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Functional Regulation of $P2X_6$ Receptors by *N*-Linked Glycosylation: Identification of a Novel $\alpha\beta$-Methylene ATP-Sensitive Phenotype

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ABSTRACT

Investigation of rat recombinant $P2X_6$ receptors has been limited because of the difficulty in obtaining functional expression in heterologous systems. In this study, we demonstrate glycosylation-dependent regulation of recombinant $P2X_6$ receptor function and associated conferral of a novel phenotype that is sensitive to the $P2X_1$ and $P2X_3$ receptor agonist, $\alpha\beta$-methylene ATP. In cells functionally expressing $P2X_6$ receptors, ATP and $\alpha\beta$-methylene ATP evoked slowly desensitizing inward currents (EC_{50} values, 0.5 and 0.6 μM, respectively) with slow kinetics of current decay on agonist washout. 2′,3′-*O*-(2,4,6-trinitrophenyl ATP) and iso-pyridoxalphosphate-6-azophenyl-2′-5′-disulfonate were effective antagonists (IC_{50} values, 0.8 and 22 μM, respectively); however, suramin was relatively ineffective. Reverse transcription-polymerase chain reaction analysis confirmed the absence of other P2X receptor subunits. Western analysis of membrane fractions from functional and nonfunctional clones confirmed the presence of $P2X_6$ at the cell membrane but revealed a difference in apparent molecular mass of immunoreactive products (~70 and ~60 kDa, respectively). *N*-glycosidase F treatment of both functional and nonfunctional receptor cell membranes increased the electrophoretic mobilities of immunoreactive products, with both proteins migrating at ~55 kDa, demonstrating an increased level of glycosylation of the $P2X_6$ receptor in functional compared with nonfunctional cells. This study demonstrates that nonfunctional rat recombinant $P2X_6$ receptors 1) are expressed on the membrane surface of human embryonic kidney cells and 2) are glycosylated. Expression of the novel functional receptor phenotype is associated with further glycosylation, resulting in an apparently larger molecular mass. These results suggest that $P2X_6$ receptor subunits contribute to $\alpha\beta$-methylene ATP sensitivity.

P2X receptors for ATP comprise a family of ligand gated ion channels produced from the homo- or heterotrimeric assembly of the seven cloned P2X receptor subunits ($P2X_{1–7}$). Although the properties of heterologously expressed, recombinant P2X receptors have been well characterized (for an excellent review, see North, 2002), the functional phenotypes of several native P2X receptors do not correlate to any recombinant homomeric or heteromeric P2X receptor assembly yet described. For example, responses in the central nervous system are commonly elicited by the ATP analog $\alpha\beta$-methylene-ATP ($\alpha\beta$-meATP) and desensitize relatively slowly (Harms et al., 1992; Chessell et al., 1997; Sansum et al., 1998; Patel et al., 2000). $\alpha\beta$-meATP is a potent full agonist at homomeric and heteromeric receptors that incorporate recombinant $P2X_1$ or $P2X_3$ receptor subunits (Valera et al., 1994). However, it is thought that neither of these subunits is present to a great extent in the central nervous system, and they have been considered unlikely to contribute significantly to $\alpha\beta$-meATP–evoked responses in the central nervous system. In addition, $\alpha\beta$-meATP fails to elicit significant responses from cells expressing recombinant $P2X_2$ (Brake et al., 1994), $P2X_4$ (Bo et al., 1995), $P2X_5$ (Collo et al., 1996), $P2X_7$ (Surprenant et al., 1996), $P2X_{2/6}$ (King et al., 2000), or $P2X_{4/6}$ receptors (Lê et al., 1998).

The $P2X_6$ receptor was originally cloned from rat brain and when heterologously expressed in HEK-293 cells was described as being functionally similar to the rat $P2X_4$ receptor (i.e., insensitive to $\alpha\beta$-meATP; Collo et al., 1996). However,

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ABBREVIATIONS: $\alpha\beta$-meATP, $\alpha\beta$-methylene ATP; HEK, human embryonic kidney; PPADS, pyridoxalphosphate-6-azophenyl-2′-5′-disulfonate; LC, locus ceruleus; TNP-ATP, 2′,3′-*O*-(2,4,6-trinitrophenyl ATP); RT, reverse transcription; PCR, polymerase chain reaction.

other investigators have attempted to replicate these findings but have failed to record ATP-induced responses from recombinant $P2X_6$ receptors expressed in either *Xenopus laevis* oocytes or HEK-293 cells (Lê et al., 1998; Khakh et al., 1999; Torres et al., 1999). It is worth noting that Collo et al. (1996) reported functional responses from less than 5% of cells transiently transfected with recombinant $P2X_6$, whereas much greater transfection efficiencies were observed when cells were transfected with $P2X_1$-$P2X_4$ receptors. These studies have led to the commonly held view that rat $P2X_6$ receptors do not readily form functional homomeric receptors when expressed in heterologous systems. In some areas of the central nervous system, $P2X_6$ receptors are colocalized with $P2X_2$ and/or $P2X_4$ receptors (Collo et al., 1996; Rubio and Soto, 2001). In these regions, $P2X_6$ receptor subunits may exist as part of heteromeric assemblies of $P2X_{4/6}$ (Lê et al., 1998), $P2X_{2/6}$ (King et al., 2000), or potentially $P2X_{2/4/6}$ and may contribute to the P2X-mediated responses recorded from areas such as the medial habenula (Edwards et al., 1992), locus ceruleus (LC; Harms et al., 1992; Sansum et al., 1998), hippocampus (Wong et al., 2000), and medial vestibular nucleus (Chessell et al., 1997).

In this study, we generated a number of HEK-293 cell lines stably expressing the $P2X_6$ receptor to 1) investigate the mechanisms regulating the expression of functional $P2X_6$ receptors and 2) characterize the pharmacological properties of $P2X_6$ receptors. Our studies demonstrate that the glycosylation state of the receptor is important in determining the production of functional ion channels and suggest that fully glycosylated $P2X_6$ receptor subunits may contribute to $\alpha\beta$-meATP–sensitive P2X receptors.

Materials and Methods

DNA Constructs. Rat $P2X_6$ cDNA (provided by G. Buell, Serono International, Geneva, Switzerland) was subcloned from pcDNA3 into two vectors: pcDNA3.1His and pcDNA4HisMax (Invitrogen, Groningen, The Netherlands) to add an N-terminal His tag and Xpress epitope. The receptor cDNA was excised using KpnI and NotI (Roche Palo Alto LLC, Palo Alto, CA) restriction digests, and the resulting insert was ligated into the multiple cloning site of the vector using T4 DNA ligase (72 h, 15°C). The pcDNA4HisMax includes an SP163 transcription enhancer (QBI Enterprises, Nes Ziona, Israel) (Stein et al., 1998) in addition to the cytomegalovirus promotor.

Transfection of HEK-293 Cells. Human embryonic kidney (HEK)-293 cells were transiently transfected with tagged $P2X_6$ receptor cDNA by incubation with 0.5 μg of DNA, 3 μl of LipofectAMINE (Invitrogen), and 200 μl of Dulbecco's modified Eagle's medium (serum free; Invitrogen, Paisley, UK) for 5 h at 37°C. Stable cell lines were created 24 to 48 h after transfection by the addition of G418 (0.6 mg/ml; Invitrogen) or zeocin (0.25 mg/ml; Invitrogen, Groningen, The Netherlands) for cells transfected with pcDNA3.1His or pcDNA4HisMax, respectively. Cells were dilution cloned at least 4 weeks after antibiotic addition and maintained in Dulbecco's modified Eagle's medium/10% fetal bovine serum (Invitrogen, Paisley, UK) in a water-saturated atmosphere of 95% air/5% CO_2 at 37°C in 75-cm^2 flasks. HEK-293 cells stably expressing $P2X_{2/3}$, $P2X_2$, or $P2X_4$ receptors were maintained in culture as for stable $P2X_6$ cell lines.

RT-PCR. RNA was prepared using a micro RNA isolation kit (Stratagene, La Jolla, CA) and Reverse-iT 1st Strand synthesis kit (ABgene, Epsom, Surrey, UK). Forward and reverse primers were designed to recognize $P2X_1$-$P2X_7$ receptors. For each reaction, 2 μl of first-strand reaction product was incubated with 400 ng of each primer, 200 μM dNTP, and 5 U of DyNAzyme (Finnzymes Oy, Espoo, Finland) in optimized reaction buffer with a final Mg^{2+} concentration of 1.5 mM. In each reaction set, PCRs were carried out using both +RT and −RT samples from the reverse transcriptase reactions as well as a no DNA control (in which water was substituted for the first-strand product). Reactions using plasmids containing $P2X_1$-$P2X_7$ as the template (1 μg per reaction) were also carried out to act as positive controls. Reaction products were resolved in a 1% agarose gel.

Western Analysis. Membrane fractions were prepared from cells transfected with $P2X_6$ receptors, resolved on 10% polyacrylamide gels, and electrophoretically transferred onto nitrocellulose (0.22 μm). Blots were blocked overnight at 4°C in Tris-buffered saline supplemented with 0.1% Tween 20 and 5% dried milk. Protein was detected using the antiXpress antibody (1 h, 22°C) and anti-mouse–horseradish peroxidase-conjugated secondary antibody (1 h, 22°C) and visualized using enhanced chemiluminescence detection according to the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ).

Electrophysiology. Whole-cell patch-clamp recordings were made from single cells or small rafts of 3 to 10 cells and voltage clamped at −70 or −90 mV. Cells were continuously perfused with an extracellular solution containing 145 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, and 10 mM D-glucose (pH 7.3; osmolarity, 300 mOsM). Patch electrodes with resistances of 3 to 5 MΩ were fire polished and back-filled with internal solution containing either 145 mM cesium aspartate, 11 mM EGTA, 5 mM HEPES, 2 mM NaCl (pH 7.3; osmolarity, 290 mOsM) or 140 mM potassium gluconate, 5 mM NaCl, 10 mM HEPES, or 10 mM EGTA (pH 7.3 adjusted by KOH). Currents were recorded using an Axopatch 200B amplifier (Axon Instruments Inc., Union City, CA) and filtered with a corner frequency of 1 to 5 kHz (8-pole Bessel filter), digitized at 2 to 10 kHz using a Digidata 1200A (Axon Instruments Inc.) interface, and stored on a computer. For the construction of concentration-effect curves, 1- to 2-s applications of agonist (ATP or $\alpha\beta$-meATP; Sigma Chemical, Poole, Dorset, UK) were separated by wash periods of 5 ($P2X_4$) or 2 min ($P2X_2$). $P2X_6$ receptor-mediated responses had prolonged time courses, and the rate of closure of the channels after washout of agonists was considerably prolonged (see *Results*); to get complete return to baseline current and record reproducible responses, an interval of 10 to 15 min was required between agonist applications. For concentration-response data, responses (mean ± S.E.M) are expressed as a percentage of the maximum response to 100 μM ATP. For antagonist experiments, the cells were presuperfused with antagonist solution for 10 min before the concomitant application of ATP (3 μM; an EC_{90} concentration) and the antagonist. In all experiments, drugs were applied using a computer controlled fast-flow U-tube application system (Nakazawa and Ohno, 1997). 2′,3′-*O*-(2,4,6-trinitrophenyl ATP) (TNP-ATP) was purchased from Molecular Probes (Eugene, OR), and iso-pyridoxalphosphate-6-azophenyl-2′-5′-disulfonate (iso-PPADS) and suramin were obtained from Sigma Chemical.

***N*-Glycosidase F Treatment.** For deglycosylation of recombinant $P2X_6$ receptor protein expressed in HEK-293 cells, an *N*-glycosidase F deglycosylation kit (Roche Applied Science, Indianapolis, IN) was employed. Cell membranes were prepared as described previously (Castro et al., 1996), and protein levels per sample were determined using the copper reduction/bicinchoninic acid protein assay (Pierce Chemical, Rockford, IL) as per the manufacturer's instructions. One hundred micrograms of protein was used for each deglycosylation reaction. Samples were incubated at 95°C for 3 min with reduced denaturing buffer (20 mM phosphate-buffered saline, 1% sodium dodecyl sulfate, and 1% 2-mercaptoethanol; pH 8.6) to denature the proteins. *N*-glycosidase F (2.4 U per sample) and reaction buffer (20 mM phosphate-buffered saline, 10 mM EDTA, and 0.5% *n*-octylglycopyranoside; pH 7.2) were added, and the tubes were incubated at 37°C for 1 h. Reactions were terminated by the addition of Laemmli sample buffer, and samples were analyzed on 10% polyacrylamide gels (see above).

Data Analysis. Where appropriate, concentration-effect curves were fitted using a three-parameter logistic equation (GraphPad Prism; GraphPad Software Inc., San Diego, CA). EC_{50} and IC_{50} values are expressed as geometric mean ± 95% confidence intervals, whereas individual data are expressed as mean ± S.E.M. Statistical comparisons were made using the Student's paired or unpaired *t* test where appropriate, and the null hypothesis was rejected when $P < 0.05$.

Results

Stable Expression of $P2X_6$ Receptors at the Cell Membrane. Immunocytochemical detection of tagged recombinant $P2X_6$ receptors in HEK-293 cells transiently transfected with $P2X_6$ with either plasmid (pcDNA3.1 or pcDNA4) demonstrated the ability of the $P2X_6$ receptor to be heterologously expressed (data not shown). Transfection efficiencies and expression levels were found to be variable between individual transfections and lower than those observed with other P2X receptors ($n > 3$ for each plasmid; 5–40% of cells expressing $P2X_6$ after transfection). Cell lines stably expressing tagged $P2X_6$ receptors were therefore constructed using each of the plasmids. Each cell line was dilution-cloned, and clones were individually screened for protein expression using fluorescence microscopy. Immunofluorescence was detected at the cell membrane using confocal microscopy (data not shown). Clonal cell lines found to express recombinant $P2X_6$ receptor protein were then screened for function electrophysiologically (response to a 2-s application of 100 μM ATP). As described previously by Collo et al. (1996), functional responses were recorded from less than 5% of cells tested. Two cell lines were selected for further investigation, one expressing functional $P2X_6$ and one expressing nonfunctional $P2X_6$ receptor protein, both transfected with the $P2X_6$/pcDNA4HisMax construct.

Functional and Nonfunctional $P2X_6$ Receptors Are Differentially Glycosylated. Western analysis of membrane preparations from the functional and nonfunctional $P2X_6$ stable cell lines confirmed the presence of recombinant $P2X_6$ receptors in membrane fractions of both lines. However, immunoreactive products from functional and nonfunctional $P2X_6$ cells had different apparent molecular masses (~70 and ~60 kDa, respectively; Fig. 1A). Figure 1A also shows the immunoreactive products identified from membrane fractions of cells transiently transfected with recombinant $P2X_6$ receptors and representing a mixed population of cells. In this case, three bands are clearly visible at approximately 70, 60, and 50 kDa.

Treatment of $P2X_6$ cell membranes with *N*-glycosidase led to a decrease in the apparent molecular mass of the 70-kDa product to ~55 kDa. The band at 62 kDa observed in the nonfunctional $P2X_6$ membrane preparation was also shifted in size to ~55 kDa after deglycosylation, and an additional product of 50 kDa was apparent (Fig. 1B). This would suggest that functional $P2X_6$ and nonfunctional $P2X_6$-expressing cells differentially glycosylate the $P2X_6$ receptor.

$P2X_6$ Receptors Have a Novel Phenotype. Whole-cell patch clamp was used to functionally characterize the $P2X_6$-mediated currents. ATP induced rapid inward currents in all of the cells tested. The current-voltage relationship was typical of a nonselective cation channel, with a reversal potential of approximately zero (−4.8 mV with cesium aspartate internal solution) and showed a slightly inwardly rectifying profile (Fig. 2D). Little desensitization was observed with agonist applications of up to 2 s (87 ± 5% response remaining after 2-s application of 30 μM ATP, $n = 10$). Currents recorded in $P2X_6$ cells were found to return slowly to baseline levels: the time for the current to ATP (3 μM) to decay by 50% at the end of agonist application was 8.7 ± 0.6 s ($n = 5$), and return to baseline current usually took >1 min. This rate of decay of the current is significantly prolonged compared with application of ATP (3 μM) to recombinant $P2X_{2/3}$ receptors (time to 50% decay at the end of the pulse, 0.8 ± 0.2 s; $n = 5$; $P < 0.05$; Fig. 2C). Mean maximum current amplitudes were smaller from cells expressing $P2X_6$ receptors (420 ± 31 pA, $n = 50$) compared with $P2X_{2/3}$ receptors (4101 ± 790 pA, $n = 5$) but were larger than those from cells expressing $P2X_4$ receptors (258 ± 40, $n = 29$).

Concentration-effect curves were determined for two P2 receptor agonists, ATP and αβ-meATP, to allow comparison between the $P2X_6$ receptor-mediated currents with those recorded from native tissue and also with previously characterized P2X receptors. ATP was found to be significantly more potent at $P2X_6$ [EC_{50} = 0.5 (0.4–0.6) μM, $n = 6$] than at $P2X_4$ [EC_{50} = 5.5 (2.6–11.6) μM, $n = 7$] or $P2X_2$ receptors]EC_{50} = 33 (30–36) μM, $n = 6$; Fig. 2]. αβ-meATP, the ATP analog that has been widely used in the study of native responses, was found to act as a full agonist with respect to ATP at cells expressing $P2X_6$ receptors (Fig. 3). αβ-meATP had equivalent potency with ATP at $P2X_6$ receptors [EC_{50} = 0.6 (0.4–1.0) μM, $n = 3$], which is in stark contrast to its lack of effect at cells expressing rat $P2X_4$ receptors. It is also interesting to note that the time course of the decay of $P2X_6$ currents on removal of agonist was faster for αβ-meATP compared with an equivalent concentration (3 μM) of ATP (5.1 ± 0.6 s versus 8.7 ± 0.6 s, respectively; $n = 5$ for each, $P < 0.05$; Figs. 2 and 3). The agonists α,β-methylene ADP

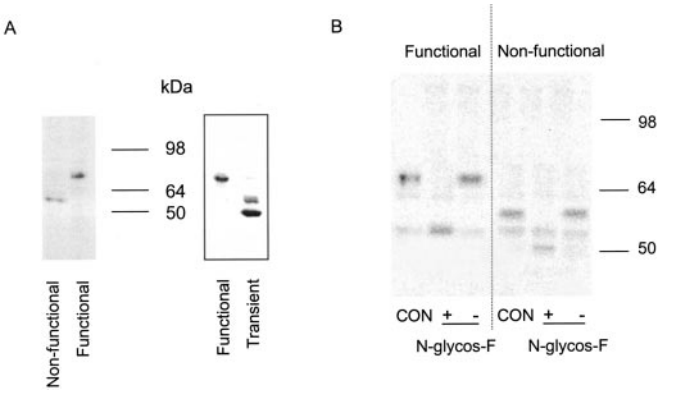

Fig. 1. Western analysis of membrane fractions from cell lines stably expressing recombinant $P2X_6$ receptors (A) highlighted a significant difference in apparent molecular mass of the immunoreactive species from nonfunctional and functional $P2X_6$ cells (~60 and ~70 kDa, respectively; A, left). Western analysis of membrane fractions from cells transiently expressing $P2X_6$ (Transient) resulted in bands equivalent in size to those from the two stable cell lines, with an additional species running at ~50 kDa (A, right). Deglycosylation of membrane fractions from cells expressing functional or nonfunctional $P2X_6$ receptors resulted in an increase in electrophoretic mobility of the immunoreactive products detected (B). Membranes were incubated in the presence (+) and absence (−) of *N*-glycosidase F for 1 h at 37°C and compared with nontreated membranes (CON). Recombinant protein was detected using the antiXpress antibody (1 h, room temperature, 1:750) and horseradish peroxidase-conjugated anti-mouse secondary antibody (1 h, room temperature, 1:2500). Marker proteins are also shown (kilodaltons).

and benzoyl benzoyl ATP were inactive at $P2X_6$ receptors at concentrations up to 100 μM (data not shown).

We determined the effect of three P2X receptor antagonists on responses to an EC_{90} concentration of ATP (3 μM; Fig. 4). TNP-ATP and iso-PPADS were effective antagonists of the ATP response with IC_{50} values of 0.83 (0.16–4.4) and 21.8 (11.3–41.9) μM, respectively. In contrast, the response to ATP was relatively suramin-insensitive with only a 27 ± 4.6% reduction in response (n = 4) to the maximal concentration of suramin tested (100 μM). Similar antagonist effects were seen when $\alpha\beta$-meATP (3 μM) was the agonist (80.9 ± 6% inhibition by 10 μM TNP-ATP, 89.2 ± 0.8% inhibition by 100 μM iso-PPADS, and 30.5 ± 2.4% inhibition by 100 μM suramin, n = 3 for each).

$P2X_6$-Expressing Cells Do Not Contain Other P2X Subunits. The observed pharmacological differences between responses in $P2X_6$-expressing cells compared with those reported previously (Collo et al., 1996) led to concerns that the responses we describe could be caused by receptors other than homomeric $P2X_6$. It has been suggested that under certain conditions, HEK-293 cells can endogenously express certain P2X receptor subunits (Worthington et al., 1999b). It was thus theoretically possible that heteropolymerization between endogenous P2X receptors with recombinant $P2X_6$ could form a novel heteromeric P2X receptor responsible for $P2X_6$ responses to ATP. With this in mind, RT-PCRs were carried out on $P2X_6$-expressing cells (Fig. 5). Positive controls (pcDNA3 plasmids containing cDNA encoding each of the individual receptor subunits) show the successful completion of each PCR, whereas negative controls demonstrate a lack of genomic contamination of $P2X_6$ cell DNA samples (−RT control) and a lack of external contamination in the reactions (no DNA control). RT-PCRs detected the presence of mRNA encoding $P2X_6$ but failed to detect the presence of mRNA for any of the other known P2X receptors. Immunocytochemical studies using the commercially available antibodies against P2X receptors confirmed the absence of $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, and $P2X_7$ (data not shown).

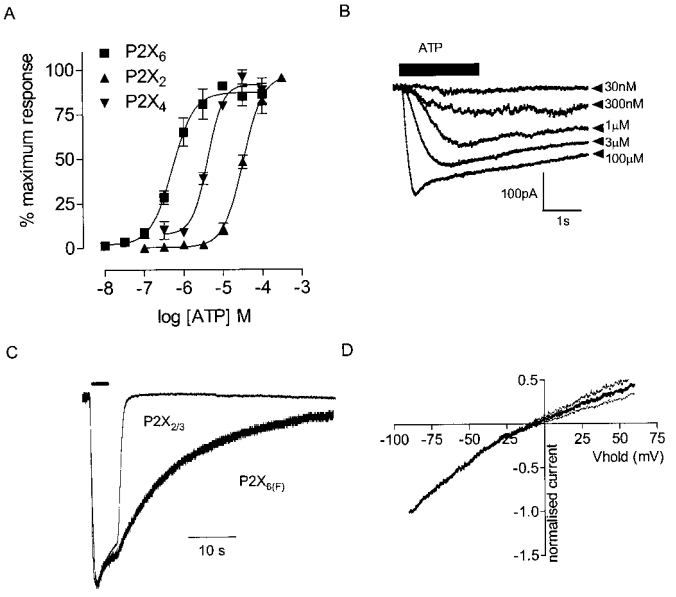

Fig. 2. Concentration-effect curves for ATP acting at rat $P2X_6$ (■) compared with rat $P2X_4$ (▼)and rat $P2X_2$ (▲) receptors (A). Serially increasing concentrations of ATP were applied for 1 s at 15- ($P2X_6$, n = 6), 5- ($P2X_4$, n = 7), or 2-min ($P2X_2$, n = 6) intervals. Data are mean ± S.E.M. from n experiments. A representative trace from cells expressing rat $P2X_6$ receptors is given (B). $P2X_6$ receptors show a very slow recovery to baseline current on removal of agonist (C). A representative trace shows the off rate after ATP (3 μM) application, normalized to the peak response for $P2X_6$- and $P2X_{2/3}$-expressing cell lines (C) The voltage-current relationship for $P2X_6$ receptors is also shown (D). Voltage ramps from −90 to +60 mV were carried out on single cells using the whole-cell patch-clamp technique. Data are mean ± S.E.M. from four experiments.

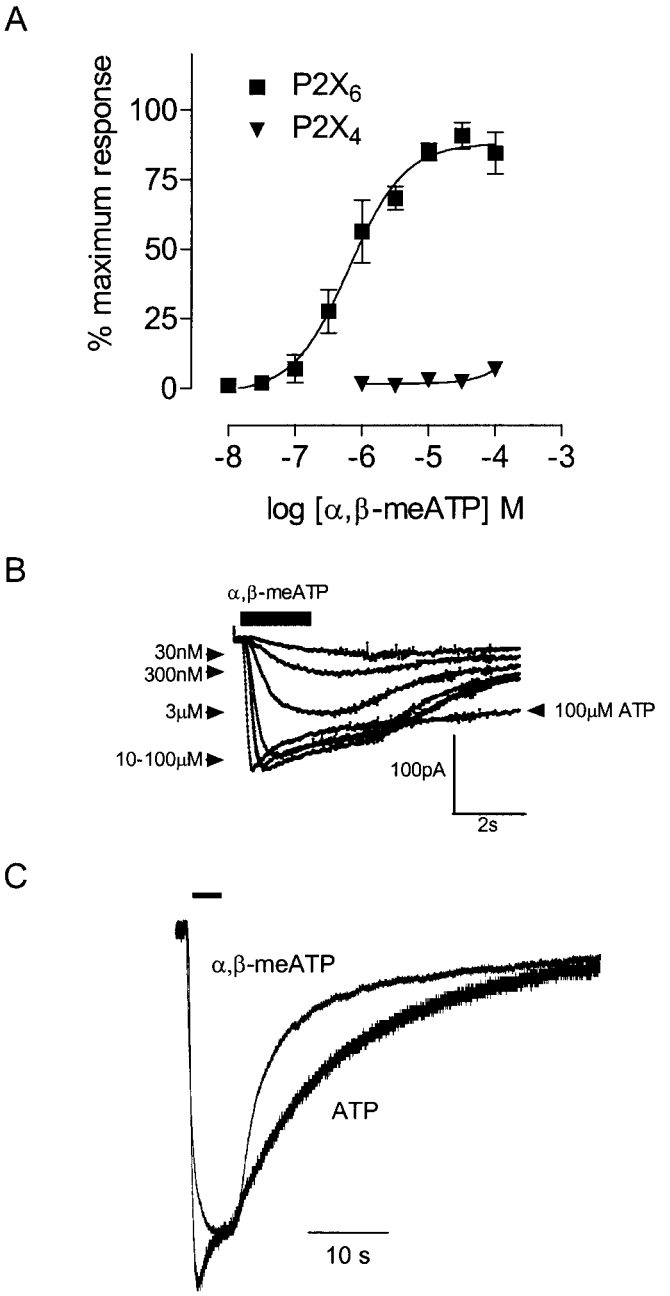

Fig. 3. Concentration-effect curves for $\alpha\beta$-meATP acting at rat recombinant $P2X_6$ (■) compared with rat $P2X_4$ (▼) receptors (A). Serially increasing concentrations of $\alpha\beta$-meATP were applied for 1 s at 15- ($P2X_6$, n = 3) or 5-min ($P2X_4$, n = 6) intervals. Data are mean ± S.E.M. from n experiments. A representative trace from cells expressing rat $P2X_6$ is given below (B). The rate of decay of $P2X_6$ currents is faster for $\alpha\beta$-meATP compared with ATP (C; both agonists applied at 3 μM, traces normalized to peak at the end of drug application).

Discussion

In this study, we have demonstrated that glycosylation plays a central role in the regulation of functional $P2X_6$ receptors. Cell-surface expression and function, contingent on post-translational *N*-linked glycosylation, is consistent with that observed for other P2X receptors (Rettinger et al., 2000) and for $P2X_6$ yields a slowly desensitizing, $\alpha\beta$-meATP-sensitive phenotype.

Few data exist on the expression and properties of recombinant homomeric $P2X_6$ receptors, because functional responses to ATP have proved elusive after transient expression of the protein in heterologous systems. It has been suggested that $P2X_6$ is poorly expressed in non-native systems but here, we show that although transient transfection efficiencies were variable and lower than expected, recombinant, albeit frequently nonfunctional $P2X_6$ receptors can be expressed in stable cell lines. In line with the original study (Collo et al., 1996), functional responses were recorded from less than 5% of cells expressing recombinant $P2X_6$ receptor protein. Previous studies have not investigated the membrane localization of $P2X_6$ receptors in recombinant systems, partly because of the absence of a reliable antibody against $P2X_6$. As demonstrated in this study (Fig. 1), the dominant immunoreactive product detected using Western analysis of transiently transfected cells had an apparent molecular mass of ~50 kDa, the expected size of a nonglycosylated $P2X_6$ receptor subunit, based on primary sequence. In this study, we have provided evidence that at least partial glycosylation of the receptor is required for cell membrane expression and that expression of a functional phenotype is associated with further glycosylation, explaining the absence of functional responses in the majority of cells transiently expressing recombinant $P2X_6$ receptor protein.

$P2X_6$ receptor protein has been detected as an ~49-kDa band from whole rat brain homogenates (5 days postnatal; Rubio and Soto, 2001) and as an ~70-kDa product from adult

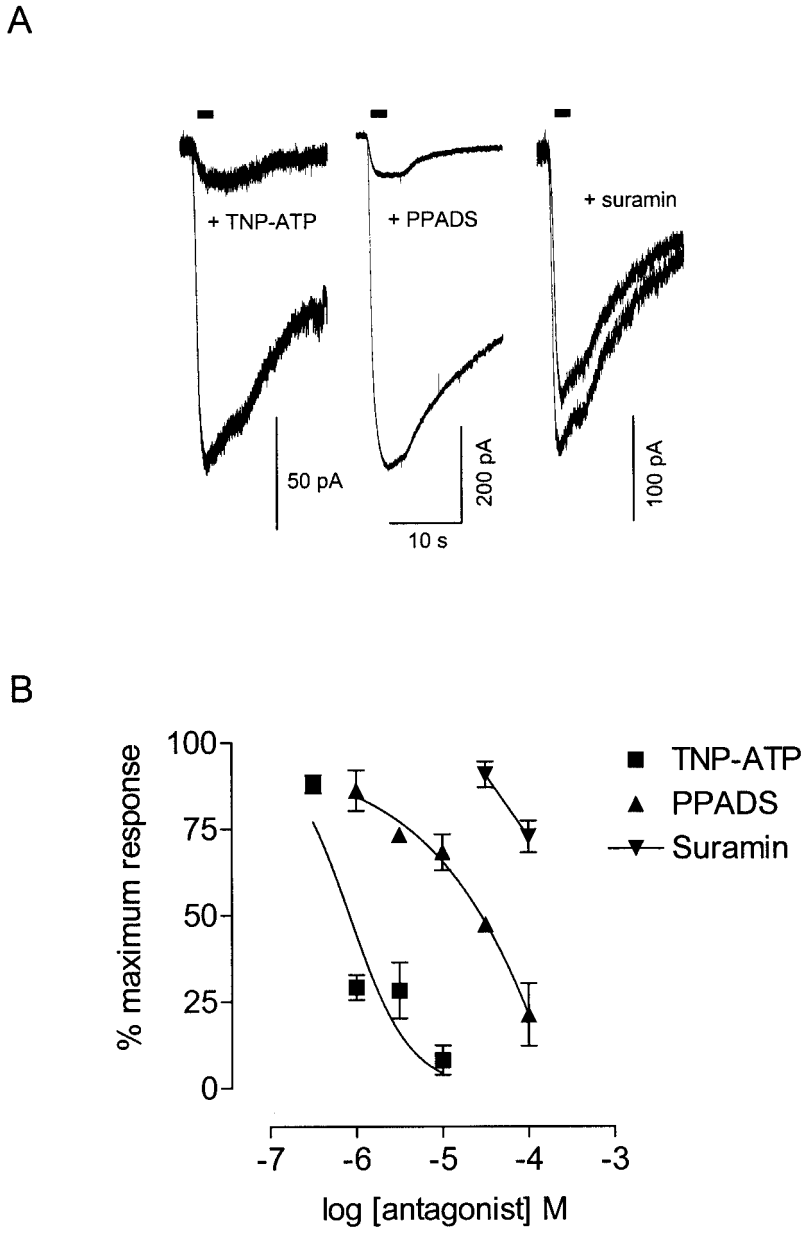

Fig. 4. The effects of purinergic antagonists at $P2X_6$ receptors. Sample traces (A) of the effects of TNP-ATP (10 μM), iso-PPADS (100 μM), and suramin (100 μM) on responses to an EC_{90} concentration of ATP (3 μM). Antagonists were presuperfused for 10 min, and the bar indicates the period of ATP application in control conditions or coapplied with antagonist. Summary of the inhibitory effects of TNP-ATP (■), iso-PPADS (▲), and suramin (▼) on responses to 3 μM ATP (B). Symbols are mean ± S.E.M. (n = 3–4).

rat midbrain homogenate (Worthington et al., 1999a), atrium, bladder, kidney (Worthington et al., 1999b), and thymocytes (Glass et al., 2000). It has also been reported at ~60 kDa from human superior cervical ganglia, HEK-293 cells, rat ventricle, bladder, and kidney (Worthington et al., 1999b). Yunaev et al. (2000) also report $P2X_6$ immunoreactive products running at ~50, 55, 60, and 70 kDa from the rat urinary bladder, so it seems possible that the recombinant $P2X_6$ species detected in stable $P2X_6$ receptor cell lines and transiently transfected cells may have physiological correlates in some tissues. To resolve this issue, a detailed study of $P2X_6$ receptor expression and glycosylation status in different brain regions and in animals of different ages is required. Age-related differences in P2X-mediated currents have been reported from the LC (Wirkner et al., 1998) in which 2-methylthioATP–induced currents increase with age, reaching maturity at ~18 days postnatal. It is therefore possible that the 49-kDa $P2X_6$ receptor protein detected from rat brain (Rubio and Soto, 2001) represents an immature form of the receptor and that increased levels of glycosylation and increased function occur with age.

The finding that $P2X_6$ receptors exist in a number of different glycosylation states is consistent with previous work carried out on recombinant $P2X_1$ (Nicke et al., 1998; Rettinger et al., 2000), recombinant $P2X_2$ (Newbolt et al., 1998; Torres et al., 1998a,b), and cardiac $P2X_4$ receptors (Hu et al., 2002). The $P2X_1$ subunit possesses five putative *N*-glycosylation sites (Asn-X-Ser, where X is any amino acid except proline). The elimination of three of the four usable sites leads to impaired protein expression, whereas elimination of all four abolished expression of the receptor (Newbolt et al., 1998). The $P2X_6$ sequence has three putative sites for *N*-linked glycosylation at amino acids 157, 187, and 202. In the present study, nonfunctional $P2X_6$ receptor protein was found to be glycosylated and at the cell membrane. This presence of *N*-linked glycans on nonfunctional receptors is thus in keeping with previous reports, which suggest that a minimum amount of glycosylation is absolutely required for P2X receptor expression at the cell surface; the molecular correlates of these sites remain to be determined.

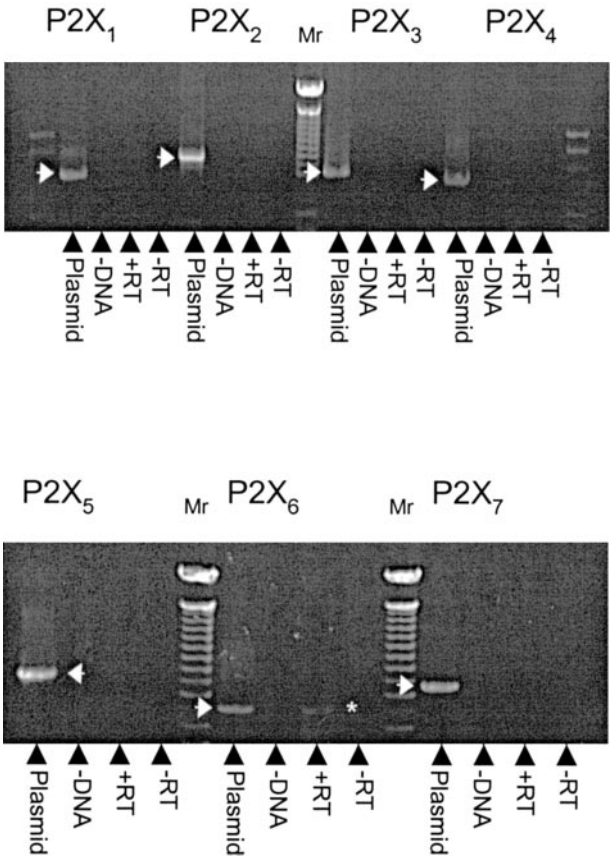

Fig. 5. RT-PCRs were carried out on cells expressing functional $P2X_6$ receptors to confirm the absence of $P2X_1$ through $P2X_5$ and $P2X_7$ receptor mRNA. Primers specific to each P2X receptor were used in seven sets of reactions. For each set, a plasmid containing the relevant P2X receptor was used as a positive control (plasmid), whereas a PCR reaction in the absence of DNA was carried out as a negative control (−DNA). Bands of the expected size were observed in all positive controls (white arrows). RNA from cells expressing $P2X_6$ was incubated in the presence (+RT) and absence (−RT) of reverse transcriptase. The results confirm that mRNA for $P2X_6$ receptor is present (asterisk) and that no $P2X_1$ through $P2X_5$ or $P2X_7$ mRNA was detected. Relative molecular mass markers are also shown (M_r).

One question remains: why do functional recombinant $P2X_6$ receptors only form in a small proportion of cells? It is clear that the glycosylation state plays a key role in receptor function, but the factors that regulate the degree of glycosylation remain to be determined. It is interesting that Torres et al. (1999) reported that coimmunoprecipitation of $P2X_6$ receptor subunits was below the limit of detection but $P2X_6$ receptors could be coimmunoprecipitated with $P2X_1$, $P2X_2$, $P2X_4$, and $P2X_5$ receptor subunits, suggesting that these other subunits could assist in the correct assembly of the receptor. The low rate of functional $P2X_6$ receptors could theoretically have been caused by heteropolymerization with endogenously expressed $P2X_4$ receptors that have been reported in a small number of HEK-293 cells (Worthington et al., 1999b). However, in the present study, non-$P2X_6$ receptor subunits were below the limit of detection by RT-PCR or immunohistochemically. It thus seems unlikely that these non-$P2X_6$ receptor subunits contribute to the observed phenotype of $P2X_6$ receptors. However, it is possible that an additional auxiliary subunit is required for the functional expression of $P2X_6$ receptors.

Pharmacological characterization of recombinant $P2X_6$ receptors revealed a novel phenotype. This was significantly different from that reported in the only other study where recombinant $P2X_6$ receptors have been described previously (Collo et al., 1996). In particular, the responses differ in terms of time course and sensitivity to $\alpha\beta$-meATP. Responses reported by Collo et al. (1996) are very similar to those of rat recombinant $P2X_4$ receptors. $P2X_4$ receptor RNA and protein can be expressed in native HEK-293 cells; however, no one has reported P2X-like responses from nontransfected HEK-293 cells (North, 2002). This raises the possibility that in the study of Collo et al. (1996), endogenous $P2X_4$ receptor subunits may have combined with $P2X_6$ receptors to form functional channels. Alternatively, differential glycosylation, post-translational processing, or the action of auxiliary subunits may account for the differences in properties between the work of Collo et al. (1996) and the present study.

In the present study, ATP (100 μM) induced inward currents and was found to be significantly more potent at rat $P2X_6$ receptors than at rat $P2X_4$ or $P2X_2$ receptors. $P2X_6$ receptor responses were relatively nondesensitizing during agonist application; however, the time taken for the response to return to baseline on agonist washout was considerably

prolonged compared with most other P2X receptors and was similar to that described for $P2X_7$ receptors (Rassendren et al., 1997). In addition, the rate of response decline was also dependent on the agonist activating the receptor. The mechanism for the slow recovery on agonist washout and the agonist-specific variations remain to be determined but could result from a long mean open time of the $P2X_6$ receptor channel, very slow unbinding rate of the agonist, or, as for the $P2X_7$ receptor, the formation of a dilating pore. $P2X_6$ receptors were also found to be highly sensitive to the ATP analog, $\alpha\beta$-meATP, which acted as a full agonist with respect to ATP. To date, only homomeric $P2X_1$ (Valera et al., 1994), $P2X_3$ (Chen et al., 1995), and heteromeric $P2X_{2/3}$ (Lewis et al., 1995) and $P2X_{1/5}$ (Lê et al., 1999) have been described as sensitive to this agonist. $P2X_{4/6}$ receptors coexpressed in *X. laevis* oocytes were reported to show an increased sensitivity to $\alpha\beta$-meATP compared with homomeric $P2X_4$ receptors (Lê et al., 1998), but the increase was small and in our own studies, found to be nonsignificant (Jones et al., 2001). This finding is therefore potentially very important, and the expression of $P2X_6$ receptor subunits could explain the incidence of $\alpha\beta$-meATP–induced responses from brain areas in which $P2X_1$ and $P2X_3$ receptors are lacking. However, two pieces of evidence suggest that it is unlikely that the majority of $\alpha\beta$-meATP–sensitive brain P2X receptors correspond to homomeric $P2X_6$ receptors. First, in the present study, $P2X_6$ receptors were relatively insensitive to the antagonist suramin, which has proved effective at many neuronal P2X receptors (e.g., Edwards et al., 1992; Chessell et al., 1997; Sansum et al., 1998); and second, $P2X_6$ receptors were insensitive to $\alpha\beta$-methylene ADP, the ATP analog shown to elicit inward currents in the LC (Sansum et al., 1998; Patel et al., 2000) and medial vestibular nucleus (Chessell et al., 1997). Therefore, although $P2X_6$ receptors may contribute $\alpha\beta$-meATP sensitivity to heteromeric channels in the central nervous system, the subunit stoichiometry of these remains to be elucidated.

Overall, this study has described the first investigation of functional homomeric $P2X_6$ receptors stably expressed in HEK-293 cells and provides evidence that nonfunctional $P2X_6$ receptors are expressed at the cell surface and are glycosylated. We suggest that although the presence of a minimum number of *N*-glycans is required for receptor expression, further glycosylation is required to confer subunit function. Finally, functional homomeric $P2X_6$ receptors were found to be slowly desensitizing and sensitive to the ATP analog, $\alpha\beta$-meATP, and thus show a novel operational phenotype. We propose that slowly desensitizing $\alpha\beta$-meATP–mediated responses in tissue lacking $P2X_1$ and $P2X_3$ receptors could be accounted for by the contribution of fully glycosylated $P2X_6$ receptor subunits to heteromeric channels.

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