A P2X purinoceptor cDNA conferring a novel pharmacological profile

Xuenong Bo[a], Yi Zhang[a], Mohammed Nassar[b], Geoffrey Burnstock[a], Ralf Schoepfer[b,*]

[a]*Department of Anatomy and Developmental Biology, University College London, Gower Street, London, WC 1E 6BT, UK*
[b]*Wellcome Laboratory for Molecular Pharmacology and Department of Pharmacology, University College London, Gower Street, London WC 1E 6BT, UK*

Received 25 September 1995

Abstract **We have cloned $P2X_4$, a member of the P2-purinoceptor family, which has a new pharmacological profile. Rat $P2X_4$ is distantly related to $P2X_1$, $P2X_2$ and $P2X_3$ and is expressed in brain, spinal cord, lung, thymus, bladder, adrenal, testis and vas deferens. This ligand gated ion channel is activated by ATP and analogs with the potency order of ATP > ATPγS > 2-methylthio ATP > ADP ≈ $\alpha\beta$-methylene ATP. However, none of the currently used P2X purinoceptor antagonists suramin, reactive blue 2 and PPADS blocked ATP evoked currents; in contrast their application resulted in potentiation of the agonist response. Due to lack of any known antagonist for $P2X_4$ it is unlikely that native $P2X_4$ has previously been recognized as a P2X purinoceptor.**

Key words: Purine receptor; P2-purinoceptor; ATP; Suramin; CNS

1. Introduction

ATP is a widespread neurotransmitter [1]. Following the classification of P1- (adenosine as the endogenous ligand) and P2-purinoceptors (ATP and ADP as the endogenous ligands) for extracellular adenine nucleotides [2], many subtypes of P2-purinoceptors have been identified based on a large amount of pharmacological, electrophysiological and molecular biological studies [3]. Lately these receptors have been reclassified into P2X (ligand-gated) and P2Y (G-protein coupled) purinoceptor families [4,5]. Whereas P2Y receptors have the bonafide structure of G-protein coupled receptors [6,7], recent cloning of P2X-purinoceptors identified them as a new family of ligand gated ion channels that is related to the inward rectifiers of potassium channels [8]. The three known P2X cDNAs are only about 35% identical to each other and have a somewhat narrow pattern of expression: $P2X_1$ [9] shows a high level expression in vas deferens and a low expression in other tissues, $P2X_2$ [10] has been found in PC12 cells and at lower levels in other tissues and the expression of $P2X_3$ [11] is limited to dorsal root ganglia.

ATP and 2-methylthio ATP (2-MeSATP) are the most potent agonists for recombinant $P2X_{1-3}$ purinoceptors. Suramin, reactive blue 2 (RB-2) and PPADS (pyridoxalphosphate-6-azophenyl-2',4'-disulphonic acid) are all efficient antagonists for those receptors that where tested [9,10,11].

ATP mediated responses in the CNS have been identified in the spinal cord [12]. More recently Edwards et al. [13] described fast synaptic transmission by ATP in the medial habenula suggestive of a role for ATP gated ion channels of the P2X type (reviewed by [14]). Autoradiographic localization of [^{3}H]α,β-methylene ATP binding sites also indicates the presence of P2X-purinoceptors in the brain and spinal cord [15].

In this paper we describe the isolation of $P2X_4$, a new P2X cDNA from rat brain. $P2X_4$ was found to be widely expressed. Recombinant $P2X_4$ purinoceptors are not blocked by currently used ATP purinoceptor antagonists and therefore define a new pharmacological type of P2X purinoceptors.

2. Materials and methods

2.1. Isolation of cDNA clone

Standard DNA techniques were used [16]. A hippocampal cDNA library was screened with ^{32}P-labeled probes derived from the $P2X_1$ [9] and $P2X_2$ [10] cDNA clones. The probes covered coding sequences only. Hybridization stringency was controlled by 20% formamide at 42°C. Low stringency washing was controlled by the final wash in 1× SSPE at 45°C. Positive clones were sequenced in both directions with ABI's (Applied Biosystems) cycle sequencing kit and ABI 373 sequencer.

2.2. Northern blot analysis

Poly(A)$^+$ mRNAs were isolated using the Fast-Track II kit from Invitrogen (San Diego, USA) from fresh tissues of male Sprague Dawley rats (250 g). Nominally 3.2 μg of poly(A)$^+$ mRNAs was size-separated through a formaldehyde-containing 1% agarose gel and transferred onto nylon membranes (Hybond N, Amersham Intl., Amersham, UK). A 600 bp fragment (30nt 5'UT segment through codon of aa 195) and 370 bp PCR derived fragment (codon for aa 354 through stop codon +270nt 3'UT segment) were used as ^{32}P-labeled probes at high stringency. Hybridization was in 50% formamide at 42°C, and final wash was at 0.3× SSPE at 65°C. Both probes gave identical patterns of signals. Some blots were further washed at 0.1× SSPE which did not change the pattern of detected signals.

2.3. In situ hybridization

Freshly obtained brain and spinal cord were cut to 14 μm thick sections on a cryostat. Sections were thaw-mounted onto silanized glass slides and fixed immediately in 4% paraformaldehyde following the protocol of refs. [17,18]. Prehybridization in 50% formamide, 37°C for 3 h, followed by overnight hybridization at 55°C, 50% formamide. Final wash was in 0.1× SSC, 50% formamide, 55°C 3 × 30 min. Same DNA segments as in Northern blots were used as probes. However, for in situ hybridization, digoxigenin labeled RNA probes (Dig-probes) were used. These Dig-probes were obtained as run-off transcripts on linearized plasmid DNA following manufacturer's instructions (Dig RNA labeling kit, Boehringer Mannheim). Hybridization was revealed through anti-Dig-alkaline phosphatase antibodies and visualized as dark brown color (DigNucleic acid detection kit, Boehringer Mannheim) followed by microphotography. Four repetition of in situ hybridization experiments gave identical results.

2.4. Electrophysiology

cRNA was obtained as previously described [19]. *Xenopus* oocytes (stage V) were manually isolated, followed by a collagenase (Sigma, type V) treatment (0.33 mg/ml for 1 h at about 22°C). Oocytes were then defolliculated and injected (Drummond Injector) with 23 nl of cRNA (approximately 0.01–0.1 μg/μl). For expression, the oocytes were incubated in modified Barth's solution (88 mM NaCl, 1 mM KCl, 2.4 mM

*Corresponding author. University College London, Department of Pharmacology, Gower Street, London, WC 1E 6BT, UK.
Fax: (44) (171) 380 7245; E-mail: r.schoepfer@ucl.ac.uk

0014-5793/95/$9.50 © 1995 Federation of European Biochemical Societies. All rights reserved.
SSDI 0014-5793(95)01203-6

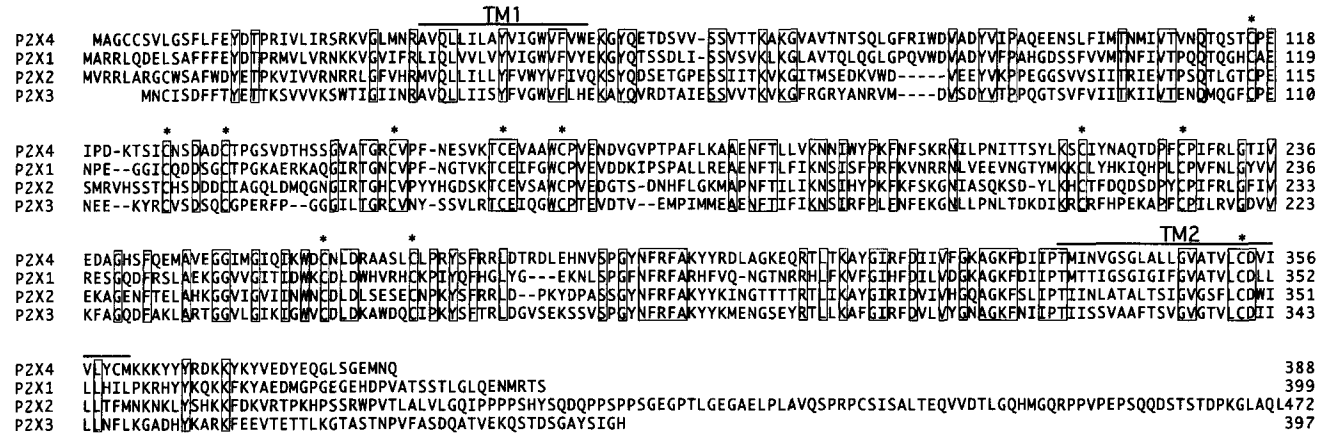

Fig. 1. Alignment of deduced $P2X_4$ sequence with $P2X_1$ [9], $P2X_2$ [10] and $P2X_3$ [11]. The two putative transmembrane segments are indicated by TM1 and TM2 bars. There are eleven conserved cysteines, marked by *. Percent similarity between $P2X_4$ and P2X1, $P2X_2$ or $P2X_3$ is 49%, 43% or 44%, respectively. The nucleotide sequence of P2X4 has been submitted to the EMBL database under number: X91200.

$NaHCO_3$, 0.33 mM $Ca(NO_3)_2$, 0.82 mM $MgSO_4$, 0.41 mM $CaCl_2$, 15 mM Tris-Cl, adjusted to pH 7.4 with HCl, supplemented with 50 IU/ml penicillin (BRL) and 50 μg/ml streptomycin (BRL)) at 19°C for 3 days followed by storage at 4°C until electrophysiological measurements. Injected oocytes were analyzed in the two electrode voltage clamp configuration (TEC05 amplifier, NPI electronics, Tamm, Germany) perfused with NFR (115 mM NaCl, 2.5 mM KCl, 10 mM Hepes adjusted to pH 7.2 with HCl and supplemented with 0.18 mM $CaCl_2$ unless otherwise noted). Data were recorded simultaneously to a chart recorder and to a computer hard disk. For chart like digital data recording data were typically filtered at 100 Hz, digitized at 300 Hz (oversampling) and reduced to 5 Hz before storage. Pulse-like data recording was done with synchronous DA and AD conversions at 1000 Hz. Application of perfusion solutions as well as clamp voltage was computer controlled.

2.5. Drugs

ATP, ADP and AMP (adenosine tri-, di- and mono-phosphate respectively), adenosine, αβ-methylene ATP, ATPγS (adenosine 5′-O-(3-thio)triphosphate), A4pA (diadenosine tetraphosphate), UTP (uridine triphosphate) and RB-2 (Reactive Blue 2) were all from Sigma, 2-methylthio ATP from Research Biochemicals Intl. (Natick, USA), suramin from Bayer AG (Strawberry Hill, Newbury, UK), PPADS (pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid) was a gift from Prof. G. Lambrecht (University of Frankfurt, Germany). All drugs were first made up as a stock solution in water and diluted in NFR to the stated concentration.

3. Results

3.1. Cloning of the $P2X_4$ purinoceptor

Low stringency library screening identified cDNA clones whose deduced amino sequence are clearly, but distantly related to the $P2X_1$ and $P2X_2$ sequences. The deduced amino acid (Fig. 1) predicts a protein of 43.5 kDa which is about equidistantly related to the three other known P2X sequences. The $P2X_4$ sequence has the two putative transmembrane segments and the 11 conserved cysteine residues considered characteristic for this gene family.

3.2. Tissue distribution of expression

$P2X_4$ was isolated from a hippocampal rat brain cDNA library. High specific RNA levels were detected in cerebellar Purkinje cells and spinal cord motor neurons (Fig. 3) and medulla oblongata (not shown). Weaker in situ hybridization signals were detected in the hippocampus and olfactory bulb (not shown). The medial habenula revealed no detectable $P2X_4$ hybridization signal in our experiments.

Northern blotting revealed widespread expression of $P2X_4$ A^+ RNA of a length of 1.9 kb. All of the tested tissues revealed comparable amounts of specific mRNA. With the exception of RNA isolated from testis, only a single RNA species was detected.

3.3. Recombinant expression

Xenopus oocytes injected with $P2X_4$ RNA yielded consistently robust instant currents after ATP application. Even at

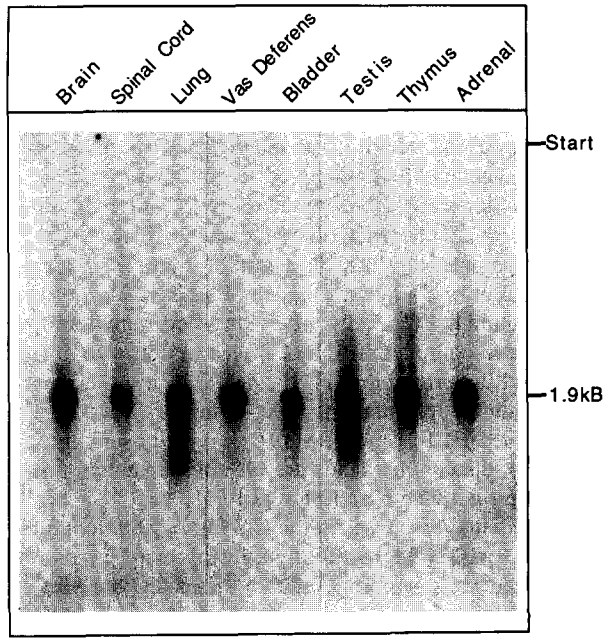

Fig. 2. Expression of $P2X_4$ specific mRNA detected by Northern blot. 3.2 μg of A^+ RNA was loaded per lane, except for RNA from bladder (1.6 μg). No signal was detected on 3.2 μg of brain total RNA (not shown).

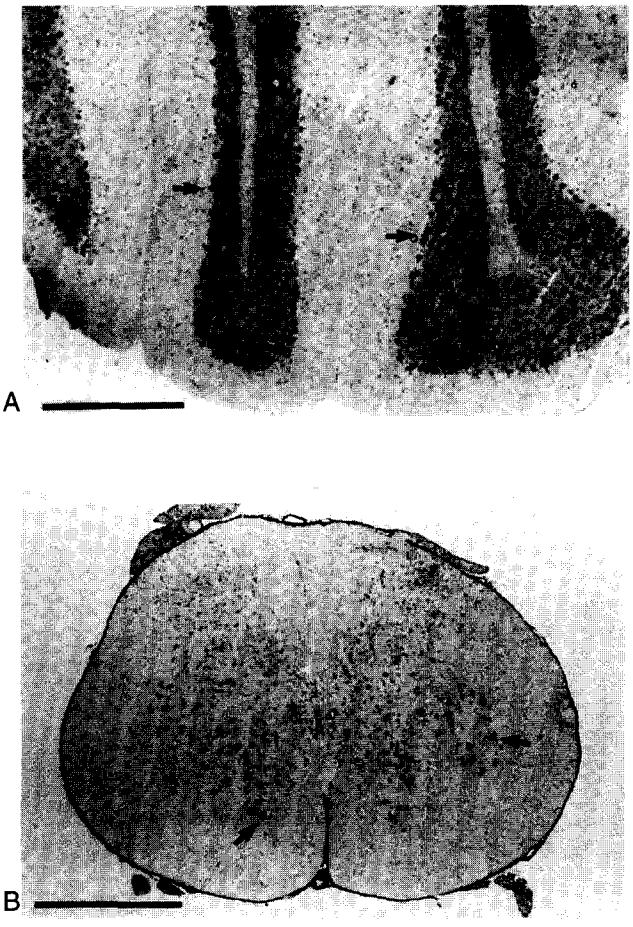

Fig. 3. $P2X_4$ Dig probes label individual Purkinje cells in cerebellar cortex (A) and motor neurons in spinal cord (B). (A) is a coronal section; scale bar: 500 μm. (B) is a transversal section of the lumbar spinal cord; scale bar = 1000 μm.

low ATP concentrations, responses showed partial desensitization. $P2X_4$ purinoceptors showed prolonged periods necessary for recovery from desensitization. We found that wash periods of 10–15 min were necessary to allow for full recovery (data not shown). This slow recovery seriously hampered experiments. All other compounds that acted as agonists (Table 1) showed this kinetic pattern. We tested whether the ATP evoked response is abolished by unrecovered desensitization from previously applied α,β-MeATP. We found that pre-application of 100 μM α,β-MeATP for 3 min resulted in a reduced ATP evoked response (still >50% of control with 3 μM ATP), but not in a complete suppression (data not shown).

We found further that the observed desensitization was identical under low, 180 μM, Ca^{2+} concentration (Fig. 4a) or Ca^{2+} substitution by 1800 μM Ba^{2+} (not shown) in the perfusion solution. These conditions should have minimized artifacts through Ca^{2+} activated chloride channels. $P2X_4$ channels displayed an identical inward rectification in low Ca^{2+} or standard Ba^{2+} conditions (Fig. 4b). We did not attempt to determine permeability of the $P2X_4$ channel for Ca^{2+} ions.

The apparent concentration for half maximal response (EC_{50}) was 10 μM for ATP ($n = 4$) using the initial peak response after agonist application (Fig 5). The precision of this

Table 1
Agonist potency ratios

Compound ($n = 4$)	Relative potency ratio
ATP	1
ATPγS	0.435 ± 0.046
2-Methylthio ATP	0.136 ± 0.016
ADP	0.036 ± 0.003
$\alpha\beta$-Methylene ATP	0.030 ± 0.002
AMP	No response
Adenosine	No response
UTP	No response
Diadenosine tetraphosphate	No response

No response: evoked currents < 10 nA with compounds tested up to 300 μM.

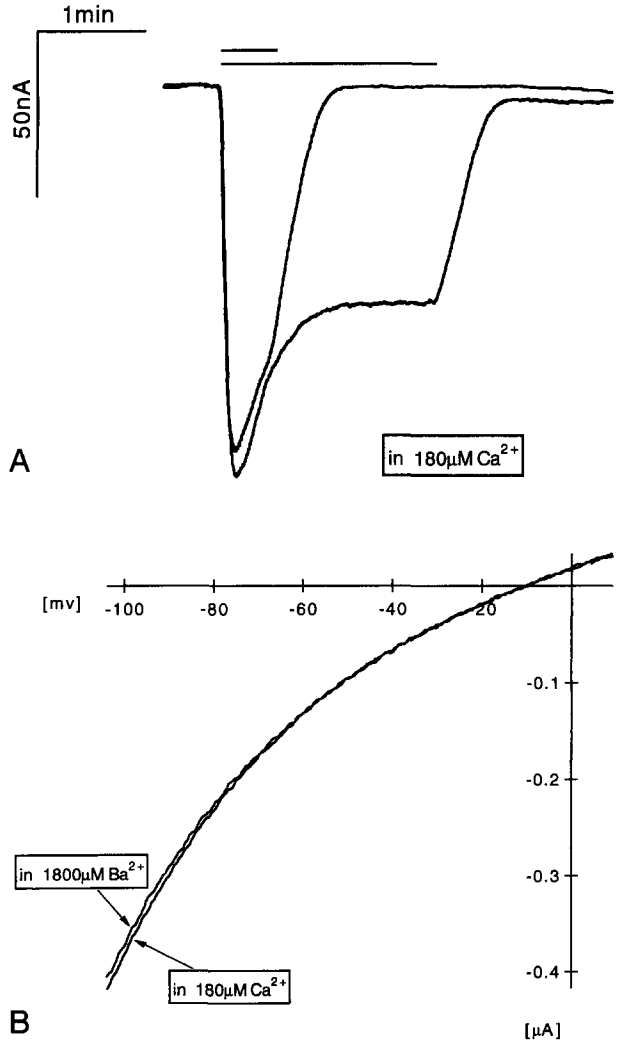

Fig. 4. (A) ATP-evoked currents in *Xenopus* oocytes injected with $P2X_4$ cRNA. 10 μM ATP was applied to oocytes perfused with NFR containing 180 μM Ca^{2+}. Same time course of current response was obtained when NFR contained 1800 μM Ba^{2+} (not shown). Holding potential −60 mV. No ATP evoked responses were detected in uninjected oocytes ($n > 5$) or oocytes injected with NMDA receptor cRNA. (B) *I–V* curves of $P2X_4$ receptors obtained through voltage ramps. Voltage ramps were applied during the plateau phase of the agonist (3 μM ATP) response. Leakage current was subtracted. Leakage current was determined from an identical ramp just before agonist application. Only the ramp segment from +5 mV to −105 mV, duration 1 s, is displayed. cRNA injection was adjusted to yield lower macroscopic currents in (A) than in (B)

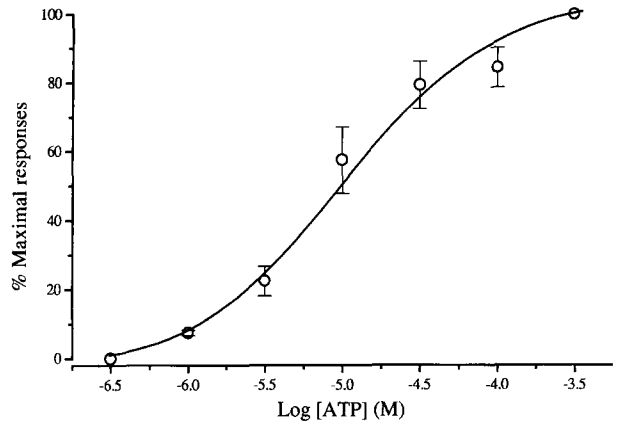

Fig. 5. Dose–response curve for ATP. The initial peak value of the current response was used. $n = 4$.

value is limited in view of the significant desensitization at higher ATP concentration, the fact that the maximal response could not be clearly determined and the relative slow solution exchange rates inherent to oocyte whole cell configurations. Several ATP-related substances activated $P2X_4$ channels. We determined the following rank order of potency: ATP > ATPγS > 2-MeSATP > ADP ≈ $\alpha\beta$-MeATP at low concentrations (Table 1). At high concentration both 300 μM ADP and 100 μM $\alpha\beta$-MeATP were less efficacious than ATP. Other compounds used to discriminate purinoceptors such as UTP, AMP, adenosine and A4pA did not evoke any measurable response at high or low concentrations (Table 1).

We further tested commonly used purinoceptor antagonists on recombinant $P2X_4$ purinoceptors. Neither PPADS, which is considered selective for P2X purinoceptors, nor the non-specific P2-purinoceptor antagonist suramin nor RB-2 antagonized (all tested up to 50 μM) ATP evoked responses (Fig. 6); nor did they act as agonists. Prolonged pre-application (up to 15 min) of those compounds still did not reveal any antagonistic effect (not shown). On the contrary, all the P2X 'antagonists' used showed some potentiation of ATP evoked responses (Fig. 6).

4. Discussion

We propose to call the newly identified purinoceptor $P2X_4$ following the proposed nomenclature for P2X purinoceptor subunits [20]. $P2X_4$ is about equidistantly related to P2X1, $P2X_2$ and $P2X_3$ and is not a closely related family member to any those subunits.

The deduced P2X sequence would predict the expected two membrane spanning topology, with both, the C and N termini located intracellularly. Remarkably all cysteines located within the proposed extracellular domain are conserved throughout the P2X family, suggesting a role for maintaining the tertiary structure of the mature protein.

$P2X_4$ is the first P2X subunit that is highly expressed in the CNS, specifically in the cerebellum and the spinal cord. Lower expression levels have been detected in the hippocampus. Radioligand binding studies [21] and autoradiography [15] using $\alpha\beta$-methylene ATP have suggested the presence of P2X receptors in the brain and spinal cord. $P2X_4$ expression pattern overlaps with $\alpha\beta$-MeATP labeling, but there are areas like the thalamus which are strongly marked by $\alpha\beta$-MeATP but do not reveal $P2X_4$ RNA expression. These data suggest that there is an additional, as yet unidentified P2X subtype in the brain. ATP mediated fast synaptic transmission in the medial habenula can be blocked by suramin [22]. $P2X_4$ message was not detected in this brain area and recombinant $P2X_4$ are not blocked by suramin.

The lack of blockade of $P2X_4$ receptors by suramin, PPADS and RB-2 was totally unexpected. All these compounds block recombinant $P2X_{1-3}$, where tested [8,9,10,11]. Those compounds have been widely used to discriminate P2 receptors [23,24]. We found that suramin, PPADS and RB-2 all potentiate ATP evoked recombinant $P2X_4$ responses at least when expressed in *Xenopus* oocytes. It would be intriguing to speculate whether the facilitation of hippocampal potentials by suramin described by Wieraszko [25] are mediated through suramin potentiation of $P2X_4$ receptors.

The newly cloned $P2X_4$ receptor has the rank order of potency of ATP > ATPγS > 2-MeSATP >> α,β-MeATP ≈ ADP, which is different from the other cloned P2X receptors for which 2-MeSATP and ATP are the two most potent agonists. The low potency of α,β-MeATP might explain the weak cross

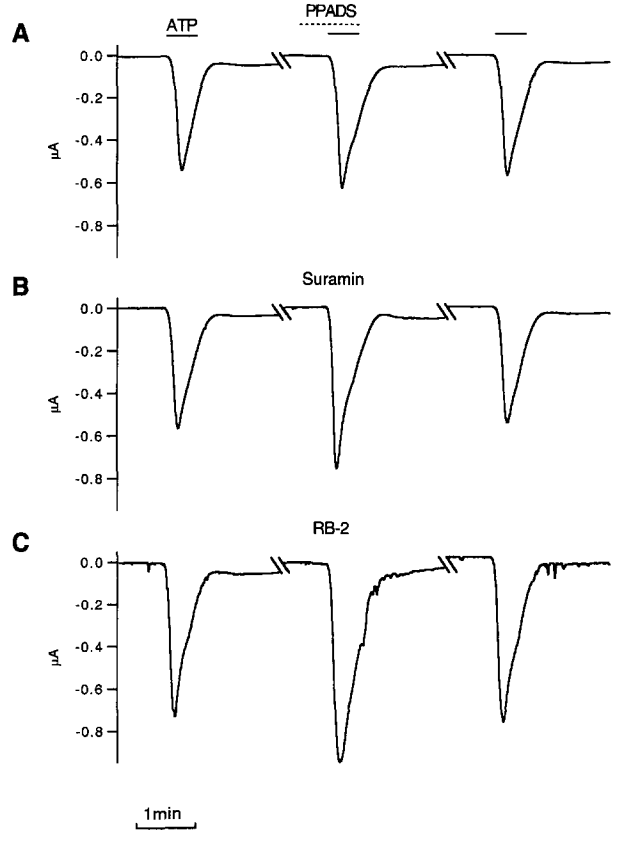

Fig. 6. Effects of PPADS, suramin and RB-2 on ATP evoked responses in oocytes expressing $P2X_4$ receptors. Wash period between ATP pre-application and ATP + substance test application was 10 min, 2nd wash period was 20 min. ATP was 3 μM, other substances 50 μM. All displayed data are from one single oocyte. Total duration of experiment was 3 h. Holding potential −60 mV. No leakage current subtraction was performed. The instability of measured current seen in (C) after RB-2 application was seen consistently. In control experiment on recombinant $P2X_2$ purinoceptors these compounds acted as published as antagonists (not shown).

desensitiztion caused by this compound. α,β-MeATP cross desensitization has been used to discriminate P2X-type of responses from other purinoceptor mediated [5]. This approach does not appear to be useful for identifying purinoceptors of the $P2X_4$ subtype.

Ueno et al. [26] described an ATP gated current in primary neuronal cultures of rat nucleus solitarii with a rank order of agonists similar to the one we determined for the $P2X_4$ type.

Outside of the CNS, purinoceptor mediated responses have been detected with a profile of agonist potency similar to that of $P2X_4$ [27]. $P2X_4$ message was found to be widely expressed throughout many different tissues; thus the $P2X_4$ subtype appears to be the most widespread member of the P2X family. This is strikingly in contrast to the very restricted expression of the $P2X_3$ RNA. Particularly interesting is the co-expression of $P2X_1$ and $P2X_4$ in vas deferens which could give raise to heteromeric ATP purinoceptors. Whereas the presence of P2X purinoceptors in lung, vas deferens and bladder has been generally accepted after numerous pharmacological experiments, the evidence for presence of purinoceptors of the P2X type in testis, thymus and adrenals was more tentative. We have now found $P2X_4$ message in all these tissues. A fast ATP evoked response [27] with an agonist potency profile already discussed above and that was potentiated by RB-2 has been described in guinea-pig intracardiac neurons which the authors tentatively classified as a P2Y-type response. However, in view of our $P2X_4$ data, it is quite likely that this response was mediated by a $P2X_4$-type of purinoceptor. Barajas-Lopez et al. [28] found an ATP activated current in myenteric neurons that is potentiated by suramin and therefore might well be mediated through the P2X4 type.

We have described a new P2X purinoceptors subtype cDNA which confers a novel and unique pharmacology. This type of pharmacology was unexpected and suggests that some of the previously uninterpretable data can now be classified as genuine P2X purinoceptor mediated events. The current lack of any $P2X_4$ antagonist makes the full evaluation of the significance of the $P2X_4$ purinoceptor subtype difficult. However, the cloning of the $P2X_4$ cDNA will be helpful in developing the necessary tools. The widespread $P2X_4$ mRNA expression reinforces the view that P2X receptors are involved in a large number of physiological processes.

Acknowledgements: We thank Dr. D. Julius, UCSF for $P2X_2$ cDNA, the Glaxo Institute for Molecular Biology, Geneva for $P2X_1$ cDNA and Dr. John Wood, UCL for $P2X_3$ data prior to publication. R.S. is a Senior Wellcome Trust Fellow. X.B. holds a British Heart Foundation fellowship. Grants support was from the Wellcome Trust (to R.S.) and the British Heart Foundation (to G.B.).

References

[1] Burnstock, G. (1993) Drug. Dev. Res. 28, 195–206.
[2] Burnstock, G. (1978) in: R.W. Straub and L. Bolis (Eds.), Cell membrane receptors for drugs and hormones: a multidisciplinary approach, Raven Press, New York, pp. 107–118.
[3] Fredholm, B.B., Abbracchio, M.P., Burnstock, G., Daly, J. W., Harden, T.K., Jacobson, K.A., Leff, P. and Williams, M. (1994) Pharmacol. Rev. 46, 143–156.
[4] Abbracchio, M.P. and Burnstock, G. (1994) Pharmacol. Ther. 64, 445–475.
[5] Burnstock, G. and Kennedy, C. (1985) Gen. Pharmacol. 16, 433–440.
[6] Lustig, K.D., Shiau, A.K., Brake, A.J. and Julius, D. (1993) Proc. Natl. Acad. Sci. USA 90, 5113–5117.
[7] Barnard, E.A., Burnstock, G. and Webb, T.E. (1994) Trends Pharmacol. Sci. 15, 67–70.
[8] Surprenant, A., Buell, G. and North, R.A. (1995) Trends Neurosci. 18, 224–2229.
[9] Valera, S., Hussy, N., Evans, R.J., Adami, N., North, R. A., Surprenant, A. and Buell, G. (1994) Nature 371, 516–519.
[10] Brake, A.J., Wagenbach, M.J. and Julius, D. (1994) Nature 371, 519–523.
[11] Chen, C., Akopian, A.N., Sivilotti, L., Colquhoun, D., Burnstock, G. and Wood, J.N. (1995) Nature 377, 428–431.
[12] Jahr, C.E. and Jessell, T.M. (1983) Nature 304, 730–733.
[13] Edwards, F.A., Gibb, A.J. and Colquhoun, D. (1992) Nature 359, 144–147.
[14] Zimmermann, H. (1994) Trends Neurosci. 17, 420–426.
[15] Bo, X. and Burnstock, G. (1994) NeuroReport 5, 1601–1604.
[16] Sambrook, J., Fritsch, E. and Maniatis, T. (1989) Molecular Cloning, CSH Laboraratory Press.
[17] Bartsch, S., Bartsch, U., Dorries, U., Faissner, A., Weller, A., Ekblom, P. and Schachner, M. (1992) J. Neurosci. 12, 736–749.
[18] Zhang, Y., Campell, G., Anderson, P.N., Martini, R., Schachner, M. and Lieberman, A.R., J. Comp. Neurol., in press.
[19] Behe, P., Stern, P., Wyllie, D.J.A., Nassar, M., Schoepfer, R. and Colquhoun, D., Proc. Roy. Soc. B., in press.
[20] Burnstock, G., in: CIBA Symposium Vol. 198, John Wiley, in press.
[21] Michel, A.D. and Humphrey, P.P. (1993) Naunyn Schmiedebergs Arch. Pharmacol. 348, 608–617.
[22] Edwards, F.A. and Gibb, A.J. (1993) FEBS Lett. 325, 86–89.
[23] Dalziel, H.H. and Westfall, D.P. (1994) Pharmacol. Rev. 46, 449–466.
[24] Voogd, T.E., Vansterkenburg, E.L., Wilting, J. and Janssen, L.H. (1993) Pharmacol. Rev. 45, 177–203.
[25] Wieraszko, A. (1995) J. Neurochem. 64, 1097–1101.
[26] Ueno, S., Harata, N., Inoue, K. and Akaike, N. (1992) J. Neurophysiol. 68, 778–785.
[27] Allen, T.G. and Burnstock, G. (1990) Br. J. Pharmacol. 100, 269–276.
[28] Barajas-Lopez, C., Barrientos, M. and Espinosa, L.R. (1993) Eur. J. Pharmacol. 250, 141–145.