Expression of P2X₂ and P2X₃ receptors in the rat carotid sinus, aortic arch, vena cava, and heart, as well as petrosal and nodose ganglia

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Abstract With single- and double-labeling immunofluorescence techniques, the distribution patterns and morphological characteristics of P2X₂- and P2X₃-immunoreactive nerve fiber terminals and neuronal bodies have been studied in the main circulatory system baroreceptors and the nodose and petrosal ganglia of rats. A high density of P2X₂- and P2X₃-immunoreactive nerve fiber terminals was detected in the carotid sinus. P2X₂- and P2X₃-immunoreactive nerve fiber terminals were also distributed widely in the aortic arch, atrium, vena cava, and ventricles. Almost all the P2X₂-immunoreactive nerve fiber terminals were immunoreactive for P2X₃ receptors. P2X₂- and P2X₃-immunoreactive neuronal bodies were also detected in the nodose and petrosal ganglia, which are the sources of the P2X₂- and P2X₃-immunoreactive nerve terminals. P2X₂ and P2X₃ receptors were expressed in the same ganglionic neurons. These data indicate that extracellular ATP, via the homomeric P2X₂ and P2X₃ receptors, and heteromeric P2X_{2/3} receptor in the sensory receptors of carotid sinus, aortic arch, atrium, and

vena cava, may be involved in the regulation of systematic circulation blood pressure.

Keywords Purinoceptor · Baroreceptor · Immunohistochemistry · Rat

Introduction

Extracellular ATP has been identified as an excitatory neurotransmitter, neuromodulator, or humoral factor which acts via P2 purinoceptors [1]. P2 purinoceptors belong to two major families: a P2X family of ligand-gated ion channel receptors and a P2Y family of G protein-coupled receptors. Currently, seven P2X receptor subtypes (P2X_{1–7}) and eight P2Y receptor subtypes (P2Y_{1, 2, 4, 6, 11–14}) are recognized [1]. There are increasing data to show that P2X receptors are involved in the function of sensory nerves [2]. ATP is a neurotransmitter for taste reception cells in the taste buds, where it transduces the taste signals to the afferent taste nerves by activating P2X₃ receptors [3]. In the carotid body, ATP is a key transmitter released by chemoreceptor cells to activate P2X_{2/3} receptors on endings of the sinus nerve afferent fibers [4]. The intraganglionic laminar endings equipped with P2X₂ and P2X₃ receptors in the gastrointestinal tract act as mechanosensors [5, 6]. Mechanosensory nerve endings were also found in the wall of the atrium of the heart, vena cava, aortic arch, and carotid sinus, sensitive to stretching of the wall resulting from increased pressure from within and functioning as the receptor of central reflex mechanisms that tend to reduce that pressure [7]. Do these mechanosensory nerve endings in the circulatory system express P2X receptors?

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In the present study, the expression of P2X₂ and P2X₃ receptors in baroreceptors have been studied with immunohistochemistry and it was found that high levels of P2X₂ and P2X₃ receptors were expressed in these mechanosensory receptors in the carotid sinus, the heart, vena cava, and aortic arch.

Materials and methods

All experimental procedures were approved by the Institutional Animal Care and Use Committee at Second Military Medical University. Six rats (250–300 g) were used. The rats were killed by asphyxiation with CO₂ and perfused through the aorta with a 0.9% NaCl solution and 4% paraformaldehyde in 0.1 mol/L phosphate buffer pH 7.4. The carotid sinuses and petrosal ganglia were dissected out and immersed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.2) for 2–4 h. The petrosal ganglia were then transferred to 25% sucrose in PBS and kept in the solution until they sank to the bottom. Thereafter, the petrosal ganglia were rapidly frozen and were cut (20 µm in thickness) with a Leica cryostat and mounted on gelatin-coated slides. After fixation, the carotid sinuses were washed three times with PBS, then the superficial connective tissues of the carotid sinus adventia were discarded carefully and the rest of carotid sinuses were used as whole-mount preparations.

Immunohistochemistry for P2X_{1–6} receptors was performed by using rabbit polyclonal antibodies against the unique peptide sequences of the P2X receptor subtypes provided by Roche Bioscience, Palo Alto, CA. The specificity of the antisera was verified by immunoblotting with membrane preparations from CHO K1 cells expressing the cloned P2X receptors. As previously reported by Oglesby et al., no cross-reactivity is observed with other P2X antisera [8].

For the immunostaining of P2X receptors in the whole-mount preparation of the carotid sinuses, the preparations were incubated with the primary antibodies of P2X_{1–6} diluted 1:200 (rabbit anti-rat IgG, dilution, Roche Palo Alto, CA, USA) in the antiserum dilution solution overnight at 4°C. Subsequently, the preparations were incubated with Cy3-conjugated donkey anti-rabbit (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted 1:400 in antiserum dilution solution for 1 h at room temperature. All the incubations were separated by 3×5-min washes in PBS.

For double-immunostaining of P2X₃ receptors with the P2X₂ receptors, the sections of the ganglia and whole-mount preparation of carotid sinuses, after being pre-incubated in an antibody dilution solution for 30 min, were incubated with the P2X₂ receptor antibody (1:200

dilution, Roche Palo Alto) and P2X₃ receptor antibody (1:600 dilution, guinea pig anti-rat, Novus) overnight. The samples were subsequently incubated with FITC-conjugated donkey-anti-rabbit IgG (1:200 dilution, Jackson ImmunoResearch) for 1 h to visualize P2X₂ receptors, Cy3-conjugated donkey anti-guinea pig IgG (1:400 dilution) for 1 h to visualize P2X₃ receptors, respectively. All staining procedures were carried out at room temperature, and all the incubations were separated by three washes in PBS, 5 min each. Some sections were counter-stained with 5 µg/ml Hoechst 33342.

The control experiments were carried out with P2X antiserum absorbed with P2X peptides at a concentration of 25 µg/ml. The amino acid sequences of these peptides were synthesized by Roche Bioscience, Palo Alto. No staining was observed in those specimens incubated with the antibody solutions pre-absorbed with P2X peptides (Fig. 2f). A further negative control of omitting the primary antibody was also carried out.

Images were taken with the Nikon digital camera DXM1200 (Nikon, Japan) attached to a Nikon Eclipse E600 microscope (Nikon). Images were imported into a graphics package (Adobe Photoshop). The two-channel readings for green and red fluorescence were merged by using Adobe Photoshop. The focal plane on the microscope was not adjusted whilst determining whether a particular cell or fiber co-localized both P2X₂ receptor and P2X₃ receptor. Only neurons or fibers that demonstrated the same morphology, orientation and position when viewed under the two different filters (in the same focal plane) for the detection of Cy3 and FITC were deemed to co-localize both P2X₂ receptor and P2X₃ receptor.

The whole-mount preparations immunostained with P2X₂ or P2X₃ receptor antibodies were used to perform a quantitative analysis of the sensory terminal density. Briefly, positively immunostained sensory terminals in the whole-mount preparations were counted per visual field (area of 0.36 mm²). Two to three randomly chosen fields in each whole-mount preparation and one to two preparations of each rat were analyzed. At least ten rats were used for P2X₂ or P2X₃ receptors. The density of P2X₂ or P2X₃ receptor staining at the terminals was calculated and expressed as mean±standard error of the mean. As all terminals with P2X₂ receptor immunoreactivity were also labeled with P2X₃ receptor immunoreactivity, the data are expressed as average values from both receptor subtypes.

Results

P2X₂ and P2X₃ receptor immunoreactivity (ir) occurred in clusters of punctiform and lamellar structures at the surfaces of nerve terminals in the carotid sinus, aortic arch,

Fig. 1 Expression of P2X₂ and P2X₃ receptors in the carotid sinus and aortic arch of the rat. **a** P2X₂ receptor-ir in sensory terminals at high magnification in the carotid sinus. An *arrow* shows a lamellae structure with strong P2X₂ receptor-ir, which is a typical sensory terminal. **b** P2X₃ receptor-ir in sensory terminals in the carotid sinus. **c**, **d** P2X₂ and P2X₃ receptor-ir in terminals in the aortic arch (*Ar*), respectively. Scale bars=50 μ m in (**a**) and (**b**) and 200 μ m in (**c**) and (**d**)

Fig. 2 Expression of P2X₂ and P2X₃ receptors in the right atrium of the rat heart, vena cava, ventricles, and in the control experiments. **a** P2X₂ receptor-ir in sensory terminals in the right atrium (*Atr*). **b** P2X₃ receptor-ir in sensory terminals in the inferior vena cava (*IVC*). **c** P2X₂ receptor-ir in sensory terminals in the superior vena cava (*SVC*). An *arrow* shows a flower-like structure with strong P2X₂ receptor-ir. **d**, **e** P2X₃ and P2X₂ receptor-ir in terminals in the right and left ventricles, respectively. **f** The result from a control experiment carried out with the P2X₃ antiserum pre-absorbed with P2X₃ peptide; note that no staining was observed in the control specimen. Scale bars=200 μ m in (**a**–**f**)

Table 1 The density and size of the sensory terminals with P2X₂ or P2X₃ receptor immunoreactivity in the carotid sinus, aortic arch, atrium, vena cava, and ventricles of the rat

Regions	Density (0.36 mm ²)	Size (μm)
Carotid sinus	42±11	120±30
Aortic arch	36±15	65±25
Atrium	12±7	61±16
Vena cava	8±4	58±20
Ventricle	3±2	39±18

atrium, vena cava, and ventricles (Figs. 1 and 2). They varied considerably in size, from punctiform structures that were 1–2-μm across and were, thus, similar to the varicosities of peripheral autonomic axons, to lamellae that were as much as 20 μm across (Figs. 1 and 2). Most lamellae were about 3–5 μm across. Lamellae had irregular shapes. The lamellae occurred in clusters that covered regions of carotid sinus, aortic arch, atrium, vena cava, and

ventricles, leaving other parts of these regions uncovered. The numerous axon terminals were organized into a well-defined discoid end organ, referred to as the ‘baroreceptor unit’. Baroreceptor units measure 100 to 150 μm in diameter [9].

In the carotid sinus, the density, and size of the sensory terminals with P2X₂ or P2X₃ receptor-ir were high or larger than that in other regions examined in this study, such as aortic arch, atrium, vena cava (inferior and superior), and right and left ventricles (Figs. 1 and 2). In the aortic arch, the size of the sensory terminals with P2X₂ or P2X₃ receptor-ir was much less than that in the carotid sinus, although the density was similar with that in the carotid sinus (Fig. 1c, d). In the right atrium (Fig. 2a), the density of the sensory terminals with P2X₂ or P2X₃ (not shown) receptor-ir was lower than that in the carotid sinus and aortic arch (see Fig. 1), but higher than that in the inferior and superior vena cava and right and left ventricles (Figs. 2a and 4d–f). In the vena cava (inferior and superior), the density of these units was lower than that in the carotid

Fig. 3 Coexistence of P2X₂ and P2X₃ receptors in the carotid sinus (CS) and inferior vena cava (IVC) of the rat. **a, b** P2X₃ receptor-ir (red) and P2X₂ receptor-ir (green) in sensory terminals in the CS, respectively. **c** The merged image from (a) and (b). Note that almost all the P2X₂ receptor-immunoreactive terminals (green) were also immunostained by the P2X₃ receptor antibody (yellow/orange). **d, e** P2X₃ receptor-ir (red) and P2X₂ receptor-ir (green) in sensory terminals in the IVC, respectively. **f** The merged image from (d) and (e). Note that all the P2X₂ receptor-immunoreactive terminals (green) were also immunostained by the P2X₃ receptor antibody (yellow/orange). All the scale bars in (a–f)=100 μm

Fig. 4 Coexistence of P2X₂ and P2X₃ receptors in the aortic arch (*Ar*) and right atrium (*Atr*) of the rat. **a, b** P2X₃ receptor-ir (*red*) and P2X₂ receptor-ir (*green*) in sensory terminals in the *Ar*, respectively. **c** The merged image from (**a**) and (**b**). Note that almost all the P2X₂ receptor-immunoreactive terminals (*green*) were also immunostained by the P2X₃ receptor antibody (*yellow/orange*). **d, e** P2X₃ receptor-ir (*red*) and P2X₂ receptor-ir (*green*) in sensory terminals in the *Atr*, respectively. **f** The merged image from (**d**) and (**e**). Note that almost all the P2X₂ receptor-immunoreactive terminals (*green*) were also immunostained by the P2X₃ receptor antibody (*yellow/orange*). All the scale bars in (**a–f**)=100 μ m

sinus, aortic arch, and atrium but higher than that in the left and right ventricles (Fig. 2b, c). The average density and size of the baroreceptor units in the regions examined are summarized in Table 1.

In the controls experiments, no immunostaining was observed in those specimens incubated with the antibody solutions pre-absorbed with P2X₂ or P2X₃ peptides, as shown in the carotid sinus (Fig. 2f). No immunostaining was also observed in a further negative control experiment of omitting the primary antibody.

Double immunofluorescence technique showed that almost all the P2X₂ receptor-positive terminals were labeled with P2X₃ receptor-ir and vice versa in all regions examined, as shown in the carotid sinus, aortic arch, atrium, superior and inferior vena cava, and right ventricle (Figs. 3, 4, and 5). Double immunofluorescence technique was also used to detect coexistence of P2X₂ and P2X₃ receptor-ir in the petrosal and nodose ganglia, which are believed to innervate the regions examined in this study. These results show that almost all the P2X₂ receptor-immunoreactive neurons and

fibers were also labeled with P2X₃ receptor-ir, although some P2X₃ receptor-immunoreactive neurons were not labeled with P2X₂ receptor-ir, as shown in the petrosal ganglion (Fig. 6).

Discussion

In the present study, the distribution patterns and morphological characteristics of P2X₂- and P2X₃-immunoreactive nerve fiber terminals and neuron cell bodies have been studied in the main baroreceptor circulation system and in the nodose and petrosal ganglia of rat. The results showed that P2X₂- and P2X₃-immunoreactive nerve fiber terminals are distributed widely in the carotid sinus, aortic arch, atrium, vena cava, and ventricles, as well as in neurons in the petrosal and nodose ganglia. A high density of P2X₂- and P2X₃-immunoreactive nerve fiber terminals was detected, particularly in the carotid sinus. Almost all the P2X₂-immunoreactive nerve fiber terminals were also

Fig. 5 Coexistence of P2X₂ and P2X₃ receptors in the superior vena cava (SVC) and right ventricle (RV) of the rat. **a, b** P2X₃ receptor-ir (red) and P2X₂ receptor-ir (green) in sensory terminals in the SVC, respectively. **c** The merged image from (**a**) and (**b**). Note that almost all the P2X₂ receptor-immunoreactive terminals (green) were also immunostained by the P2X₃ receptor antibody (yellow/orange). **d, e** P2X₃ receptor-ir (red) and P2X₂ receptor-ir (green) in sensory terminals in the RV, respectively. **f** The merged image from (**d**) and (**e**). Note that all the P2X₂ receptor-immunoreactive terminals (green) were also immunostained by the P2X₃ receptor antibody (yellow/orange). All the scale bars in (**a–f**)=100 μ m

labeled with P2X₃ receptor-ir. It is not clear why some P2X₂-immunoreactive nerve fiber terminals are not also immunoreactive for the P2X₃ receptor, but we can speculate: (1) that the neurons only labeled with P2X₂ receptor-ir do not send processes to this system; (2) that the cell bodies of these neurons contain too low a concentration of P2X₃ receptor protein to be seen, but their terminals contain relatively high concentrations.

There has been increasing interest in the role of ATP via P2X purinoceptors in peripheral sensory transduction. ATP activates sensory neurons via P2X receptors, a family of ligand-gated ion channels [10, 11]. Two P2X subunits, P2X₂ and P2X₃, forming either homomeric P2X₂ and P2X₃ or heteromeric P2X_{2/3} receptors, mediate the rapid excitation of sensory neurons by ATP [12–14]. P2X₃ is critical for peripheral pain responses and afferent pathways controlling urinary bladder voiding reflexes [15]. The P2X₂ subunit or heteromeric P2X_{2/3} receptors play a pivotal role in carotid body function and in mediating ventilatory responses to hypoxia; in this process, ATP is a key neurotransmitter released by chemoreceptor cells

of the carotid body to activate endings of the sinus nerve afferent fibers [4]. ATP via P2X_{2/3} receptors links taste buds to the nervous system and acts as the key neurotransmitter between taste receptor cells and sensory terminals [16]. The sensory functions mentioned above are involved in the transduction mechanism of ATP via P2X₂, P2X₃, or P2X_{2/3} receptors. The present results are consistent with the view that this transduction mechanism also exists in rat sensory terminals of the cardiovascular system. Recently, Liang's lab reported that the P2X₃ receptor could be thought of as a new target for treating myocardial ischemic injury and cardiac arrhythmia and inhibiting nociceptive transmission of myocardial ischemic injury [17]. P2X₂ and P2X₃ receptor-immunoreactive terminals in the ventricles, as demonstrated by the present study, may be involved in the nociceptive transmission of myocardial ischemic injury.

It is well known that the main baroreceptors in the circulation system are in the carotid sinus and aortic arch, which cause reflex bradycardia and a reduction in systemic vascular resistance following stimulation [18, 19]. These

Fig. 6 Coexistence of P2X₂ and P2X₃ receptors in the petrosal ganglion (PG) and nodose ganglion (NG) of the rat. **a, b** P2X₃ receptor-ir (red) and P2X₂ receptor-ir (green) in neuronal bodies and processes in the PG, respectively. **c** The merged image from (**a**) and (**b**). Note that almost all the P2X₂ receptor-immunoreactive terminals (green) were also immunostained by the P2X₃ receptor antibody, although a small proportion of P2X₃ receptor-immunoreactive neuronal bodies were not labeled by the P2X₂ receptor antibody. An arrow shows a double-labeled neuron (yellow). **d, e** P2X₃ receptor-ir (red) and P2X₂ receptor-ir (green) in neuronal bodies and processes in the NG, respectively. **f** The merged image from (**d**) and (**e**). Note that majority of the P2X₂ receptor-immunoreactive terminals (green) were also immunostained by the P2X₃ receptor antibody. An arrow shows a double-labeled neuron (yellow). All the scale bars in (**a–f**)=100 μ m

two baroreceptors are innervated by the vagus nerve and the sinus nerve (the branch of the glossopharyngeal nerve), with afferent fibers from the nodose ganglion and petrosal ganglion, respectively. With immunofluorescence technique we found that P2X₂, P2X₃, or P2X_{2/3} receptors are expressed in the afferent terminals of carotid sinus and aortic arch baroreceptors and sensory neuronal bodies of nodose and petrosal ganglia. These data imply that ATP acts on P2X receptors and acts as a neurotransmitter, involved in the regulation of blood pressure.

It is likely that ATP is a signaling molecule in the carotid sinus and aortic arch. There are reports that indirectly support this view. For example, ATP, that acts on P2X₃ and P2X_{2/3} receptors on sensory nerve endings, is released by mechanical distortion from urothelial cells during distension of bladder and ureter and from mucosal epithelial cells during distension of the colorectum [2]. ATP is also probably released from odontoblasts in tooth pulp [20], from epithelial cells in the tongue taste buds [16], from the neighboring epithelial supporting cells or the olfactory

neurons of olfactory epithelium [21] and from glomus cells in the carotid body [22]. As the mouse sensory terminals are localized in the adventitia and the medio-adventitial border [9], the possible candidates that might be releasing ATP in the carotid sinus and aortic arch of the mouse might be fibroblast cells, smooth muscle cells, even Schwann cells, as the majority of the baroreceptor terminal surfaces were wrapped up by the plasma of the Schwann cell [9].

In conclusion, using single- and double-labeling immunofluorescence techniques, the present study demonstrated that P2X₂- and P2X₃-immunoreactive nerve fiber terminals are widely distributed in the carotid sinus, aortic arch, atrium, vena cava and ventricle, as well as in neurons in the nodose, and petrosal ganglia of rats. P2X₂- and P2X₃-immunoreactive nerve fiber terminals, with a high density, were detected, particularly in the carotid sinus. Almost all of the P2X₂-immunoreactive nerve fiber terminals in the regions we examined were also immunoreactive for P2X₃ receptors and vice versa. These data suggest that extracellular ATP, via the homomeric P2X₂ and P2X₃ receptors and heteromeric

P2X_{2/3} receptors in the carotid sinus, aortic arch, atrium, and vena cava, may be involved in the regulation of systematic circulation blood pressure.

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