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Full Length Research Paper

Characterization of an intrinsic serotonergic system in rat heart

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The objective of this study was to characterize biochemically and immunohistochemically an intrinsic serotonergic system in rat heart. Male Wistar rats were used with an average body weight of 250 ± 10 g. Rats were adapted for 2 weeks to standard animal laboratory conditions. Hearts were later obtained to determine the principal components of the serotoninergic system using immunohistochemistry and Western blot for tryptophan-5-hydroxylase (TPH) 1 or 2, serotonin transporter (SERT) and serotoninergic receptors 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2B} and 5-HT_4. Activity of TPH, L-tryptophan (L-Trp), 5-hydroxytryptamine (serotonin, 5-HT) and 5-hydroxyindolacetic acid (5-HIAA) were also determined by high-resolution liquid chromatography. Immunopositive cardiomyocytes for both isoforms of TPH were observed. There was a greater expression of TPH2in relation to TPH1. Immunoreactivity for SERT and 5-HT_{1B}, 5-HT_{2A}, 5-HT_{2B} and 5-HT_4receptors was also observed in cardiomyocytes. TPH activity, L-Trp, 5-HT and 5-HIAA concentrations were also demonstrated in heart tissue.These findings support the fact that cardiomyocytes synthesize, utilize and reuptake 5-HT, which constitutes an intrinsic serotoninergic system that actively participates as an autocrine and paracrine modulation system of heart activities.

Key words: Cardiomyocytes, serotonin, tryptophan-5-hydroxylase, SERT, serotoninergic receptors

INTRODUCTION

5-Hydroxytryptamine (5-HT, serotonin) in the body regulates multiple functions. In the brain, its role as a trophic factor, neuromodulator and neurotransmitter has been widely studied (Jacobs and Azmitia, 1992; Mercado and Hernandez, 1992; Lauder, 1993). At the peripheral level, 5-HT is also synthesized from L-tryptophan (L-Trp) by two biochemical reactions: the first is catalyzed by L-tryptophan-5-monooxygenase (TPH) (Boadle-Biber, 1993; Walther and Bader, 2003) that transforms L-Trp into 5-hydroxytryptophan (5-HTP) and the second reaction is catalyzed by decarboxylase of aromatic L-amino acids to convert 5-HTP to 5-HT (Hernandez and Illnerova, 1970; Jacobs and Azmitia, 1992; Boadle-Biber, 1993).

Serotonin carries out its functions by means of its interactions with specific receptors found in the plasma

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membrane. Different serotoninergic receptors (5-HT_{1A}, 5-HT_{2A}, 5-HT_3, 5-HT_4, and 5-HT_7) participate in cardiovascular function and may induce bradycardia or tachycardia, hypotension or hypertension and vasodilation or vasoconstriction in the heart (Frishman and Grewall, 2000; Nebigil and Maroteaux, 2001; Hoyer et al., 2002; Cöte et al., 2003; Qvisgstad et al., 2005; Ramage and Villalon, 2008). These receptors are located in the nerve ends or in the cardiomyocytes (Frishman and Grewall, 2000; Nebigil and Maroteaux, 2001; Hoyer et al., 2002; Cöte et al., 2003; Qvisgstad et al., 2005; Ramage and Villalon, 2008). Expression of the serotonin transporter (SERT) has also been observed in fetal heart and participates in the processes of remodelation and development of the heart; however, it has not been shown in the adult myocardium and has been observed only in the interstitial cells of the heart valves (Sari and Zhou, 2003; Gustafsson et al., 2005; Mekontso et al., 2006; Pavone et al., 2007).

Serotonin plays also an important role in the pathophysiology of various cardiovascular diseases, among which is valvular dysfunction associated with the activation of SERT and/or to the increase of plasma 5-HT concentration, an aspect demonstrated in patients with carcinoid syndrome or in those who receive treatment with 5-HT_{2B} receptor agonists or with selective serotonin reuptake inhibitors (Robiolio et al., 1995; Connolly et al., 1997; Fitzgerald et al., 2000; Elangbam et al., 2005; Mekontso et al., 2006; Levy, 2006;). It is also known that activation of the 5-HT_{2B} receptor located in heart tissue produces fibroblastic mitogenesis and subendocardiac fibrosis (Elangbam et al., 2005; Mekontso et al., 2006).In addition, it has been observed that cardiomyocytes from rats with heart failure are regulated by 5-HT through 5-HT_{2A} and 5-HT_4 receptors (Qvisgstad et al., 2005; Brattelid et al., 2012).The positive inotropic effect exerted by 5-HT in the heart is through myosin light chain phosphorylation. Activation of the 5-HT_{1B} receptor located in the fibroblasts also induces collagen production (Mekontso et al., 2006). Recently, the presence of cardiac mast cells producing 5-HT and expression of TPH during cardiogenesis has been reported (Manjarrez et al., 2009) along with interstitial cells located on the adult heart valves (Disatian et al., 2010). These findings together allowed us to consider that in the heart there is an intrinsic serotonergic system that actively participates in autocrine and paracrine regulation of cardiac activity.

MATERIALS AND METHODS

In the present study we used 60 male Wistar rats weighing 250 ± 10 g. Rats were obtained from the animal facilities of the Research Center and Advanced Studies of the National Polytechnic Institute (IPN) (CINVESTAV-IPN). The protocol was authorized by the Research and Ethics Committees of the National Commission of Ethics in Health of the IMSS. General management of experimental animals was carried out in the Department of Production and Experimentation of Laboratory Animals (UPEAL) of the CINVESTAV-IPN under the health standards and ethics of the Internal Committee for the Care and Use of Laboratory Animals (CICUAL) according to the Official Mexican Guidelines (NOM-062-ZOO-1999).

Rats were maintained for 2 weeks under standard environmental conditions of animal facilities with a controlled climate at 22 ± 2°C, 12-h periods of light and darkness, and relative humidity 50-60%. Animal handling was kept to a minimum. During this period, rats were fed with a Lab Diet 5P14 (PMI Feeds, Richmond, IN) and water *ad-libitum.* The diet was composed of 23% proteins, 56.9% carbohydrates, 4.5% fat, 6% fiber and 8% ash, vitamins and minerals as well as lysine, thiamine, α-tocopherol, calcium, ferrous sulfate, manganese, among other micronutrients, providing a caloric density of 4.05 kcal/g of food.

At the end of the adjustment period, rats were sacrificed by cervical dislocation. Hearts were dissected and washed three times with a Hartman solution for biochemical and immunohistochemical assays. Also, in order to decrease the circadian variation, heart dissections were always done between 9:00 and 11:00 AM.

Biochemical Assays

L-Trp, 5-HT and 5-HIAA in the heart were determined by high-performance liquid chromatography (HPLC) according to the method of Peat and Gibb, 1983. Briefly, the hearts were homogenized with $HClO_4$ 0.1 M solution + 4 mM sodium metabisulfite. The homogenate was centrifuged at 15,000 rpm for 15 min at 4°C. Subsequently, 20 µl of the supernatant was injected into the HPLC (Waters Corporation) using a reverse phase C_{18} symmetry column (5 µm particle size, 3.9 x 150 mm length). A binary system of a monobasic potassium phosphate solution was used (2 mM, pH 3.40 + heptanosulfonic acid 1 g/L of solution and methanol/water mixture at a ratio of 3:2 v/v) at the rate of 1 mL/min. Determinations of L-Trp, 5-HT and 5-HIAA were carried out with a fluorometric detector (Waters Model 474) with an excitation of 290 nm and emission of 330 nm. The response was obtained using an Epower 2 (Waters Corporation) analog system, and the concentrations were considered as the maximum highs of the signals according to a standard curve of known quantities of L-Trp, 5-HT and 5-HIAA and expressed in nmol/g of tissue.

TPH activity in the heart was evaluated by 5-HTP determination using HPLC with a fluorometric detector (Waters model 474) (Johansen et al., 1995; Johansen et al., 1996). This method consists of incubating 300 µg of tissue protein in the presence of a buffer solution of Tris-HCl 50 mM, pH 7.40, EGTA 1.0 mM, 15µg catalase, and

200µM 2-amino-4-hydroxy-1-methyl tetrahydrobiopterine. The reaction was incubated at 37°C for 10 min and was stopped by the addition of $HClO_4$ 6 M + 5 mM of EDTA and 0.1% of ascorbic acid. Twenty µL of the reaction media was injected into HPLC. A C_{18} symmetry column (5 µm particle size, 3.9 x 150 mm) was used. The mobile phase was prepared with sodium acetate (40 mM, pH 3.30) and acetonitrile at a ratio of 95:5, respectively, and was run at 1 mL/min. Excitation and emission wavelengths used for detection of 5-HTP were 280 nm and 340 nm, respectively.

Immunotransference of TPH1 and 2, SERT, 5-HT_{1B}, 5-HT_{2A} 5-HT_{2B} and 5-HT_4 by Western blot.

Hearts were homogenized for 30 sec at 4°C in a Tris-HCl 50 mM solution, pH 7.4, plus protease inhibitors (Protease Inhibitor Cocktail, Sigma-Aldrich). Samples were later centrifuged at 10,000 rpm for 15 min at 4°C. Protein concentration was quantified by the Bradford method; 30 µg of protein was then placed in each of the1-mm-thick channels of 12% SDS-polyacrylamide gel. Electrophoretic run was done at 100 V for 2 h. For electrotransference of the proteins, gel was mounted on nitrocellulose membranes and the run was carried out at 10 V, 1.30 mA for 45 min. Nitrocellulose membranes with the transferred proteins were placed in a blocking solution (Millipore Chemiluminescent blocker) at 50% for 30 min. Membranes were then incubated with the primary antibody specific for TPH1 (Rabbit monoclonal IgG, Gene Tex Inc., Irvine, CA), TPH 2 (Rabbit polyclonal, Merck-Millipore) and antibodies for SERT (Rabbit polyclonal Santa Cruz Biotechnology, Santa Cruz, CA), and 5-HT_{1B}, 5-HT_{2A} 5-HT_{2B} and 5-HT_4 (Rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:500 in the same blocking solution. On the following day, membranes were incubated with Goat anti-rabbit IgG (H+L)-HRP conjugate secondary antibody (Bio Rad., Laboratories 2000. Alfred Nobel. Dr Hercules, CA 94547) at a dilution of 1:5000 in the same blocking solution. Membranes were immediately incubated with the ABC Elite system (Vector Laboratories, Burlingame, CA) (Naish et al., 1989) for 1 h. After the membranes were washed with PBS, they were revealed with diaminobenzidine (DAB). Internal control was glyceraldehyde-3-phosphate dehydrogenase (GADPH).

Immunohistochemistry for 5-HT, TPH1 and 2, SERT, 5-HT_{1B}, 5-HT_{2A} 5-HT_{2B} and 5-HT_4.

Hearts were placed in a 4% p-formaldehyde solution for 1 week at 4°C and then embedded in paraffin to obtain 5-µM-thick cuts. Cuts were then placed on electrocharged slides. After the cuts were deparaffinized, antigenic unmasking was done using a 0.1 M, pH 6.0, citrate buffer solution (DECLERE, Cell Marque) in a microwave oven for three 2-min cycles. Endogenous peroxidase activity was immediately inhibited with H_2O_2 at 5%. After washing, the slices were incubated with the primary antibody specific for TPH1 (Rabbit monoclonal IgG, Gene Tex Inc., Irvine, CA), TPH2 (Rabbit polyclonal, Merck-Millipore) and antibodies for SERT (Rabbit polyclonal Santa Cruz Biotechnology, Santa Cruz, CA), and 5-HT_{1B}, 5-HT_{2A} 5-HT_{2B} and 5-HT_4 (Rabbit polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:500 in a PBS solution (0.1 M, pH 7.4, Triton X100) at 0.3% and 3% goat serum for 1 h at 4°C. The cuts were washed the following day and incubated with the secondary antibody (mouse/rabbit immunodetector biotin link) for 15 min. They were washed with PBS and subsequently, the tissues were incubated with the mouse/rabbit immunodetector HRP label complex for 15 min at room temperature. They were then washed and the activity was revealed with a commercial kit (3,3-diaminobenzidine and H_2O_2) according to the Vector Laboratory protocol (Naish et al., 1989). Microphotographs were taken with a digital camera (Coolpix 995, Nikon) with a 40X lens.

RESULTS

Specific activity of TPH in the heart is shown in the representative chromatogram (blue line in Figure 1A). L-Trp and 5-HTP are shown with a black line. The enzyme activity of 5-HTP/mg of protein/h was 62 ± 7.6 fmol. In order to confirm the presence of TPH in the heart, Western blot experiments were carried out. Two immunoreactive bands of TPH were observed: 1) 51 kDa that corresponded to TPH1 and 53 kDa corresponding toTPH2 and 2) 37 kDa for GADPH, which served as internal control for each of the experiments (see Figure 1B).

In addition, immunopositive cardiomyocyte heart tissue for TPH1 and TPH2 was observed. Immunoreactivity was localized in intracytoplasmic granules and conglomerates were observed in some zones, especially in the membranes (arrows in Figure 2). Interestingly, TPH2 showed a greater immunoreactivity in relation to TPH1 both in heart tissue as well as in the Western blot experiments (see Figure 1B and Figure 2).

5-HIAA, L-Trp and 5-HT in the heart are shown in the chromatogram of Figure 3A (black line). The respective standards are shown in the blue line. Concentrations were 5-HIAA 1.93 ± 0.23, L-Trp 10.88 ± 1.34 and 5-HT 3.44 ± 0.23 nmol/g of tissue. Figure 3B shows heart tissue where cardiomyocytes immunoreactive to 5-HT are observed. Immunopositivity was demonstrated in intracytoplasmic granules similar to those shown for both isoforms of the TPH. There were also conglomerates of the immunoreactivity in some areas of the myocardium (see arrows), but in other areas they were not observed (arrowheads Figure 3B).

SERT expression in the heart is illustrated in Figure 4A.

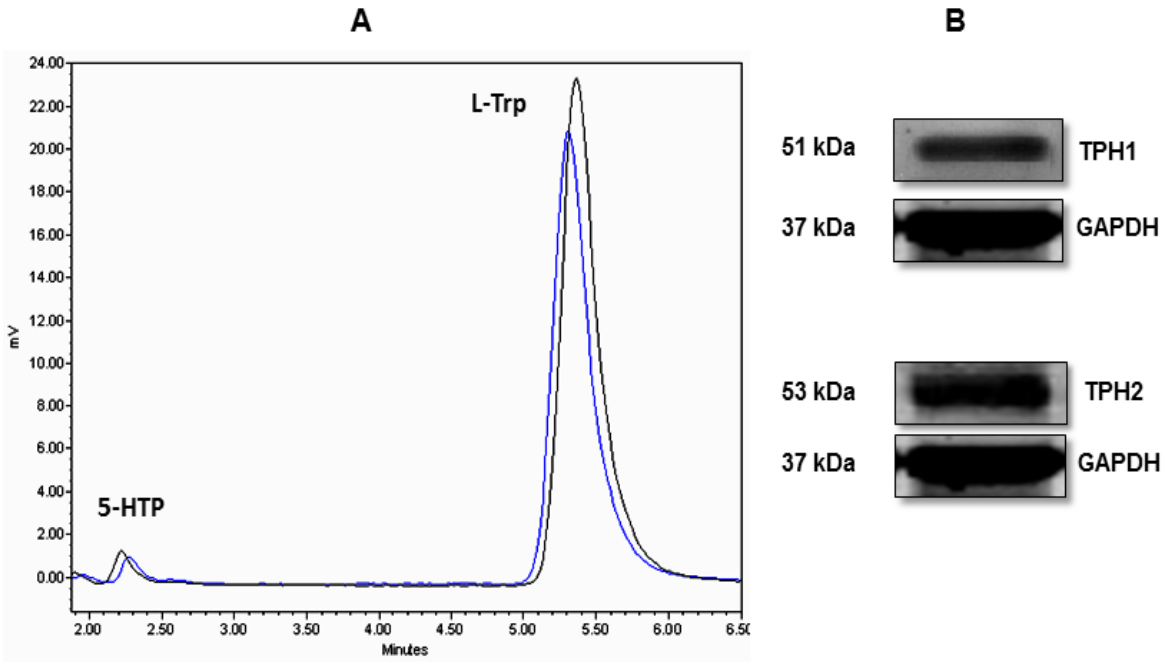

Figure 1A. Chromatogram representative of a standard 5-HTP and L-Trp (black line) and of the specific activity of TPH obtained in rat heart (blue line). (B) Expression of TPH1 and 2 in rat heart by Western blot. Immunoreactivity was detected with antibodies specific for each of the proteins. One 51-kDa band for TPH1 was observed and another band of 53 kDa that corresponded to TPH2 and GADPH of 37 kDa as internal control.

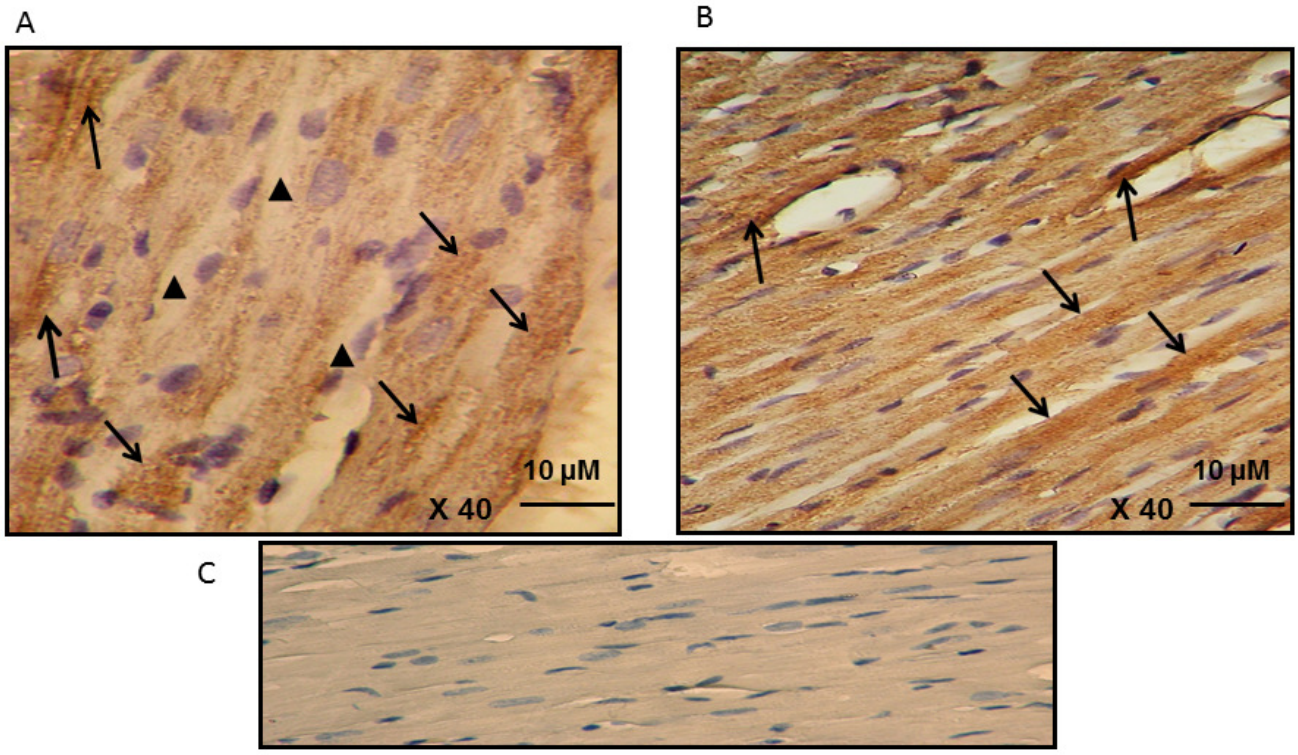

Figure 2. Photomicrographs of longitudinal cuts of the heart that show immunopositive cardiomyocytes for tryptophan-5-hydroxylase (TPs). A) TPH1 and B) TPH2. Cuts were incubated with monoclonal antibodies specific for each of the isoforms (dilution 1:500) and immunoreactivities were detected with secondary antibodies conjugated with peroxidase and revealed with 3,3-diaminobenzidine. Arrows show the immunoreactivity in cytoplasmic conglomerates and in the membranes. Arrowheads point to the sites where there was no immunopositivity of the enzymes.C) Negative control

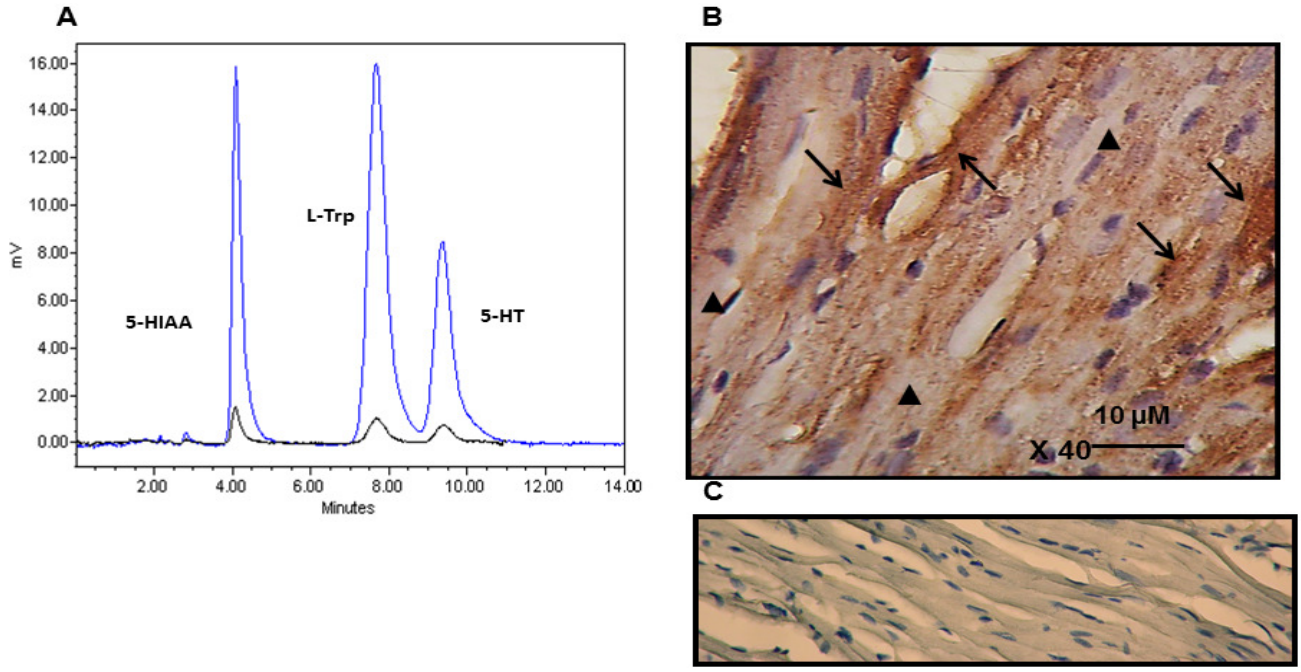

Figure 3 A. Chromatogram representative of the presence of 5-HIAA, L-Trp, and 5-HT in the heart (black line) and the response of the respective standards (blue line). (B) Photomicrograph of a longitudinal slice of the heart showing 5-HT immunopositive cardiomyocytes. The heart tissue was incubated with polyclonal antibody for the amine (dilution 1:1000) and immunoreactivity was detected with a secondary antibody conjugated with peroxidase and revealed with 3,3-diaminobenzidine. Arrows show immunoreactivity in conglomerates and arrowheads point to the zones with no immunopositivity. C) Negative control.

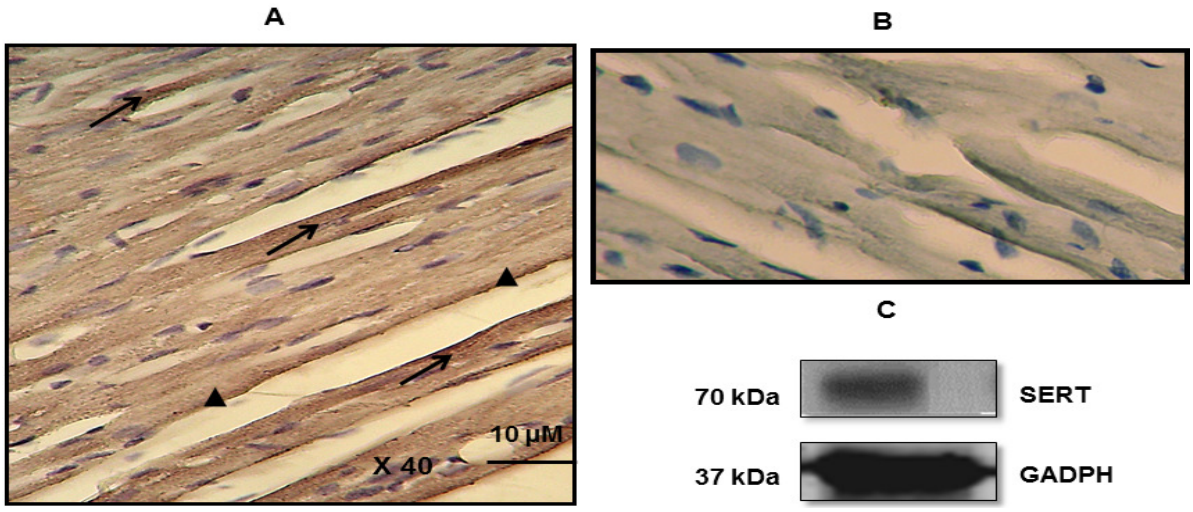

Figure 4 A. Photomicrograph of a longitudinal cut of the heart that shows immunopositive cardiomyocytes for SERT. The cut was incubated with protein-specific monoclonal antibody (dilution 1:500) and immunoreactivity was detected with secondary antibody conjugated to peroxidase and revealed with 3,3-diaminobenzidine. Arrows show immunoreactivity with cytoplasmic conglomerates and in the membranes. Arrowheads show the zones where there was no immunopositivity in the cardiomyocytes. (B) Negative control. C) Expression of SERT by Western blot; immunoreactivity was detected with specific antibodies. A 70-kDa band for SERT and another 37-kDa band was observed, which corresponded to GADPH as internal control for the experiment.

Note that the immunopositivity was located in intracytoplasmic granules and in cellular membranes of the cardiomyocytes (arrows in Figure 4A). It was also observed that immunoreactivity predominated in certain regions of the myocardium compared to other sites where it did not exist (arrowheads in Figure 4A). Figure 4B

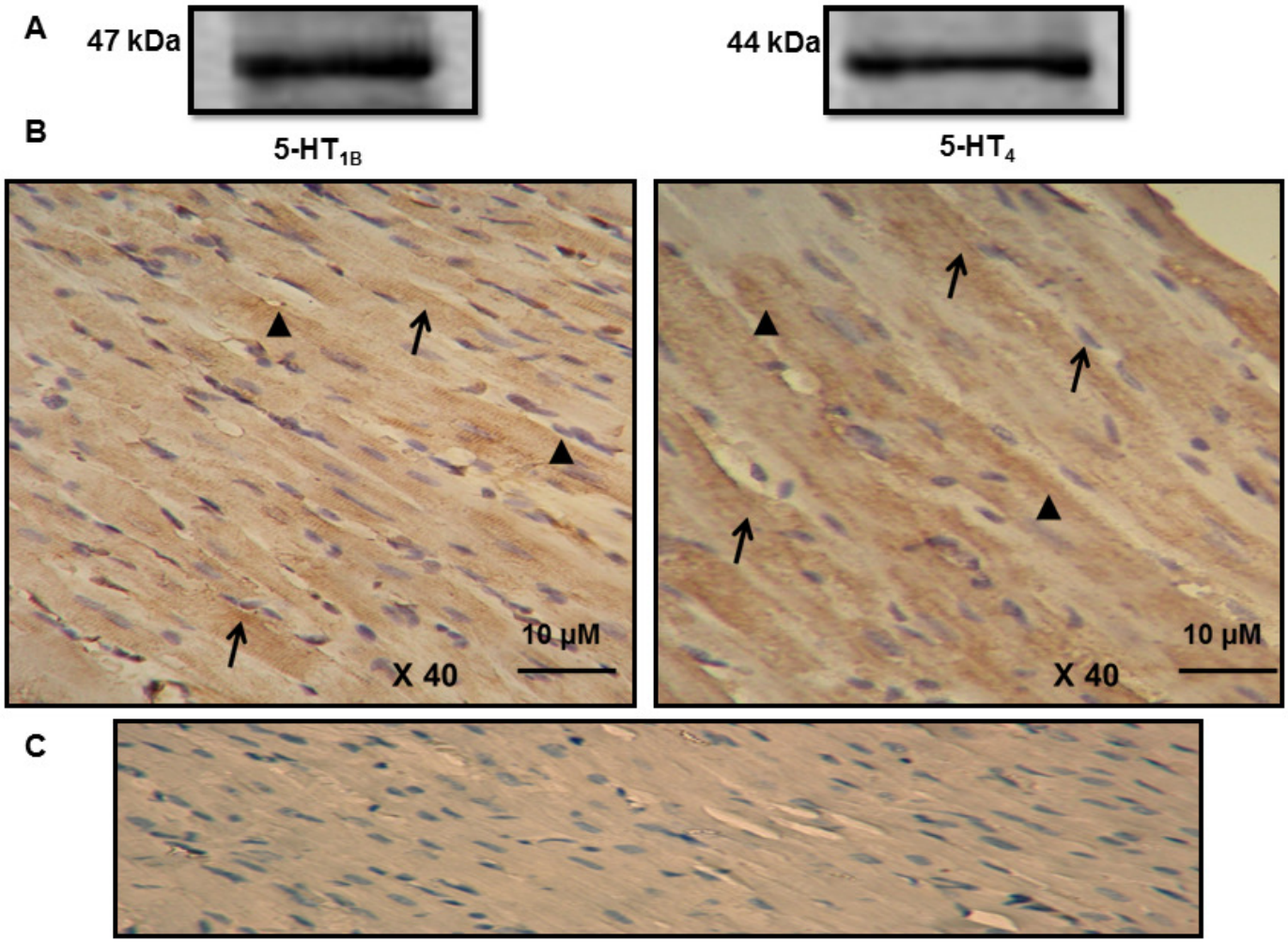

Figure 5A. Expression of 5-HT_{1B} and 5-HT_4 receptors in the heart by Western blot; immunoreactivity was detected with specific antibodies for each of the receptors. A 47-kDa band was observed for 5-HT_{1B} and another band of 44 kDa, which corresponded to 5-HT_4. (B) Photomicrographs of longitudinal cuts of the heart show cardiomyocytes immunopositive for receptors. Cuts were incubated with specific monoclonal antibodies (dilution 1:400) and immunoreactivity was detected with secondary antibody conjugated to peroxidase and revealed with 3,3-diaminobenzidine. Arrows show conglomerates of the immunoreactivity in the cardiomyocytes. Arrowheads show immunopositivity in the membranes. C) Negative control

illustrates SERT expression through Western blot and GADPH as internal control. Two bands were present: one 70-kDa band that corresponded to SERT and another 37-kDa band for GADPH.

Expression of the serotoninergic receptors 5-HT_{1B} and 5-HT_4can be seen in Figure 5. A strong expression of both receptors in cardiomyocytes was distinguished. Immunoreactivity of both receptors was located diffusely throughout the cytoplasm (Figure 5 arrows) and, in some zones, was located in the membranes of the cardiomyocytes (Figure 5 arrow head). With regard to the expression of the receptor proteins, two bands were observed: a 47-kDa band that corresponded to 5-HT_{1B} and a 44-kDa band corresponding to 5-HT_4 (see Figure 5A).

Expressions of the 5-HT_{2A} and 5-HT_{2B} receptors are shown in Figure 6. They were demonstrated in two immunopositive bands: a 53-kDa band that corresponded to the 5-HT_{2A} receptor and a 55-kDa band corresponding to 5-HT_{2B}. In addition, the same figure shows the immunopositivity that expressed both receptors in cardiomyocytes and increased immunoreactivity of the 5-HT_{2B} receptor. Immunopositivity of the 5-HT_{2A} receptor was located in certain areas of the myocardium in conglomerates (Figure 6 arrows) and in the membranes (Figure 6 arrowheads). It is important to point out that the immunoreactivity shown by the 5-HT_{2B} receptor was localized in intracytoplasmic granules around the cell nuclei (Figure 6 arrows).

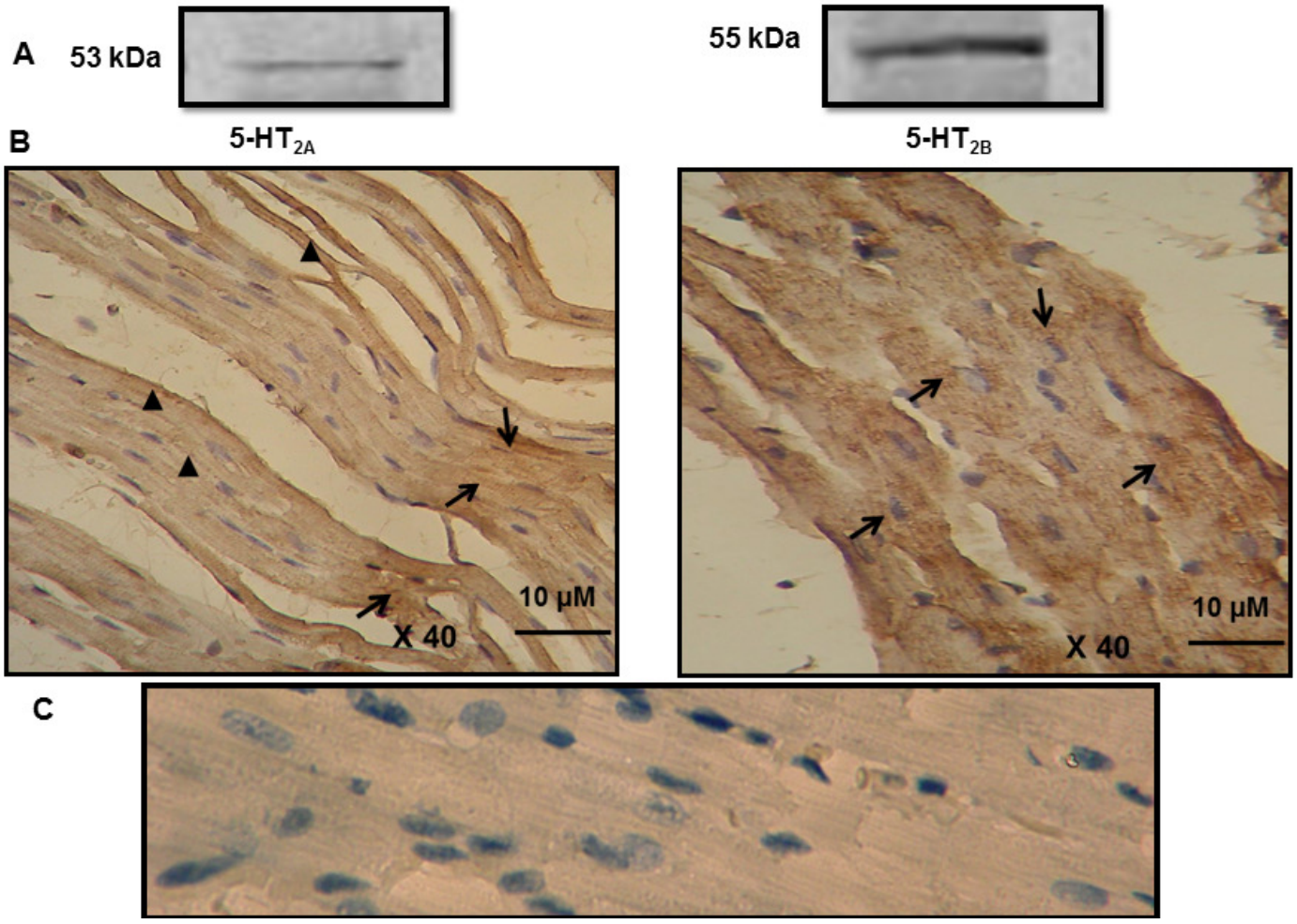

Figure 6A. Expression of 5-HT_{2A} and 5-HT_{2B} receptors in the heart of the adult rat by Western blot; immunoreactivity was detected with antibodies specific for each of the receptors. A 53-kDa band was observed for 5-HT_{2A} and another band of 55 kDa that corresponded to 5-HT_{2B}. (B) Photomicrographs of longitudinal cuts of the heart that show cardiomyocytes immunoreactive for the 5-HT_{2A} and 5-HT_{2B} receptor. Cuts were incubated with monoclonal antibodies specific for each (dilution 1:500) and immunoreactivity was detected with a secondary antibody conjugated to peroxidase and revealed with 3,3-diaminobenzidine. Arrows show immunoreactivity in conglomerates and around the nuclei. Arrowheads show immunopositivity located in the membranes. C) Negative control

DISCUSSION

It has currently been shown that 5-HT plays an important role in cardiac muscle contraction (Buccino, 1967; Naish, 1989; Qvisgstad et al., 2005; Ramage and Villalon, 2008*)* and in fetal heart development (Nebigil et al., 2000; Nebigil et al., 2001; Cöte et al., 2003; Sari and Zhou, 2003; Pavone et al., 2007; Brattelid et al., 2012). It has been observed that with replacement of the TPH1 gene by the β-galactosidase gene (Cöte et al., 2003) or ablation of the 5-HT_{2B} receptor, various cardiovascular defects are produced (Choi et al., 1997; Nebigil et al., 2001).Also, 5-HT participates in the pathophysiology of a variety of cardiac diseases of the adult heart (Robiolio et al., 1995; Connolly et al., 1997; Fitzgerald et al., 2000; Gustafsson et al., 2005; Elangbam et al., 2005; Qvisgstad et al., 2005*;* Mekontso et al., 2006; Levy, 2006; Brattelid et al.,2012). Recently, the presence of TPH-immunopositive cells during early cardiogenesis has been reported. The phenotype of these cells appears to correspond to mastocytes that probably synthesize 5-HT, suggesting that they participate in the differentiation of cardiomyocytes (Manjarrez et al., 2009). The presence of activity and expression of TPH in the fetal heart has also been shown (Manjarrez et al., 2009).

The purpose of this study was to characterize, both biochemically and immunohistochemically, an intrinsic serotoninergic system in rat heart. The results of the present study support the fact that cardiomyocytes have the molecular and biochemical apparatus for synthesis, utilization and reuptake of 5-HT. TPH activity was demonstrated as well as expressions of both isoforms. TPH1 showed a molecular mass of 51 kDa and TPH2 of 53 kDa. The presence of both isoforms, but interestingly

with predominance of expression of TPH2, was observed in comparison to THP1. This suggests the probability that two subtypes of cardiomyocytes may exist or that both isoforms are localized in the same cell, an aspect currently being investigated.

Synthesis and release of 5-HT to the interstitium is also suggested by our results taken up from blood circulation through SERT(Harrison, 2005; Mercado and Kilic, 2010). Therefore, free 5-HT in the cytosol of cardiomyocytes probably also unites to actin and myosin through transglutaminase activity and modifies its function, as has been demonstrated in platelets (Shirakawa et al., 2000; Walther et al., 2003; Mercado and Kilic, 2010). This indicates that intracellular 5-HT has functions independent from those produced by its interaction with serotoninergic receptors. Therefore, it may be proposed that 5-HT in the cytosol of cardiomyocytes modifies the contractile proteins (actin and myosin), thus participating in the autocrine and paracrine modulation of the heart muscular tone.

A key protein involved in the removal of plasma 5-HT is SERT and it is responsible for bioamine internalization (Levy, 2006; Pavone et al., 2007; Murphy and Lesch, 2008). It has been reported that SERT is expressed in cardiomyocytes during fetal life (Nebigil et al., 2000; Sari and Zhou, 2003; Pavone et al., 2007) and has been demonstrated only in endocardium and endothelium of the coronary and capillary arteries, suggesting that these cells have a high capacity for uptake of the circulating 5-HT for its degradation (Mekontso et al., 2006; Pavone et al., 2007). Interestingly, in this study the presence of SERT in cardiomyocytes is demonstrated (see Figure 4). These observations strongly suggest that, in addition to pulmonary and cardiac endothelial cells, cardiomyocytes have the ability to remove 5-HT from the blood through SERT and modulate the effects of 5-HT over heart activity. Expression of the serotoninergic receptors (5-HT_{1B}, 5-HT_{2A}, 5-HT_{2B} and 5-HT_4) in cardiomyocytes suggests that 5-HT exercises various direct effects on the heart. It has been shown that the 5-HT_4 receptoris implicated in control of electrical activity of cardiomyocytes and, with the 5-HT_{2A} receptor, participates in the regulation of the strength of ventricular contraction during acute heart failure (Qvisgstad et al.,2005*).* It has recently been demonstrated that the 5-HT_4 receptor also participates in the genetic programming of heart development and its reactivation during heart failure (Brattelid et al., 2012). It has also been observed that ablation of the 5-HT_{2B} receptor during cardiogenesis causes diverse structural and functional changes (Choi et al., 1997; Nebigil et al., 2001) and that its overexpression participates in the development of cardiac hypertrophy (Nebigil et al., 2003). With regard to the 5-HT_{1B} receptor, it has been detected in the subendocardial cells of heart valves where its stimulation causes fibroblastic proliferation (Seuwen et al., 1988), however, its role in heart development of valve fibrosis has not been demonstrated.

In conclusion, our findings demonstrate the existence of an intrinsic serotoninergic system in rat heart not described up to this point. The study provides evidence indicating that cardiomyocytes have the biochemical machinery capable of synthesis, utilization and reuptake of 5-HT, which supports the hypothesis of paracrine and autocrine functions of 5-HT along with better understanding of its participation in some pathophysiological aspects of the heart.

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