Differential gene expression patterns and colocalization of ATP-gated P2X6/P2X4 ion channels during rat small intestine ontogeny

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Abstract
Gene coding for ATP-gated receptor ion channels (P2X1–7) has been associated with the developmental process in various tissues; among these ion channel subtypes, P2X6 acts as a physiological regulator of P2X4 receptor functions when the two receptors form heteroreceptors. The P2X4 receptor is involved in pain sensation, the inflammatory process, and body homeostasis by means of Mg(2+) absorption through the intestine. The small intestine is responsible for the absorption and digestion of nutrients; throughout its development, several gene expressions are induced that are related to nutrients received, metabolism, and other intestine functions. Previous work has shown a differential P2X4 and P2X6 protein distribution in the small intestine of newborn and adult rats; however, it is not well-known at what age the change in the relationship between the gene and protein expression occurs and whether or not these receptors are colocalized. In this work, we evaluate P2X4 and P2X6 gene expression patterns by qPCR from embryonic (E18, P0, P7, P17, P30) to adult age in rat gut, as well as P2X6/P2X4 colocalization using qRT-PCR and confocal immunofluorescence in proximal and distal small intestine sections. The results showed that P2X6 and P2X4 gene expression levels of both receptors decreased at the embryonic-perinatal transition, whereas from ages P17 to P30 (suckling-weaning transition) both receptors increased their gene expression levels. Furthermore, P2X4 and P2X6 proteins were expressed in a different way during rat small intestine development, showing a higher colocalization coefficient at age P30 in both intestine regions. Those results suggest that purinergic receptors may play a role in intestinal maturation, which is associated with age and intestinal region.

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1. Introduction

The gene expression of the ATP-gated receptor ion channels known as P2X purinergic receptors is important in several cell functions. These receptors were first proposed in the 1970s by Professor Geoffrey Burnstock. P2X receptors respond to extracellular adenosine 5'-triphosphate (ATP) and mediate permeability to small monovalent cations and Ca2+. ATP is more than an intracellular energy carrier; it is a neurotransmitter, cotransmitter, or neuromodulator that binds to ligand-gated P2 receptors and regulates diverse cellular activities under physiological conditions such as development and aging (Burnstock, 2012; Rong et al., 2009; Xiang and Burnstock, 2005). In addition, P2X receptors are expressed by neuronal and non-neuronal systems (Yu et al., 2010; Brandle et al., 1999). Sensory nerve endings in the gastrointestinal tract express P2X receptors with key roles in synaptic transmission of the enteric nervous system (ENS) and these receptors are involved in the control of gastrointestinal motility, mucosal secretion, and absorption (Galligan, 2004; Burnstock, 2014; Loera-Valencia et al., 2014).

There is growing evidence to suggest that P2X receptors play important roles in the developmental process. Cheung et al. (2005) demonstrated that there is a differential expression of P2X receptors, including the P2X6 receptor, during rat brain development. Later studies showed changes in the relaxant response mediated by multiple P2 receptor subtypes on the longitudinal muscle of the...
mouse gastrointestinal tract during postnatal development (Giaroni et al., 2006). Interestingly, purinergic signaling is gaining recognition in cell differentiation processes such as neuronal and fiber cell differentiation (Glaser et al., 2013; Suzuki-Kerr et al., 2008).

P2X receptors are homo- or heterotrimeric complexes produced by an assembly of the seven cloned P2X receptor subunits (P2X1−7) (Torres et al., 1999). The P2X6 subtype is unable to form functional homomeric receptors, but readily forms heteromers with P2X2 and P2X4, producing receptors with properties different from the original homomeric receptors (Antonio et al., 2014; North, 2002). P2X6 receptors contribute to the ATP sensitivity of heterotrimeric channels and in this way regulate the physiological function of P2X2 and P2X4 subtypes (Le et al., 1998; King et al., 2000). It has been reported that subunits P2X4 and P2X6 are coexpressed in many areas of the central nervous system and in other parts external thereto, such as vascular endothelial cells and renal epithelial duct cells (Kukley et al., 2001; Glass and Burnstock, 2001; Turner et al., 2003).

Gene expression of the P2X4 subtype is important because it is involved in the sensation of pain and inflammation, which has been of particular interest in the medical community as it targets for anti-inflammation and analgesic therapies (Young, 2010). Interestingly, purinergic signaling is gaining importance due to early life events that could affect the responsiveness of the intestine in later life and their modulation by P2X receptors (Drozdowski et al., 2010; Burnstock, 2014) associated with evidence of dietary changes and in particular obesity (Misawa et al., 2010; Mizuno et al., 2014). Interestingly, from a physiological point of view, gene expression during gut ontogeny is important for the adult intestine due to early life events that could affect the responsiveness of the intestine in later life and their modulation by P2X receptors (Drozdowski et al., 2010; Burnstock, 2014) associated with evidence of dietary changes.

The aim of this work was to determine whether or not gene expression and colocalization of P2X4 and P2X6 could be influenced during the time periods in which the most important feeding changes occur in rat small intestine development.

2. Results

2.1. P2X6 and P2X4 transcript levels oscillate during rat gut development

The relative mRNA levels of P2X6 and P2X4 transcript levels of the whole intestine were measured using quantitative RT-PCR (qRT-PCR) and the housekeeping gene β-actin as a reference. E18 (embryonic day 18) intestine was evaluated also in order to determine whether or not the transcription of these genes is present since embryonic age. The results indicated that P2X6 and P2X4 transcripts were differentially expressed during ontogeny and the changes were significant (ANOVA GLM P < 0.05) compared with other ages and sections studied (Fig. 1). In addition, both P2X6 and P2X4 expression were greater in the proximal region (ANOVA GLM P < 0.05) than in the distal region of the rat small intestine. Expression of the P2X6 gene was seen since the embryonic stage (E18); nevertheless, it decreased after the birth of the rat (P0) and increased at age P7, remaining constant until age P30. Finally, at adult age, P2X6 gene expression was downregulated and expressed at levels similar to those of P0 (p > 0.05). This behavior was observed in the distal region (Fig. 1A–B). By comparison, both P2X4 and P2X6 transcripts were detected at age E18, decreasing at age P0, but from ages P7 to P30, P2X4 transcript levels increased and then decreased at adult age. These changes were observed in both gut regions (Fig. 1).

2.2. Confocal microscopy enabled visualization of P2X6 and P2X4 protein distribution in the proximal and distal intestine regions during rat gut development

The results showed intense immunoreactivity of the P2X6 receptor subunit in the enteric vessels, while the P2X4 receptor subunit was distributed in the endothelial cells at age P0 (Fig. 2A). Nevertheless, at age P30 both receptor subunits were detected again with a similar localization in the enteric vessels (Fig. 2D); this receptor expression was also observed at adult age for both P2X6 and P2X4 receptor subunits (Fig. 2E). Fig. 3 shows that P2X6 immunoreactivity was not detected in distal rat intestine at ages P0
Fig. 2. Distribution of P2X6 and P2X4 receptors in the proximal intestine region during rat guts development. Mucosa of proximal intestine sections were double-labeled, anti-P2X6 (in green) and anti-P2X4 (in red) antibodies. The merge of these receptors is displayed in panel 3, together with the corresponding light image (T-PMT). (A) Figure displays the absence of both, P2X4 and P2X6 subunits at embryo age. (B) Shows the presence of P2X6 subunit in the enteric vessels (EV) and in the endothelial cells (ECs) for P2X4 subunit at age P0. (C) Rat intestine proximal section at age P7. (D) P17 proximal rat intestine section. P2X6 and P2X4 receptor immunoreactivity was not detected for P7 and P17 ages. (E) Rat intestine proximal section at age P30. (F) Adult rat intestine proximal section. P30 and adult age exhibit slight immunoreactivity for P2X6 and P2X4 in enteric vessels.
Fig. 3. P2X6 and P2X4 protein distribution in distal intestine section during rat gut development. Dual-labeling with anti-P2X6 (in green) and anti-P2X4 (in red) antibodies was performed in the mucosa of distal intestine sections. The images were merged together with a bright field image, which was taken by a photomultiplier of transmitted light (T-PMT).

(A) Figure shows P2X6 and P2X4 immunoreactivity absence at E18 age. (B) Only P2X4 receptor was expressed at age P0 in the endothelial cells (ECs). (C) At age P7 P2X4 receptor was still expressed in ECs. (D) Figure displays P2X6 and P2X4 immunoreactivity absence at P17 age. (E) Shows moderate immunoreactivity for P2X6 and P2X4 receptors in the enteric vessels at P30. (F) This immunoreactivity increased at adulthood.
and P7, while P2X4 immunoreactivity was detected in endothelial cells at both ages (Fig. 3A–B), although P17 had no P2X6 and P2X4 immunoreactivity (data not shown). At P30 (Fig. 3C), as well as adult age, both receptors were found in the enteric vessel (Fig. 3D).

2.3. Densitometry and a coexpression study indicated different expression patterns for P2X6 and P2X4

Densitometry analyses of the proximal gut region exhibited the highest P2X6 and P2X4 immunoreactivity at P0; then, at P7 and P17 protein expression was absent, but appeared again at P30 lower by ~25% (p < 0.05) compared to P0. Significant differences were not found (p > 0.05) between P30 and adult age for P2X6 protein expression. Furthermore, P2X4 immunoreactivity was significantly lower (p < 0.05) compared to P2X6 at P0 and P30, whereas at adult age it was not statistically different (Fig. 4A). P2X6 immunoreactivity in the distal gut region was only detected at P30 and adult age, without statistical difference (p > 0.05). P2X4 protein expression was detected from P0 onwards, showing a significant increase (p < 0.05) with age: 20% at P7, 32% at P30, and 40% at adult age. At adult age, P2X4 immunoreactivity levels were not statistically different (p > 0.05) compared to the P2X6 subunit (Fig. 4B).

Co-expression studies were performed using the lambda mode of confocal fluorescence microscopy, in order to avoid cross-talk between secondary antibodies. The results indicated a weak co-expression of P2X6 and P2X4 in the mucosa of rat small intestine. For the proximal region at P0 (Fig. 4C) we found statistical differences (p < 0.05) between receptor colocalization percentages, with a higher P2X6 with P2X4 (12%) compared to P2X4 with P2X6 (5%). Interestingly, at P30, P2X6 and P2X4 receptor colocalization increased compared to P0 in both intestine regions. In the proximal gut region, the P2X6 and P2X4 receptor colocalization did not show a statistical difference (p > 0.05) between the percentages (30% P2X6 with P2X4 colocalization and 39% P2X4 with P2X6), while in the distal region a statistical difference (p < 0.05) was observed (15% P2X6 with P2X4 and 30% P2X4 with P2X6). Finally, at adult age P2X6 with P2X4 receptor colocalization decreased (p < 0.05) by 10% compared to P30. In the proximal region, no statistical difference was observed (p > 0.05) between P2X6 with P2X4 and P2X4 with P2X6. However, in distal regions P2X6 with P2X4 colocalization increased by 40%, while P2X4 with P2X6 colocalization significantly decreased (p < 0.05) by 25% compared to P30 (Fig. 4C).

3. Discussion

We found high levels of P2X6 and P2X4 gene expression at embryonic age and then a decrease at age P0 as well as at adult age. The results are consistent with those observed in brain by Da Silva. The high levels of P2X6 gene expression could be associated with a possible role in ENS differentiation, due to its role in intracellular calcium mobilization (Glaser et al., 2013). Furthermore, Hao et al. (2011) reported that mouse ENS responds to ATP since embryonic ages. In addition, at P0, P2X6 and P2X4 gene expression decreased compared to E18 and this is consistent with the expression observed in other tissues, such as human bladder (O’Reilly et al., 2001) and rat liver (Xiang et al., 2006). In addition, the high P2X6 and P2X4 protein expression in the proximal region, as well as the weak colocalization, may be associated with possible P2X4 homomers, whereas the high levels of P2X6 gene expression could be associated with other purinergic subunits forming hetero-receptors, e.g. P2X2 as reported by Schwindt et al. (2011) in neurosphere cultures.

P2X6 protein expression was absent in the distal region, which suggests a differentiated physiological action dependent on bowel region. In the distal gut region, P2X4 may regulate sodium transport in the mucosa of rat small intestine (Wildman et al., 2005),

Fig. 4. Developmental changes of P2X6 and P2X4 protein expression and colocalization in mucosa of rat small intestine. (A) Densitometric analysis of P2X6 and P2X4 immunolabeling of proximal intestine section. (B) Densitometric analysis of P2X6 and P2X4 immunolabeling of distal intestine section. (C) Colocalization study of P2X6 receptor immunoreactivity with P2X4 receptor in the mucosa of proximal intestine section. (D) Colocalization study of P2X6 receptor immunoreactivity with P2X4 receptor in the mucosa of distal intestine section. ND, not detected. *P < 0.05, ns, not significant, letters denote values that are statistically different, two-way ANOVA followed by a Tukey’s multiple comparison test. The bars represent mean ± SEM (standard error). Co-expression studies were performed using the lambda method (see Image analysis).
while P2X6 may be associated with neuronal differentiation in the ENS (Glaser et al., 2013). Finally, previous works have reported a disparity in distribution of P2X6 and P2X4 in other rat tissues such as the thyroid and retina (Suzuki-Kerr et al., 2008; Glass and Burnstock, 2001).

P2X6 and P2X4 gene expression increases at P7 and P17 compared to P0; this behavior has also been observed during brain development (Cheung et al., 2005). Nevertheless, P2X6 protein expression was not detected in the intestinal mucosa of either region. Therefore, the P2X6 receptor could be only expressed in the ENS at those ages as the P2X3 subunit in rat gastric myenteric neurons, which appears at P1 and shows maximal protein expression at P14 (Xiang and Burnstock, 2005). It is important to note that the gene expression results were obtained from the whole rat intestine, while protein expression was only evaluated in the intestinal mucosa. Taken together, these results may suggest a possible role of these receptors in postnatal neurogenesis in both CNS and ENS. Moreover, the absence of P2X4 and P2X6 protein expression, despite the high gene expression, could also be explained by the mRNAs not always being translated to protein due to cellular mechanisms such as storage and degradation (Lee et al., 2007). Additionally, discrepancies between gene expression and protein function for P2X subunits has been reported in dorsal root ganglion (DRG) neurons (Kobayashi et al., 2005; Hamilton and McMahon, 2000).

Interestingly, at P30 we found high levels of P2X6 and P2X4 gene expression in the whole small intestine, along with the highest protein colocalization in the intestinal mucosa of both receptors in the proximal gut region. Thus, at P30, P2X6 and P2X4 could be forming P2X6/P2X4 heteroreceptors in order to regulate intestinal contraction due to changes in the food composition, from milk to solid food after weaning age (P21) as observed by Giaroni et al. (2006) regarding P2Y1 purinergic receptor functionality. In the distal intestine region, a decrease of P2X6 with P2X4 colocalization was observed compared to the proximal region; this may be associated with the possibility that P2X6 forms heteromereric receptors with other subunits as a possible function of differential distribution (Drozdowski et al., 2010).

Finally, at adult age, P2X6 and P2X4 gene expression was reduced compared to P30, and did not show a statistical difference (p > 0.05) compared to P0, in agreement with previous results observed in guinea pig gut (Loera-Valencia et al., 2014). Despite the low P2X6 and P2X4 transcript levels in the whole small intestine, we found high P2X6 and P2X4 protein expression in intestinal mucosa in both intestine regions, and we also observed P2X6 and P2X4 colocalization. Therefore, it is possible that at early ages, the P2X6 regulatory subunit is involved in ENS differentiation processes, while at P30 and adult age the functions of the intestinal mucosa may be associated with dietary changes.

P2X6 and P2X4 purinergic receptors may be regulated by dietary changes through two possible mechanisms: 1) epigenetic mechanisms such as histone acetylation or histone methylation (Inoue et al., 2015); 2) the potential role of P2X6 and P2X4 purinergic receptors in the functional development of intestinal enzyme activity or transporters. For example, it has been reported that milk composition could modify lactase-phlorizin hydrodase (LPH) activity and mRNA abundance in human small intestine. Interestingly, LPH activity has a proximal-to-distal gradient along the small intestine (Drozdowski et al., 2010). Perhaps for that reason P2X4 and P2X6 receptors have a differential distribution dependent on intestine region due to differential LPH activity. The regulation of enzyme activity induced by purinergic receptors may be due to Ca(2+) entry activation of Ca(2+)-/calmodulin-dependent protein kinases (CaMKs), which constitute an important molecular mechanism for controlling intestinal motility (Gao et al., 2012).

In addition, other neurotransmitter receptors such as serotonin receptors 5-HT2C and 5-HT5A have shown modulation in their protein expression by dietary changes (Lopez-Esparza et al., 2015).

The results of this study indicate differential P2X6 and P2X4 gene expression and protein and colocalization patterns in the small intestine in developmental stages, associated with changes such as neuronal differentiation and suckling-weaning transition. Taken together, our results suggest a possible role played by purinergic receptors intestine metabolism associated with age and intestinal region, as well as a possible modulation of the expression of these receptors by feeding changes during the development of rat small intestine regions.

4. Experimental procedures

4.1. Animals

Adult Sprague-Dawley rats were given free access to food and water, and were housed under a light cycle with 12 h of darkness. The newborn rat pups were kept with their mothers for hours and then killed by decapitation and the intestines were dissected and divided into proximal and distal regions. The P17, P30, and adult rats were anesthetized with sodium pentobarbital (40 mg/kg, IP). As soon as they were unresponsive to nociceptive stimuli, they were killed by cervical dislocation. The small intestine was quickly removed and divided into proximal (near to stomach) and distal regions; then the tissues were washed in cold phosphate-buffered saline (PBS), and frozen immediately in liquid nitrogen and stored at −80 °C until the experiments were performed. Tissues were obtained from embryonic day 18 (E18) and postnatal rats on day 0, 7, 17, 30 (P0–P30), and adult rats. International guidelines for the care and use of animals were followed in accordance with the Guidelines for Animal Experimentation of Laboratory Animal Science.

4.2. Real-time RT-PCR

A portion (100 mg) of the small intestine for each age was used for the total RNA extraction. Total RNA was isolated using a High Pure RNA Tissue Kit (Roche, Mannheim, Germany) according to the manufacturer’s protocol. To remove potential genomic DNA contamination, the samples were treated with DNase I amplification grade (Invitrogen, USA). Total RNA was quantified using a Nano-Drop spectrophotometer (ND-1000) and then 1 μg of total RNA was subjected to reverse transcription employing a Transcriptor First Strand cDNA Synthesis Kit (Roche, Mannheim, Germany), using an oligo dT primer (Roche, Mannheim, Germany) according to the manufacturer’s protocol. P2X6 receptor and the housekeeping gene β-actin cDNAs (2 μl of each reaction) were amplified by real-time PCR and were performed in triplicate for each sample using 2 μl of cDNA reaction in the Light Cycler system (Roche, Mannheim, Germany) using the LightCycler FastStart DNA Master SYBR Green 1 (Roche, Mannheim, Germany). The primer sequences were as follows: rat P2X6: forward primer 5’TGA-CACCAGCTCAAGTCCAG-3′ and reverse primer 5’-CACCATGTACGTAGCAAG-3′ (Gen Bank Accession No. NM_012721.2); rat P2X4: forward primer 5’TATGGCTACTGCGTCTGTCA-3′ and reverse primer 5’-GAGAGGGAGGAGGATCTTG-3′ (Gen Bank Accession U470311); rat β-actin: forward primer 5’-AGCCATGGTACGTCGCTGTC-3′ and reverse primer 5’-TCTCAGCTGTGGTGGTGAA-3′ (Gen Bank Accession No. NM_031144.2). Amplification conditions were as follows: pre-activation at 95 °C for 15 min, denaturation at 95 °C 5 s, annealing at 63°C 5 s, and extension at 72°C 9 s for 40 cycles.
4.3. Immunofluorescence

In order to assess developmental trends in the distribution of the P2X6 receptor, proximal and distal sections were collected from E18, P0, P7, P17, P30 and adult rat small intestine. Tissues were fixed in 4% paraformaldehyde at 4 °C, cryoprotected in 30% sucrose and embedded in Tissue-Tek at 80 °C until cryosectioning. Sections (12 μM) were cut using a cryostat (Leica CM 1850, USA) and collected on slides; finally, samples were stored at −20 °C. The slide preparations were washed three times for 10 min in PBS, and then incubated for 30 min at room temperature in blocking solution (3% w/v bovine serum albumin and 2 mg/ml poly-L-lysine in PBS with 0.5% Triton X-100, PBT) to reduce non-specific binding, followed by incubation with P2X6 produced in goat and P2X4 produced in donkey-anti-rabbit IgG (Invitrogen, USA), diluted 1:1000 in PBT for 4.4. Image analysis

Slides per rat were quantified (viii of the small intestine mucosa) by the ROI method (Region Of Interest) of the ZEN software. The mean was used to represent each rat and three rats were quantified.

4.5. Statistical analysis

Densitometry and co-expression studies were analyzed by two-way ANOVA and a post-hoc Tukey’s test at a 95% confidence level. For the analysis of the qRT-PCR data, a full factorial design was employed. Four factors were used: The age of the rat (E18, P0, P7, P17, P30, adult), the region of the small intestine (distal, proximal), replication number, and the number of the rat. A test of residual normality was conducted using the Kolmogorov-Smirnov statistic at a confidence level of 95%. Subsequently, the general linear model was used to determine the significance of the variables. All tests were performed randomly in triplicate. The statistical package Minitab 16® was used to perform the analysis.

Conflict of interest

The authors declare that they have no conflicts of interest.

Ethical approval

National guidelines for the care and use of animals were followed.

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