Immunohistochemical identification of cells expressing ATP-gated cation channels (P2X receptors) in the adult rat thyroid

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ABSTRACT

We carried out immunohistochemistry and western blotting of fresh frozen sections and crude extracts from adult rat thyroids. The histochemical and immunoblotting studies were performed with P2X receptor antibodies from 2 different sources. P2X-immunopositive cells were identified by fluorescence double labelling and confocal microscopy. Results of the western blotting experiments showed double bands of approximately 70 kDa and 140 kDa for all 7 P2X receptor subtypes with both sets of antibodies. Histochemical stains with antibodies from both sources also gave essentially identical results. $P2X_1$, $P2X_2$ and $P2X_6$ receptors were detected exclusively in vascular smooth muscle; $P2X_5$ and $P2X_7$ receptors were also present on vascular smooth muscle. Endothelial cells stained for $P2X_3$, $P2X_4$ and $P2X_7$ receptors. Thyroid follicular cells displayed immunoreactivity for $P2X_3$, $P2X_4$ and $P2X_5$ receptors. No immunostaining for P2X receptors was observed on C-cells. Possible roles for the broad expression of P2X receptor subtypes in the rat thyroid are discussed.

Key words: Western blotting; rat; immunohistochemistry.

INTRODUCTION

Extracellular nucleotides have been shown to play an important role in cell signalling of excitable and non-excitable cells (Abbracchio & Burnstock, 1998). Nucleotides are recognised as neurotransmitters of the central and the peripheral nervous systems (Burnstock, 1997, 1999 *a*), and have been shown to be messengers in cellular growth and differentiation (Abbracchio, 1996) and to mediate apoptosis (Chow et al. 1997).

Two families of receptors for extracellular nucleotides have been cloned. P2Y receptors are G protein-coupled receptors responding to purine and pyrimidine nucleotides, whereas P2X receptor are ATP-gated, nonselective cation channels (Abbracchio & Burnstock, 1994; Ralevic & Burnstock, 1998). The family of P2X receptors can be further subdivided into 7 subtypes, named $P2X_1$ to $P2X_7$ (Burnstock & King, 1996; North & Surprenant, 2000). These P2X receptor subtypes seem to assemble to homomeric and heteromeric oligomers, which form the functional channel (Torres et al. 1999). The different P2X receptor homomers and heteromers were shown to have different pharmacological properties. The ability of P2X receptors to assemble to oligomers and the paucity of selective agonists and antagonists for P2X receptors make the pharmacological identification difficult. To identify the expression of P2X receptors in native tissues and cell cultures specific antibodies for all 7 P2X receptors subtypes have been generated (Oglesby et al. 1999) and recently antibodies for P2X receptors from commercial sources have also become available.

In the present study we investigate the expression of P2X receptors in thyroid glands from adult rats. The thyroid is a bilobed organ surrounding the trachea just below the larynx. The thyroid consists of large (300 μm diameter) cavities, the thyroid follicles, which contain a single layer of epithelial cells, the thyroid follicular cells. The follicular cells secrete thyroid hormone precursors into the follicular lumen, where the
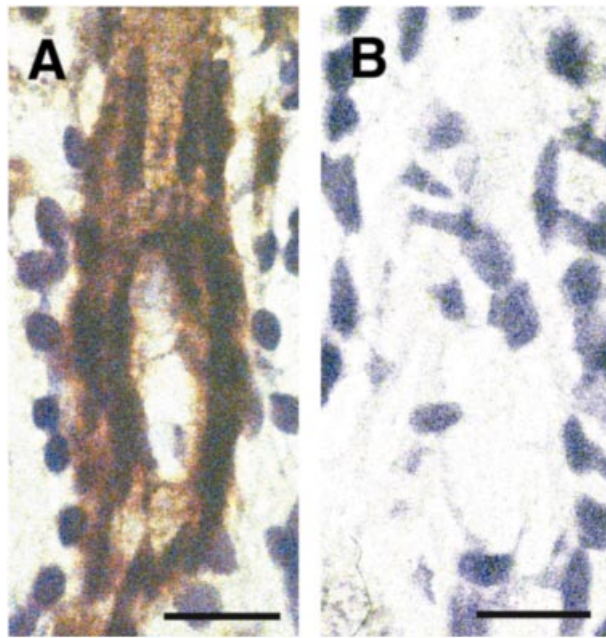
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Fig. 1. Immunohistochemical staining for P2X receptors with Alomone antibody. (*A*) Alomone P2X$_1$ antibody labels the vascular media; (*B*) Labelling with Alomone P2X$_1$ is completely pre-absorbable. Bars, 10 μm.

sympathetic and sensory nerves (Grunditz et al. 1988) and also consists of connective tissue (Werner & Ingbar, 1996). Thyroid hormone affects body growth, cellular catabolism, has effects on body temperature and heart rate, and has behavioural effects.

Interest arose in purinergic signalling in the thyroid after cell culture studies showed that ATP has profound effects on thyroid follicular cells, related to hormone synthesis and secretion (Raspé et al. 1989; Sato et al. 1992). Further studies showed that extracellular ATP also had comitogenic effects on the follicular cells (Törnquist et al. 1996). Recently, a mechanism for purinergic control of thyroid hormone secretion was proposed (Bourke et al. 2000). However, in these pharmacological studies only purinergic signalling via P2Y receptors could be proven. In the current study, we show for the first time that P2X receptors are expressed in the rat thyroid and discuss their possible functions.

hormone synthesis is completed. Dispersed between the thyroid follicles is another type of hormone secreting cell, the calcitonin secreting parafollicular cell or C-cell (Werner & Ingbar, 1996), participating in the control of the body's calcium homeostasis stimulating bone formation. The thyroid is highly vascularised, is innervated by sympathetic, para-

MATERIALS AND METHODS

Animals

Tissues were taken from 250 g, male Sprague–Dawley rats (n = 12). Animals were kept at a constant 12 h/12 h light-dark cycle with water and food ad libitum. Rats were killed with increasing concentrations of carbon dioxide and death was confirmed by cervical dislocation.
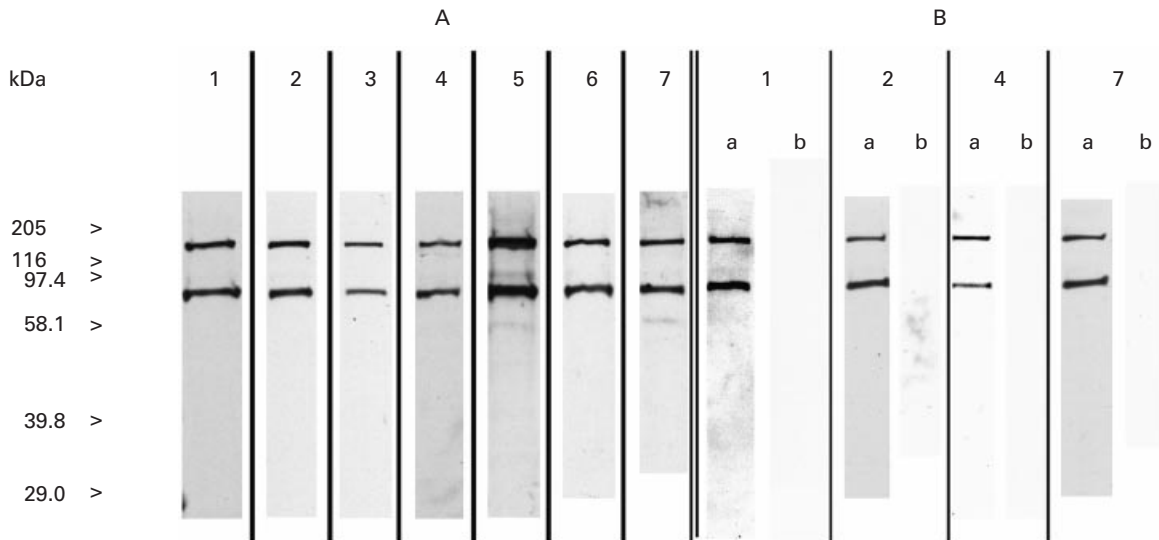

Fig. 2. Western blots of crude extracts from rat thyroid. Gels were run under reducing conditions, proteins were transferred onto nitrocellulose, incubated with antibodies for P2X receptors and were visualised using chemiluminescence. In panel *A* P2X receptor antibodies from Roche Bioscience were tested (subtypes 1 to 7); in panel *B* antibodies for P2X receptors from Alomone Labs were used (subtypes 1, 2, 4, and 7). Antibodies from Alomone Labs were also preabsorbed in immunoblotting, lanes *a* showing the immunodetection, lanes *b* giving results from the preabsorption experiments. Molecular weight markers are indicated by chevrons and the respective molecular weights are given in kDa. The number on each lane indicates for the respective P2X receptor subtypes; (*1*) P2X$_1$, (*2*) P2X$_2$, (*3*) P2X$_3$, (*4*) P2X$_4$, (*5*) P2X$_5$, (*6*) P2X$_6$ and (*7*) P2X$_7$.

Immunohistochemistry

Tissue handling. Thyroids were removed quickly and put immediately in ice-cold Hanks' Balanced Salts Solution, pH 7.5 (Gibco–BRL, UK). Unfixed tissues were embedded in Tissue-Tek (Sakura Finetek, Netherlands) and frozen in isopropanol, precooled in liquid nitrogen. Cryostat-sections (0.7 µm) were cut for immunohistochemical staining and conventional fluorescence microscopy. Sections (14 µm) were cut for immunofluorescence detection by confocal microscopy. The sections were placed on gelatine coated slides.

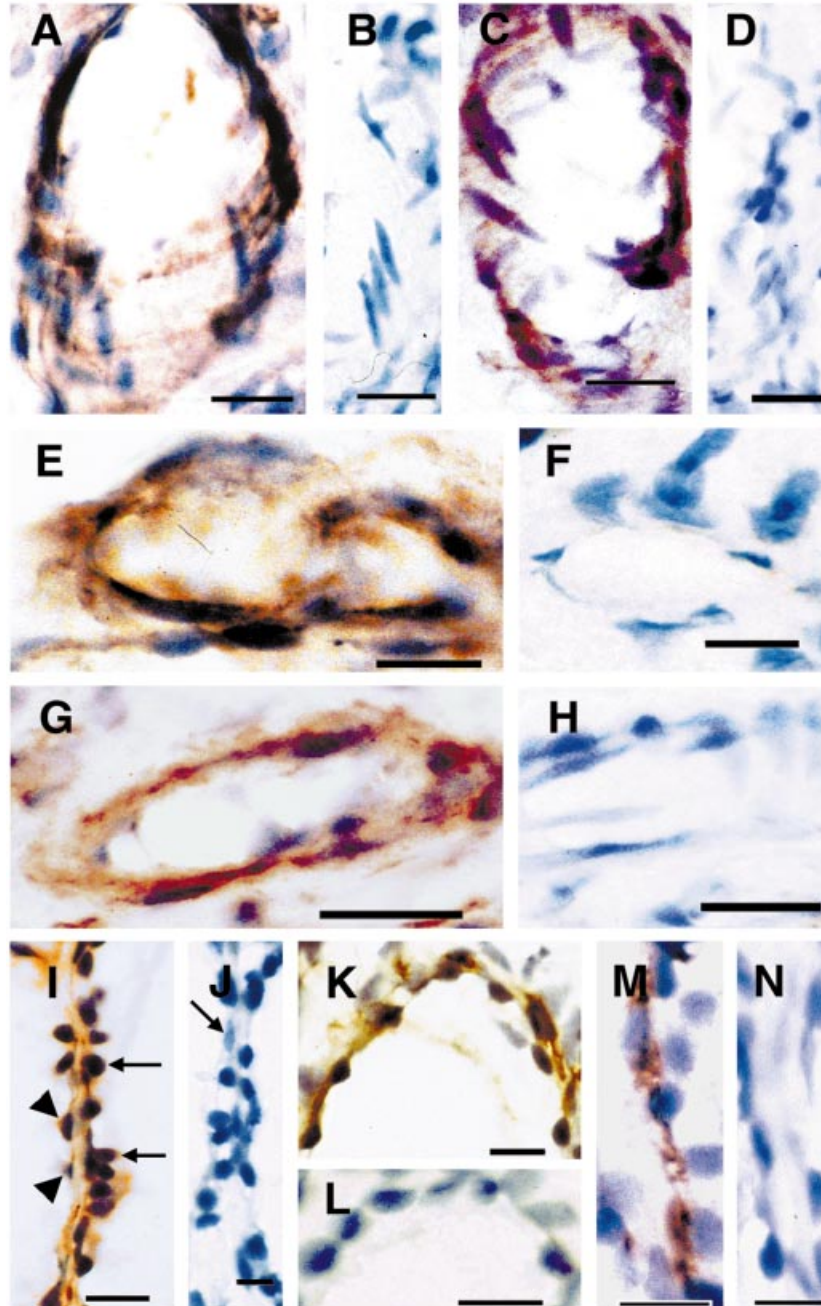Tissue-sections were postfixed for 2 min at room

Fig. 3. Light microscopical detection of immunohistochemical labelling with antibodies against P2X receptors. Staining was performed with antibodies against P2X receptor subtypes 1–7. Preabsorption experiments were carried out for each P2X antibody on sections that were consecutive to those used for immunostaining. (*A*) Vascular smooth muscle stained with $P2X_1$ antibody; (*B*) preabsorption of $P2X_1$; (*C*) immunostaining for $P2X_2$ receptors on vascular smooth muscle; (*D*) preabsorption of $P2X_2$ antibody; (*E*) vascular smooth muscle immunopositive for $P2X_5$ receptors; (*F*) preabsorbed $P2X_5$ antibody; (*G*) $P2X_6$ antibody on vascular smooth muscle; (*H*) staining with $P2X_6$ antibody was preabsorbable; (*I*) staining for $P2X_3$ receptors on endothelial cells (arrowheads) of a vessel, that is adjacent to a follicle (arrows) which is also immunopositive; (*J*) staining for $P2X_3$ receptors is preabsorbable (endothelial cells are still present, arrow); (*K*) immunoreactivity for $P2X_4$ receptors on endothelial cells; staining for $P2X_4$ is preabsorbed in (*L*); (*M*) $P2X_7$ antibody staining an endothelial cell; (*N*) $P2X_7$ antibody preabsorbed. Bars, 10 µm.

temperature (RT) in 4% formaldehyde (BDH Laboratory Supply, UK) in 0.1 M phosphate buffer. Inactivation of endogenous peroxidase was carried out in 50% methanol and 0.3% H_2O_2 (30 min at RT) for immunohistochemistry. Tissue-sections for immunofluorescence labelling were incubated for 10 min (at RT) in cyanoborohydrate coupling buffer (Sigma, UK), containing 3 g cyanoborohydrate per litre of phosphate-buffered saline (PBS). Sections were washed in PBS prior to immunostaining.

Immunohistochemical staining and counterstaining. Nonspecific binding sites were blocked by pre-incubation with 10% normal horse serum (NHS) (Harlan Sera-Lab, UK) in PBS for 20 min at RT. An indirect immunohistochemical method with 3 layers of antibodies was used. P2X receptor antibodies raised in rabbit were diluted in PBS/10%NHS and incubated over night at RT. The primary antibodies were allowed to react with biotinylated donkey antirabbit antibody (Jackson, UK) and were detected with avidin coupled horseradish-peroxidase (Sigma, UK) and diaminobenzidine (DAB; Sigma, UK), giving a brown colour precipitate.

The specificity of P2X antibodies was determined immunohistochemically by replacement of the primary antibody with nonimmune rabbit serum and by preabsorption with their cognate antigens. Preabsorption and immunohistochemical-staining experiments were carried out in parallel, using corresponding areas on consecutive sections for staining and preabsorption.

P2X receptor antibodies. One set of P2X antibodies (for P2X receptors 1 to 7) was obtained from Roche Bioscience (Palo Alto, CA). The P2X subtype-selective antibodies were raised in rabbit against a specific 15 amino acid residue at the carboxy-terminus of each purinoceptor molecule (Oglesby et al. 1999). A second set of antibodies (against P2X receptors 1, 2, 4 and 7) was purchased from Alomone Labs, Israel. The P2X receptor antibodies from Alomone Labs were also raised in rabbit against an antigenic sequence at the carboxy-terminus of the receptors. All Alomone Labs antibodies were purchased as affinity purified antibodies.

Comparison of antibodies for P2X receptors. The Alomone Labs antibodies were made monospecific, by elution against their cognate peptides. Thus this set of antibodies contains mainly immunoglobulins with high avidity for P2X receptors. The antibodies from Roche Bioscience consist of purified immunoglobulin, but have not been eluted against their cognate peptides. Comparing the data from western blotting and immunostaining with both antibody-sets can give clues whether the polyclonal Roche antibodies consist of high-avidity immunoglobulins, specific for P2X receptors. Staining and preabsorption of the Alomone $P2X_1$ receptor antibody is shown (Fig. 1).

Immunohistochemistry using 'high salt' medium. Primary antibodies were diluted in phosphate buffer/10% NHS containing 1.5% (w/v) NaCl (high-salt medium). In experiments using high-salt medium the washing step after incubation with primary antibody was carried out in phosphate buffer containing 1.5% (w/v) NaCl. It has previously been shown that variations in the salt concentration of the medium used for antibody dilution can have profound effects on antibody binding (Labrousse et al. 1994). Comparing both sets of antibodies after incubation in isotonic and 'high salt' medium can provide information on their vulnerability to changes in electrostatic interactions with their antigen.

Comparison of conventional immunofluorescence with tyramide-amplified immunofluorescence. Roche P2X receptor antibodies for conventional immunofluorescence were used at 2.5 to 6 µg protein/ml in 10% NHS. P2X receptor antibodies (Roche) for tyramide amplification (Renaissance, TSA indirect, NEN, USA) were used at 0.4 to 0.625 µg/ml in blocking solution according to the manufacturer's instructions. Primary antibodies were coupled to biotinylated donkey anti-rabbit IgG (Jackson, UK) and were detected with Streptavidin-FITC (Amersham, UK). Conventional immunofluorescence experiments often show good sensitivity of the method, but may give relatively high background. Immunofluorescent labelling after tyramide amplification can yield high sensitivity and low background, as reported previously by Shindler & Roth (1996).

Immunofluorescence and double-labelling. Three different immunofluorescence double-labelling techniques for P2X receptor detection and cell identification were used.

Firstly, P2X receptor antibodies (from Roche) were applied as for conventional histochemical staining (as described above). The primary antibody was detected by biotinylated donkey antirabbit antibody (Jackson, UK), applied together with mouse monoclonal antibodies directed against α-smooth muscle actin (Sigma, UK) to identify smooth muscle cells (Shalli et al. 1988), or against cytokeratins 1, 4, 5, 6, 8, 10, 13, 18, and 19 (pan-cytokeratin antibody; Sigma, UK) to identify epithelial cells (Dockhorn-Dworniczak et al. 1987). Slides were then (simultaneously) incubated with streptavidin-coupled Texas red (TR) (Amersham, UK) and goat antimouse immunoglobulin G (IgG; Sigma, UK). Finally, the antimouse IgG

was detected with fluorescein-isothiocyanate (FITC) coupled to chicken antigoat antibody (ICN, CA). For colabelling with endothelial cells the P2X antibodies were used as above, but no other primary antibody was applied. Endothelial cells were identified with FITC-coupled lectin from *Bandeira simplicifolia* (Sigma, UK), as previously described by Bankston et al. (1991).

In a second immunfluorescence double-labelling experiment primary antibody against P2X receptors (from Roche) was enhanced with tyramide signal amplification (as described above). P2X receptors were then again visualised with strepavidin-coupled TR. Labelling of vascular smooth muscle, epithelial cells and endothelial cell was performed as above.

In a third series of experiments, colocalisation studies for C-cells and P2X receptors were performed, using 2 primary antibodies raised in the same species. The experiments were carried out as described by Shindler & Roth (1996). Briefly, P2X receptors were detected by tyramide amplification using P2X antibody concentrations below the detection limit of a fluorophore-coupled secondary antibody (goat anti-rabbit, FITC-coupled; Nordic, NE). C-cells were then identified by incubation with rabbit anti-CGRP antibody (Affinity, UK) and by FITC-coupled goat antirabbit antibody (Arias et al. 1989).

Photography. Images of histochemical stains and immunofluorescence-labelling were taken with a Zeiss Axioplan microscope (Zeiss, Germany). Films were scanned with a Nikon LS-1000 scanner using Adobe-Photoshop 5.0. Prints were made with an Epson Stylus Photo 700 printer. Immunostaining of colocalisation experiments was analysed with an MRC 600 confocal microscope (Bio-Rad, CA), using filters for double labelling with TR and FITC, and Kalman filtering. All images were taken with a $\times 60$ objective, the aperture less than one-third open and the gain set between 8 and 9. The 2-channel readings for green and red fluorescence were merged using Confocal Assistant software (Bio-Rad, CA) and processing of the merged image was as described above.

Western blots

Thyroids were taken from three 250 g, male Sprague Dawley rats. The organs were immediately snap-frozen in liquid nitrogen and chopped up under liquid nitrogen, using a mortar and pestle. The tissue powder was then dissolved in RIPA buffer, containing 1% Igepal, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 100 µg/ml phenylmethyl-sulphonyl-fluoride (PMSF), 30 µl/ml Aprotinin and 1 mM sodium orthovanadate (all purchased from Sigma, UK). The homogenate was pulled through a 21 gauge needle to shear DNA and then cleared by centrifugation at 10 000 g for 30 min at 4 °C. The supernatant was used for western blotting in a Mini-Protean 2 Electrophoresis and Trans-Blotting Cell (Bio-Rad, USA) according to the manufacturer's instructions.

Proteins were loaded on Tris-HCl Ready Gels (10% gel; Bio-Rad, USA) and run under reducing conditions (10% SDS and 26 mM dithiothreitol). Biotinylated molecular weight markers were obtained from Sigma (UK) and from Amersham (UK). Proteins were transferred onto a Hybond ECL-nitrocellulose membrane (Amersham, UK). Nitro-celluloses were then blocked (blocking medium: PBS containing 3% nonfat milk powder and 0.05% Tween 20) at RT and incubated in blocking medium containing 2.5 µg/ml Roche antibodies or a dilution of 1:200 of the Alomone Labs antibodies. Pre-absorption of the Alomone antibodies was carried out according to the manufacturer's instructions. For detection of the immunoblots, the ECL chemilumi-nescence method was performed using a peroxidase-linked donkey antirabbit IgG, peroxidase-linked streptavidin and ECL Western Blotting Reagents (all purchased from Amersham, UK). The signal was visualised on a Hyperfilm ECL (Amersham, UK).

RESULTS

Western blotting

Both sets of antibodies for P2X receptors gave identical results in western blotting (Fig. 2). Anti-bodies from Roche Bioscience against $P2X_1$ through $P2X_7$ receptors and antibodies from Alomone Labs against $P2X_1$, $P2X_2$, $P2X_4$ and $P2X_7$ receptors showed clear bands of approximately 70 kDa and of approxi-mately 140 kDa. As the antibodies from Alomone Labs are supplied with sufficient antigenic peptide, preabsoptions of the immunoblots were carried out with this set of antibodies. Immunostaining of both bands of P2X receptors was completely pre-absorbable.

Comparison of immunohistochemical staining for P2X receptors with Alomone Labs and Roche antibodies

Results of the immunohistochemical experiments were principally identical. However, Alomone Labs $P2X_2$ antibody produced broad nuclear staining and
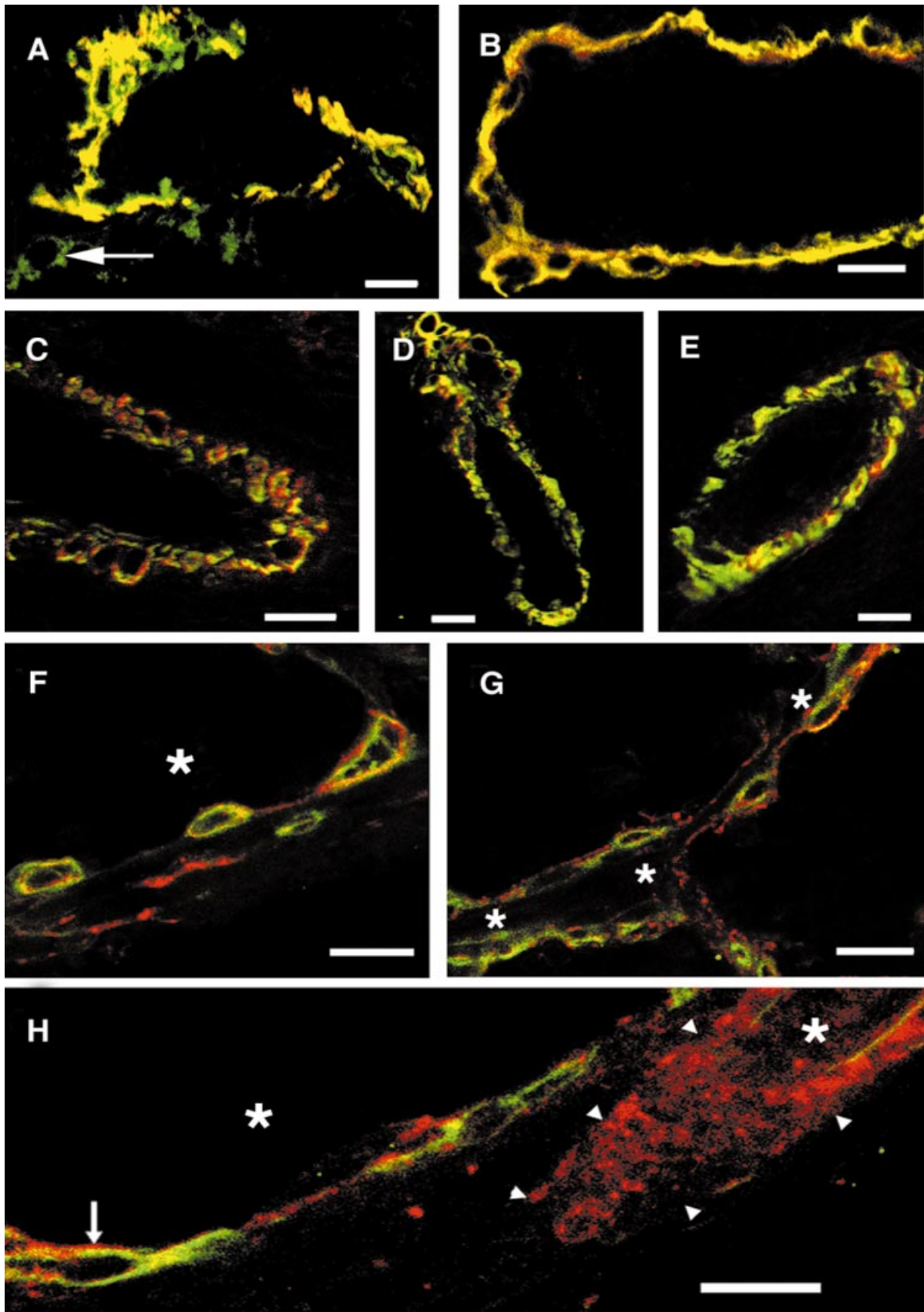
Fig. 4. Colocalisation study, identifying P2X receptors on vascular smooth muscle and endothelial cells by confocal microscopy. Sections from rat thyroid were incubated with antibodies for P2X receptors and were visualised with Texas red (red fluorescence). Parallel staining with a marker for vascular smooth muscle a marker for rat endothelial cells was performed, the markers were detected with FITC (green fluorescence). Areas of colocalisation (red and green fluorescence overlap) appear yellow. (*A*) P2X$_1$ receptors are expressed on vascular
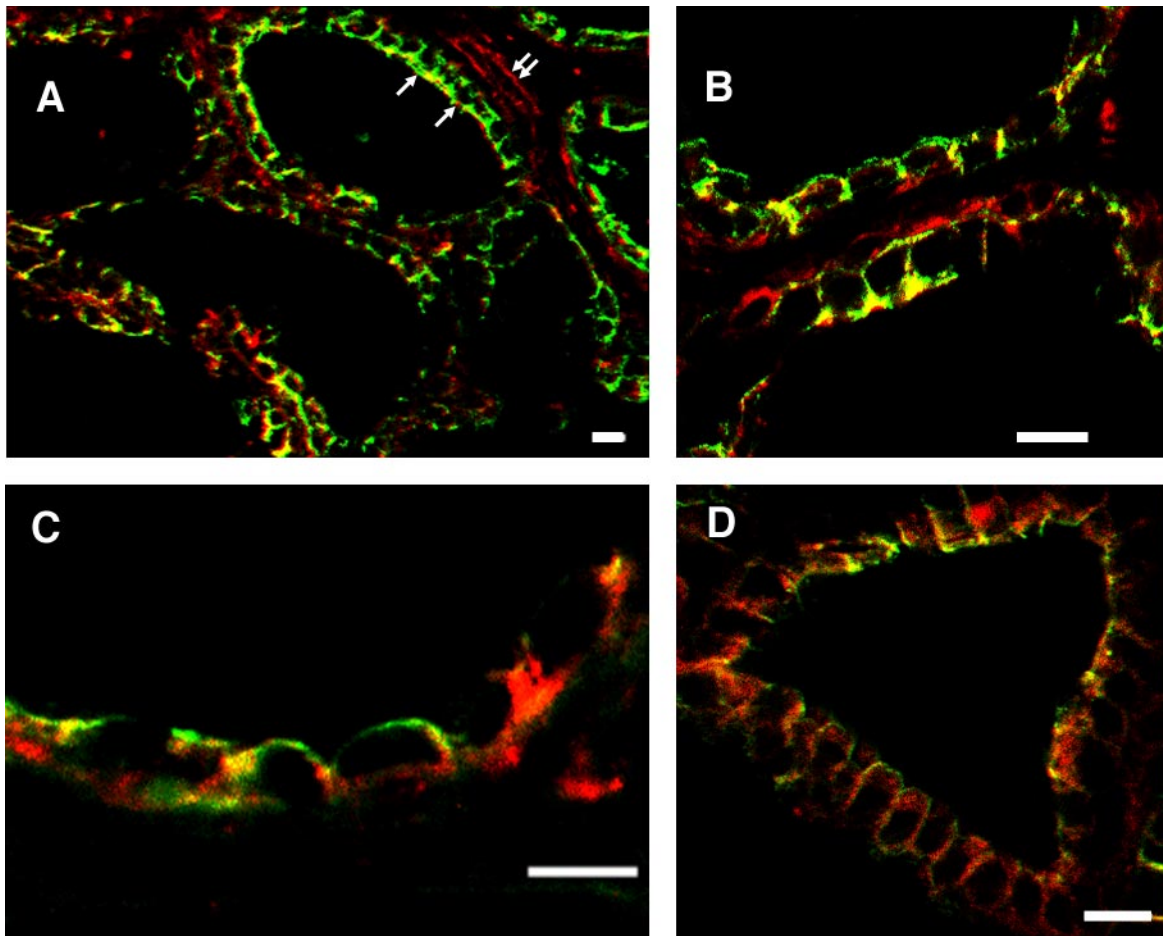
Fig. 5. Identification of P2X receptor immunopositive epithelial cells by confocal microscopy. Sections were incubated with antibodies for P2X receptors and visualised by red fluorescence. Epithelial cells were identified by a pancytokeratin marker, which was coupled to green fluorescence. Areas of colocalisation appear yellow. (*A*) Widespread immunoreactivity for $P2X_3$ receptors is seen on thyroid follicular cells (single arrows) and in some nearby endothelial cells (double arrows). (*B*) Higher magnification showing immunoreactivity for $P2X_3$ receptors in thyroid follicular cells. (*C*) Thyroid follicular cells stain for $P2X_4$ receptors. (*D*) $P2X_5$ receptors were detected on thyroid follicular cells. Bars, 10 µm

Alomone Labs $P2X_7$ antibody was not completely preabsorbable.

Immunohistochemistry using 'high salt' medium

The labelling appeared now slightly weaker, but no qualitative differences in immunostaining for P2X receptors were observed.

Conventional immunofluorescence experiments

Background staining obtained after 'conventional immunofluorescence' experiments was relatively high. However, incubation with cyanoborohydride-

coupling buffer significantly reduced thyroid autofluorescence.

Tyramide amplified immunofluorescence

The background obtained with 'conventional immunofluorescence' was greatly reduced after using tyramide amplification.

P2X receptors on blood vessels

The media of blood vessels was found to be immunopositive for $P2X_1$ receptors and the staining was completely preabsorbable (Fig. 3 *A*, *B*). Results of preabsorption and immunostaining experiments were

smooth muscle cells, but not all smooth muscle cells express $P2X_1$ (arrow); (*B*) $P2X_2$ receptors colocalise with vascular smooth muscle cells; (*C*) vascular smooth muscle cells express $P2X_5$ receptors; (*D*) $P2X_6$ receptors could be detected in vascular smooth muscle; (*E*) immunoreactivity for $P2X_7$ receptors was seen in vascular smooth muscle; (*F*) endothelial cells stain for $P2X_3$ receptors (vascular lumen is indicated by asterisk); (*G*) $P2X_4$ receptors were detected on endothelial cells (vascular lumen is indicate by asterisks); (*H*) immunostaining for $P2X_7$ receptors was observed in endothelial cells (arrow) and in vascular smooth muscle (arrowheads, lumina of the vessels are indicated by asterisks). Bars, 6 µm.

generally shown for corresponding locations of the tissue. The media of blood vessels was immunopositive for $P2X_2$ receptors and the immunostaining was again fully preabsorbable (Fig. 3 *C*, *D*). Thyroid vessels displayed immunoreactivity for $P2X_5$ and $P2X_6$ receptors in the median layer, which was completely abolished after preabsorption. Staining for $P2X_7$ receptors was also regularly found in the media of small and large vessels of the thyroid.

Immunolabelling for $P2X_3$ and $P2X_4$ receptors was found in the intima of thyroid vessels and in some capillaries. Antibodies against $P2X_7$ receptors consistently gave immunostaining not only of the media, but also of the intima of thyroid blood vessels and appeared to label the endothelium of interfollicular capillaries. All intimal staining for P2X receptors was preabsorbable.

Immunostaining for P2X receptors was also observed in follicle-like structures. $P2X_3$, $P2X_4$ and $P2X_5$ receptors were observed on the thyroid follicular epithelium, which was preabsorbable.

Immunofluorescence double labelling

Thyroid vascular smooth muscle was shown to express $P2X_1$, $P2X_2$, $P2X_5$, $P2X_6$ and $P2X_7$ receptors (Fig. 4). Colocalisation experiments of P2X receptor antibodies with a marker for endothelial cells showed that thyroid endothelial cells express $P2X_3$, $P2X_4$ and $P2X_7$ receptors (Fig. 4). A colocalisation study with antibodies for P2X receptors and a marker for cytokeratins revealed that thyroid follicular epithelial cell express $P2X_3$, $P2X_4$ and $P2X_5$ receptors (Fig. 5).

Colocalisation experiments for P2X receptors and C-cells. No labelling for P2X receptors could be detected in the interfollicular C-cells.

DISCUSSION

The specificity of antibodies used in this study was shown by western blotting of crude organ extracts, by comparing different sets of antibodies and several immunohistochemical techniques.

Western blotting

Antibodies from Roche Bioscience and from Alomone Labs showed distinct bands of 70 kDa and 140 kDa in western blotting; both bands were preabsorbable. Identical molecular weights for P2X receptors have been reported earlier (Glass et al. 2000). P2X receptors have been described as highly glycosylated molecules,

which can form dimers (Vulchanova et al. 1997) and were shown to have molecular weights between 57 kDa and 64 kDa in transfected cells (Vulchanova et al. 1997; Lê et al. 1998). In situ glycosylation of the homomeric receptor and dimerisation of the glycosylated subunits can account for the higher molecular weights of P2X receptors shown in the present study.

P2X receptors on vascular smooth muscle

$P2X_1$, $P2X_2$, $P2X_5$, $P2X_6$ and $P2X_7$ receptors were seen in this study in the media of blood vessels. Colocalisation studies revealed that these P2X receptor subtypes were expressed in vascular smooth muscle cells. Previously, $P2X_1$, $P2X_2$ and $P2X_4$ receptors have been found expressed on vascular smooth muscle (Nori et al. 1998) and recently immunolabelling for $P2X_5$ and $P2X_6$ receptors was detected on smooth muscle of the bladder (Lee et al. 2000). In human vascular smooth muscle $P2X_7$ receptors mediate cell contraction and cell lysis (Cario-Toumaniantz et al. 1998). P2X receptors on vascular smooth muscle are involved in the regulation of vascular tone by sympathetic innervation (Burnstock, 1990; Abbracchio & Burnstock, 1998). The regulation of vasoconstriction by sympathetic neurons is by noradrenergic and purinergic cotransmission to noradrenaline receptors and $P2X_1$, $P2X_2$ and possibly $P2X_4$ receptors on vascular smooth muscle (Abbracchio & Burnstock, 1998; Nori et al. 1998). The greater number of P2X receptor subtypes in the thyroid may reflect a necessity for increased fine tuning of vascular tone. The synthesis and secretion of thyroid hormone is controlled by thyrotrophin (or thyroid stimulating hormone; TSH), reaching the thyroid via the circulation. Alterations of thyroid vascular tone may affect the amount of TSH reaching the hormone-producing cells. A different, but not alternative view is that thyroid vascular tone can be altered by the vasodilatory actions of thyroid hormone on vascular smooth muscle (Ojamaa et al. 1993). $P2X_6$ receptors may also participate in vascular fine tuning in the thyroid, although this receptor subunit does not form functional homomeric receptors, but assembles with other P2X subunits to form heteromeric receptors (Torres et al. 1999). The heteromeric assembly of P2X receptor subunits can provide an increased diversity of the pharmacological response to ATP and may allow increased fine tuning of vascular tone.

The thyroid vessels may undergo constant remodelling during normal thyroid function, but have to maintain the blood supply for thyroid follicles,

which can undergo profound changes in shape and size during hormone synthesis and storage or in hormone secretion (Werner & Ingbar, 1996). Thus the thyroid vasculature may have a high plasticity, increasing or reducing vascular branches to support the multiple changes in organ and follicle morphology. $P2X_5$ receptors have been associated with cellular differentiation (Gröschel-Stewart et al. 1999) and $P2X_7$ receptors are established as mediating apoptosis (Ferrari et al. 1999). An elevated vascular turnover may show elevated cell differentiation and increased vascular apoptosis, which would be reflected by the abundance of $P2X_5$ and $P2X_7$ receptors.

P2X receptors on endothelial cells

$P2X_3$, $P2X_4$ and $P2X_7$ receptors were detected on endothelial cells in the present study. Previously we have described the presence of $P2X_3$ receptors on vascular endothelial cells in the thymus by light-microscopy (Glass et al. 2000) and of $P2X_2$ receptors on endothelial cells by electron-microscopy (Loesch & Burnstock, 2000). Confocal microscopy has shown an abundance of $P2X_2$ receptors on endothelial cells (Hansen et al. 1999).

Capillaries surround the thyroid follicles in a 3-dimensional basket-like structure (Imada et al. 1986). The regulation of endothelial barrier function in these capillaries is crucial for the delivery of hormones to and from the thyroid follicles. The morphology of thyroid capillaries undergoes profound changes in response to stimulation of the thyroid (Imada et al. 1986) and endothelial cells accelerate protein synthesis in response to goitrogenic stimuli (Ericson & Wollman, 1980). One might speculate that the abundance of P2X receptors in endothelial cells of the thyroid reflects a tighter regulation of endothelial barrier function and a greater excitability to external stimuli than for many other organs. Extracellular ATP may affect thyroid blood flow and endothelial permeability via P2X receptors, as has been described for other substances such as 5-hydroxytryptamine and histamine (Melander et al. 1975). $P2X_7$ receptors are likely to be involved in endothelial cell apoptosis after follicular involution. $P2X_7$ receptor-mediated endothelial apoptosis has been described (von Albertini et al. 1998) and follicular involution is frequently seen in thyroids with normal physiology (Werner & Ingbar, 1996).

P2X receptors on thyroid follicular cells

$P2X_3$, $P2X_4$ and $P2X_5$ receptors were found on thyroid follicular cells (an epithelial cell type) in the present study. C-cells are also an epithelial cell type (Hardy, 1988), which appear scattered between the thyroid follicles; from colocalisation studies with a specific C-cell marker (CGRP), however, P2X receptors were absent from C-cells.

Studies on the rat thyroid cell line FRTL-5 have shown that thyroid follicular cells respond to extracellular ATP by activation of several second messenger systems, possibly through a P2Y receptor-mediated effect (Sato et al. 1992). Martin (1992) reported that ATP activated a Ca^{2+}-dependent Cl^- current in FRTL-5 cells, which was probably mediated through a P2Y receptor, but ATP also activated a Ca^{2+} influx pathway (Aloj et al. 1993). Most recent evidence for ion fluxes initiated after application of ATP was found on pig thyroid epithelial cells by Bourke et al. (2000).

$P2X_4$ and $P2X_5$ receptors have already been shown to be involved in the mediation of Cl^- and K^+ secretion in airway epithelia (Taylor et al. 1999). Ion fluxes across membranes of thyroid follicular cells are important in trapping iodide for thyroid hormone synthesis (Werner & Ingbar, 1996) and Cl^- and K^+ fluxes are thought to participate in mediating thyroid hormone secretion (Yap et al. 1993). $P2X_3$ receptors were also found expressed on thyroid epithelial cells. $P2X_3$ receptors have so far been associated with purinergic signalling in sensory neurons (Lewis et al. 1995), but mRNA for $P2X_3$ receptors has also been detected in several lung-derived epithelial cells (Taylor et al. 1999). The $P2X_3$ receptor subunits may form homodimeric receptors of unknown function on the follicular cells, or they may assemble to heterodimeric receptors with the $P2X_5$ receptor subunits (Torres at al. 1999).

Extracellular ATP could derive from various sources. The thyroid is extensively innervated by sympathetic, parasympathetic and sensory neurons (Grunditz et al. 1988) and ATP can be coreleased with noradrenaline from sympathetic nerves (Abbracchio & Burnstock, 1998). Sympathetic innervation controlling thyroid hormone secretion has previously been described (Nilsson & Karlberg, 1983; Riedel & Burke, 1988). Thus sympathetic nerves may stimulate P2X receptors on thyroid follicular cells and may be involved in some thyroid functions.

Another possible source for extracellular ATP is the calcitonin-secreting C-cells. Ekelund et al. (1980) found that C-cells fluoresce after labelling with quinacrine that was later established as a marker for vesicular-stored ATP (Crowe & Burnstock, 1982). Some coupling of calcitonin secretion by C-cells to thyroid hormone secretion by follicular cells has been

described (Riedel & Burke, 1988). The C-cells may provoke a response from the follicular cells by secreting ATP, which could act on the follicular P2X receptors.

P2X receptors may also be excited via autocrine stimulation. Thyroid follicles expand greatly in size as they accumulate thyroid hormone (Werner & Ingbar, 1996). During this expansion the thyroid follicular epithelial cells become stretched, which can be detected as a change in cell morphology. ATP release may be induced by this distension (Burnstock, 1999*b*) and the ATP may then act in an autocrine fashion on the follicular epithelial cells. This model may explain how thyroid hormone synthesis/storage and secretion can both be controlled by the same chemical messenger, TSH. In empty thyroid follicles the epithelial cells are relaxed and no stretch-induced secretion of ATP may occur. TSH may then be the predominate chemical messenger for stimulating thyroid hormone synthesis and storage. However, in a fully expanded follicle the epithelial cells would be stimulated by extracellular ATP and TSH together, which may cause thyroid hormone secretion.

In summary we have reported an abundance of P2X receptors on thyroid vascular smooth muscle, thyroid endothelial cells and thyroid follicular epithelial cells. The thyroid expresses a wider range of P2X receptors on its vasculature than in any other organ reported so far. This may reflect the constant dynamic changes in thyroid morphology and the special need to regulate thyroid vascular tone. The high level of expression of P2X receptors on thyroid endothelial cells could reflect the need for tight control of endothelial barrier function in this endocrine gland and may reflect the high turnover of thyroid capillaries. P2X receptors on thyroid follicular epithelial cells may be associated with the control of thyroid hormone secretion by stimulation in a neurocrine, paracrine and/or autocrine way.

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