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A loss-of-function polymorphism in the human P2X4 receptor is associated with increased pulse pressure

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Abstract

The P2X4 receptor is involved in endothelium-dependent changes in large arterial tone in response to shear stress and is, therefore, potentially relevant to arterial compliance and pulse pressure. Four identified non-synonymous polymorphisms in *P2RX4* were reproduced in recombinantly-expressed human P2X4. Electrophysiological studies showed that one of these, the Tyr315>Cys mutation (rs28360472), significantly reduced the peak amplitude of the ATP-induced inward current to 10.9% of wild-type P2X4 receptors in transfected HEK-293 cells (10 μmol/L ATP, n = 4-8 cells, $P<0.001$). Concentration-response curves for ATP and the partial agonist BzATP demonstrate that the 315Cys-P2X4 mutant had an increased EC_{50} value for both ligands. Mutation of Tyr315>Cys likely disrupts the agonist binding site of P2X4 receptors, a finding supported by molecular modelling based on the zebrafish P2X4 receptor crystal structure. We tested inheritance of rs28360472 encoding the Tyr315>Cys mutation in *P2RX4* against pulse pressure in 2874 subjects from the Victorian Family Heart Study. The minor allele frequency was 0.014 (1.4%). In a variance components analysis we found significant association with pulse pressure ($P = 0.023$ for total association) where one minor allele increased pulse pressure by 2.84 mmHg (95% CI 0.41 to 5.27). This increase in pulse pressure associated with inheritance of 315Cys-$P2X_4$ receptors might reflect reduced large arterial compliance as a result of impaired endothelium-dependent vasodilation in large arteries.

Keywords: P2X4 receptor, polymorphism, SNP, P2RX4, pulse pressure, ATP

Introduction

Pulse pressure is recognised as an independent risk factor for cardiovascular disease [1] and is determined by large artery compliance as well as by cardiac stroke volume and reflected pressure waves. Endothelial cell responses to blood flow have an important role in the control of vascular tone and it is well documented that endothelial cells release ATP in response to shear stress produced by the flow of blood [2]. ATP is the physiological ligand for the purinergic receptor family comprising seven ligand-gated ion channels (P2X1-P2X7) and seven metabotropic P2Y receptors (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y14) [3]. P2X4 receptors are expressed in vascular endothelial cells where this receptor functions in shear stress-induced calcium signalling and the generation of nitric oxide (NO) [4-6]. P2X4 receptors show fast activation kinetics with high permeability to Ca^{2+} ions [7] and is the only P2X receptor to show potentiation by the pharmacological agent ivermectin [8]. Anti-sense knockdown of P2X4 receptors abolishes shear stress-induced Ca^{2+} signals in endothelial cells [9] and the P2X4 receptor knockout mouse exhibits reduced shear stress-mediated Ca^{2+} influx into cultured endothelial cells and reduced production of NO [10]. Endothelial responses to shear stress are important in regulating the compliance of large arteries, an intrinsic property of the vessel wall with effects particularly on pulse pressure. Thus it could be hypothesized that functional variations in the human P2X4 receptor may play an important role in shear stress related parameters within the vasculature.

The human P2X4 receptor gene (*P2RX4*) is located on chromosome 12q24.32 [11] and contains a number of non-synonymous single nucleotide polymorphisms (SNPs). Here we determined functional effects of four non-synonymous coding SNPs in *P2RX4* and focussed our investigation on one SNP (rs28360472) that leads to loss of P2X4 receptor function through disruption of the agonist binding site and is associated with increased pulse pressure.

Methods

Detailed methods are available in the online supplement (please see http://hyper.ahajournals.org).

For the Sydney cohort, genomic DNA was prepared from peripheral venous blood as described previously [12] and the study was approved by Sydney West Area Health Service HREC Nepean with informed written consent obtained from all participants. Four non-synonymous SNPs in *P2RX4* were genotyped in the Sydney cohort to determine minor allele frequencies; rs1044249, rs28360470, rs25644, and rs28360472. For the Victorian cohort, the details of the recruitment of participants for the Victorian Family Heart Study (VFHS) have been published previously [13, 14] and the study was approved by the Ethics Review Committee of the Alfred Hospital, Melbourne with informed consent obtained from all participants. Blood pressure measurements on participants of the VFHS were taken carefully using a calibrated mercury sphygmomanometer by trained observers. Pulse pressure was calculated as the difference between systolic and diastolic pressure.

HEK-293 cells were transfected with wild-type or mutated EGFP-tagged human P2X4 receptor and functional responses measured by whole cell patch clamping[15]. Surface staining was performed on transfected HEK-293 cells using a rabbit anti-human P2X4 antibody.

Peripheral venous blood was taken from healthy volunteers carrying no mutations in *P2RX4* (wild-type) or carrying one allele of rs28360472. $CD14^+$ monocytes were isolated and differentiated to macrophages. For functional experiments, data are presented as mean ± S.E.M. Statistical analysis was performed using t-tests or ANOVA where appropriate with Instat version 3 (GraphPad Software Inc.).

A molecular model of the human P2X4 receptor was built by homology to the zebra-fish P2X4 receptor structure (pdb code 3I5D [16]) using SwissModel [17] to explore the putative ATP-binding pocket in wild-type P2X4 and 315Cys-P2X4 receptors.

For the VFHS cohort statistical association between genotype and pulse pressure was assessed using variance components modelling and the quantitative trait linkage disequilibrium (QTLD) approach.

Results

Polymorphic variants of the P2X4 Receptor

Four non-synonymous coding SNPs in the *P2RX4* gene were identified in NCBI dbSNP database (using build 126). The mutations were introduced into a human P2X4 receptor plasmid and expressed in HEK-293 cells to ascertain whether these mutations altered functional responses. We measured ATP-induced inward currents by whole cell patch clamp (Figure 1A) and found only the Tyr315>Cys mutation (rs28360472) to have a significant effect, reducing the amplitude of the P2X4 response to 10.9% of wild-type P2X4 receptors (n=4-8 cells, $P<0.001$). The mutations altering Ala6>Ser (rs1044249), Ile119>Val (rs28360470) and Ser242>Gly (rs25644) had no major effect (n=4-8 cells, Figure 1). As expected all P2X4 receptor constructs displayed potentiation by the allosteric modulator ivermectin (data not shown). We found the cell surface expression of 315Cys-P2X4 receptors was reduced compared to wild-type P2X4 receptors where mean fluorescent intensity was 62.7% of wild-type P2X4 receptors (please see supplemental figure S1 http://hyper.ahajournals.org). The 315Cys-P2X4 mutant exhibited a right-shifted concentration-response curve and approximately 40-fold higher EC_{50} value relative to the wild-type P2X4 receptor (Table 1) whereas the other P2X4 mutations showed no significant difference (Table 1). Tyrosine 315 is thought to contribute to an agonist binding domain in the P2X4 receptor [18] therefore we used BzATP, a known partial agonist at P2X4 receptors [19], which also showed a right-shifted concentration-response curve and an increased EC_{50} value in the 315Cys-P2X4 receptor compared to the wild-type P2X4 receptor (Figure 1C).

The Tyr315>Cys polymorphism affects P2X4 receptor ectodomain structure

P2X receptors have 10 conserved cysteine residues in the ectodomain, thought to stabilise the structure through five disulphide bonds [20, 21]. The Tyr315>Cys mutation in the P2X4 receptor introduces an additional cysteine residue into the ectodomain which may destabilise the structure of the receptor and permit aberrant disulphide bonding contributing to the reduced

maximal response. We mutated the Tyr315 residue to serine, which cannot form disulphide bonds, and this 315Ser-P2X4 mutant showed a similar right-shifted ATP concentration-response curve and higher EC_{50} value (EC_{50} ATP = 243 μmol/L) compared to the wild-type P2X4 receptor but did not show a reduced maximal response to ATP (Figure 2A, B). Cell surface expression of 315Ser-P2X4 receptors was similar to 315Cys-P2X4 receptors (please see supplemental figure S2 http://hyper.ahajournals.org). Therefore we suggest there are two distinct effects with a mutation of Tyr315 to cysteine; mutation away from tyrosine affects agonist potency and the introduction of an extra cysteine residue reduces the maximum response. We investigated this further by using a short treatment of the reducing agent DTT to disrupt disulphide bonds and found this rescued the maximum response in 315Cys-P2X4 receptors to a level similar to that of wild-type P2X4 and 315Ser-P2X4 receptors (Figure 2C, D). Similar DTT treatment did not affect peak current response to ATP for wild-type P2X4 receptors (data not shown) and DTT had no effect on P2X4 receptor surface expression (please see supplemental figure S3 http://hyper.ahajournals.org).

Inheritance of one copy of rs28360472 impairs P2X4 receptor responses

We genotyped 200-430 normal Caucasian subjects from the greater Sydney area (Sydney cohort) at each of the four identified polymorphic positions. We found no subjects carrying the variant allele at rs1044249 or rs28360470 although these have been reported by others [22]. rs25644 was found to have an allele frequency of 0.116 (n= 268 subjects) and the loss-of-function SNP rs28360472 was found to have a frequency of 0.011 (n=430 subjects, Table 2).

rs28360472 was found only in heterozygous dosage and therefore to determine whether inheritance of one allele encoding Tyr315>Cys would have a significant functional effect, we performed co-transfection experiments in HEK-293 cells to mimic a heterozygous state. Co-expression reduced the ATP-induced inward current to 40% of wild-type P2X4 receptors alone (39.8 ± 9.6 pA/pF for WT/315Cys compared to 111.1 ± 11.2 pA/pF for WT P2X4 receptors

alone, n= 4-8 cells, $P<0.01$, Figure 3A,B). To determine if this reduction in P2X4 response was also seen in native cells, we recorded ATP-induced inward currents from monocyte-derived macrophages isolated from wild-type P2X4 and 315Cys-P2X4 heterozygous subjects. We found the mean current density for the P2X4 response in 315Cys heterozygote macrophages was 40% of the P2X4 response in wild-type macrophages (0.53 ± 0.19 pA/pF (n=13 cells, 315Cys heterozygotes) compared to 1.32 ± 0.13 pA/pF (n=15 cells, WT subjects), $P<0.01$, Figure 3C,D).

Inheritance of rs28360472 (Tyr315>Cys) is associated with increased pulse pressure

A second cohort of 2874 subjects from the Victorian Family Heart Study [14] were genotyped for rs28360472 to determine if inheritance of this SNP was associated with pulse pressure. The variant allele was found to have an allele frequency of 0.014 in this cohort (Table 2) and was present in 89 heterozygotes and one homozygote. The variant allele was in Hardy-Weinberg equilibrium ($P = 0.42$) and there was no evidence of population stratification ($P = 0.73$). Using variance components models and the total association test we found that rs28360472 was associated with a slightly higher systolic and slightly lower diastolic blood pressure and was associated with a greater pulse pressure ($P = 0.023$). We estimated that one or more copies of the variant allele increased pulse pressure by 2.84 mmHg (95% CI 0.41 to 5.27, Table 3). Almost identical results were obtained for the Quantitative Trait Linkage Disequilibrium test (P=0.025).

Discussion

In this study we present evidence that an uncommon genetic variant, rs28360472, encoding amino acid change Tyr315>Cys, in the human P2X4 receptor gene reduces the receptor functional response and shows an association with increased pulse pressure in a large Caucasian cohort. These observations are consistent with the hypothesis that the loss-of-function variant depresses the normal endothelial vasodilatory mechanisms involving the P2X4 receptor and results in reduced large arterial compliance and an increase in pulse pressure.

The Tyr315>Cys mutation has two distinct effects on the ATP-induced human P2X4 receptor response. The first is a reduction in agonist potency as demonstrated by an increase in EC_{50} values for the primary agonist ATP and the partial agonist BzATP. This confirms that Tyr315 contributes to the agonist binding site on the P2X4 receptor, as shown by several mutagenesis studies on rat P2X receptors [18, 23, 24] and also by molecular modelling (Figure 4) based on the recently published crystal structure of the zebrafish P2X4 receptor [16]. Our molecular modelling analysis of the human P2X4 receptor structure shows that Tyr315 contributes to a putative ATP-binding pocket at the subunit interface lined by several other residues that have been implicated in ATP binding [16]. Computational docking of ATP at this pocket revealed that, relative to wild-type P2X4 receptors, docking to 315Cys-P2X4 receptors showed a different preferred pose that would clash with the Tyr315 side-chain in the wild-type P2X4 receptor (Figure 4). Furthermore no cluster of poses were observed when docking to the 315Cys-P2X4 receptor that were similar to the preferred docking pose for the wild-type P2X4 receptor. Similar results were observed with Tyr315>Ser and Tyr315>Ala models of the P2X4 receptor (data not shown) consistent with the hypothesis that Tyr315 makes a direct contribution to the ATP-binding site that is lost with mutation to cysteine, serine or alanine.

The second effect of the Tyr315>Cys mutation is a reduction in the P2X4 receptor maximum response. The presence of an extra cysteine residue in the large ectodomain in

addition to the conserved 10 cysteines important for P2X receptor structure may permit aberrant disulphide bond formation leading to a disruption in ion channel structure. This hypothesis is supported by experiments using the Tyr315>Ser P2X4 receptor mutant which cannot form disulphide bonds and does not show a reduced maximum response, only a reduced agonist potency (Figure 2). In addition, experiments with the reducing agent DTT could rescue the reduction in 315Cys-P2X4 maximum response, apparently by rescuing non-functional receptors at the cell surface rather than by increasing cell surface expression. The simplest explanation of this data would be that aberrant disulphides involving 315Cys render some P2X4 receptors non-functional but this is reversible with reduction by DTT.

Genotyping of two groups of Caucasian subjects revealed that rs28360472 (Tyr315>Cys) in *P2RX4* was found in only 9 of 430 subjects in the Sydney cohort (minor allele frequency 0.011, Table 2) and in 90 of 2874 subjects in the Victorian cohort (minor allele frequency 0.014, Table 2). The loss-of-function polymorphism was predominantly found in heterozygous dosage therefore to mimic heterozygous inheritance of the Tyr315>Cys mutation we co-transfected HEK cells with a mixture of wild-type P2X4 and 315Cys-P2X4 receptor plasmids. This reduced the P2X4 response to 40% of wild-type suggesting that this functional defect would not be fully rescued by the presence of a wild-type P2X4 receptor allele. This reduction was also replicated in monocyte-derived macrophage from 315Cys-P2X4 heterozygote subjects.

Our previous studies have identified a modest but significant genetic contribution to pulse pressure in the Victorian Family Heart Study [25]. Recent genetic analyses of population blood pressures have revealed the existence of common (population frequency >10 %) alleles of small effect (<1 mmHg) [26] and rare alleles (population frequencies < 0.1%) of modest effect (up to 6 mmHg) [27]. The former are typically in non-coding regions of DNA while the latter are generally in amino acid coding sequences. Interestingly, rs28360472, encoding the Tyr315>Cys

amino acid mutation, falls somewhere between the common and rare alleles in terms of expected frequencies and estimated effect sizes.

Pulse pressure represents the amplitude of the arterial pressure waveform and is recognised as a risk factor for cardiovascular disease, independent of systolic and diastolic blood pressures *per se* [28]. Pulse pressure is determined by the compliance of large arteries, by the cardiac stroke volume and by reflected pressure waves. The P2X4 receptor has an important role in determining large vessel tone through endothelial-dependent mechanisms, such that P2X4 receptor activation by ATP leads to endothelial cell NO release and arterial smooth muscle relaxation. Such reduced large arterial tone would increase vascular compliance and in the context of a dysfunctional 315Cys-P2X4 receptor, reduced endothelial NO release would favour decreased large arterial compliance and account for the higher pulse pressure seen in carriers of rs28360472.

Perspectives

Many of the genetic correlates of blood pressure have focussed on renal mechanisms that control fluid and electrolyte balance. This study instead turns attention to vascular haemodynamic control mechanisms as defined by the actions of ATP that are mediated by the P2X4 receptor. The frequency of rs28360472 and the estimated magnitude of the inherited functional effect distinguish themselves from the often-reported common variants of small effect and the more rare mutations of moderate effect. These observations will stimulate further investigations into the role of ATP and P2X4 receptors in blood pressure and vascular homeostasis and a more detailed physiological study into the mechanisms by which pulse pressure and its underlying phenotypes might be altered by the dysfunctional 315Cys-P2X4 receptor.

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Disclosures

None.

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Figure Legends

Figure 1: The Tyr315>Cys polymorphism in human P2X4 receptors shows a loss-of-function effect. (A) Representative inward current traces for human P2X4 receptor expressed in HEK-293 cells in response to 10 μmol/L ATP applied for 5 seconds denoted by black solid bars. Inset: schematic diagram of a single P2X4 receptor subunit and location of four SNPs altering amino acids. (B) Concentration-response curve for wild-type and mutant P2X4 receptors for ATP and (C) BzATP. Responses were normalised to cell capacitance and are plotted as mean current density (pA/pF) (n=3-8 cells per point). Due to the desensitising nature of P2X4 receptors, experiments were performed by applying a single concentration of ATP per cell. Key: Wild-type (closed black squares), Ala6>Ser (open white circles), Ile119>Val (closed black triangles), Ser242>Gly (open diamonds) and Tyr315>Cys (open inverted triangles).

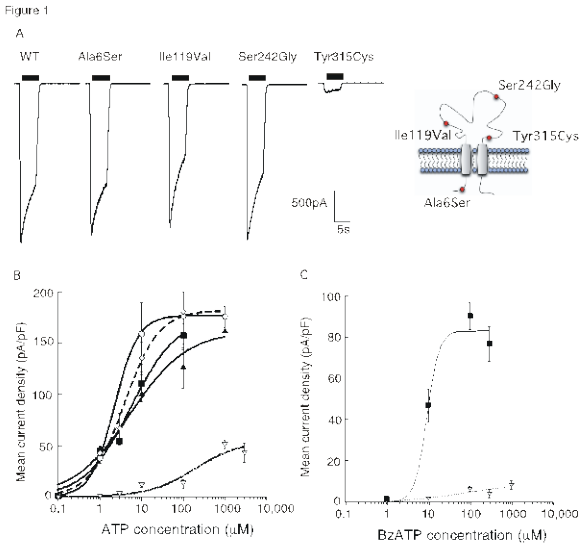


Figure 2: The Tyr315>Cys mutation disrupts P2X4 receptor ectodomain structure

(A) Representative inward current traces for wild-type, 315Cys-P2X4 and 315Ser-P2X4 receptors in response to 1 mmol/L ATP (black bars) and (B), concentration-response curves to ATP for wild-type (WT) (black closed squares), 315Cys-P2X4 (open white circles) and 315Ser-P2X4 (black closed triangles). (C) Representative inward current traces for wild-type (WT), and 315Cys-P2X4 compared to 315Cys-P2X4 receptor treated with 1 mmol/L DTT for 15 mins. (D) Responses to 1 mmol/L ATP from 3-4 cells are plotted as mean current density (pA/pF).

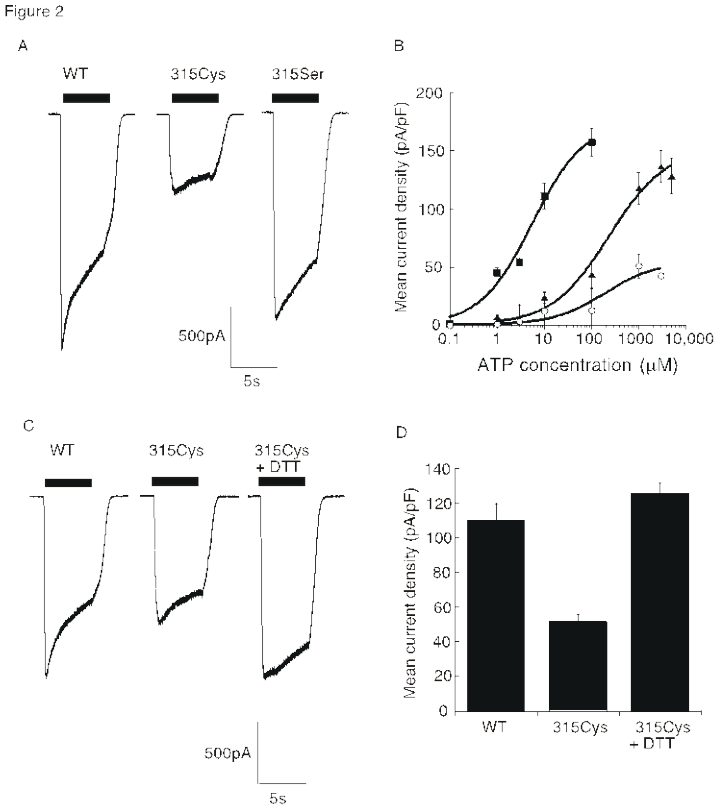


Figure 3: Heterozygosity for Tyr315>Cys P2X4 receptors. (A) Representative inward current traces for WT, Tyr315Cys-P2X4 and a 1:1 mixture of WT plus Tyr315Cys-P2X4 receptor DNA transfected into HEK-293 cells. Responses to 10 μmol/L ATP applied for 5 seconds are shown and are denoted by black bars. (B) Responses were normalised to cell capacitance and plotted as mean current density (pA/pF) (n=3-4 cells) ** P<0.01, * P<0.05 ANOVA. (C) Representative inward current traces to 100 μmol/L ATP applied for 5 seconds to macrophages from wild-type (n=3) or 315Cys heterozygote subjects (n=2). Responses were potentiated by 3 μmol/L ivermectin. (D) P2X4 responses were normalised to cell capacitance and plotted as mean current density (pA/pF) (n=13-15 cells) ** P<0.01.

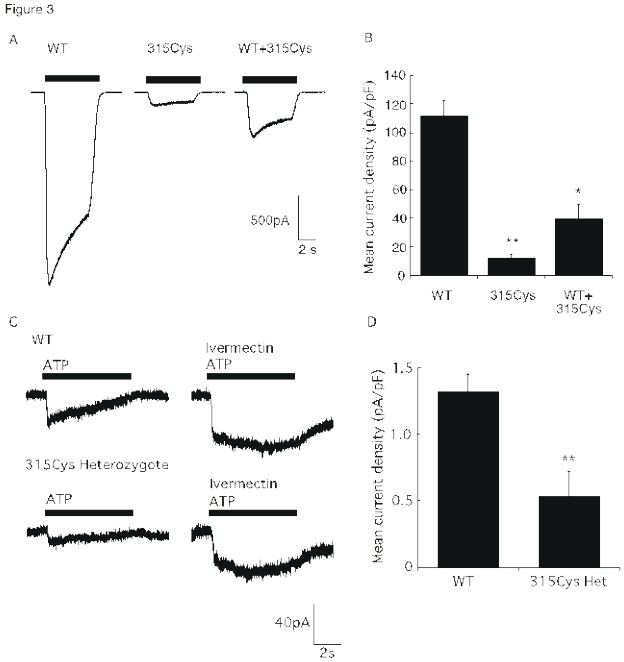
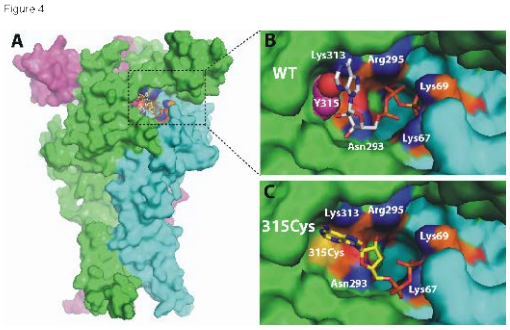


Figure 4: Molecular model of ATP-binding pocket in wild-type and 315Cys-P2X4 receptors. The molecular model of human P2X4 receptors was built by homology to zebra-fish P2X4 receptor (pdb code 3I5D, (20)) with ATP computationally docked at the putative ligand-binding pocket. (A) Surface rendering of the overall structure of the trimeric receptor model colored by subunit, oriented with the membrane-inserted domain at the bottom of the picture with the membrane normal to the plane of the page. ATP can be seen docked at the putative binding site at the interface between the green and the blue subunits. (B) An enlargement of the ATP-binding pocket for the wild-type receptor with key residues labelled and coloured by atom type. Tyr315 is shown as spheres with C atoms coloured magenta. Docked ATP is shown as bonds, colored by atoms, with C atoms white. (C) The same view as for B for the 315Cys-P2X4 mutant receptor with the 315Cys atom visible as a yellow surface patch. Docked ATP (with C atoms yellow) showed a different preferred docking pose relative to the wild-type P2X4 receptor.



Tables

Mutation	10 μM ATP Peak Amplitude (pA ± SE)	% Desensitisation to 10 μM ATP*	ATP EC_{50} μM)†	BzATP EC_{50} (μM)‡
Wild-type	111.1 ± 11.1	30.3	5.7	9.4
Ala6>Ser	159.3 ± 30.3	29.2	2.3	*nd*
Ile119>Val	101.3 ± 8.4	30.2	6.8	*nd*
Ser242>Gly	135.1 ± 16.1	31.2	4.0	*nd*
Tyr315>Cys	12.2 ± 2.3**	20.3*	198.7	71.8

Table 1: Characterisation of four non-synonymous single nucleotide polymorphisms in the human P2X4 receptor.

*Desensitisation was measured as percentage decline over 4 seconds from the peak of the inward current as described in (8). †EC_{50} value for ATP was determined from concentration-response curves generated from application of a single concentration of ATP per cell to eliminate any effect of desensitisation. ‡EC_{50} value for BzATP was determined from concentration-response curves generated from application of a single concentration of BzATP per cell to eliminate any effect of desensitisation. *nd* = not determined. ** $P<0.01$, * $P<0.05$.

dbSNP id	Amino acid change	Minor Allele frequency	Number of subjects
rs1044249	Ala6>Ser	0	200*
rs28360470	Ile119>Val	0	200*
rs25644	Ser242>Gly	0.116	268*
rs28360472	Tyr315>Cys	0.011	430*
rs28360472	Tyr315>Cys	0.014	2874†

Table 2: Frequencies of four non-synonymous SNPs in *P2RX4*.

*Subjects were recruited from the greater Sydney area for genotyping.
†Subjects were part of the Victorian Family Heart Study

Variable	rs28360472 A/A (n=2784)	rs28360472 A/G or G/G (n=90)
Age (years)	39.8 ± 15.8	36.68 ± 15.69
Weight (kg)	71.7 ± 14.0	72.57 ± 13.02
Height (mm)	1690 ± 93	1706.3 ± 89.0
Systolic BP (SBP)	123.3 ± 15.4	124.8 ± 15.1
Diastolic BP (DBP)	72.6 ± 11.6	71.3 ± 11.6
Pulse Pressure (PP)	50.7 ± 12.0	53.5 ± 12.5*

Table 3: Blood pressure measurements correlated with *P2RX4* genotype at rs28360472 for 2874 subjects from the Victorian Family Heart Study.

* represents $P<0.05$