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Changes in $P2Y_2$ receptor localization on adrenaline- and noradrenaline-containing chromaffin cells in the rat adrenal gland during development and aging

Mekbeb Afework [a,b], Geoffrey Burnstock [a,*]

[a] *Autonomic Neuroscience Centre, Royal Free and University College Medical School, Rowland Hill Street, London NW3 2PF, UK*
[b] *Department of Anatomy, Faculty of Medicine, Addis Ababa University, P.O. Box 9086, Addis Ababa, Ethiopia*

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Abstract

Using immunohistochemistry, the occurrence and age-related changes of the $P2Y_2$ receptor was investigated in the adrenal gland of rat at different ages, ranging from embryonic day E16 to 22 months. Immunoreactivity for the $P2Y_2$ receptor was present in chromaffin cells and nerve fibres at all ages examined. Double labeling with the antibody against phenyl ethanolamine-*N*-methyltransferase, which marks adrenaline-producing chromaffin cells, revealed that only a few of the $P2Y_2$-immunoreactive cells were adrenaline producing at embryonic day E16, the vast majority being noradrenaline-containing cells. However, immunoreactivity for adrenaline-containing cells in the $P2Y_2$ receptor-labeled chromaffin cells increased with increasing age and at 1 week post-natal almost all chromaffin cells were positive for both $P2Y_2$ and phenyl ethanolamine-*N*-methyltransferase, while noradrenaline-containing cells were minimal. At 2 weeks, there was a dramatic drop in $P2Y_2$-immunoreactive chromaffin cells and this was maintained in adult rats, noradrenaline-containing cells dominating. In the aging rat adrenals, $P2Y_2$ receptor-immunoreactivity was localized in subpopulations of both adrenaline and noradrenaline-producing cells. Intrinsic neurones were also visible that were positively labeled with the $P2Y_2$ receptor antibody in the adrenals of both adult and aging rats. $P2Y_2$-immunoreactive nerve fibres formed a plexus around the adrenal cortical cells of zona glomerulosa in the post-natal, but not in adult or aging rats.

In conclusion, this study suggests that ATP, acting through $P2Y_2$ receptors, may influence the phenotypic expression of chromaffin cells during the development and aging of the rat adrenal gland. However, during early development, when the chromaffin cells are actively dividing and during aging, when the adrenal medullary cells are known to show hyperplastic lesions, ATP acting through $P2Y_2$ receptors may be involved in other physiological activities, such as proliferation and/or differentiation of the chromaffin cells associated with their adrenaline or noradrenaline phenotype.

Keywords: Adrenal gland; $P2Y_2$ receptor; Immunohistochemistry; Rat; Development; Aging

1. Introduction

The function of adenosine 5′-triphosphate (ATP) as an extracellular cell-to-cell mediator is now well established; it is involved in neurotransmission and neuromodulation, inflammatory and immune responses as well as exocrine and endocrine secretion (see Burnstock, 1972, 1997; Burnstock and Knight, 2004). In mammals, ATP acts through two families of receptors that are widely distributed among different cells. These are the P2X family of receptors that is comprised of ligand-gated ion channel receptors and the P2Y family, metabotropic G protein-coupled receptors (Abbracchio and Burnstock, 1994; Ralevic and Burnstock, 1998). Seven subtypes of receptor have been cloned and characterized for the P2X receptor family and eight for the P2Y receptor family (Burnstock, 2003).

It has long been known that a large amount of ATP is co-stored and released with catecholamines from the adrenal

Abbreviations: ATP, adenosine 5′-triphosphate; E, embryonic day; IgG, Immunoglobulin G; PBS, phosphate-buffered saline; PNMT, phenyl ethanolamine-*N*-methyltransferase

* Corresponding author. Tel.: +44 207 830 2948; fax: +44 207 830 2949.
E-mail address: g.burnstock@ucl.ac.uk (G. Burnstock).

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medullary chromaffin cells (Hillarp et al., 1955; Blaschko et al., 1956; Winkler and Westhead, 1980; Coupland, 1989). However, it is relatively recently that evidence has emerged to show ATP as a regulator of secretion in both the medulla and cortex of the adrenal gland. In the medulla, it has been found to be involved in both facilitation (Chern et al., 1988; Kim and Westhead, 1989) and inhibition (Chern et al., 1987) of catecholamine secretion, while in the cortex, ATP has been shown to cause steroidogenesis from the adrenocortical cells of the zona glomerulosa (Jurányi et al., 1997) and zona fasciculata (Kawamura et al., 1991; Matsui, 1991; Niitsu, 1992).

We have previously identified the presence of P2X receptors in the adrenal gland of both rat and guinea-pig using immunohistochemistry (Afework and Burnstock, 1999, 2000a). In the rat, all seven P2X receptor subtypes were identified, while in the guinea-pig, only $P2X_1$, $P2X_2$, $P2X_5$ and $P2X_6$ receptor subtypes were found. Furthermore, there was an increased localization of $P2X_5$ receptor subtype in the adrenal chromaffin cells of the rat during early development, while during aging, the occurrence of the $P2X_4$ receptor subtype was increased in the adrenal chromaffin cells, but decreased in the adrenal cortical cells (Afework and Burnstock, 2000b). This suggests that there are distinct functional roles for ATP acting through the P2X receptors during development as well as in aging. Changes in the expression of other messenger molecules by adrenal gland cells as well as in their innervation are also known to occur during development and aging (Ito et al., 1986; Henion and Landis, 1990; Tomlinson and Coupland, 1990; Afework et al., 1994; Afework and Burnstock, 1995).

In this study, we have investigated the localization of $P2Y_2$ receptor in the adrenal glands of the rat at different age groups ranging from embryonic development to old age. In addition, in order to verify whether localization of $P2Y_2$ receptor shows any preference to adrenaline or noradrenaline-producing cells of adrenal medulla, double-labeling studies of the $P2Y_2$ receptor with phenyl ethanolamine-*N*-methyltransferase (PNMT), the enzyme that selectively marks the adrenaline-containing cells, were performed.

2. Experimental procedures

2.1. Animals

The study was conducted on male (post-natal) and either sex (pre-natal) Sprague–Dawley rats. The animals were housed under normal conditions at 21 °C, 12-h light:12-h darkness. Principles of good laboratory animal care were followed and animal experimentation was in compliance with the specific national laws and regulations. Adrenal glands from five rats of each of the following ages were studied: developing rats of embryonic day (E) 16, E18, E20 and post-natal 1, 4, 7, 14 and 21 days of age; adult rats of 12 weeks and aging rats of 22 months.

Pregnant rats were sacrificed with increasing concentration of CO_2 and fetuses were removed and their adrenals quickly dissected. Similarly, all other rats were sacrificed with CO_2 and their adrenal glands were removed. The adrenals were frozen in liquid nitrogen. Frozen sections were cut at 14 μm in a cryostat (Leica CM 1800, Germany) and thaw-mounted onto gelatinized slides.

2.2. Immunohistochemistry

Immunohistochemistry in the rat adrenal gland was performed with the indirect immunofluorescence method as previously described with minor modifications (Afework et al., 1994). Sections were fixed in 4% formaldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4, for 10 min at room temperature. Non-specific binding sites were blocked by incubation for 20 min with 10% normal horse serum (NHS) in PBS-containing 0.05% merthiolate. Sections were incubated overnight at room temperature in a humid chamber with the polyclonal antibody against $P2Y_2$ receptor subtype (Alomone Labs, Jerusalem, Israel), at a dilution of 1.5 μg/ml in 10% NHS, which was found to be optimal by titration. The sites of antibody–antigen reaction were visualized by incubation with biotinylated donkey anti-rabbit IgG (Jackson Immunoresearch, PA, USA) at a dilution of 1:500 in 1% NHS in PBS-containing 0.05% merthiolate for 1 h and then with streptavidin fluorescein (Amersham Bioscience UK Ltd., Buckinghamshire, UK) at a dilution of 1:200 in PBS-containing 0.05% merthiolate for 1 h. After each incubation, sections were washed in PBS (3 × 5 min).

In order to investigate whether $P2Y_2$ receptors have any preference to the adrenaline or noradrenaline-containing chromaffin cells, double-labeling experiments were also performed by incubation of the tissue sections overnight with a mixture of the $P2Y_2$ receptor antibody, at the dilution and procedure described above and sheep anti-PNMT (Chemicon International, Temecula, CA) at a dilution of 1:1000. The sites of $P2Y_2$ receptor antibody binding were localized by incubation with biotinylated donkey anti-rabbit IgG followed by streptavidine fluorescein as described above, while that of PNMT binding sites were identified by the application of Cy 3-conjugated donkey anti-sheep IgG (Jackson Immunoresearch) at a dilution of 1:300. Finally, the sections were washed (3 × 5 min) in PBS and mounted with citifluor mountant (Citifluor Ltd., London, UK).

For control experiments, omission of the primary antibodies, replacement of the primary antibodies with rabbit pre-immune IgG and absorption of the primary antibodies with their respective homologous peptide antigens were performed. Immunoprocessed sections were studied using a Zeiss Axioplan light microscope, (Germany) and images of representative positive immunolabelings were printed using Adobe PhotoShop (Version 5).

3. Results

No positive staining was seen in any of the control adrenal gland sections where the primary antibodies were omitted, after primary antibodies were pre-absorbed with the respective peptide antigen or replaced by rabbit pre-immune IgG. In contrast, specific positive stainings were seen in the adrenal gland sections of the rats that were incubated with the primary antibodies against the $P2Y_2$ receptor subtype and PNMT as described below.

3.1. Developing rat adrenal gland

Immunoreactivity for $P2Y_2$ receptors was already present in the chromaffin cells and nerve fibres of the developing adrenal gland at E16 (Fig. 1(a)). The $P2Y_2$-immunoreactive chromaffin cells were found as groups in the regions of developing medulla and as islands intermingled with the developing cortex. The $P2Y_2$-immunoreactive cells and nerve fibres also appeared crossing the capsule and migrating towards the centre of the gland. The adrenomedullary blastema that were located near the medial border of the gland was also positively immunoreactive for the $P2Y_2$ receptor antibody. Relatively, an increasing number of positively labeled cells and nerve fibres were visible in the adrenal glands from the E18- and E20-old rats.

After birth, at days 1 and 4, the relative number of positively labeled chromaffin cells and nerve fibres increased and it appeared that at day 4, nearly, all of the chromaffin cells were labeled with the $P2Y_2$ receptor antibody (Fig. 1(b)). The relative occurrence of labeled chromaffin cells then progressively decreased afterwards with increasing ages until 3 weeks after birth.

Double labeling with the antibody against PNMT in order to investigate whether $P2Y_2$ immunolabeling has any preference to the adrenaline or noradrenaline-containing chromaffin cells showed that during the pre-natal period at E16, the $P2Y_2$ receptor labeling was in the chromaffin cells that showed no or only a little immunoreactivity for PNMT (Fig. 1(a)). Following this, there was an increase in the occurrence of $P2Y_2$ receptor labeling in PNMT-immunoreactive chromaffin cells at E18 and E20 in a progressive manner.

After birth, at days 1 and 4, the occurrence of $P2Y_2$ immunoreactivity in the PNMT-immunoreactive chromaffin cells was progressively increased and at day 4, almost all the chromaffin cells were positively labeled for both $P2Y_2$ receptors and PNMT (Fig. 1(b)). Following this, at 7 days, some chromaffin cells that were immunoreactive for only $P2Y_2$ receptors were observed (Fig. 1(c)). Such $P2Y_2$-positive, but PNMT-negative, cells were located at the periphery of the medulla near the corticomedullary junction. The occurrence of $P2Y_2$ immunoreactivity in the non-PNMT-immunoreactive cells increased progressively at 2 and 3 weeks and at the latter age, $P2Y_2$ immunoreactivity became restricted to the non-PNMT-immunoreactive, noradrenaline-producing cells (Fig. 1(d)). This was also accompanied by a relatively increasing appearance of chromaffin cells that were immunoreactive only for PNMT progressively at 2 and 3 weeks.

$P2Y_2$-immunoreactive nerve fibres were seen mainly as bundles and nerve fibres traversing the cortex towards the medulla. Nerve terminals that were associated with chromaffin cells in the medulla were also visible. In addition, several nerve fibres that were immunoreactive for $P2Y_2$ receptors formed plexuses around the cortical cells of the zona glomerulosa in the post-natal developing rat adrenals (Fig. 1(e and f)).

3.2. Adult rat adrenal gland

In the adrenal glands of the 12-week-old rat, immunoreactivity for $P2Y_2$ receptors was observed in chromaffin cells that were located as groups at different regions of the medulla. Double labeling with the antibody against PNMT, showed that the $P2Y_2$-immunoreactive chromaffin cells were non-PNMT-immunoreactive noradrenaline cells (Fig. 2(a)).

$P2Y_2$ immunoreactivity was also observed in some intra-adrenal neurones that occurred in groups among both $P2Y_2$-labeled and unlabeled chromaffin cells (Fig. 2(a)).

Several $P2Y_2$-positive nerve fibres were present as bundles, nerve fibres and terminals within the adrenal medulla (Fig. 2(a)). Some nerve bundles that are positively immunoreactive for $P2Y_2$ receptors were also visible traversing adrenal cortex towards the medulla. However, unlike the adrenal glands of the developing rats described above, no immunoreactive nerve fibres formed plexus in close association with the cortical cells (Fig. 2(b)).

3.3. Aging rat adrenal gland

In the adrenal glands from the 22-month-old rats, several chromaffin cells that were seen both in groups as well as singly dispersed in the medulla were positively labeled for the $P2Y_2$ antibody. As seen from the double-labeling studies, $P2Y_2$ immunoreactivty occurred in both PNMT-immunoreactive adrenaline-producing and non-immunoreactive noradrenaline-producing subpopulations of the adrenal chromaffin cells (Fig. 2(c)). In addition, $P2Y_2$ immunoreactivity was observed in some intra-adrenal neurones and nerve fibres. No $P2Y_2$-labeled nerve fibres were seen closely associated with adrenal cortical cells (Fig. 2(d)).

4. Discussion

The absence of any labeling in the control experiments (where primary antibodies were omitted or replaced by rabbit pre-immune IgG or pre-absorbed with the respective peptide antigens) indicates the specificity of the staining

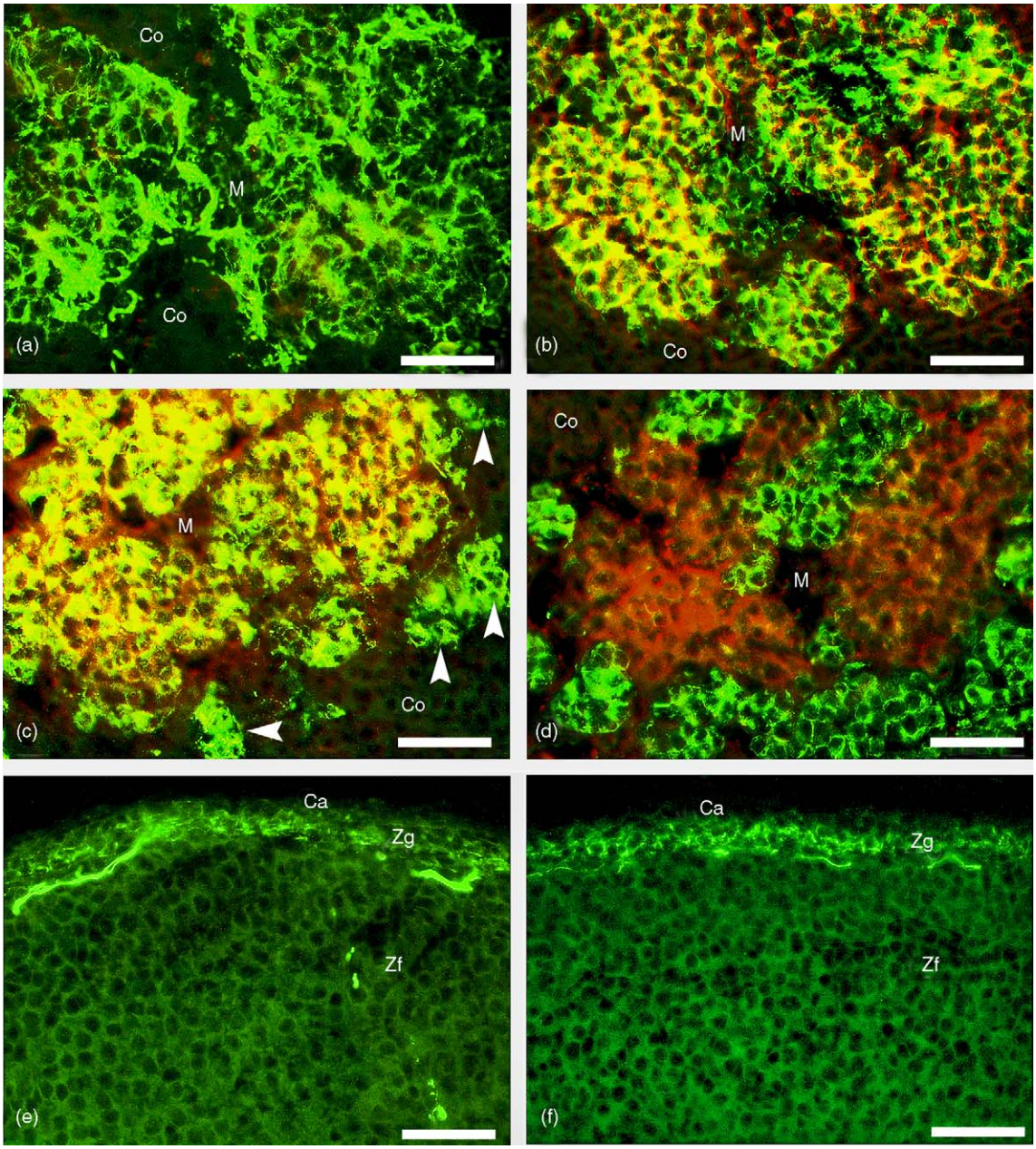

Fig. 1. Photomicrographs of embryonic rat adrenal medulla at E16 (a) and post-natal adrenal medulla at 4 days (b), 7 days (c) and 3 weeks (d) and cortex at 1 day (e) and 3 weeks (f) showing immunoreactivity for $P2Y_2$ receptors (green fluorescence) or PNMT (red fluorescence) separately or colocalization of the two antibodies (yellow fluorescence). Note that at E16 (a), $P2Y_2$ receptor-immunoreactivity is localized in developing chromaffin cells that show little double labeling with PNMT. At day 4 (b), almost all chromaffin cells are double-labeled for the antibodies against $P2Y_2$ receptors and PNMT. At 7 days (c), some chromaffin cells (arrow heads) located in the region of the corticomedullary junction are immunoreactive only for $P2Y_2$ receptors. These cells increase in their occurrence at 3 weeks (d) and immunoreactivity for $P2Y_2$ receptors is localized in non-PNMT-immunoreactive noradrenaline-containing cells. $P2Y_2$ receptor-immunoreactive nerve fibres found as plexus and bundles are visible in the region of developing zona glomerulosa of the cortex at day 1 (e) and 3 weeks (f). Ca: capsule; Co: cortex; M: medulla; Zf: zona fasciculate; Zg: zona glomerulosa. Bars = 50 μm.

observed with both the $P2Y_2$ receptor as well as the PNMT antibodies.

In the present study, we found that $P2Y_2$ receptors were localized in the adrenal gland of the rat and showed significant variation in distribution during development and aging. This suggests a changing functional involvement of ATP acting on $P2Y_2$ receptors in the adrenal gland during development and aging. Fig. 3 is a schematic summary of the relative expression of $P2Y_2$ receptors on adrenaline- and noradrenaline-containing chromaffin cells during pre- and post-natal development and in old age.

The dramatic changes in expression of $P2Y_2$ receptors on adrenaline- and noradrenaline-containing cells may indicate that they mediate events associated with early development and old age, such as chromaffin cell proliferation and/differentiation into different chromaffin cell phenotypes.

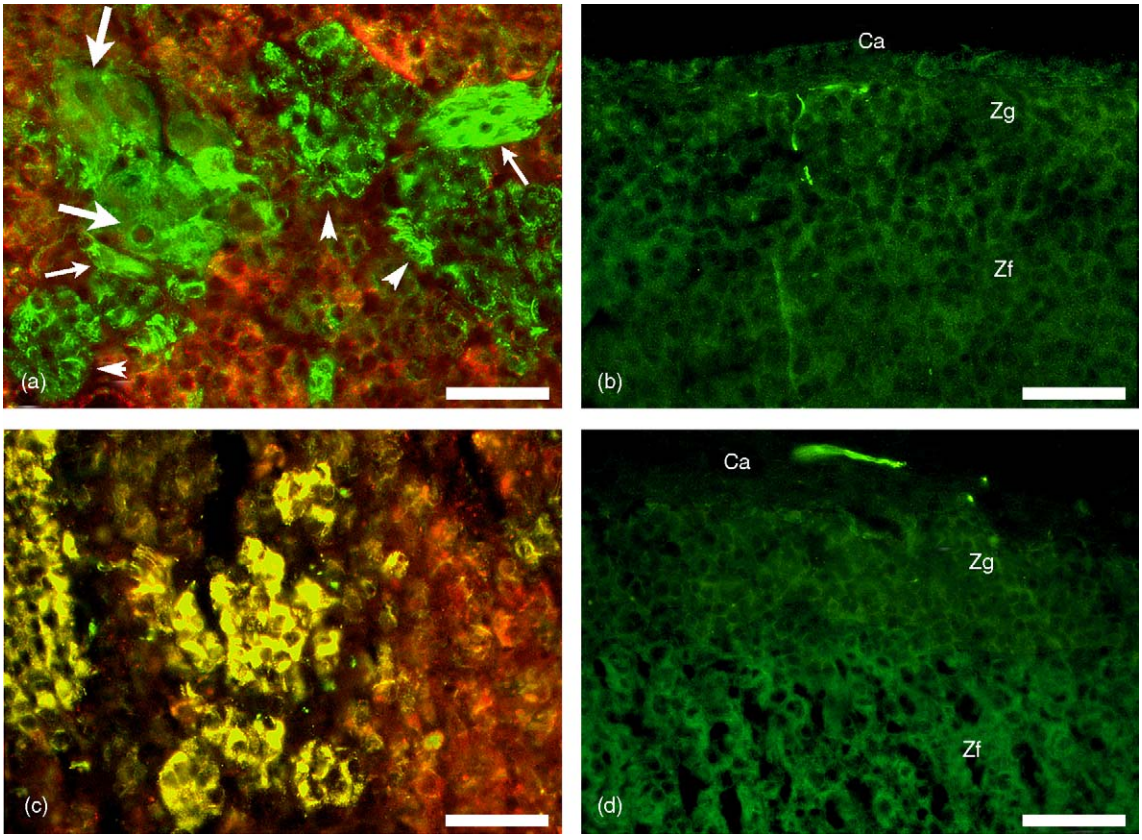

Fig. 2. Photomicrographs of adult rat (12-week old) adrenal medulla (a), cortex (b), aging rat (22-month old) adrenal medulla (c) and cortex (d) showing immunoreactivity for $P2Y_2$ receptors (green fluorescence) or PNMT (red fluorescence) separately or colocalization of the two antibodies (yellow fluorescence). Note that in the adult rat medulla (a), immunoreactivity for $P2Y_2$ receptors is localized in the non-PNMT-immunoreactive noradrenaline-containing chromaffin cells (arrow heads), intrinsic neurons (large arrows) and nerve fibres and bundles (small arrows). In the aging rat medulla (c), $P2Y_2$ receptor-immunoreactive chromaffin cells are also positively immunoreactive for PNMT. Note also in the region of zona glomerulosa of adrenal cortex of the adult (b) and aging (d) rat gland, there is only a little $P2Y_2$-immunoreactive nerve fibres as compared with those of the developing rats as shown in Fig. 1(e and f). Ca: capsule; Zf: zona fasciculata; Zg: zona glomerulosa. Bars = 50 μm.

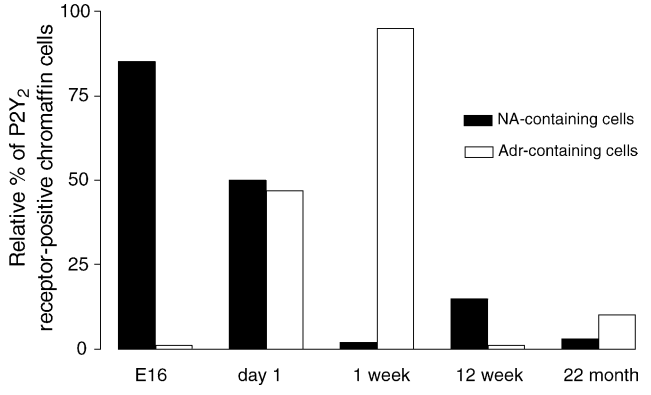

Fig. 3. Schematic summary based on semi-quantitative data of $P2Y_2$ receptor localization among the chromaffin cells of adrenal medulla showing the relative proportions of noradrenaline or adrenaline phenotype. Note that in pre- and post-natal development (up to 7-day old) $P2Y_2$ receptors are found in the majority of the chromaffin cells of the medulla, which were mainly of noradrenaline phenotype initially, but switches to adrenaline phenotype later. Afterwards, the relative occurrence of $P2Y_2$ receptor labeling is reduced and by 3 weeks, the phenotype frequency changes back to noradrenaline-containing cell dominancy. This remains unchanged in the adult, but changes again in old age, where subpopulations of both noradrenaline- and adrenaline-containing chromaffin cells express the $P2Y_2$ receptor.

Consistent with this possibility, ATP has been implicated in cellular development, including proliferation, mitosis and DNA synthesis, sometimes acting synergistically with growth factors (Neary et al., 1996; Abbracchio and Burnstock, 1998; Burnstock, 2002). In addition, as shown previously, immunoreactivity for $P2X_5$ receptors, which is thought to be involved in cell differentiation (Gröschel-Stewart et al., 1999; Ryten et al., 2002) shows a progressive increase in rat adrenal chromaffin cells during the first week and subsequently, transiently disappears during the second and third weeks post-natally (Afework and Burnstock, 2000b).

The presence of $P2Y_2$ receptors on the chromaffin cells may also suggest that ATP and nucleotides are involved in the modulation of secretion from the gland. Indeed, functional studies by several investigators have already implicated ATP to cause both facilitation (Chern et al., 1988; Kim and Westhead, 1989) and inhibition (Chern et al., 1987) of catecholamine secretion. This dual modulatory role of ATP acting on the chromaffin cells appears to be due to the presence and activation of different types of purinoceptors. When ATP activates the P2X receptors located on

chromaffin cells, it induces a non-selective cation current that causes Ca^{2+} influx followed by exocytosis (Castro et al., 1995; Lin et al., 1995; Reichsman et al., 1995; Otsuguro et al., 1996). However, ATP activation of P2Y receptors on chromaffin cells inhibits catecholamine secretion by inhibition of Ca^{2+} channels (Lin et al., 1995; Currie and Fox, 1996; Harkins and Fox, 2000; Powell et al., 2000; Ulate et al., 2001). Although the $P2Y_2$ receptor is found in both adrenaline and noradrenaline storing cells in early stages of development and later during aging, it is confined to the noradrenaline storing cells by 3 weeks and this is maintained in the adult, when the gland is presumably at its optimum secretory activity. It, therefore, appears that the receptor is involved in preferential modulation of noradrenaline secretion from the gland and gives an example where noradrenaline secretion is regulated by a distinct signaling system from that of adrenaline in the rat adrenal gland. In line with this, there are previous studies that indicated differential secretion of two stress hormones from the gland (Ungar and Phillips, 1983; Matsui, 1984; Yadid et al., 1992) as well as distinct localizations of various cell surface receptors on either adrenaline or noradrenaline cells in the gland (see Aunis and Langley, 1999).

In the aging adrenal gland, unlike that of the young and adult rats, the absence of $P2Y_2$ receptors from some noradrenaline cells and its expression in some adrenaline-producing cells is interesting. The adrenal gland of aging rat shows several changes, such as proliferative lesions of chromaffin cells (Tischeler et al., 1985, 1988; Coupland and Tomlinson, 1989) and differentiation of the noradrenaline cells towards neural phenotype (Coupland and Tomlinson, 1989). The catecholamine secretory activity of the adrenals is also altered in aging (Sato et al., 1987; Seals and Esler, 2000). Either one of these or combinations of them may be associated with the observed changes in the expression of the $P2Y_2$ receptor in the chromaffin cells during aging.

The presence of $P2Y_2$ receptor-immunoreactivity in the intra-adrenal neurones and nerve fibres indicate that ATP may also have effects on the activity of the gland by influencing its functional innervation. The $P2Y_2$ receptor-positive intra-adrenal neurones should at least partly account for the nerve fibres that were also immunoreactive for the $P2Y_2$ receptor antibody within the gland. However, the abundance of the $P2Y_2$ receptor-immunoreactive nerve fibres that occur specially as bundles as well as nerve terminals that are closely associated with the chromaffin cells points to an additional extradrenal source of nerve fibres. It is known that the adrenal gland of the rat is richly innervated by nerve fibres that come from different sources, including the splanchnic preganglionic neurones, the sensory root ganglia and the vagal ganglia (Parker et al., 1993). Either one of these or combination of them may, therefore, be the source of some of the nerve fibres seen in the gland with $P2Y_2$ receptor-immunoreactivity. The presence of the nerve fibres and plexus in the zona glomerulosa during the developmental period may indicate the involvement of $P2Y_2$ receptors in the proliferation and/or differentiation of the nerve fibres innervating the cortical cells during this period. It is also possible that ATP is involved in modulation of secretion from the gland at this time. ATP has been found to stimulate the production of aldosterone by the cortical cells of the zona glomerulosa (Jurányi et al., 1997; Szalay et al., 1998). In the adrenal cortex, ATP has also been shown to promote glucocorticoid secretion from adrenocortical cells of the zona fasciculata through activation of the P2Y receptors (Kawamura et al., 1991, 2001; Niitsu, 1992; Nishi, 1999; Xu and Enyeart, 1999). However, $P2Y_2$-immunoreactive nerve fibres innervating the cortical cells are absent during adulthood and aging; it is not known, whether this is because of the age-related disappearance of the nerve fibres that express the receptors or whether it is the result of down-regulation in the expression of the receptors.

In conclusion, this study suggests that ATP, acting through $P2Y_2$ receptors, may influence the phenotypic expression of chromaffin dells during development and aging of the rat adrenal gland, although it is possible that the changes in expression of $P2Y_2$ receptors on adrenaline- and noradrenaline-containing chromaffin cells are concomitant with changes controlled by other mechanisms. During early developmental ages when the chromaffin cells are actively dividing and during aging, when the adrenal medullary cells are known to show hyperplastic lesions, ATP acting through $P2Y_2$ receptors may be involved in other physiological activities, such as proliferation and/or differentiation of the chromaffin cells associated with their adrenaline or noradrenaline phenotype.

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