20 µmol CaCl₂ for 5 to 10 min. The atria were then cut off and stirred to obtain cells.

All solutions to record whole-cell I_{CaL} have been previously published [5, 18]. Briefly, for whole cell experiments, the external solution contained (in mM): N-methyl-D-glucamine 130, TEA-Cl 20, CaCl₂ 1.8, MgCl₂ 2, HEPES 10, D-glucose 10 (pH=7.4 with TEA-OH). Internal solution contained (in mM): CsCl 120, TEA-Cl 20, ATPMg 5, HEPES 5, EGTA 3 (pH=7.2 with CsOH).

Whole-cell recording techniques and standard voltage clamp protocols were done as described previously [5]. Briefly, recordings were performed with pipettes through a voltage clamp amplifier (Dagan Corp., Minneapolis, MN, USA). Junction potential was always compensated. Membrane currents were low pass filtered at 2 kHz, digitized with a sampling frequency of 5 kHz, and stored in a computer for subsequent analysis. To record I_{CaL}, all K currents were blocked with intracellular and extracellular cesium and TEA [5]. Fast Na current was blocked by

a prepulse to -40 mV in the presence of $50 \mu\text{M}$ tetrodotoxin. Cells were depolarized every 4 or 8 sec from a holding potential of -80 mV to a prepulse level of -40 mV for 100 msec and subsequently to a test pulse of 0 mV for 200 msec to avoid the possible existence of T-type Ca current in these cells.

Results

mRNA expression and functional evaluation of the cardiac 5-HT₄ serotonergic receptor in the human foetal heart

One requisite to be satisfied, if antibodies to the 5-HT₄ receptor relate to the development of CHB, is to demonstrate the expression of this receptor in the human foetal heart, since it was cloned from the right atrial appendages of hearts from individuals aged 11–74 years [19]. This was accomplished by RT-PCR. First strand synthesis was performed using mRNA isolated separately from the atrium and ventricle of a 24-week foetal heart. The resulting cDNA was amplified with primers flanking bases 3–20 and 1127–1145. As previously reported [16], a transcript of 1.14 kb was amplified from the atrium (Figure 1). No transcript was identified in the ventricular tissue. A 1.0 kb transcript corresponding to full-length 52 kD SSA-A/Ro mRNA and a 0.3 kb transcript corresponding to GAPDH were seen in both the atrium and ventricle, confirming the accuracy in RNA estimation and gel loading techniques of the ventricular mRNA.

In adult atrial myocytes, the activation of 5-HT₄ receptors induces the cAMP mediated activation of L-type calcium channels which results in an increase of I_{CaL} [20]. Initial experiments were performed to identify whether human foetal atrial cells express functional 5-HT₄ receptors. This was accomplished by examining the effects of serotonin (5-hydroxytryptamine, 5-HT), a general 5-HT receptor agonist, and 5-methoxytryptamine (5-MeOT), a specific 5-HT₄ agonist, on I_{CaL} in myocytes obtained from 4 different foetal hearts (18–23 weeks). Serotonin effects on I_{CaL} were evaluated in the presence of $1 \mu\text{M}$ propranolol to block β -adrenergic receptors and exclude the possibility that 5-HT may stimulate β -adrenergic receptors. Activation of 5-HT₄ receptors by $1 \mu\text{M}$ 5-HT increased I_{CaL} by $153.9 \pm 46.5\%$ ($N=7$, $P=0.03$). Figure 2A shows original tracings of I_{CaL} recorded at 0 mV from a holding potential of -40 mV during control conditions and after cell superfusion with 5-HT. Figure 2B shows the time course of I_{CaL} in the presence of 5-HT. In addition, the use of $1 \mu\text{M}$ of 5-MeOT also increased I_{CaL} by 235.5% from 656 pA to 2200 pA. Both the general 5-HT receptor agonist (5-HT) and the specific 5-HT₄ agonist (5-MeOT) increased I_{CaL} amplitude, indicating that these receptors are functional.

To further verify that the observed 5-HT response is mediated through 5-HT₄ receptors, a potent selective 5-HT₄ antagonist, GR 113808 [21], and an additional selective 5-HT₄ agonist, 2-[1-(4-Piperonyl)piperazinyl] benzothiazole (which also blocks 5-HT₃ receptors)

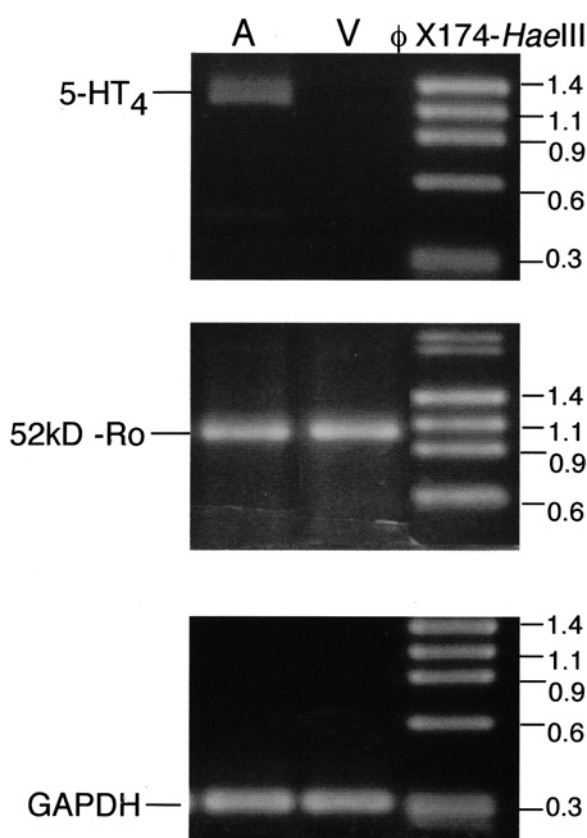


Figure 1. RT-PCR analysis of 5-HT₄ receptor mRNA in the human foetal heart. Using the sense primer spanning bases 3–20 and an antisense primer covering bases 1127–1145 of the known sequence for the 5-HT₄ receptor, a fragment corresponding to 1.14 kb was amplified from cDNA isolated from the atrium (A) of a 24-week foetal heart. No amplification was noted from cDNA amplified from the ventricle (V). mRNA expression of both full-length 52 kD SSA/Ro and GAPDH were demonstrated in the atrium and ventricle.

[22], were employed. Exposure of single human atrial myocytes to the specific 5-HT₄ agonist, 2-[1-(4-Piperonyl)piperazinyl] benzothiazole ($1 \mu\text{M}$), resulted in an increase of I_{CaL} (Figure 3A). This increase was antagonized by the potent selective 5-HT₄ antagonist, GR 113808 ($1 \mu\text{M}$). Review of averaged data on I_{CaL} changes during control, application of 5-HT₄ agonist, and application of 5-HT₄ agonist plus 5-HT₄ antagonist, in a total of 5 atrial myocytes, demonstrated that 5-HT₄ stimulation of I_{CaL} was reversed to control levels with antagonist (Figure 3B). These results taken together support the expression of functional 5-HT₄ receptors in the human foetal atrium.

Maternal health status of the cohort and reactivity of the antisera with full-length 52 kD SSA/Ro and peptide aa-365–382

Sera from 116 mothers whose children have CHB were evaluated. The health status of the mothers is as follows: 31 are asymptomatic, 21 have SLE, 6 have SLE

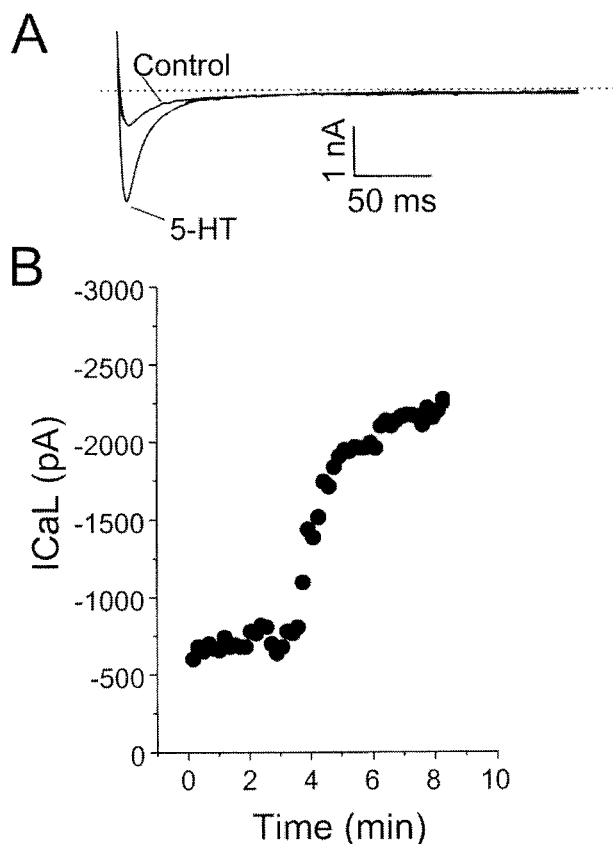


Figure 2. Effect of 5-HT receptor stimulation on L-type calcium current, I_{CaL} from a human foetal atrial myocyte. Panel A shows selected I_{CaL} tracings recorded from a holding potential of -40 mV to a test potential of 0 mV, during control conditions and in the presence of $1 \mu\text{M}$ 5-HT. Panel B illustrates the time course of I_{CaL} stimulation by $1 \mu\text{M}$ 5-HT. $1 \mu\text{M}$ propranolol was added to the external solution to block β -adrenergic receptors.

and SS, 27 have SS, 13 have UAS, and 1 has scleroderma. For 17 of the mothers, medical records were not available for classification. Ninety-nine of these sera were demonstrated to have anti-SSA/Ro and/or anti-SSB/La antibodies by commercial ELISA. With rare exception, the 17 seronegative samples were from mothers whose children had heart block either detected after birth or associated with structural abnormalities. This was not surprising, since both of these latter features are not characteristic of autoantibody-associated CHB. Of the 99 sera containing antibodies to SSA/Ro, 84% were reactive with the 52 kD SSA/Ro component by immunoblot of MOLT4 (anti-52 kD SSA/Ro was initially identified on immunoblot, and its recognition is still generally dependent on this technique [15]), and 16% were non-reactive. Of 76 sera tested with anti-52 kD SSA/Ro antibodies by immunoblot and 16 sera without this reactivity, 15 (20%) and 2 (13%), respectively, contained antibodies against the peptide aa365–382 of 52 kD SSA/Ro as assessed by ELISA (88% of positives had OD between 0.32 and 0.59, 2–5 SD > normal healthy donors).

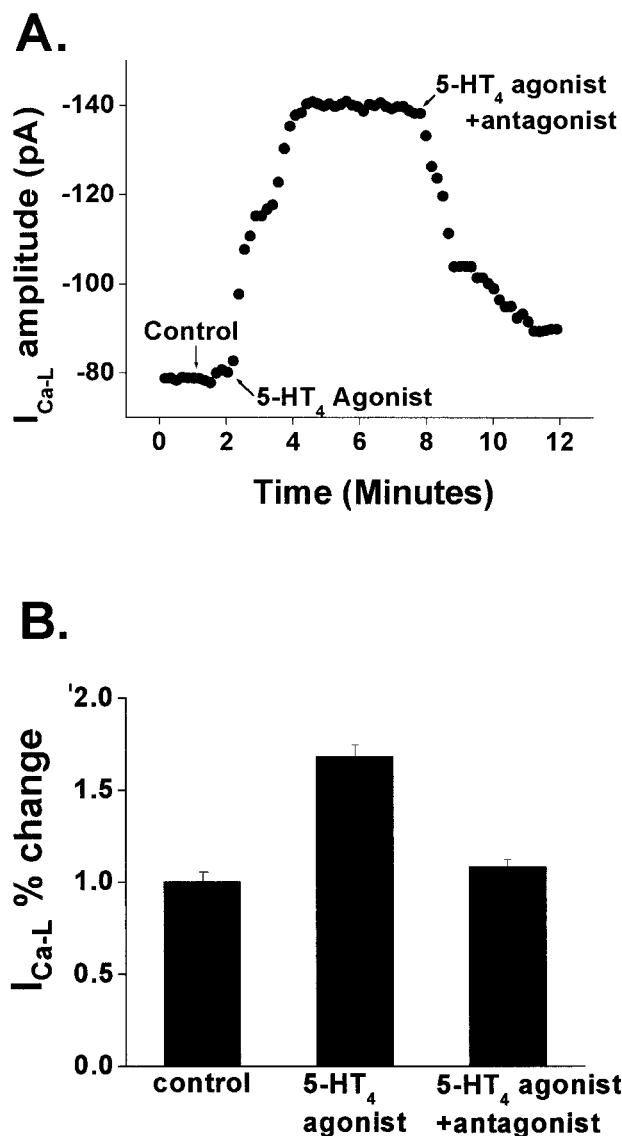


Figure 3. Effect of a selective 5-HT₄ agonist, 2-[1-(4-Piperonyl)piperazinyl]benzothiazol, and a potent selective 5-HT₄ antagonist, GR 113808, on L-type calcium current (I_{CaL}) from human foetal atrial myocytes. Panel A shows the time course of I_{CaL} stimulation by $1 \mu\text{M}$ 2-[1-(4-Piperonyl)piperazinyl]benzothiazole and its antagonism by $1 \mu\text{M}$ GR 113808. Panel B illustrates average data of I_{CaL} changes during control, the application of 5-HT₄ agonist, and the application of 5-HT₄ agonist plus 5-HT₄ antagonist in a total of 5 atrial myocytes. $1 \mu\text{M}$ propranolol was added to the external solution to block β -adrenergic receptors.

Antibody reactivity against the cardiac 5-HT₄ serotonergic receptor

Given the expected high frequency of anti-52 kD SSA/Ro antibodies in the mothers of children with CHB, these sera were subsequently tested for anti-5-HT₄ receptor antibodies. Rabbit antisera generated by immunization with the 5-HT₄ peptide served as the positive control. The results are summarized in Table 1. In an ELISA using peptide aa165–185 of 5-HT₄ and blocking with BSA, the mean OD of 31 sera from healthy donors was 0.084 ± 0.087 SD. Two of these sera

Table 1. Summary of antibody responses to the 5-HT₄ receptor peptide in the 3 groups of mothers whose children have CHB and healthy controls, as well as in preimmune and postimmune rabbits

Sera tested (1:600)	(N)	5-HT ₄ peptide (aa165–185)		
		Blocking with BSA		Blocking with soy milk*
		Mean OD±SD	N Positive (>2 SD)	N Positive (>2 SD)
Healthy Controls	(31)	0.08±0.09	2	0
CHB (Ro+**, 52+***)	(83)	0.06±0.06	1	0
CHB (Ro+, 52-)	(16)	0.13±0.27	1	0
CHB (Ro-)	(17)	0.09±0.10	2	0
Preimmune rabbit		0.10	0	0
Postimmune rabbit		2.0	1	1

(N)=number of patients tested for reactivity against the 5-HT₄ receptor.

N=number of patients whose reactivity was >2 SD above the mean for the healthy control group,

*All sera that contained antibodies to the 5-HT₄ peptide when BSA was used as the blocking reagent were subsequently tested using soy milk. Except for the postimmune rabbit antisera, none remained positive.

**Reactivity to SSA/Ro is evaluated by commercial ELISA which uses calf thymus SSA/Ro and does not contain 52 kD SSA/Ro.

***Reactivity to 52 kD SSA/Ro is evaluated by SDS-immunoblot of MOLT4.

readings were greater than 2 SD above the mean. Only 4 of the 116 sera from the 'CHB' mothers were positive for reactivity with aa165–185, with one of substantially high reactivity, OD=1.11 (approximately 50% of that obtained with the rabbit anti-5-HT₄ antibody). However, when the four 'positive' sera and the rabbit anti-5-HT₄ antibody were tested for reactivity with the peptide receptor and soy milk was substituted in the blocking step, only the rabbit antibody remained reactive.

Rabbit anti-5-HT₄ receptor antibodies did not recognize the peptide aa365–382 of 52 kD SSA/Ro as assessed by ELISA, or the full-length recombinant 52 kD SSA/Ro protein by ELISA or immunoblot.

Discussion

In all large studies to date, antibodies reactive with the 52 kD SSA/Ro protein have been identified in over 75% of mothers whose children have isolated heart block detected *in utero* [10–12]. A molecular definition for the 52 kD Ro antigen has been provided by the isolation of cDNA clones encoding the full-length protein which consists of 475 amino acids [23, 24]. The full-length protein, 52 α , has three distinct domains: an N-terminal region rich in cysteine/histidine motifs containing two distinct zinc fingers known as RING finger and B-box; a central region containing two coiled coils with heptad periodicity, one being a leucine zipper, both with potential for intermolecular dimerization; and a C-terminal 'rfp-like' domain. In addition, we have described an alternative 52 mRNA transcript derived from the splicing of exon 4 encoding aa 168–245 inclusive of the leucine zipper, which results in a smaller protein, 52 β , with a predicted m.w. of 45 kD [16]. This isoform is maximally expressed in

the human foetal heart between 14 and 16 weeks of gestation just prior to the clinical detection of CHB [25]. Although the function of 52 kD SSA/Ro is undefined at present, several of its protein motifs suggest a role in transcriptional regulation.

One approach to linking the anti-52 kD SSA/Ro response with a molecular mechanism that accounts for CHB is B cell epitope mapping and subsequent identification of crossreactive targets specific to the foetal heart. Several groups have evaluated B-cell epitopes of the 52 kD SSA/Ro protein [26]. Virtually all studies [27–34] have identified a commonly recognized epitope in the central region spanning aa183–232. Of particular relevance to the current work, Ricchiuti and colleagues, using 39 overlapping peptides, identified reactivity with the N-terminal region aa365–382 in 19% of 89 patients with SS and 62% of 26 patients with SLE and SS [35]. Ricchiuti concluded that this peptide is displayed at the surface of the intact RoRNP particle based on inhibition of antisera binding to native 52 kD SSA/Ro in the fluid phase [36]. In the current study, reactivity against this same peptide aa365–382 of 52 kD SSA/Ro was present in ~20% of the mothers whose children have CHB, two of which did not recognize 52 kD SSA/Ro on immunoblot. Of interest are the findings by Ricchiuti *et al.* that a rabbit antiserum raised against aa365–382 did not recognize the full-length 52 kD SSA/Ro recombinant protein [36], and the observation of Eftekhari that one serum which recognized the peptide also did not recognize the full-length protein [9]. Notably, it is this C-terminal peptide aa365–382 which was found by Eftekhari and colleagues to be crossreactive with antibodies to the 5-HT₄ receptor peptide. Review of the amino acid sequences reveals that 4 residues of aa365–382 52 kD SSA/Ro are identical to those present in the second extracellular loop of the human 5-HT₄ receptor aa165–185.

The recent finding of antibodies reactive with a cardiac serotonergic receptor in 18 of 26 SLE patients (many of whom also had antibodies to 52 kD SSA/Ro, in particular reactivity to the potentially crossreactive epitope aa365–385) led to the novel hypothesis that this response is directly relevant to the development of CHB. Sera from mothers of affected children were not tested for anti-5-HT₄ reactivity in that study. Serotonin 5-HT₄ exerts positive chronotropic, inotropic and lusitropic effects in the human atrium after binding to the pharmacologically defined 5-HT₄ receptor. Engagement of the receptor leads to stimulation of adenyl cyclase activity, activation of cAMP-dependent protein kinase A and phosphorylation of several key proteins involved in excitation-contraction coupling. One of these proteins is the L-type calcium channel. In isolated cells from human right atrial appendage, 5-HT₄ induces stimulation of the cardiac L-type calcium current [20].

This study is the first to establish the existence of 5-HT₄ receptors in human foetal atria both functionally and at the gene transcriptional level, extending the observation of Eftekhari *et al.* [37] who recently demonstrated the 5-HT₄ receptor in the murine foetal heart by immunohistochemistry. Antibodies reactive with the 5-HT₄ receptor can be deleterious to cell electrical activity by inhibition of stimulated-I_{CaL}. This inhibition could be critical for the developing foetal heart in which L-type calcium channels play a major role in excitation-contraction coupling and conduction at the AV node [5].

Screening a large number of sera from a national Registry of mothers whose sera contain anti-52 kD SSA/Ro antibodies and whose children have CHB did not substantiate the 'serotonin' hypothesis postulated by Eftekhari *et al.* [9]. Specific reactivity with the single 5-HT₄ receptor peptide tested, aa165–185, could not be demonstrated in any of these mothers. A potential explanation may be sought in the maternal diagnosis, given that Eftekhari *et al.* examined only patients with SLE [9], the disease most commonly present with regard to reactivity with the crossreactive 52 kD SSA/Ro peptide reported by Ricchiuti *et al.* [35]. However, in the cohort reported herein, none of the 27 SLE patients studied had detectable antibodies to the 5-HT₄ receptor peptide. The discrepancy in reactivity, therefore, cannot be explained by maternal health status. Moreover, autoimmune-CHB is associated with anti-SSA/Ro antibodies and is independent of maternal diagnosis. Inhibition of inward calcium currents due to the blockade of serotonergic responses by antibody binding to the putative 5-HT₄ receptor does not appear to be either necessary or sufficient for the development of CHB. Further studies are required to delineate the specific contribution of 5-HT₄ antibodies and/or anti-SSA/Ro-SSB/La antibodies to the pathogenesis of CHB.

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