Characterisation of the R276A gain-of-function mutation in the ectodomain of murine P2X7

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Abstract The cytolytic P2X7 purinoceptor is widely expressed on leukocytes and has sparked interest because of its key role in the activation of the inflammasome, the release of the pro-inflammatory cytokine IL-1 β and cell death. We report here the functional characterisation of a R276A gain-of-function mutant analysed for its capacities to induce membrane depolarisation, calcium influx and opening of a large membrane pore permeable to YO-PRO-1. Our results highlight the particular sensitivity of R276A mutant to low micromolar adenosine triphosphate (ATP) concentrations, which possibly reflect an increased affinity for its ligands, and a slower closing kinetics of the receptor channel. Our findings support the notion that evolutionary pressures maintain the low sensitivity of P2X7 to ATP. We also believe that the R276A mutant described here may be useful for the

generation of new animal models with exacerbated P2X7 functions that will serve to better characterise its role in inflammation and in immune responses.

Keywords P2X7 receptor · ATP · Inflammation · Apoptosis · Gain-of-function · Mutagenesis · Evolution

Abbreviations

Bz-ATP	2',3'-O-(4-benzoyl)-benzoyl-ATP
HEK	Human embryonic kidney
IL	Interleukin
KN-62	(1-[N,O-bis(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine)
mAb	Monoclonal antibody
PE	Phycoerythrin

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Introduction

Extracellular adenosine triphosphate (ATP) has emerged as an important signalling molecule that can regulate numerous biological processes [1–5]. It is released into the extracellular milieu by passive mechanisms accompanying cell death, as well as in the context of active physiological processes like muscle contraction. The actions of extracellular ATP are mediated through ionotropic P2X and metabotropic P2Y purinoceptors [6–8]. Among purinoceptors, P2X7 is widely expressed on cells of hematopoietic origin and plays important roles in inflammation and apoptosis [9–11]. Activation of P2X7 evokes ionic currents resulting from the opening of a membrane channel that allows influx of calcium and sodium as well as efflux of potassium and chloride ions [10, 12–15]. Prolonged activation of the receptor is accompanied by the formation

of a non-selective membrane pore that allows the passage of larger molecules of up to 900 Da. Formation of this membrane pore can be monitored by the incorporation of DNA-staining dyes like YO-PRO-1 and is considered to be a typical hallmark of P2X7 activation. Among P2X receptors, pore formation is unique to P2X7 and is linked to the very long C-terminal cytosolic tail of this receptor [10, 14–18]. This trait was long viewed as an intrinsic feature of the P2X7 receptor itself which was believed to form a channel able to dilate upon continuous stimulation. New data instead have implicated pannexin-1, a distinct membrane protein structurally and functionally related to gap junction proteins, which can form non-selective hemi-channels [19]. However, inhibition of pannexin-1 expression or function only abrogates the fast initial phase of dye uptake, leaving the possibility that the P2X7 receptor itself or another yet unknown protein may also partially account for a slower uptake of DNA-staining dyes [20].

P2X7 differs from other P2X receptors by its relatively low sensitivity to ATP. Indeed, while most P2X receptors are activated with micromolar ATP concentrations, stimulation of P2X7 is only achieved with concentrations ranging from 100 μ M to 5 mM [7, 21]. As ATP-catabolising enzymes like CD39 can very efficiently degrade this molecule, it has been postulated that such high ATP concentrations may only be reached in the vicinity of dying cells or within wounded tissues. Interestingly, we have characterised an alternative mechanism leading to P2X7 activation and operating with low micromolar concentrations of nicotinamide adenine dinucleotide (NAD) [14, 22]. This pathway involves the ART2.2 ecto-enzyme that catalyses the transfer of an ADP-ribose group from NAD to target proteins at the cell surface. This post-translational protein modification, called ADP-ribosylation, is a well-known enzymatic reaction responsible for the deleterious effects of various bacterial toxins, as for instance the agents responsible for diphtheria and cholera. A family of toxin-related ARTs has been discovered and characterised in mammals and has been shown to display a similar conserved protein fold [23, 24]. Remarkably, while ADP-ribosylation catalysed by bacterial toxins usually results in the functional inactivation of the target proteins, ADP-ribosylation of P2X7 by ART2.2 on the surface of mouse T lymphocytes results in its activation [14]. We recently identified the arginine residues modified by ADP-ribosylation in the P2X7 ectodomain and have proposed that modification of R125 by a covalently linked ADP-ribose group provides a ligand structurally related to ATP that accommodates into the nucleotide-binding pocket [22]. In accordance with the covalent nature of this modification catalysed by ART2.2, even a brief exposure to micromolar NAD concentrations can lead to prolonged activation of P2X7 [14, 25].

Prolonged activation of P2X7 either by ATP or by NAD-dependent ADP-ribosylation elicits several distinctive

effects apart from ion and dye uptake, i.e. exposure of phosphatidyl serine (PS) on the outer leaflet of the plasma membrane, loss of mitochondrial potential, membrane blebbing, release of lactate dehydrogenase, DNA fragmentation and ultimately cell death [10, 14, 26].

P2X7 has been proposed to function as a key regulator of inflammation and plays a crucial role in the ATP-dependent processing and release of the leader-less cytokines IL-1 β , IL-1ra and IL-18 [27–32]. Other important functions where P2X7 has been implicated include killing of mycobacteria and Chlamydia residing inside macrophages [33–35], apoptosis of immune cells [14, 36, 37], cell fusion [38, 39] and shedding of the CD62L homing receptor and other membrane proteins including CD23 and CD27 [14, 40, 41].

Several natural polymorphisms in the primary sequence of the gene coding for human P2X7 have been shown to partially account for the observed variation of its functionality. In laboratory mice, we have previously described an impairment-of-function polymorphism, P451L, which affects pore formation by cells from the widely used C57BL/6 mouse strain [42]. In the human population, single amino acid polymorphic substitutions (R307Q, T357S, E496A, I568N) and a 5' intronic splice site polymorphism have been shown to result in reduced or absent receptor functions [43–48]. Moreover, splice variants leading to a receptor lacking the cytoplasmic tail have been detected. By antagonising the function of the normal variant, these may account for reduced P2X7 functions in some normal tissue as well as in tumour cells over-expressing such variants [49]. To date, only one polymorphism in the sequence of the human P2X7 (i.e. H155Y) has been linked to a gain-of-function phenotype [50]. However, both allelic variants occur at frequencies around 50%, and although historically the H155 variant was discovered first and therefore considered to be the reference sequence, alignment with the P2X7 sequences from rat and mouse suggests that the Y155 variant is the one conserved in evolution, and H155 thus represents another impairment-of-function polymorphism.

Considering that P2X7 is a key player in inflammation, selection in the human population of numerous variants displaying reduced function may confer some benefit against the potentially deleterious effects of exaggerated inflammation. The very low sensitivity of P2X7 to extracellular ATP, as compared to other P2X receptors, may in itself also reflect the action of selective pressures acting to lower the level of inflammation. In the course of our site-directed mutagenesis study aiming to identify arginine residues in mouse P2X7 that are ADP-ribosylated by ART2.2, we discovered that mutations at three positions (R206, R276 and R277) independently conferred ten- to 30-fold enhanced sensitivity to extracellular ATP [22]. We

describe here the first detailed functional characterisation of one of these mutants, namely R276A. Our findings show that point mutations can generate P2X7 mutants responding to low micromolar ATP concentrations, a situation more comparable to the sensitivity of other P2X receptors. Our results may aid in understanding the relationship between P2X7 structure and function as well as the evolutionary pressures that maintain its low sensitivities to ATP.

Experimental procedures

Cloning of expression vectors, generation of P2X7 mutants and cell transfection The full-length complementary DNA (cDNA) sequence for wild-type P2X7 was PCR-amplified from BALB/c mouse splenocyte cDNA and cloned into the pCDNA6 vector (Invitrogen) under the control of the CMV promoter as described [42]. Mutants were generated by site-directed PCR mutagenesis using the QuickChange system (Stratagene) [22, 52]. Expression constructs (5 µg per 10⁶ cells) were transfected into HEK cells with the jetPEI transfection reagent (Q-Biogen). Expression levels were systematically evaluated 40 h after transfection by anti-P2X7 antibodies that were generated by genetic immunisation as described previously [51, 52]. Antibodies were conjugated to Alexa-488 (Molecular Probes/Invitrogen) according to the manufacturer's instructions.

Immunoblot analyses Cells were harvested by trypsinisation 40 h post-transfection, and washed cells were lysed in phosphate-buffered saline (PBS), 1% Triton-X100 and 1 mM AEBSF (Sigma) for 20 min at 4°C. Insoluble material was pelleted by high-speed centrifugation (15 min 13,000×g) and soluble proteins in total cell lysates (1×10⁵ cell equivalents/lane) were size-fractionated on pre-cast SDS-PAGE gels (Invitrogen) and blotted onto PVDF membranes. P2X7 was detected with rabbit anti-P2X7 antibody (1:1,000) directed against a conserved peptide present in the extracellular loop of P2X7 (Alomone) and peroxidase-conjugated anti-rabbit IgG (1:5,000) using the ECL system (Amersham).

Evaluation of the functionality of the mutants by cytometry Cells were harvested by trypsinisation 40 h post-transfection and were stained with Alexa-488 conjugated anti-P2X7 antibodies (1 µg/2×10⁵ cells/100 µl) to assess P2X7 expression levels. Cells were subjected to different assays to evaluate the functionality of the P2X7 receptor. For flow cytometry assays of ATP-induced calcium flux, cells were loaded with Fluo-4 (Molecular Probes) for 30 min at RT. Cells were then washed, resuspended in 10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) pH 7.5, 140 mM NaCl, 5 mM KCl, 1 mM CaCl₂ and 10 mM glucose and kept on ice until analysis. For measure-

ments of ATP-induced calcium flux, cells were resuspended in 20-fold excess of pre-warmed (37°C) medium. Following measurement of background fluorescence, cells were briefly removed from the flow cytometer for addition of ATP or Bz-ATP and returned immediately, and measurements were continued for 3 min. A warm pack was placed around the tubes to prevent temperature drop during measurement. All measurements were performed on a FACSCalibur and analysed with Cellquest-Pro software (Becton Dickinson). For graphical representation, data were exported to Excel software and MFI values of Fluo-4 fluorescence were plotted against time. For evaluation of P2X7-dependent YO-PRO-1 uptake using flow cytometry, cells were incubated in the absence or presence of the indicated concentrations of ATP or agonist in 10 mM HEPES pH 7.5, 140 mM NaCl, 5 mM KCl and 10 mM glucose for 60 min at 37°C in the presence of 1 µM YO-PRO-1 (Molecular Probes). Cells were then washed and analysed for their level of fluorescence with a FACSCalibur. In some experiments, the kinetic of YO-PRO-1 incorporation was measured over 10 min. In these experiments, concentrated cells were resuspended in pre-warmed (37°C) buffer containing Bz-ATP and 1 µM YO-PRO-1. Changes in fluorescence levels were then monitored over time by cytometry as described for calcium assays.

Measuring cell surface expression of P2X7 following treatment with ATP Cells were harvested by trypsinisation 40 h post-transfection with P2X7 variants. After washing, cells were resuspended in PBS buffer supplemented with 1 mM CaCl₂ and incubated for various times with different concentrations of ATP. Cells were then washed and stained with a rabbit polyclonal anti-P2X7 antiserum (K1G) followed by a polyclonal secondary anti-rabbit antibody coupled to PE (Dianova, Hamburg, Germany).

Evaluation of the functionality of the mutants by electrophysiological recordings Transiently transfected HEK cells were analysed using the whole-cell configuration of the patch-clamp technique. To this end, cells were continuously superfused at room temperature as described in [12] with a solution containing 147 mM NaCl, 2 mM KCl, 0.3 mM CaCl₂, 10 mM HEPES and 12 mM sucrose (pH 7.4). Patch pipettes were pulled from thin-walled borosilicate glass using a DMZ puller (Zeitz). Internal pipette solution contained 154 mM NaCl, 11 mM EGTA and 5 mM HEPES (pH 7.2). Currents were recorded using an EPC9 patch-clamp amplifier controlled by PULSE software (HEKA). The program PULSEFIT (HEKA) was used to analyse current traces, and the data obtained were further exported to Prism4 (GraphPad Software). All experiments were performed with a holding potential of -60 mV. Calculation of the time constant associated with current decay after ATP removal was estimated by fitting a single exponential function using PULSEFIT software.

Results

1. Substitution of R276 in the ectodomain of P2X7 by alanine results in increased sensitivity to ATP-induced down-modulation from the cell surface of transiently transfected HEK cells

In experiments initially designed to study the role of conserved arginine residues in mouse P2X7 and to identify the residue responsible for P2X7 activation upon its ADP-ribosylation [22], we substituted each of the 11 conserved arginine residues in the ectodomain of P2X7 to alanine or lysine. We then assayed each mutant for its expression level and functionality after transfection into HEK cells. During this study, we noticed that two mutants, namely R276A and R294A, had remarkable phenotypes. Mutant R276A displayed higher sensitivity to ATP than the wild-type receptor, while the R294A mutant completely lost its function. Analyses of the total expression levels by immunoblotting using a commercial antibody against a conserved peptide of P2X7 showed a reduced total expression of the R276A variant compared to the WT and the R294A variant (Fig. 1a). Cell surface expression was further evaluated by flow cytometry using polyclonal

(K1G) and monoclonal antibody (Hano43) raised against the native conformation of mouse P2X7 [52]. The results confirmed that R276A is expressed at lower density at the cell surface than the wild-type receptor while, the non-functional R294A mutant was detected on the cell surface at slightly higher levels than wild-type (Fig. 1b).

As we had previously noted the down-modulation of P2X7 from the cell surface of transfected cells upon treatment with effective doses of ATP [52], we hypothesised that the lower expression level of the R276A mutant may reflect its loss from the cell surface upon encounter with low doses of ATP during cell culture or cell preparation. To determine whether low doses of ATP can indeed induce a detectable reduction in P2X7 levels at the cell surface, we transfected HEK cells with the different variants of P2X7 and measured their cell surface expression by flow cytometry after incubation of cells in the presence or absence of ATP (Fig. 2). Cells transfected with the R294A mutant did not change their level of P2X7 surface expression upon incubation with even high concentrations of ATP, consistent with the lack of reactivity of this mutant. In marked contrast, cells transfected with either the wild-type P2X7 or the R276A mutant displayed significantly decreased P2X7-density upon exposure to extracellular

Fig. 1 Overall and cell surface expression levels of P2X7 mutants in transfected HEK cells. **a** HEK cells were transfected with expression constructs coding for wild-type or mutated variants of P2X7. Cells were harvested 40 h post-transfection, washed and lysed. Proteins in cell lysates were size-fractionated by SDS-PAGE and subjected to immunoblot analyses with an antibody directed against a conserved extracellular peptide of P2X7. **b** Cells were harvested 40 h post-transfection and stained either with fluorochrome-conjugated rat

monoclonal anti-P2X7 antibody Hano43 (*top panels*), or with polyclonal rabbit anti-P2X7 serum K1G followed by a fluorochrome-conjugated secondary antibody (*bottom panels*). The fluorescence intensities of transfected cells (*filled histograms*) were compared to those of cells transfected with an irrelevant control vector (*open histograms*). Numbers indicate the mean fluorescence intensity (MFI) of the total population and the percentage of cell present in the indicated gated region

Fig. 2 Dose response (a) and kinetics (b) of ATP-induced down-modulation of P2X7 from the cell surface of transfected HEK cells. HEK cells transfected with wild-type P2X7 (open diamonds), with the R294A mutant (open squares) or with the R276A mutant (filled triangles) were treated with the indicated concentrations of ATP for

60 min (a) or with 1 mM ATP for the indicated times (b). Washed cells were then stained at 4°C with anti-P2X7 polyclonal antibody K1G followed by a PE-conjugated secondary antibody. The results are expressed as the percentage of the mean fluorescence intensity (MFI) of vital cells in the absence of ATP

ATP. Twenty-fold lower concentrations of ATP sufficed to induce down-modulation of mutant R276A from the surface of transfected cells in comparison to wild-type P2X7 (Fig. 2a). Wild-type and R276A mutant P2X7 did not differ in the kinetics of ATP-induced down-modulation from the cell surface (Fig. 2b). These findings suggest that the apparent lower overall expression of mutant R276A may result from its activation during cell culture and/or manipulation. However, we cannot exclude the possibility that its lower expression reflects an intrinsic lower stability and/or a higher turnover of this variant. Moreover, these results show a ten- to 30-fold higher sensitivity of mutant R276A to ATP (Fig. 2a) despite a three- to fivefold lower surface expression level than wild-type P2X7 (Fig. 1b).

2. Micromolar concentrations of ATP induce calcium flux and YO-PRO-1 uptake in HEK cells transfected with the R276A variant

To further examine the functionality of the R276A and R294A variants, we measured the calcium influx triggered upon activation of P2X7 with ATP or with the more potent agonist Bz-ATP. To this end, HEK cells transiently transfected with either variant were loaded with the calcium-sensitive dye Fluo-4 and analysed by flow cytometry for changes in intracellular calcium levels in response to ATP or Bz-ATP. In our standardised assay, wild-type mouse P2X7 responded to concentrations of Bz-ATP at and above 100 μ M (Fig. 3a). Treatment of R294A-transfected cells with the potent agonist Bz-ATP did not induce any detectable calcium influx even at very high (3 mM) concentrations, consistent with a complete loss of function of this mutant (Fig. 3c). In marked contrast and in accordance with previous experiments, the threshold for

induction of calcium influx was reduced ten- to 30-fold in cells transfected with the R276A mutant compared to wild-type (Fig. 3a, b). In the same assay, ATP also triggered calcium influx by R276A transfectants at concentrations as low as 10–30 μ M, while ten- to 30-fold higher concentrations were required to induce similar effects with wild-type P2X7 transfectants (Fig. 3d, e). After normalisation against the maximal response attained 100 s after the addition of ATP, the EC_{50} was calculated as 250 μ M for the WT P2X7 and 25 μ M for the R276A mutant (Fig. 3f).

Activation of P2X7 not only results in a rapid influx of sodium and calcium ions, but upon its continuous stimulation also triggers the opening of a larger pore allowing the entry of fluorescent dyes like YO-PRO-1. As the kinetics of dye accumulation inside the cell reflects the level of P2X7 stimulation as well as the efficiency of coupling to downstream effector molecules, we compared the capacity of the individual P2X7 variants to induce pore formation. To this end, cells transfected with each P2X7 variant were incubated with different concentrations of Bz-ATP in the continuous presence of YO-PRO-1, and accumulation of the fluorescent dyes inside the cell was monitored over time by flow cytometry. The R294A variant never responded in this assay (data not shown). The R276A mutant responded to tenfold lower concentrations of Bz-ATP (i.e. 3 vs. 30 μ M) and with faster kinetics than the wild-type receptor (Fig. 4a, b). Indeed, even at saturating doses of Bz-ATP wild-type P2X7 necessitated a continuous stimulation lasting for a least 200 s to elicit significant dye uptake, while the R276A mutant responded almost immediately to concentrations as low as 10 μ M Bz-ATP (Fig. 4a, b).

3. Mutant R276A retains the rank order of potency of P2X7 agonists and sensitivity to inhibition by KN-62

Fig. 3 Sensitivity of P2X7 mutants to ATP- and Bz-ATP-induced calcium flux in transiently transfected HEK cells. HEK cells were harvested 40 h post-transfection and loaded with the calcium-sensitive fluorochrome Fluo-4 before live cell FACS analyses. Following measurement of background fluorescence, tubes were briefly removed for addition (indicated by the arrow) of the indicated concentration of Bz-ATP (a, b, c) or ATP (d, e). Tubes were then returned to the flow

cytometer and measurements were continued for a total of 3 min. Data obtained in (d) and (e) were further analysed by comparing the mean fluorescence intensity (MFI) obtained 100 s after addition of ATP (f). Results are expressed as the percentage of the maximum response obtained for the wild-type P2X7 (dashed line) or the R276A mutant (solid line) plotted against the concentration of ATP

Fig. 4 Comparison of Bz-ATP-induced YO-PRO-1 uptake in cells transfected with wild-type or R276A mutant P2X7. HEK cells transfected with wild-type P2X7 (a) or with R276A mutant (b) were resuspended in pre-warmed buffer containing 1 μ M YO-PRO-1 and

the indicated concentrations of Bz-ATP. Incorporation of YO-PRO-1 was then immediately and continuously monitored over 10 min by flow cytometry

Fig. 5 Comparison of YO-PRO-1 uptake induced by different P2X7 agonists in cells transfected with wild-type or R276A mutant P2X7. HEK cells transfected with wild-type P2X7 (a) or with R276A mutant (b) were treated with the indicated concentrations of agonist and incubated in the presence of 1 μ M YO-PRO-1 at 37°C for 60 min. Cells were then washed and analysed for their fluorescence levels by flow cytometry

Site-directed mutagenesis can potentially affect ligand binding (affinity) and/or subsequent conformational changes leading to channel formation (gating) [8]. Studying the response elicited by partial agonists and evaluating the blocking effect of known inhibitors can help to better interpret the effects of a given mutation. Therefore, we evaluated the rank order of potency of known P2X7 agonists on cells transfected with wild-type and R276A mutant P2X7 (Fig. 5). The results show that the R276A mutant displays enhanced sensitivity not only to Bz-ATP and ATP but also to all known P2X7 agonists. Remarkably, even partial agonists that were unable to trigger activation of the wild-type receptor in our assay elicited detectable dose-dependent responses in the gain-of-function R276A mutant (Fig. 5a, b). The rank order of potency of the agonists corresponded to the described pharmacological profile known for wild-type P2X7 (i.e. Bz-ATP > ATP > ATP- γ S > MeS-ATP > β , γ -ATP) [10, 21]. We further

analysed the capacity of KN-62—a well-known P2X7 inhibitor—to block calcium influx elicited by half-maximum ATP concentrations. The results reveal equivalent inhibitory curves for wild-type and the R276A mutant P2X7 (Fig. 6a–c). The precise mechanisms that underlie inhibition of P2X7 function by KN-62 still remain uncertain [53], but possibly involve allosteric modulation of the receptor rather than direct interference with the ligand-binding site. Our data indicate that the R276A mutant has retained a normal sensitivity to inhibition by KN-62, while it has gained considerable sensitivity to all known P2X7 agonists.

4. The kinetics of membrane depolarisation differs between wild-type and R276A mutant

Membrane currents elicited in mutant P2X7 receptors may differ from the wild-type receptor with respect to the opening

Fig. 6 Inhibition of wild-type and R276A mutant P2X7 by KN-62. HEK cells were transfected and prepared as in Fig. 3. Cells were pre-incubated 5 min with the indicated concentrations of KN-62 before measuring ATP-induced calcium influx. For proper comparison of the inhibitory effect of KN-62, ATP concentrations used to trigger the calcium influx were chosen to match the EC_{50} values determined in

Fig. 3f for wild-type (300 μ M) and R276A (30 μ M). Data obtained in (a) and (b) were further analysed by comparing the mean fluorescence intensity (MFI) obtained 100 s after addition of ATP (c). Results are expressed as the percentage of the response obtained in the absence of KN-62 plotted against the concentration of KN-62

and closing times of the channel, as well as the maximum current elicited [21, 54]. To further examine the effect of the R276A mutation on the kinetics of membrane depolarisation, HEK-293 cells transfected either with wild-type or mutant P2X7 receptors were subjected to patch-clamp analyses during perfusion with sequentially increasing doses of ATP (Fig. 7). The R294A mutant did not show any detectable currents under these conditions (data not shown). In contrast, both wild-type and R276A mutant receptors showed inward currents of increasing magnitude with increasing doses of ATP (Fig. 7a, b). Consistent with the data shown in Figs. 3 and 5, the concentration of ATP necessary to elicit a response was one order of magnitude lower for the R276A mutant (30 μ M) than for the wild-type receptor (300 μ M). For wild-type P2X7, increasing doses of ATP primarily increased the maximum current achieved and shortened the lag interval between agonist application and depolarisation. The wild-type P2X7 displayed rather fast channel opening and channel closing kinetics during addition and washout of ATP, respectively. By contrast, the R276A channel showed slower opening at low ATP concentrations (30 and 100 μ M ATP) that which was significantly accelerated (300 and 1,000 μ M) (Fig. 7b). Interestingly, current decay after washout of ATP was significantly slower for the R276A mutant than for the wild-type (Fig. 7a, b), reflected by a four- to fivefold higher time constant associated with the exponential current decay for mutant vs. wild-type P2X7 (Fig. 7c). These results show that the R276A mutation influences both opening and closing kinetics of the receptor. The prolonged open state

of the channel after ATP washout may reflect a higher affinity for ATP or a shift in the equilibrium between the open and closed states of the channel in favour of the open state.

Discussion

In the absence of crystallographic data to help resolve the 3-dimensional structure of P2X receptors, alanine scanning mutagenesis has been largely employed to study the relationship between P2X receptor structure and function. To date, such studies have highlighted the key roles of a number of conserved amino acids in determining ATP potency, folding and/or allosteric regulation by ions [8, 55]. Systematic mutagenesis studies often result in the generation of many mutants displaying little or no difference to the wild-type receptor regarding the sensitivity to ATP and only a minority of mutants showing an impaired response to agonists [8, 55–57]. Very few examples have been reported concerning P2X mutants displaying gain-of-function phenotypes [56]. In the course of our own mutagenesis study initially designed to identify the Arg residue responsible for activation of P2X7 through its covalent ADP-ribosylation by ART2.2, we mutated the 11 conserved Arg residues present in the extracellular loop of mouse P2X7 to alanine and to lysine [22, 52].

Interestingly, mutations at three of 11 positions analysed resulted in mutants with striking gain-of function pheno-

Fig. 7 Comparative analyses of ATP-induced currents in HEK cells transfected with wild-type or R276A mutant P2X7. HEK cells expressing wild-type P2X7 (**a**) or the R276A mutant (**b**) were grown on coverslips. Forty hours post-transfection, cells were subjected to perfusion with increasing doses of ATP from 10 μ M to 3 mM as indicated. ATP was added in the close vicinity of the recorded cell at time 2.5 s and replaced with buffer at time 7.5 s. After each single measurement, the cell was allowed to recover for 15 s before starting a new measurement with a different dose of ATP. The plots illustrate responses for individual cells transfected with wild-type (**a**) or mutant

(**b**) P2X7 and are representative for >10 (WT) and >40 (R276A) transfectants analysed. **c** The time constant associated with the decay phase was calculated using a single exponential equation fitting the decay phase of the curve between 7.5 to 15 s (after removal of ATP). The calculated time constant obtained in different cells subjected to electrophysiology measurements ($n=48$ for R276A mutant and $n=13$ for wild-type P2X7) are shown. The mean time constants were found to be significantly different between the two groups ($P < 0.0001$, Mann–Whitney’s non-parametric statistical test)

types (i.e. R206 to A or K, R276 to A or K and R277K; [22] and our unpublished results).

Our results reveal ten- to 30-fold enhanced sensitivities of the R276A variant vs. wild-type P2X7 to ATP and Bz-ATP in assays for membrane depolarisation, calcium influx and uptake of YO-PRO-1 (Figs. 2, 3 and 7). The results are compatible with two possible scenarios: increased affinity for ATP and/or facilitation of channel gating upon receptor engagement. An increased affinity for ligands is supported by the finding that even poor agonists unable to elicit a response at the wild-type receptor triggered significant responses at the R276A mutant (Fig. 5). On the other hand, data obtained with the patch-clamp technique indicate that the R276A mutation also affects the kinetics of channel opening upon addition of ATP and appears to stabilise the open channel after removal of ATP (Fig. 7). This indicates that the R276A mutation may bias the equilibrium between the different conformational states of the receptor towards an open configuration.

A natural polymorphism exists for the residue corresponding to R276 in human P2X7 (R276H) [50]. However, HEK cells transfected with human R276H show markedly reduced expression levels and ATP responses compared to wild-type hP2X7 (our own unpublished observations). Further, substitution of the neighbouring arginine residue (R277) in mouse P2X7 to alanine did not alter ATP sensitivity, while substitution with lysine enhanced it [22]. Mutation of the corresponding residue in rat P2X4 resulted either in a slightly enhanced ATP potency (R278K) or in a strong reduction in ATP potency (R278A) [58].

We also describe here for comparison the phenotype of a lost-of-function mutant, R294A. This mutant showed robust expression at the cell surface but displayed a complete loss of sensitivity in all our assays to both ATP and to the more potent agonist Bz-ATP (Figs. 1, 2 and 3). Most likely, this residue forms an essential part of the ATP-binding site. Mutation of the residues corresponding to R294 in human P2X1 (R292) and in rat P2X2 (R291) also resulted in dramatic reductions in ATP potency [59, 60]. A more direct evidence for the participation of this residue in ATP binding has recently been provided for human P2X1, by showing that its mutation abolishes by 80% the binding of a radiolabelled ligand [56]. The conserved role of this residue in the different P2X receptors suggests that a common ATP-binding site is, at least partially, shared by these related receptors.

Last but not least, our results suggest that the low sensitivity of P2X7 to ATP, as compared to other P2X receptors, may be maintained by evolutionary pressures. As P2X7 expressed by monocytes and macrophages plays a key role in the release of IL-1 β and in the activation of the inflammasome [11, 19, 30–32], its sensitivity to ATP may have been moulded during evolution to prevent its

excessive activation. Fine tuning the sensitivity of P2X7-expressing inflammatory cells to very high levels of ATP released from cells at sites of tissue damage may reduce the risk of exaggerated inflammatory responses. Conceivably, such evolutionary pressures may also be responsible for the numerous natural impairment-of-function and loss-of-function mutations found for human P2X7 and the remarkably high frequency of these mutations in human populations [18, 43, 45]. The R276A gain-of-function mutant described here offers the possibility to test such scenarios by generating transgenic mice expressing this variant and comparing the inflammatory responses of such mice with wild-type mice in various pathophysiological conditions.

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