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REGULAR ARTICLE

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Tissue distribution of $P2X_4$ receptors studied with an ectodomain antibody

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Abstract A monoclonal antibody was developed to the extracellular domain of the rat $P2X_4$ receptor. The antibody was highly selective among all rat P2X receptor subunits, and recognised only the oligomeric, non-denatured form of the $P2X_4$ receptor. Immunohistochemistry showed an extensive pattern of distribution throughout the central and peripheral nervous systems, the epithelia of ducted glands and airways, smooth muscle of bladder, gastrointestinal tract, uterus, and arteries, uterine endometrium and fat cells. The protein was identified by Western blotting in membrane extracts of these tissues, and the ectodomain antibody immunoprecipitated a protein that was recognised with a $P2X_4$ receptor C terminus antibody. The findings indicate that the $P2X_4$ receptor subunit has a very extensive distribution among mammalian tissues, and this suggests possible new functional roles.

Keywords $P2X_4$ receptor · Purinoceptor · ATP · Immunoprecipitation · Immunohistochemistry · Rat (Wistar)

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Introduction

When extracellular ATP binds to P2X receptors it opens an integral channel that allows the net inflow of cations into the cell. This effect is observed in a very wide range of cells, ranging from bone to brain (see Ralevic and Burnstock 1998; North 2002). There is considerable diversity in the details of the ionic currents observed, and this is presumed to result from diversity in the molecular composition of the P2X receptors. The receptors are formed most likely as trimers (or hexamers) from the same or different subunits (Nicke et al. 1998; see North 2002). Seven mammalian subunit genes have been identified, and cDNAs cloned ($P2X_1$–$P2X_7$; see North 2002).

The $P2X_4$ subunit cDNA was isolated from several different tissues (hippocampus: Bo et al. 1995; brain: Séguéla et al. 1996; Soto et al. 1996; pancreatic islets: Wang et al. 1996; superior cervical ganglion: Buell et al. 1996), and the mRNA is particularly highly expressed in epithelia such as salivary glands (Collo et al. 1996). Recordings from the submandibular gland showed that current elicited by ATP was unusual in that it was not blocked by the P2X receptor antagonists suramin and pyridoxal-5-phosphate-6-azophenyl-2', 4'-disulfonic acid (PPADS) (Buell et al. 1996). The finding that the $P2X_4$ receptor expressed in HEK cells (Buell et al. 1996; Soto et al. 1996) or *Xenopus* oocytes (Bo et al. 1995; Séguéla et al. 1996; Wang et al. 1996) shares this property is consistent with it being formed from $P2X_4$ subunits in salivary glands. On the other hand, there is evidence for

$P2X_4$and $P2X_6$ subunits forming hetero-oligomers in expression systems (Lê et al. 1998; Khakh et al. 1999), and these two subunit proteins have a strongly overlapping distribution at the subcellular level in the brain (Rubio and Soto 2001).

P2X receptor subunits have intracellular N and C termini, and two membrane-spanning domains which are connected by an ectodomain some 280 amino acid residues in length. Almost all studies of the distribution of $P2X_4$ subunits have used an antibody directed against the final amino acids of the carboxyl terminus of the protein. There are several reasons for seeking a monoclonal antibody directed to the ectodomain of the subunit, and these include applications in living cells, such as FACS analysis. They include the opportunity to use the antibody to distinguish between multimeric and monomeric forms of the protein (e.g. $P2X_7$: see Kim et al. 2001a). Immunoprecipitation of the $P2X_7$ receptor has recently been used as the basis for identifying a complex of interacting proteins (Kim et al. 2001b), and such approaches are also facilitated by an antibody that recognises non-denatured protein.

In this paper we describe the production and characterisation of a monoclonal ectodomain antibody for the rat $P2X_4$ receptor. We used the antibody in immunohistochemistry and immunoprecipitation to identify the $P2X_4$ receptor subunit in a range of rat tissues.

Materials and methods

Development of anti-$P2X_4$ monoclonal antibody

Two DNA fragments encoding the segments Ile^{44} to Asp^{224} and Cys^{165} to Ala^{313} of the deduced amino acid sequence of the rat $P2X_4$ subunit were subcloned into pRSET7c. pRSET7c is derived from pRSET5c (Schoepfer 1993) and expresses a N-terminal fusion protein with an His-tag (MASMHHHHHHGI...). BL21(DE3) [pLysS] *E. coli* were transformed with the two constructs, and expression of $P2X_4$ peptides with N-terminal 6×His tags was induced with IPTG. Peptides were purified with Ni^{2+}-NTA-agarose (Qiagen, Hilden, Germany). Balb/c mice were immunised with 25 μg of each peptide and RIBI adjuvant system, followed by three boasts before bleeding. Successful immunisation was confirmed by ELISA and immunocytochemistry, and the spleen cells were fused with a myeloma cell line. Hybridoma cells were cultured in a medium with the following components: RPMI1640, 15% fetal calf serum, 5% horse serum, 2 mM glutamine, 1 mM sodium pyruvate, 10% Doma-drive (Immune Systems, Bristol, UK), 50 U/ml penicillin/streptomycin, and 50 μg/ml Fugizone (Gibco BRL). Single cell cloning was performed using immunostaining of rat cerebellum with hybridoma culture medium as a selection marker. Three rounds of single cell cloning were carried out. The isotypes of the monoclonal antibodies were determined with IsoStrip Mouse Monoclonal Antibody Isotyping Kit (Roche, Indianopolis, USA). The monoclonal antibody from a selected clone (2G11) was purified from cell culture medium using ImmunoPure Immobilized Protein L Plus (Pierce, Rockford, IL, USA).

Immunohistochemistry

Wistar rats of either sex (150–200 g) were killed by overdose of CO_2. Tissues were embedded in OTC compound and frozen immediately in isopentane precooled with liquid nitrogen. Cryosections (14 μm) were fixed in 2% paraformaldehyde and 0.2% picric acid for 2 min. Cells were permeabilised with 0.1% Triton X-100. Sections were blocked with PBS with 5% normal goat serum, 0.1 M glycine, and 0.1% BSA. The $P2X_4$ecto-Ab diluted in PBS with 5% normal goat serum and 0.1% BSA was applied to the sections and incubated at 4°C overnight. Secondary FITC-conjugated goat-anti-mouse antibody (DAKO; 1:150 dilution) was applied and fluorescence was observed with a Zeiss microscope.

Preparation of membrane fractions

Adult Wistar rats were killed by overdose of CO_2, and tissues were rapidly removed and placed in ice-cold PBS containing protease inhibitors (Complete, Roche). Tissues were homogenised by a polytron homogeniser and cell debris was removed by centrifugation at 400×*g* for 10 min at 4°C. The supernatants were subjected to ultracentrifugation at 105,000×*g* for 1 h at 4°C. The pellets (membrane fractions) were solubilised with 20 mM dodecylmaltoside (Calbiochem, San Diego, USA) in PBS containing protease inhibitors overnight at 4°C. Insoluble membrane fractions were removed by centrifugation at 25,000×*g* for 1 h at 4°C. The concentrations of protein in total membrane extracts were measured by Protein Assay kit (Bio-Rad, Hercules, CA, USA).

Immunoprecipitation

Total solubilised membrane proteins were incubated with ImmunoPure Immobilized Protein L Plus, which had been pre-equilibrated with solubilisation buffer for 3 h at 4°C for preabsorption of background proteins. $P2X_4$ecto-Ab was diluted 1:50 and incubated with the preabsorbed solubilised membrane proteins for 24 h at 4°C, and ImmunoPure Immobilized Protein L Plus was added. After overnight incubation with resin at 4°C, the resins were washed 5 times for 20 min with solubilisation buffer, and the immunocomplex of proteins was separated from the resin by boiling for 5 min in SDS sample buffer. For immunoprecipitation of EE-tagged $P2X_n$ receptors from transfected HEK 293 cells, purified membrane extracts were preincubated with GammaBind G Sepharose (Pharmacia Biotech, Uppsala, Sweden) for preabsorption, followed by incubation with EE-Ab (BabCo, Berkeley, CA) and subsequent GammaBind G Sepharose precipitation. Resin-bound proteins were separated as described above.

Immunoblotting

Total membrane fractions or immunoprecipitated samples were separated by SDS-PAGE (12%) and transferred to PVDF membranes. Membranes were rinsed in TBS buffer (20 mM TRIS, 500 mM NaCl, pH 7.5) and blocked with TBS buffer containing 5% non-fat dry milk for 1 h. After washing with TTBS (20 mM TRIS, 500 mM NaCl, pH 7.5, 0.1% Tween-20), membranes were incubated overnight with polyclonal antibodies ($P2X_4$C-Ab) from Alomone (Jerusalem, Israel) or with polyclonal antibody (EE-Ab) from Bethyl (Montgomery, TX, USA). After several washings with TTBS, the membranes were incubated for 1 h with alkaline phosphatase-conjugated goat anti-mouse or anti-rabbit antibody (Bio-Rad). Membranes were washed and immunoreactive proteins were detected with Immun-Star assay kit (Bio-Rad) following the manufacturer's instructions.

Native electrophoresis

Blue Native PAGE (BN-PAGE) was performed as described previously (Kim et al. 2001a). Solubilised membrane fractions were supplemented with BN sample buffer to a final concentration of 10% glycerol, 0.2% Coomassie blue R-250 and 50 mM Bistris (pH 7.0). For the cathode buffer, 5 mg/ml of Coomassie blue R-250 was used in 50 mM Tricine, 15 mM Bistris (pH 7.0). Molecular

weight standards (Combithek II, Roche) were applied to lanes on each border of the gel. Either linear polyacrylamide gradients from 4% to 15%, or 7.5% (w/v) gels without stacking gel, were performed at 4°C; these were transferred to PVDF membranes and immunoblotted with $P2X_4$C-Ab polyclonal antibody (1:1000). All experiments were repeated 3–5 times with similar results obtained throughout.

Results

Generation and specificity of $P2X_4$ecto-Ab

The $P2X_4$ecto-Ab was selected by immunostaining of Purkinje cells in rat cerebellum, which have been shown to express a high level of $P2X_4$ mRNA transcripts (Bo et al. 1995; Séguéla et al. 1996; Collo et al. 1996). Twelve clones were initially selected; one of these, an IgM isotype, was chosen for all subsequent studies. The antibody specifically immunoprecipitated the $P2X_4$ subunit. HEK293 cells were transfected with cDNAs encoding epitope-tagged receptors ($P2X_n$-EE), and detergent-solubilised proteins from whole cell lysates were immunoprecipitated with either EE-Ab or $P2X_4$ecto-Ab (Fig. 1A). Detection by immunoblotting with EE-Ab showed that $P2X_4$ecto-Ab was able to immunoprecipitate only $P2X_4$ subunits (Fig. 1A).

We have not been able to detect any protein by immunoblotting with the $P2X_4$ecto-Ab itself, suggesting that the antibody recognises native rather than denatured forms of the protein. We tested this by repeating the immunoprecipitation after either reduction (with dithiothreitol) or denaturation (95°C, 5 min) of the protein. Denaturation prevented the immunoprecipitation by $P2X_4$ecto-Ab, although simple reduction did not (Fig. 1B, upper panel); denaturation made no difference to the immunoprecipitation by the antibody directed at the C terminal epitope (EE-Ab) (Fig. 1B, middle panel). The existence of larger oligomeric complexes was also apparent from Blue Native gel electrophoresis (Fig. 1B, bottom panel); the $P2X_4$ receptor migrated with a molecular weight in excess of 300 kDa, and denaturation of the protein produced a major band at about 65 kDa, which is the size expected for monomeric $P2X_4$ subunits. These findings are similar to those reported for some other P2X receptors ($P2X_1$, $P2X_3$: Nicke et al. 1998; $P2X_7$: Kim et al. 2001a), and indicate that the $P2X_4$ecto-Ab recognises $P2X_4$ subunits only in native oligomeric form.

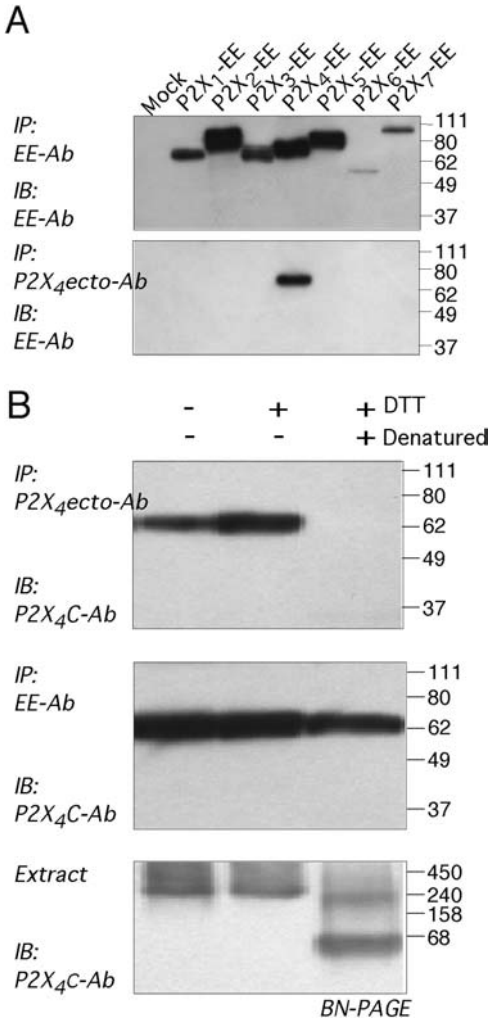

Fig. 1A, B The ectodomain antibody (*$P2X_4$ecto-Ab*) is specific among rat P2X subunits, and recognises only non-denatured protein. **A** HEK293 cells were transfected with each of the EE-tagged subunits. Each subunit was immunoprecipitated with anti-EE antibody (*top*), but only the $P2X_4$ subunit was immunoprecipitated with $P2X_4$ecto-Ab (*bottom*). **B** HEK293 cells were transfected with $P2X_4$ subunits. Membrane extract was treated with dithiothreitol (100 mM) or denatured (95°C, 5 min). These samples were immunoprecipitated with $P2X_4$ecto-Ab (*upper*) or EE-Ab (*middle*) and immunoblotted by $P2X_4$C-Ab. *Lower panel* shows Blue Native PAGE of membrane extracts. The non-denatured protein migrates as a large complex >300 kDa, which can be denatured to a monomeric form (≈65 kDa). $P2X_4$ecto-Ab does not detect the denatured monomeric form of the receptor (*upper panel*)

$P2X_4$ecto-Ab immunoreactivity is widely distributed in rat tissues

Immunoreactivity with $P2X_4$ecto-Ab was observed in many rat tissues (Fig. 2, Table 1). In the brain, the strongest signals were observed in Purkinje cells (Fig. 2A, arrows) in cerebellum, while in hippocampus, where the original $P2X_4$ was cloned from (Bo et al. 1995), the immunosignals were much weaker. Large motor neurons in the brain stem (Fig. 2B, arrows) and neurons in the deep nucleus of the cerebellum were moderately labelled. Glial cells in cerebellum were also moderately labelled (Fig. 2A). Immunopositive parallel fibres in the molecular layer of cerebellum were visible (Fig. 2A, orange coloured arrow). Cells in pia mater and small blood vessels in the meninges were strongly labelled (Fig. 2A, arrowhead). In the peripheral nervous system, neurons in pelvic ganglion, intracardiac ganglion, and superior mesenteric ganglia were moderately positive. In superior cervical ganglia, neurons were weakly labelled (Fig. 2C).

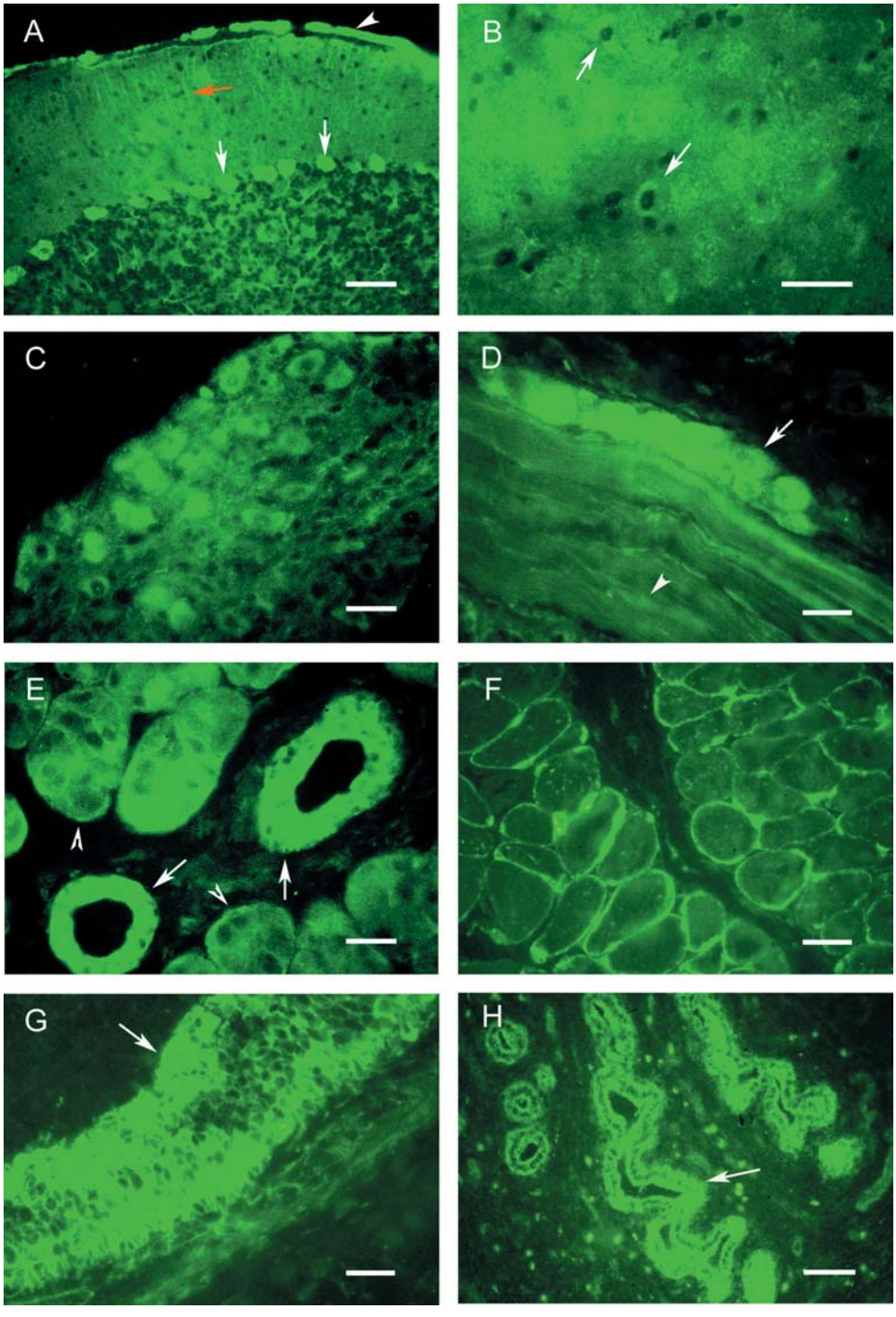

Fig. 2A–H Tissue distribution of $P2X_4$subunit by immunohistochemistry. $P2X_4$ecto-Ab was used to stain sections of fixed rat tissues. **A** Cerebellum: Purkinje cells (*arrows*), pia mater (*arrowhead*), parallel fibres (*orange arrow*). **B** Brain stem: positively labelled neurons (*arrows*). **C** Superior cervical ganglion. **D** Stomach: myenteric neurons (*arrow*), smooth muscle (*arrowhead*). **E** Parotid: interlobular duct (*arrows*), serous acini (*arrowheads*).**F** Adipose tissue. **G** Bronchus: epithelium (*arrow*). **H** Uterus: endometrial glands (*arrow*). *Scale bars* 100 μm **(A)**, 50 μm **(B–H)**

Neurons in the submucosal and myenteric nerve plexuses in the gastrointestinal tract were also positive (Fig. 2D, arrow). The immunosignals in sensory neurons of the dorsal root or trigeminal ganglia were generally undetectable using the present method. Some small neurons in the trigeminal ganglia and some nerve fibres in the dorsal root were weakly labelled.

Strong immunostaining was observed in epithelial cells in many organs. Both the serous acini and intralobular ducts in the parotid and mandibular glands were labelled, with the duct cells showing brighter fluorescence (Fig. 2E, arrow: intralobular ducts, arrowheads: serous acini). The intralobular ducts in pancreas and bile ducts in the liver were also labelled brightly. The pseudostratified ciliated epithelial cells in bronchus were strongly positive (Fig. 2G), as were endometrial glands in the uterus (Fig. 2H).

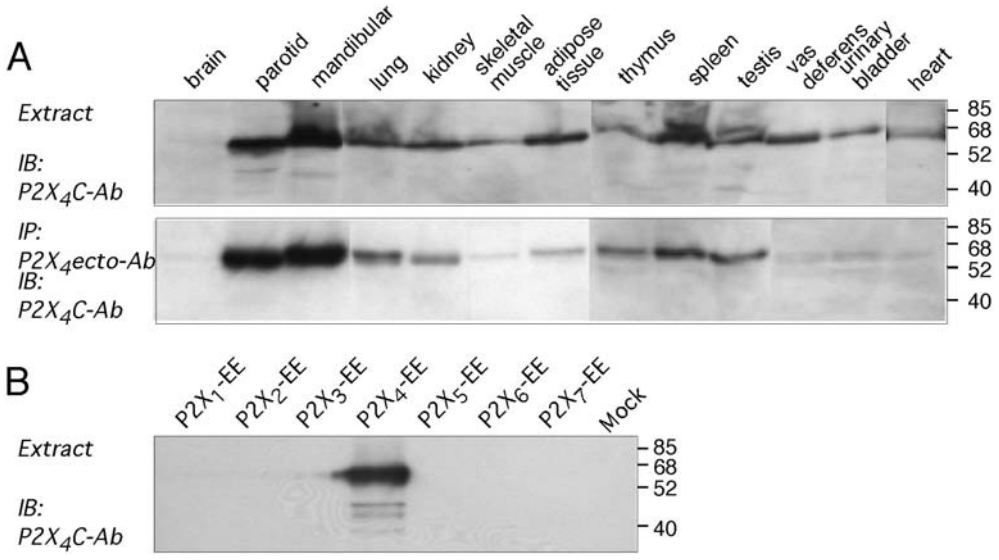

Fig. 3A, B Tissue distribution of $P2X_4$ subunit by Western blotting. **A** Total membrane extracts from various tissues were detected by $P2X_4$C-Ab antibody (*top*). Receptors were immunoprecipitated with $P2X_4$ecto-Ab from membrane extracts and detected with $P2X_4$C-Ab antibody (*bottom*). **B** Total cell extracts of HEK 293 cells transfected as indicated were immunoblotted with $P2X_4$C-Ab; other P2X subunits were not detected

Smooth muscle cells in many organs such as urinary bladder, gastrointestinal tract (Fig. 2D, arrowhead), uterus, and medium-sized arteries were weakly positive. In cardiac muscle, the intercalated discs were strongly positive, whereas immunoreactivity in other parts of the myocardium was not detected. Finally, adipocytes of the mesentery were well stained (Fig. 2F).

All major organs of rat have been examined with $P2X_4$ecto-Ab immunostaining. The relative intensities of the immunostaining are listed in Table 1.

Identification of $P2X_4$ subunits in rat tissues by Western blot and immunoprecipitation

Membrane fractions were prepared from 13 tissues, and extracted by detergent. Western blotting showed that the $P2X_4$C-Ab antibody recognised a protein of ~60 kDa in the extract from all of the tissues tested (Fig. 3). This C terminal antibody recognised a protein of the same size in the material immunoprecipitated by the new $P2X_4$ecto-Ab. On the basis of the immunoprecipitation, the $P2X_4$ subunit is most abundantly expressed in the salivary glands (parotid and mandibular); there was moderate expression in lung, kidney, fat cells, thymus, spleen, testis, vas deferens and urinary bladder, with less but still detectable protein in brain, heart and skeletal muscle.

Discussion

The $P2X_4$ subunit appears to be the most widely expressed of the seven genes. A very wide distribution pattern of mRNA transcript has been described, including vascular muscle (Nori et al. 1998), endothelium (Yamamoto et al. 2000), osteoclasts (Naemsch et al. 1999), colonic crypts (Tanaka et al. 1996), renal collecting duct cells (McCoy et al. 1999), bronchial epithelium (Buell et al. 1996), salivary glands (Buell et al. 1996; Turner et al. 1998; Tenneti et al. 1998) and the central nervous system (Bo et al. 1995; Buell et al. 1996; Soto et al. 1996; Séguéla et al. 1996). $P2X_4$ subunit protein expression has been shown by immunohistochemistry and/or Western blotting using antibodies raised against the final 15–19 amino acids of the subunit (here designated $P2X_4$C-Ab) in several laboratories. $P2X_4$immunoreactivity has been observed in Purkinje cells and hippocampal neurons (Rubio and Soto 2001), neurons of many ganglia (Xiang et al. 1998a, 1998b), thyroid follicular cells (Glass and Burnstock 2001), thymocytes (Glass et al. 2000), arteries (Lewis and Evans 2001), retina neurons (Wheeler-Schilling et al. 2001), osteoclasts (Hoebertz A et al. 2000), and B lymphocytes (Sluyter et al. 2001).

The $P2X_4$ecto-Ab developed in the present work provides a pattern of immunohistochemical staining that further extends our knowledge of the distribution of this subunit (see "Introduction"). In some of these tissues, such as salivary glands, there is already some evidence for ATP-activated ion current properties of the homomeric $P2X_4$ receptor (Buell et al. 1996); the hallmarks are the ineffectiveness of α, βmeATP as an agonist, and of suramin and PPADS as antagonists. Likewise, there is compelling evidence from anti-sense RNA depletion experiments for a role for $P2X_4$ receptors in the shear-stress-induced rise in $[Ca]_i$ in vascular endothelium (Yamamoto et al. 2000). In other tissues that we studied (e.g. Purkinje cells, heart, kidney, thymus; see references in the "Introduction") there is evidence for the $P2X_4$ subunit on the basis of mRNA expression, but there has been little or no characterisation of the functional response. And in some tissues (spleen and fat cells) we found expression of $P2X_4$ protein where it had not previously been described. Of these tissues, brown fat cells respond to extracellular ATP with a cation conductance increase (Lee and Pappone 1997), and splenic dendritic cells show an increase in $[Ca]_i$ (Nihei et al.

Table 1 Distribution of $P2X_4$immunoreactivity among rat tissues

Tissues	$P2X_4$ecto-Ab immunostaining
Hippocampus	− ~ +
Cerebral cortex	−
Cerebellum: Purkinje cell body	+++
Parallel fibres	+
Granule cells	++
Dentate nucleus	++
Olfactory bulbs, astrocyte-like cells	++
Brain stem, neurons	+ ~ ++
Pia mater	++ ~ +++
Superior cervical ganglion	+
Superior mesenteric ganglion	+
Pelvic ganglion	+
Cardiac ganglion	++
Dorsal root ganglion: neurons	−
Nerve fibres	+
Trigeminal ganglion: small neurons	+
Large neurons	−
Adrenal ganglion (inside medulla)	++
Adrenal gland	−
Myocardium: intercalated disc	+
Small arteries	+ ~ ++
Trachea and bronchial: epithelium	+
Smooth muscle	+
Pulmonary artery	++
Alveoli	++
Parotid: interlobular duct	+++
Acini	+++
Tongue: taste buds	++
von Ebner's glands	+
Skeletal muscle	−+
Oesophagus: smooth muscle	+
Basal layer of epithelium	+++
Stomach: smooth muscle	+
Myenteric neurons	++
Epithelial cells	++
Intestine: smooth muscle	+
Myenteric neurons	++
Epithelial cells	++
Pancreas: interlobular duct	++++
Liver: biliary duct	+++
Bladder: smooth muscle	+
Epithelial cells	−+
Vas deferens: smooth muscle	+
Epithelium	−+
Prostate	−+
Testis: late spermatids	++
Vagina: smooth muscle	++
Epithelial cells	++++
Uterus: smooth muscle	++
Epithelial cells	+++
Glands	+++
Arteries	+++
Ovary: follical cells of young follicles+	+++
Oviduct: smooth muscle	+++
Kidney: some tubules	++
Spleen	−+
Adipocyte	+++
Eye: cornea epithelium	+++
Ciliary body epithelium	++
Lacrymal gland (outer layer)	+++

2000). In most of the tissues positive by immunohistochemistry we can be confident of the identity of the subunit because Western blotting identified a protein of the appropriate size for the $P2X_4$ subunit in the membrane extract, and the $P2X_4$ecto-Ab was able to bring down a protein immunoreactive for $P2X_4$C-Ab. In spleen and testis, another protein band with a higher molecular weight was present in the immunoblot, which may be due to non-specific binding of the $P2X_4$C-Ab, because they are not seen on the material immunoprecipitated with the ectodomain antibody.

There is a certain discrepancy between the results from immunohistochemistry and immunoblotting in the present study. $P2X_4$ proteins were detected from skeletal muscle and spleen in immunoblotting, while no signal was detectable in these tissues in the immunohistochemical study. One reason may be that receptor proteins were enriched as membrane preparation was used in immunoblotting. Another reason may be the presence of $P2X_4$ subunits on small blood vessels in the tissues (Nori et al. 1998; Lewis and Evans 2001). In the brain, immunohistochemistry and in situ hybridisation showed the presence of $P2X_4$ subunits in many regions of the brain (Rubio and Soto 2001; Bo et al. 1995; Buell et al. 1996; Soto et al. 1996; Séguéla et al. 1996); however, the $P2X_4$ protein band in the immunoblot is quite weak. A possible reason is that although the expression level of $P2X_4$ in several brain regions is relatively high, $P2X_4$ protein was diluted as the whole brain was used for membrane preparation.

The immunohistochemical results obtained using $P2X_4$ecto-Ab in the current study are generally in agreement with those obtained with $P2X_4$C-Ab, such as the staining of Purkinje cells and hippocampal neurons (Rubio and Soto 2001), blood vessels (Lewis and Evans 2001), uterine epithelial cells (Slater et al. 2000), and neurons in peripheral ganglia (Xiang et al. 1998a). Recently, it was reported that $P2X_4$ was the major P2X subunit in the human airway epithelial cells (Zsembery et al. 2003), which confirmed our observation of intense staining of rat bronchial epithelial cells with $P2X_4$ecto-Ab. We were able to detect the $P2X_4$ subunit in rat testis, while Glass et al. (2001) reported that $P2X_4$ was absent in testis in their study. As the resources of $P2X_4$ antibodies and the methods used in different laboratories were different from each other, it is difficult to match the results from all the laboratories.

Several of the tissues that express $P2X_4$ subunits also express one or more further P2X subunits. Examples are arterial smooth muscle ($P2X_1$ and $P2X_4$: Lewis and Evans 2001), endothelium ($P2X_4$ and $P2X_7$: Ramirez and Kunze 2002), parotid gland (Tenneti et al. 1998; Turner et al. 1998), and B lymphocytes ($P2X_1$, $P2X_4$ and $P2X_7$: Sluyter et al. 2001). This raises the possibility that in native cells $P2X_4$subunits might form hetero-oligomeric channels with other subunits, in which case the widespread distribution that we have found might suggest that the $P2X_4$ subunit serves as a principal or alpha subunit of many P2X receptors. On the other hand, the present results would also be consistent with individual tissues

expressing more than one set of homomeric receptors. The monoclonal antibody that we characterise in the present work will be valuable for the isolation of $P2X_4$ subunits from a wide range of native tissues, and thus will be a useful tool to investigate questions such as actual subunit composition of native P2X receptors.

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