PERGAMON

Int. J. Devl Neuroscience 18 (2000) 515–520

INTERNATIONAL JOURNAL of DEVELOPMENTAL NEUROSCIENCE

www.elsevier.com/locate/ijdevneu

Age-related changes in the localization of P2X (nucleotide) receptors in the rat adrenal gland

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

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

Received 10 December 1999; received in revised form 25 February 2000; accepted 29 February 2000

Abstract

Observation of the changes in the occurrence and distribution of nucleotide (P2X) receptors in the adrenal gland during development and ageing, and correlation with the changes in adrenal status at similar stages may give morphological insights into the functions of purine nucleotides in the gland. Age-related changes in the localization of all seven subtypes of the P2X receptor in the adrenal gland of rat were therefore investigated immunohistochemically. In the adrenal glands of prenatal rats, immunoreactivity to P2X receptor subtypes was not observed. In glands of the postnatal rat at the developmental stages studied, only immunoreactivity for the $P2X_5$ receptor subtype was observed. A small number of faintly $P2X_5$-immunoreactive chromaffin cells were found in the adrenal glands of 1-day-old rats; the frequency of localization and intensity of staining of immunoreactive cells had increased by day 4 and was further increased at day 7. $P2X_5$ immunoreactivity was not observed in the adrenal glands from 14- and 21-day-old rats. At 8 weeks of age, immunoreactivity with a specific distribution for each of the seven receptor subtypes was observed. Except for the $P2X_4$ receptor, adrenal glands at 24 months showed a similar pattern of immunoreactivity for the receptor subtypes as that observed at 8 weeks. Immunoreactivity for $P2X_4$ was first observed in the adrenal cortical cells of the zona reticularis at 8 weeks, but was absent in 24-month-old rats. However, several $P2X_4$-immunoreactive chromaffin cells appeared at 24 months. Such immunoreactive cells were not seen in rats of any of the other ages studied. It was concluded that the greater expression of $P2X_5$ receptor at an early developmental stage and of $P2X_4$ in ageing might reflect functional roles for purines in cellular proliferation and/or differentiation, and in cellular degeneration, respectively, in adrenal glands of rat. © 2000 ISDN. Published by Elsevier Science Ltd. All rights reserved.

Keywords: Adrenal gland; P2X receptors; Immunohistochemistry; Rat; Development; Ageing

1. Introduction

Adenosine 5′-triphosphate (ATP) is involved in several extracellular activities, in addition to its function as an intracellular energy source (see [7,8]). In the extracellular activities, ATP plays roles in long term events such as embryonic development, growth, cell proliferation and differentiation [28], while in short term events it is involved in activities such as neurotransmission and/or modulation, regulation of visceral muscle contraction and neuroendocrine secretion [7,8]. Such effects of ATP are mediated through different types of receptors located on the surface of cells, consisting of two families of P2 receptors that are widely distributed among mammalian cells: P2X receptors are ligand-gated non-selective cation channels; while P2Y receptors are G protein-coupled membrane receptors [1,5,6]. So far, seven subtypes of P2X receptors and nine subtypes of P2Y receptors have been identified [1,9,17,31].

Using antisera raised against the unique peptide sequences to each of the seven subtypes of the P2X

Abbreviations: ATP, adenosine 5′-triphosphate; E, embryonic day; PBS, phosphate-buffered saline; IgG, Immunoglobulin G.

* Corresponding author. Tel.: +44-20-7-830-2948; fax: +44-20-7-830-2949.

E-mail address: g.burnstock@ucl.ac.uk (G. Burnstock).

PII: S0736-5748(00)00023-X

receptors [32], immunohistochemical localization of the receptors has been achieved recently in different regions [10,18,19], including rat adrenal gland [3]. In the adrenal gland, ATP is stored and released along with the catecholamines from chromaffin cells [38]. ATP is also released from the adrenal capsule and zona glomerulosa region of the gland [23]. Functional studies have shown that ATP can facilitate [12,25] and inhibit [11] catecholamine secretion from adrenal chromaffin cells. ATP can also stimulate steroidogenesis from adrenal cortical cells [23,24,27,30]. These observations suggest that ATP receptors located on the adrenal gland cells play important physiological roles in the activity of the gland.

During development, adrenal gland cells exhibit changes in their innervation and expression of various messenger molecules [2,4,21,37]. In addition, during ageing, adrenal medullary cells show changes in their innervation, expression of various mediator molecules, and evidence of proliferative lesions and structural changes [4,15,22,35,36]. It is not known whether changes also occur in the P2X receptor subtypes of the adrenal gland during development and ageing, and so this warrants examination. Understanding the level and pattern of any change in the occurrence of the receptors during different conditions, such as in development and ageing, would give morphological insights into the functions of purines in the gland. Using immunohistochemical techniques we have, therefore, investigated the occurrence of all seven subtypes of the P2X receptors in the adrenal glands of the rat at ten different ages, ranging from embryonic day 16 to 24 months after birth.

2. Experimental procedures

2.1. Animals

The study was conducted on male (postnatal) Sprague–Dawely rats and either sex (prenatal). The animals were housed under normal conditions at 21°C, 12 h light and 12 h darkness. Adrenal glands from five rats of each of the following ages were studied: developing rats of embryonic day (E) 16, E18, E20; postnatal rats 1, 4, 7, 14 and 21 days of age; adult rats of 8 weeks and ageing rats of 24 months.

Pregnant rats were killed by a rising concentration of CO_2, foetuses were removed and their adrenals quickly dissected out and immersion-fixed for 1 h at room temperature in 4% formaldehyde containing 0.03% picric acid in 0.1 M phosphate-buffered saline (PBS), pH 7.4. Similarly, all postnatal rats were sacrificed under CO_2 anaesthesia, their adrenals removed and immersion-fixed for 1 h. The adrenals were left overnight in 10% sucrose in PBS at 4°C. Frozen sections were cut at 14 μm in a cryostat (Leica CM 1800, Germany) and thaw-mounted onto gelatinized slides.

2.2. P2X antibodies

Immunohistochemistry for P2X receptors was carried out using rabbit polyclonal antibodies against unique peptide sequences of each of $P2X_{1-7}$ receptor subtypes provided by Roche Bioscience, Palo Alto, USA [32].

The immunogens were synthetic peptides representing 15 receptor-type specific amino acids in the C-terminal part of the receptor: $P2X_1$, amino acid 385-399 (ATSSTLGLQENMRTS); $P2X_2$, amino acid 458-472 (QQDSTSTDPKGLAQL); $P2X_3$, amino acid 383-397 (VEKQSTDSGAYSIGH); $P2X_4$, amino acid 374-388 (YVEDYEQGLSGEMNQ); $P2X_5$, amino acid 437-451 (RENAIVNVKQSQILH); $P2X_6$, amino acid 357-371 (EAGFYWRTKYEEARA); $P2X_7$, amino acid 555-569 (TWRFVSQDMADFAIL). The peptides were covalently linked to keyhole limpet haemocyanin. Rabbits were immunized with the conjugated peptides in multiple monthly injections (performed by Research Genetics, Inc., Huntsville, AL, USA). The specificity of the P2X antibodies has been verified by immunoblotting with membrane preparations from cloned $P2X_{1-7}$ receptor-expressing CHO-K1 cells or 1321N1 cells [32]. The antibodies recognize only one protein of the expected size in the heterologous expression systems and have been shown to be receptor subtype.

Immunoglobulin G (IgG) fractions were isolated from the pre-immune and immune sera ($P2X_{1-7}$) following the method of Harboe and Ingild [20]. The protein concentration was determined at 280 nm using an extinction factor of 1.43 for 1 mg/ml.

2.3. Immunohistochemistry

Endogenous peroxidase was blocked with 50% methanol and 0.4% hydrogen peroxide for 10 min. Non-specific binding sites were blocked by incubation for 20 min with 10% normal horse serum in PBS containing 0.05% merthiolate. Sections were incubated overnight at room temperature in a humid chamber with the polyclonal antibodies against $P2X_{1-7}$ receptor subtypes (see below), $P2X_{1-3}$ and $P2X_{5-7}$ at dilutions of 1 μg/ml in 10% normal horse serum, and $P2X_4$ at 0.5 μg/ml, were used. These dilutions were 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 for 1 h, and then with ExtrAvidin peroxidase conjugate (Sigma, Poole, UK) at a dilution of 1:1500 for 1 h. After each incubation, sections were washed in PBS (3 × 5 min). The nickel-intensified 3,3′-diaminobenzidine (DAB)

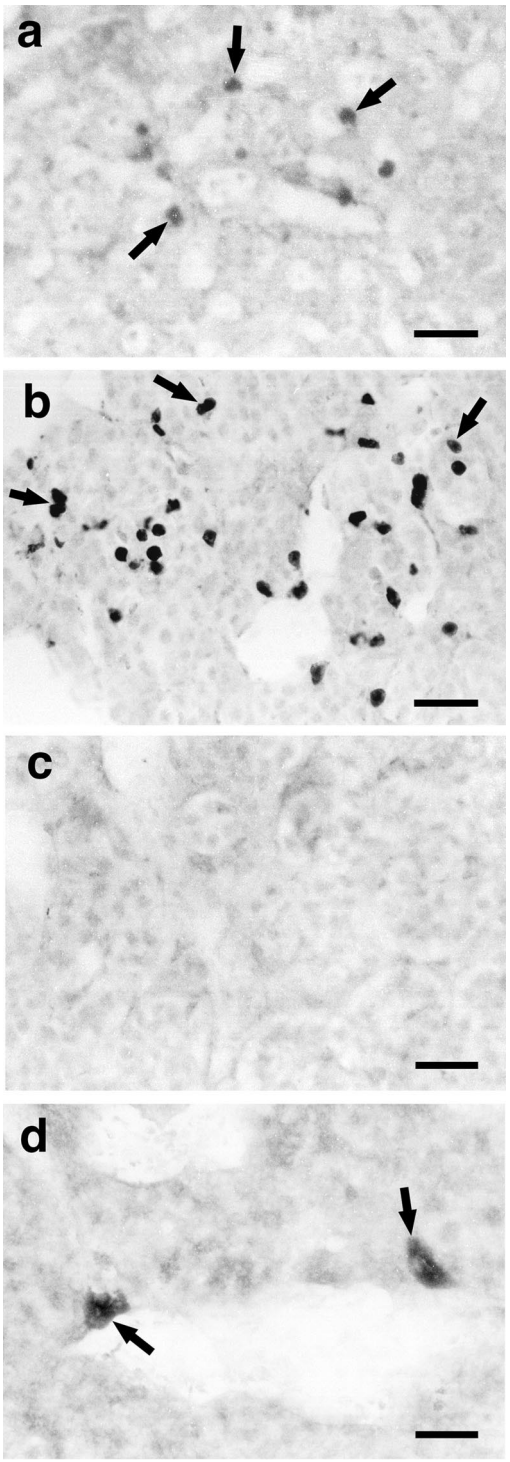

Fig. 1. $P2X_5$ receptor immunoreactivity in adrenal gland sections of 1-day (a), 7-day (b), 2-week (c) and 8-week-old (d) rats. Note that the immunoreactive chromaffin cells (arrows in (a), (b) and (d)) appeared as a small number of slightly immunoreactive cells at day 1 (a), increased in number and intensity of staining at day 7 (b), and disappeared at 2 weeks (c) to reappear in a small number of chromaffin cells at 8 weeks (d). Bar = 40 μm.

reaction was performed for 5–10 min according to the protocol developed by Llewellyn-Smith et al. [26]. Sections were dehydrated in graded alcohol, cleared in xylene and mounted with Eukitt (BDH, Leicester, UK).

Immunoprocessed sections were studied using an Edge R 400 high-definition light microscope (Edge Scientific Instrument Co., Santa Monica, CA) and photographed with Kodak TMX 100 black and white film.

Controls included: omission of the primary antibodies, replacement of the primary antibodies with rabbit preimmune IgG, and preabsorption of the primary antibodies with their respective homologous peptide antigens.

3. Results

The control experiments in which the primary antibodies were omitted or replaced by rabbit preimmune IgG or preabsorbed with the respective peptide antigens did not show any staining.

3.1. Developing rat

Immunoreactivity to the seven P2X receptor subtypes was not found in the adrenal gland sections from prenatal rats at stages E16, E18, E20.

Only $P2X_5$ immunoreactivity showed a transitory appearance during early postnatal life, while no immunoreactivity was seen for any of the remaining six receptor subtypes in the different age levels studied, until the age of 8 weeks. Immunoreactivity for $P2X_5$ receptors in the adrenal gland first appeared as some faintly labelled chromaffin cells at day 1 (Fig. 1(a)). $P2X_5$-immunoreactive chromaffin cells had increased in frequency and staining intensity in adrenal glands of 4-day-old rats and had further increased by day 7 (Fig. 1(b)). At this latter stage, the labelled cells were found dispersed singly or in groups of two or three cells among non-immunoreactive chromaffin cells. However, immunoreactivity for $P2X_5$ had disappeared at 2 and 3 weeks (Fig. 1(c)) and reappeared at 8 weeks (Fig. 1(d)).

3.2. Adult rat

In the adrenal glands of the 8-week-old rat, immunoreactivity for each of the seven receptor subtypes, including the $P2X_5$ subtype, was comparable to that of adult rat observed and reported previously [4]. Briefly, $P2X_1$ was found in the nerve fibres innervating adrenal chromaffin cells and blood vessels in the medulla. $P2X_2$ and $P2X_3$ receptor immunoreactivities were localized in the adrenal intrinsic neurones. Immunoreactiv-

ity to $P2X_2$ antibodies were also found in the smooth myocytes of capsular and subcapsular blood vessels. $P2X_4$, and $P2X_5$, $P2X_6$ and $P2X_7$ were localized in adrenal cortical cells of zona reticularis and zona fasciculata, respectively. $P2X_5$- and $P2X_7$-immunoreactivities were also found in the adrenal chromaffin cells.

3.3. Ageing rat

In the adrenal glands from 24-month-old rats, the immunoreactivity to each of the receptor subtypes, except that to the $P2X_4$ subtype, was comparable to that of the 8-week-old animals as mentioned above. However, striking changes were observed in the occurrence of the $P2X_4$ receptor. Firstly, the $P2X_4$-immunoreactive cortical cells of the zona reticularis observed at 8 weeks of age (Fig. 2(a)) were absent from the 24-month old rat (Fig. 2(b)). Secondly, several $P2X_4$-immunoreactive chromaffin cells were seen in the adrenal glands at 24 months (Fig. 2(b)). These immunoreactive chromaffin cells were localized singly, dispersed among non-immunoreactive chromaffin cells. $P2X_4$-immunoreactive chromaffin cells were not observed in rats of any of the other age groups investigated.

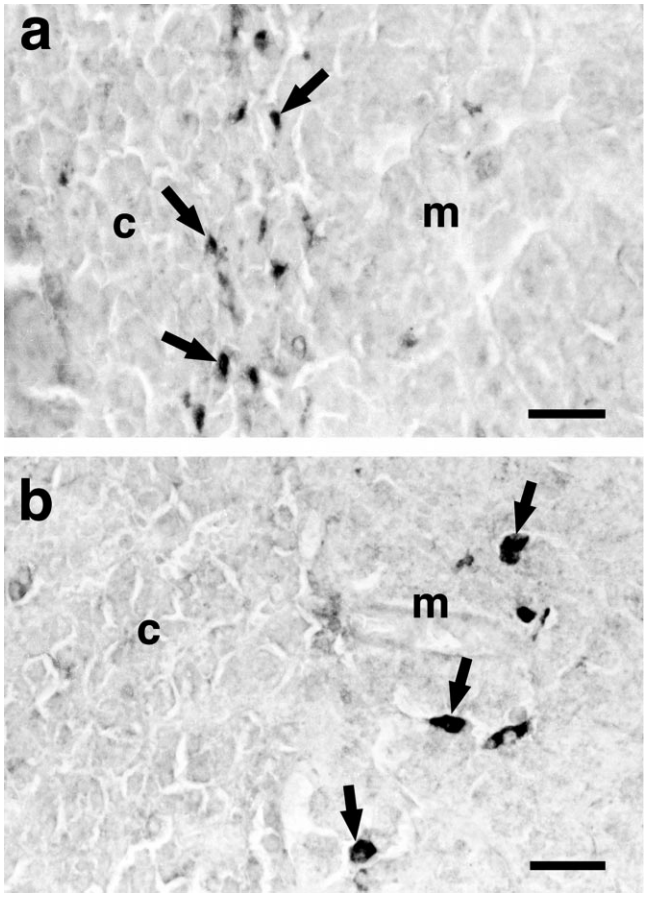

Fig. 2. $P2X_4$ receptor immunoreactivity in adrenal gland sections of 8-week (a) and 24-month-old (b) rats. Note that immunoreactive adrenal cortical cells of zona reticularis at 8 weeks (arrows in (a)) are not visible in the gland of the 24-month-old rat (b). Instead, $P2X_4$-immunoreactive chromaffin cells have appeared at 24 months (arrows in (b)), which are not found in the glands of the 8-week-old rat. c: cortex, m: medulla. Bar = 40 μm.

4. Discussion

4.1. Changes in the localization of $P2X_5$ receptor subtype

The absence of localization of P2X receptor subtypes, except the $P2X_5$, during the early postnatal development of the adrenal gland, in spite of their occurrence in the adult, suggests a lack of functional involvement of most of the receptor subtypes until the gland reaches full functional maturity. However, the progressive increase in the occurrence of the $P2X_5$ receptor subtype in the first week and the subsequent transitory disappearance at 2 and 3 weeks postnatally suggests a temporary functional significance of the $P2X_5$ receptor during the first week of development.

Developing adrenal chromaffin cells exhibit two distinct phases: an initial phase that is controlled by intrinsic genetic information and is subjected to modification by hormonal and other metabolic inputs; and a second phase of transynaptic control mediated by the acetylcholine released from splanchnic nerve terminals [33]. The second phase appears towards the end of the first week and becomes fully operational by 10 days postnatally [33]. In this present study, the increased occurrence of $P2X_5$ receptors observed during the first week, when the non-neurogenic mechanism is prevailing may indicate that purines are involved in modulating the developmental activities of the chromaffin cells during the initial phase of their development. In this connection, ATP has already been implicated in cellular development because concurrent treatment of astrocytes with extracellular ATP and fibroblast growth factors leads to a P2 receptor-mediated synaptic activation of DNA synthesis [29]. Moreover, in the skin, $P2X_5$ immunoreactivity was localized in differentiating cell layers of the epithelia [18]. The presence of $P2X_5$ receptor subtype in the adrenal chromaffin cells, at an early developmental stage, also suggests the involvement of ATP in the regulation of catecholamine secretion from the gland at this stage. A non-neurogenic mechanism has been previously suggested to precede the acetylcholine-mediated transynaptic neurogenic input from the splanchnic nerves for the control of the release of catecholamines from developing medullary chromaffin cells [33].

Since during the early developmental period chromaffin cells are known to actively divide and be smaller in size [15], it was not unexpected to find that at 8

weeks of age the $P2X_5$-labelled chromaffin cells were larger (Fig. 1(d)) than those at early postnatal stages (days 1 and 7; Fig. 1(a) and (b)).

4.2. Changes in the localization of $P2X_4$ receptor subtype

The plasticity of the occurrence of $P2X_4$ receptors seen in the ageing rat is another interesting observation of the present study. Localization of the $P2X_4$ receptor in the adrenal cortical cells of zona reticularis in the adult rat may suggest that ATP is involved in steroidogenesis in the gland. Previously, functional studies have shown that ATP facilitates steroidogenesis from adrenal cortical cells of the zona glomerulosa and fasciculata cells [23,24,27,30]. However, the reason for the absence of immunoreactivity in the cortical cells of zona reticularis of the ageing rat is intriguing, and awaits further investigation. It may be because of an age-related disappearance of the specific cortical cells possessing the receptor. Alternatively, it may be because the receptors disappear from these cells in the ageing rat. There is circadian variation of the level of corticosteroid secretion by the adrenal cortex and, thus, it could be hypothesized that the level of occurrence of purine receptors may also show changes during a 24-h period. Investigation of this will constitute a separate study in the future. However, since in the present study we investigated age-related changes at a specific time of the day, circadian rhythm is unlikely to have any influence on the current findings.

The localization of $P2X_4$ receptors in some chromaffin cells of the ageing rat, but their absence in those of the other age groups, is interesting. This may indicate the emergence of new chromaffin cells that express $P2X_4$ receptors, or perhaps the expression of the receptors in previously negative cells. It is known that during ageing there is an increased frequency of spontaneous changes in the adrenal chromaffin cells of the rat. These changes occur as proliferative lesions of adrenal medullary cells from hyperplasia, phaeochromacytoma and/or hypertrophy of chromaffin cells [15,35,36]. It is, therefore, possible that the $P2X_4$ receptor is expressed in any of the affected adrenal chromaffin cells showing such changes in ageing. The smaller size of the $P2X_4$-labelled chromaffin cells from the ageing rat (Fig. 2(b)) compared to chromaffin cells showing $P2X_5$ immunoreactivity in the 8-week-old rat (Fig. 1(d)) may also result from the proliferative changes in ageing. However, it is known that there are at least two subtypes of chromaffin cells of different sizes [14], and the possibility exists that the $P2X_4$ receptor is expressed in a small-sized subtype of chromaffin cells. Moreover, the presence of $P2X_4$ receptor in the chromaffin cells could be associated with cellular degeneration that is caused by ageing. In fact, $P2X_1$, $P2X_4$ and $P2X_7$ receptors have been previously implicated with apoptosis at different sites [13,16,34], and ATP has been found to induce cell death [39,40].

Part of this study was presented at the 4th International Congress of the Society of the Neuroscientists of Africa, 12–16 April 1999, Dakar, Senegal.

Acknowledgements

We gratefully acknowledge the support of Roche Bioscience in Palo Alto, USA We would like to thank Dr T. Cowen for providing us the adrenal glands of ageing rat. The editorial assistance of Mr R. Jordan is gratefully acknowledged.

References

[1] Abbracchio, M. P. and Burnstock, G., Purinoceptors: are there families of P2x and P2y purinoceptors? *Pharmacol. Ther.*, 1994, **64**, 445–475.
[2] Afework, M. and Burnstock, G., Calretinin immunoreactivity in adrenal gland of developing, adult and ageing Sprague-Dawley rats. *Int. J. Dev. Neurosci.*, 1995, **13**, 515–521.
[3] Afework, M. and Burnstock, G., Distribution of P2X receptors in the rat adrenal gland. *Cell Tissue Res.*, 1999, **298**, 449–456.
[4] Afework, M., Tomlinson, A. and Burnstock, G., Distribution and colocalization of nitric oxide synthase and NADPH-diaphorase in adrenal gland of developing, adult and aging Sprague-Dawley rats. *Cell Tissue Res.*, 1994, **276**, 133–141.
[5] Barnard, E. A., Burnstock, G. and Webb, T. E., G protein-coupled receptors for ATP and other nucleotides: a new receptor family. *Trends Pharmacol. Sci.*, 1994, **15**, 67–70.
[6] Bean, B. P., Pharmacology and electrophysiology of ATP-activated ion channels. *Trends Pharmacol. Sci.*, 1992, **13**, 87–90.
[7] Burnstock, G., Purinergic nerves. *Pharmacol. Rev.*, 1972, **24**, 509–581.
[8] Burnstock, G., The past, present and future of purine nucleotides as signalling molecules. *Neuropharmacology*, 1997, **36**, 1127–1139.
[9] Burnstock, G. and King, B., Numbering of cloned P2 purinoceptors. *Drug Dev. Res.*, 1996, **38**, 67–71.
[10] Chan, C. M., Unwin, R. J., Bardini, M., Oglesby, I. B., Ford, A. P. D. W., Townsend-Nicholson, A. and Burnstock, G., Localization of the $P2X_1$ purinoceptors by autoradiography and immunohistochemistry in rat kidneys. *Am. J. Physiol.*, 1998, **274**, F799–F804.
[11] Chern, Y. J., Herrera, M., Kao, L. S. and Westhead, E., Inhibition of catecholamine secretion from bovine chromaffin cells by adenine nucleotides and adenine. *J. Neurochem.*, 1987, **48**, 1573–1576.
[12] Chern, Y. J., Kim, K. T., Slaky, L. L. and Westhead, E., Adenosine receptors activate adenylate cyclase and enhance secretion from bovine adrenal chromaffin cells in the presence of forskolin. *J. Neurochem.*, 1988, **50**, 1484–1493.
[13] Chow, S. C., Kass, G. E. N. and Orrenius, S., Purines and their roles in apoptosis. *Neuropharmacology*, 1997, **36**, 1127–1139.
[14] Coupland, R. E., The natural history of the chromaffin cell — 25 years on the beginning. *Arch. Histol. Cytol.*, 1989, **52**, 331–341.
[15] Coupland, R. E. and Tomlinson, A., The development and maturation of adrenal medullary chromaffin cells of the rat in

vivo: a descriptive and quantitative study. *Int. J. Dev. Neurosci.*, 1989, **7**, 419–438.

[16] Di Virgilio, F., Bronte, V., Collavo, D. and Zanovello, P., Responses of mouse lymphocytes to extracellular ATP. *J. Immunol.*, 1989, **143**, 1955–1960.

[17] Fredholm, B. B., Abbracchio, M. P., Burnstock, G., Dubyak, G. R., Harden, T. K., Jakobson, K. A., Schwabe, U., Spedding, M. and Williams, M., Towards a revised nomenclature for P1 and P2 receptors. *Trends Pharmacol. Sci.*, 1997, **18**, 79–82.

[18] Gröschel-Stewart, U., Bardini, M., Robson, T. and Burnstock, G., Localisation of $P2X_5$ and $P2X_7$ receptors by immunohistochemistry in rat stratified squamous epithelia. *Cell Tissue Res.*, 1999, **296**, 599–605.

[19] Gröschel-Stewart, U., Bardini, M., Robson, T. and Burnstock, G., P2X receptors in the rat duodenal villus. *Cell Tissue Res.*, 1999, **297**, 111–117.

[20] Harboe, N. and Ingild, A., Immunization, isolation of immunoglobulins, estimation of antibody titre. *Scand. J. Immunol. Suppl.*, 1973, **1**, 161–164.

[21] Henion, P. D. and Landis, S. C., Asynchronous appearance and topographic segregation of neuropeptide-containing cells in developing rat adrenal medulla. *J. Neurosci.*, 1990, **12**, 3818–3827.

[22] Ito, K., Sato, Y. and Suzuki, H., Increase in adrenal catecholamine secretion and sympathetic nerve unitary activities with aging in rats. *Neurosci. Lett.*, 1986, **69**, 263–268.

[23] Jurányi, Z., Orsó, E., Jánssy, A., Szaly, K. S., Sperlágh, B., Windisch, K., Vinson, G. P. and Vizi, E. S., ATP and [^{3}H]noradrenaline release and the presence of ecto-Ca^{2+}-ATPases in the capsule-glomerulosa fraction of the rat adrenal gland. *J. Endocrinol.*, 1997, **153**, 105–114.

[24] Kawamura, M., Matsui, T., Niitsu, A., Kondo, T., Ohno, Y. and Nakamichi, N., Extracellular ATP stimulates steroidogenesis in bovine adrenocortical fasciculata cells via P2 purinoceptors. *Jpn. J. Pharmacol.*, 1991, **56**, 543–545.

[25] Kim, K. T. and Westhead, E., Cellular responses to Ca^{2+} from extracellular and intracellular sources are different as shown by simultaneous measurements of cytosolic Ca^{2+} and secretion from bovine chromaffin cells. *Proc. Natl. Acad. Sci. USA*, 1989, **86**, 9881–9885.

[26] Llewellyn-Smith, I. J., Pilowsky, P. and Minson, J. B., The tungstate-stabilized tetramethylbenzidine reaction for light and electron microscopic immunochemistry and for revealing biocytin-filled neurons. *J. Neurosci. Methods*, 1993, **46**, 27–40.

[27] Matsui, T., Biphasic rise caused by extracellular ATP in intracellular calcium concentration in bovine adrenocortical fasciculata cells. *Biochem. Biophys. Res. Commun.*, 1991, **178**, 1266–1272.

[28] Neary, J. T. and Burnstock, G., Purinoceptors in the regulation of cell growth and differentiation. *Drug Dev. Res.*, 1996, **39**, 407–412.

[29] Neary, J. T., Baker, L., Jorgensen, S. L. and Norenberg, M. D., Extracellular ATP induces stellation and increases glial fibrillary acidic protein content and DNA synthesis in primary astrocytic cultures. *Acta. Neuropathol.*, 1994, **87**, 8–13.

[30] Niitsu, A., Calcium is essential for ATP-induced steroidogenesis in bovine adrenocortical fasciculata cells. *Jpn. J. Pharmacol.*, 1992, **60**, 269–274.

[31] North, R. A. and Barnard, E. A., Nucleotide receptors. *Curr. Opin. Neurobiol.*, 1997, **7**, 346–357.

[32] Oglesby, I. B., Lachnit, W. G., Burnstock, G. and Ford, A. P. D. W., Subunit specificity of polyclonal antisera to the carboxy terminal regions of P2X receptors, $P2X_1$ through $P2X_7$. *Drug Dev. Res.*, 1999, **47**, 189–195.

[33] Slotkin, T. A., Development of the sympathoadrenal axis. In *Developmental Neurobiology of the Autonomic Nervous System*, ed. P. M. Gootman. Humana Press, Clifton, New Jersey, 1986, pp. 69–96.

[34] Tanaka, J., Murate, M., Wang, C.-Z., Seino, S. and Iwanaga, T., Cellular distribution of the $P2X_4$ ATP receptor mRNA in the brain and non-neuronal organs of rats. *Arch. Histol. Cytol.*, 1996, **59**, 485–490.

[35] Tischler, A. S., DeLellis, R. A., Nunnemacher, G. and Wolfe, H. J., Acute stimulation of chromaffin cell proliferation in the adult rat adrenal medulla. *Lab. Invest.*, 1988, **58**, 733–735.

[36] Tischler, A. S., DeLellis, R. A., Perlman, R. L., Allen, J. M., Costopoulos, D., Lee, Y. C., Nunnemacher, G., Wolfe, H. J. and Bloom, S. R., Spontaneous proliferative lesions of the adrenal medulla in aging Long-Evans rats. Comparison to PC12 cells, small granule-containing cells, and human adrenal medullary hyperplasia. *Lab. Invest.*, 1985, **53**, 486–498.

[37] Tomlinson, A. and Coupland, R. E., The innervation of adrenal gland. IV. Innervation of the rat adrenal medulla from birth to old age. A descriptive and quantitative morphometric and biochemical study of the innervation of chromaffin cells and adrenal medullary neurons in Wistar rats. *J. Anat.*, 1990, **169**, 209–236.

[38] Winkler, H. and Westhead, E., The molecular organization of adrenal chromaffin granules. *Neuroscience*, 1980, **5**, 1803–1823.

[39] Zheng, L. M., Zychlinsky, A., Lin, C.-C., Ojcius, D. M. and Young, J. D.-E., Extracellular ATP as a trigger for apoptosis or programmed cell death. *J. Cell Biol.*, 1991, **112**, 279–288.

[40] Zoeteweij, J. P., van de Water, B., de Bont, H. J. G. M., Mudler, G. J. and Nagelkerke, J. F., Involvement of intracellular Ca^{2+} and K^+ in dissipation of the mitochondrial membrane potential and cell death induced by extracellular ATP in hepatocytes. *Biochem. J.*, 1992, **288**, 207–213.