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Contribution of $P2X_1$ receptor intracellular basic residues to channel properties

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Abstract

The intracellular amino and carboxy termini of P2X receptors have been shown to contribute to the regulation of ATP evoked currents. In this study we produced, and expressed in *Xenopus* oocytes, individual alanine point mutants of positively charged amino acids (eight lysine, seven arginine and one histidine) in the intracellular domains of the human $P2X_1$ receptor. The majority of these mutations had no effect on the amplitude, time-course or rectification of ATP evoked currents. In contrast the mutant K367A was expressed at normal levels at the cell surface however ATP evoked currents were reduced by >99% and desensitised more rapidly demonstrating a role of K367 in channel regulation. This is similar to that previously described for T18A mutant channels. Co-expression of T18A and K367A mutant $P2X_1$ receptors produced larger ATP evoked responses than either mutant alone and suggests that these amino and carboxy terminal regions interact to regulate channel function.

Keywords: P2X receptors; Mutagenesis; Ion channels; ATP

P2X receptors for ATP are a distinct family of ligand gated cation channels with two transmembrane segments, intracellular amino and carboxy termini and a large extracellular ligand binding loop [1]. Genes encoding seven P2X receptor subunits ($P2X_{1–7}$) have been identified and they can form as homo- or heterotrimeric channels with a range of properties and physiological functions [2]. For example $P2X_1$ receptors contribute to the control of smooth muscle contraction [3–6], platelet responsiveness [7] and neuronal phenotypes in sympathetic neurons [8] and the auditory brainstem [9]. P2X receptors subtypes can be distinguished based on their time-course to ATP into three groups (i) rapidly desensitising $P2X_1$ and $P2X_3$ homomeric receptors (ii) moderately desensitising $P2X_4$ receptors and (iii) slowly desensitising $P2X_{2,5–7}$ receptors [2,10]. The time-course of P2X receptor currents can be regulated by the transmembrane, intracellular and extracellular segments [1]. Studies on $P2X_2$ [11–14] and $P2X_4$ [13,15] receptors have shown that positively charged amino acids in the intracellular carboxy terminus can contribute to receptor trafficking and the time-course of responses. There are eight lysine, and seven arginine residues in the intracellular amino and carboxy termini of the $P2X_1$ receptor. In addition there is a histidine residue at position 355, histidine has a pK_a of 6 and so in an acidic environment can be positively charged. In this study, we have used alanine replacement mutagenesis to determine the contribution of individual positively charged amino acids in the intracellular domains of the $P2X_1$ receptor to channel properties.

Methods

Site-directed mutagenesis. Individual point mutations of intracellular positively charged residues in the human $P2X_1$ receptor were made using the QuikChange™ mutagenesis kit (Stratagene) as described previously [16]. Production of the correct mutations and absence of coding errors in the $P2X_1$ mutant constructs was verified by DNA sequencing (Automated ABI Sequencing Service, University of Leicester).

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P2X receptor expression in Xenopus laevis oocytes. Mutant and wild type constructs were transcribed to produce sense strand cRNA (mMessage mMachine™, Ambion, Texas, USA) as described previously [16]. Manually defoliculated stage V *X. laevis* oocytes were injected with 50 nl (50 ng) of cRNA using an Inject + Matic microinjector (J. Alejandro Gaby, Genéva, Switzerland) and stored at 18 °C in ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM sodium pyruvate, and 5 mM Hepes, pH 7.5). Media was changed daily prior to recording 3–7 days later. In some studies the $P2X_1$ K367A mutant was co-injected with either WT $P2X_1$ receptor, T18A mutant $P2X_1$ receptor, or WT human $P2X_2$ receptor RNAs—in all cases the final concentration of RNA was 50 ng.

Electrophysiological recordings. Two-electrode voltage clamp recordings (−60 mV) were made from injected oocytes using a GeneClamp 500B amplifier with a Digidata 1322 analog-to-digital converter and pClamp 8.2 acquisition software (Axon Instruments, USA) as previously described [16]. ND96 contained 1.8 mM $BaCl_2$ in bath solutions replacing 1.8 mM $CaCl_2$ to prevent activation of endogenous calcium activated chloride channels. ATP (Mg salt, Sigma, Poole, UK) was applied via a U-tube perfusion system. Reproducible responses to ATP were recorded after 5 min intervals between applications. The rectification index was measured as the peak current amplitude to ATP at +60 mV/peak current amplitude to ATP at −60 mV. All data are shown as mean ± standard error of the mean with significant differences between groups calculated by one way analysis of variance followed by Dunnett's test for comparisons of individual mutants against control using the SPSS 12.0 for Windows package. The significance of any changes in the time-course of desensitisation between holding potentials of +60/−60 mV were determined with paired Student's *t* tests. *n* corresponds to the number of oocytes tested.

Western blotting. Expression levels of wild type and mutant receptors were estimated by Western blot analysis of total cellular protein and cell surface proteins. Total cellular protein samples were prepared from oocytes injected with wild type or mutant receptor cRNA homogenised in buffer H (100 mM NaCl, 20 mM Tris–Cl pH 7.4, 1% Triton X-100, and 10 μl/ml protease inhibitor cocktail) at 20 μl/oocyte. Sulpho–NHS–LC–Biotin (Pierce) labels cell surface proteins by reacting with primary amines and was used to estimate the level of wild type or mutant receptor trafficked to the cell surface membrane. Sulpho–NHS–LC–Biotin is impermeable to the cell membrane and can only biotinylate proteins available at the cell surface. Oocytes injected with wild type or mutant receptor cRNA were treated with Sulpho–NHS–LC–Biotin (0.5 mg/ml) in ND96 for 30 min and washed with ND96. Oocytes were homogenised in buffer H and spin-cleared supernatant mixed with streptavidin agarose beads (Sigma) was treated as described previously [17]. All samples were mixed with SDS sample buffer and heated to 95 °C for 5 min. Samples were run on a 10% SDS–PAGE gel, transferred to nitrocellulose and probed with $P2X_1$ primary antibody (1:500, Alomone, Jerusalem, Israel), secondary goat anti-rabbit antibody (Sigma A6154)(1:1000), developed using ECL Plus (Amersham) and exposed to Hyperfilm™ film (Amersham Biosciences, Buckinghamshire, UK).

Results

To determine the contribution of individual positively charged amino acid residues, and histidine in the intracellular domains of the $P2X_1$ receptor to receptor properties we generated a series of alanine point mutants. The majority (14/16) of these point mutations had no effect on the peak amplitude or time-course of responses to an EC_{90} concentration of ATP (10 μM) (Table 1). The mutants H355A and K367A showed decreases in peak current amplitudes of ~80 and >99% respectively. Increasing the concentration of ATP to 10 mM had no effect on the peak current amplitude for H355A or K367A demonstrating that the reduction in current amplitude does not result from a decrease in ATP potency at the receptor. Western blot analysis of total $P2X_1$ receptor levels showed that both H355A and K367A mutant channels were produced at similar levels to wild type (WT) and mutants that had no effect on peak current amplitude (e.g. K359A, Fig. 1b and c). The marked reduction in the level of cell surface expression for H355A likely accounts for the decreased peak current amplitude for this mutant and suggests that this mutation affects

Table 1
Summary of the effects of alanine mutants in the intracellular domain of the $P2X_1$ receptor

	Peak (nA)	50% Decay time of current at −60 mV (ms)	50% Decay time of current at +60 mV (ms)	Rectification index
WT	−8674 ± 587	750 ± 57	464 ± 33***	0.64 ± 0.06
R3A	−8627 ± 995	874 ± 128	548 ± 67**	0.58± 0.10
R4A	−8134 ± 457	676 ± 101	425 ± 68*	0.78 ± 0.23
R20A	−6799 ± 684	341 ± 44	347 ± 55	0.43 ± 0.08
R25A	−6559 ± 1760	610 ± 83	402 ± 60*	0.72 ± 0.09
K27A	−10733 ± 1089	679 ± 137	584 ± 81*	0.77 ± 0.15
K28A	−9689 ± 1473	726 ± 75	460 ± 42*	0.69 ± 0.31
H355A	−1810 ± 590***	619 ± 24	629 ± 49	0.20 ± 0.01*
K359A	−8119 ± 1215	630 ± 58	429 ± 51***	0.83 ± 0.13
R360A	−6279 ± 1759	469 ± 31	383 ± 30*	0.55 ± 0.12
K361A	−9326 ± 1672	900 ± 137	623 ± 121*	0.58 ± 0.14
K364A	−6135 ± 379	695 ± 175	508 ± 194***	0.76 ± 0.09
K366A	−8758 ± 1443	838 ± 172	438 ± 73*	0.62 ± 0.12
K367A	−51 ± 14***	63 ± 7***	63 ± 6	0.29 ± 0.07*
K369A	−6892 ± 1120	997 ± 126	554 ± 50*	0.75 ± 0.27
R381A	−7489 ± 1019	501 ± 31	426 ± 57*	0.65 ± 0.18
R397A	−7134 ± 682	763 ± 99	534 ± 78**	0.67 ± 0.07

Peak current amplitude to a 10 μM application of ATP. Decay time is the time for the peak current to decay by 50% and was measured from a holding potential of either −60 or +60 mV. Rectification index is the ratio of the peak current amplitude at +60 mV/peak current amplitude at −60 mV, (n = 4–20). For peak currents, time to 0% decay at −60 mV and rectification index significant differences from WT are shown, $*p < 0.05$, $***p < 0.001$, for 0% decay at +60 mV significant differences are shown relating to paired values (−60 mV and +60 mV) from either WT or mutant receptors (n = 4–20 oocytes).

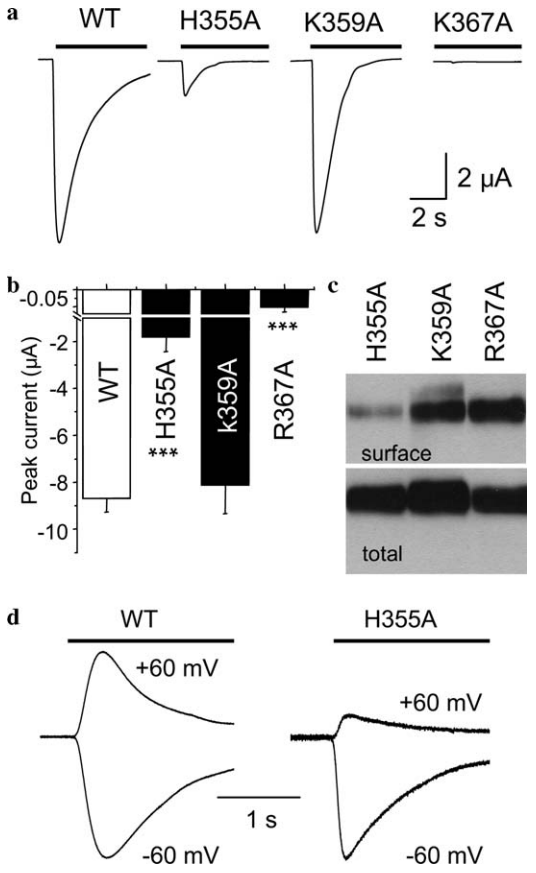

Fig. 1. Effects of mutation of intracellular positively charged residues at the $P2X_1$ receptor. (a) ATP (10 μM) evoked currents from oocytes expressing wild type (WT) and mutant $P2X_1$ receptors (application indicated by bar, holding potential −60 mV). (b) Peak amplitude of ATP (10 μM) evoked currents from WT and H355A, K359A, and R367A mutant receptors. ***$p < 0.001$ compared to WT. (c) Comparison of total and cell surface expression of mutants H355A, K359A and R367A. (d) Voltage dependence of P2X receptor currents. ATP (10 μM period indicated by bar) evoked currents at holding potentials of +60 and −60 mV for WT and H355A $P2X_1$ receptors (currents for WT and H355A were normalised to the peak response at −60 mV to show the difference in rectification).

receptor trafficking. In contrast the K367A mutant is expressed at the cell surface at similar levels to $P2X_1$ receptors that express normal amplitude currents and suggests that the functional properties of these channels have been affected. This is supported by the speeding in the time-course of the currents with a shorter time to peak and desensitisation than for WT channels.

The WT $P2X_1$ receptor channel is cation selective and has a reversal potential of ~0 mV. The $P2X_1$ receptor shows inward rectification as ions flow more easily through the channel into the cell than out of the cell; the rectification index of the WT channel (amplitude of peak outward ATP current at +60 mV/peak amplitude of inward ATP current at −60 mV) is 0.64 ± 0.06. In addition these studies showed that for the WT $P2X_1$ receptor the time-course of desensitisation of the ATP evoked current was voltage dependent with the time to 50% decay significantly faster ($p < 0.001$) at +60 mV (464 ± 33 ms) than at −60 mV (750 ± 57 ms) (Fig. 1d and Table 1). The majority of mutants (13/16) had no effect on either the rectification index or the time course of desensitisation (Table 1). For the mutant R20A there was no voltage dependent change in the time-course of the response. At the H355A and K367A mutants there was a significant increase in the rectification to 0.20 ± 0.01 and 0.29 ± 0.07 respectively ($p < 0.05$) and no voltage dependence to the time-course of the response (Fig. 1d and Table 1). These results suggest that residues R20, H355, and K367 are involved in the regulation of $P2X_1$ receptor currents.

The marked decrease in current amplitude and speeding of the time-course of the response for K367A with no effect on surface expression levels is similar to that seen when the threonine residue in a consensus protein kinase C phosphorylation sequence (T18 for the $P2X_1$ receptor) was mutated [18]. In that case the T18A subunit exerted a dominant effect and led to speeding of the time-course when co-expressed with $P2X_1$ receptors [18]. We therefore determined the effect of co-expression of K367A receptors with either T18A mutant or WT $P2X_1$ receptors. Co-expression of T18A and K367A channels (25 ng RNA each) gave fast currents, like for the individual subunits, that were ~10 fold greater in amplitude than expression of either T18A or K367A alone (50 ng of RNA for each). These results demonstrate that when a heteromeric $P2X_1$ receptor contains both T18A and K367A mutant subunits the amplitude of the response is partially recovered however the time-course is still rapid. When K367A was coexpressed with WT channels the ratio of RNAs was 9K367A:1WT. Assuming free association of subunits homo-trimeric WT channels would account for 0.1% of the resulting channels. Based on normal WT levels (peak current amplitude 6681 ± 619 nA to 100 μM ATP, $n = 10$) these homo-trimeric channels would only account for ~7 nA of current and not have a major effect on our analysis. Similarly 72.9% of channels would be predicted to be homo-trimeric K367A channels (expected current amplitude ~39 nA based on mean of 53 ± 14 nA for homotrimeric K367A channels, $n = 15$). The remaining channels would be those with one WT and two K367A subunits (24.3%) and two WT and one K367A subunits (2.7%). When K367A and WT RNAs were co injected (ratio 9:1, 50 ng total) responses were ~20 fold greater than K367A alone (Fig. 2) and the time for 50% decay of these currents was indistinguishable from the K367A mutants (83.9 ± 9.6 and 63.7 ± 7.8 ms respectively). This demonstrates that the K367A mutant dominates the time course of heteromeric $P2X_1$:$P2X_1$K367A channels and that the speeding in the time-course of the K367A mutant currents alone does not account for the reduction in current amplitude for homo-trimeric K367A mutant channels. A heteromeric channel with two WT and one K367A subunit is predicted to account for 2.7% of the subunits possible (assuming free association of subunits) and even if this gave channels with amplitudes similar to WT (expected ~200 nA) they would only give an ~4 fold increase in peak current compared to K367A alone (~50 nA). The results suggest that incorporation of

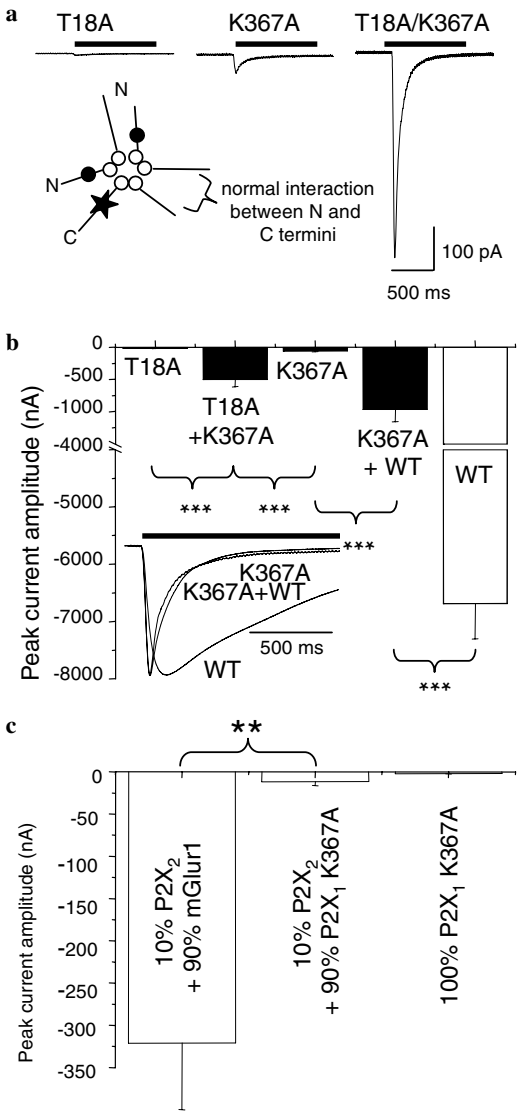

Fig. 2. The $P2X_1$ receptor mutant K367A can regulate the properties of other P2X receptor subunits. (a) Representative current traces of ATP (10 μM, application indicated by bar) evoked currents from oocytes expressing either T18A, K367A or a mixture of T18A/K367A (50:50 ratio) receptors (total 50 ng of RNA for each). Inset shows the arrangement of subunits within a heterotrimeric channel with two amino terminal (N) T18A mutants (indicated by black circles) and one carboxy terminal (C) K367A mutant (black star), note that there are adjacent amino and carboxy termini from adjacent subunits that have the WT sequence. (b) Peak current amplitudes in response to ATP (10 μM) for T18A, K367A (50 ng each) and T18A:K367A mix (25 ng of RNA each) and WT:K367A (5 and 45 ng of RNA respectively). Inset shows normalised traces for the time-course of WT, K367A and a 10:90 ratio co-expression of WT:K367A. (c) Peak current amplitudes in response to ATP (100 μM) from oocytes expressing P2X2:mGlur1a (5 and 45 ng of RNA, respectively), $P2X_2$:$P2X_1$K367A (5 and 45 ng of RNA, respectively) and $P2X_1$ K367A (50 ng of RNA).

a single WT subunit with two K367A subunits is responsible for the increase in current amplitude and speeding in the time-course of the response.

The equivalent of the K367A mutation in the $P2X_2$ receptor has been shown to co-assemble with $P2X_3$ receptors [13] and produce functional $P2X_{2/3}$ heteromeric channels. Previous studies have described heterotrimeric $P2X_{1/2}$ receptors [8,19,20]. We therefore tested whether the $P2X_1$ K367A mutant could be used to produce heteromeric $P2X_{1/2}$ receptors. We used a ratio of 9$P2X_1$K367A mutant to 1 of $P2X_2$. ATP (100 μM) evoked sustained $P2X_2$ receptor currents of 321 ± 79 nA ($n = 19$) when oocytes were injected with RNA for the human $P2X_2$ receptor and the metabotropic glutamate receptor mGluR1α in the ratio 1:9 (5:45 ng per injection, the mGluR1α was added to give the final amount of RNA to 50 ng). In comparison when $P2X_2$ and $P2X_1$K367A subunits were co-expressed (5:45 ng per injection) the peak current amplitude of the current was reduced by >95% in comparison to co-expression of $P2X_2$ and mGluR1α receptors (Fig. 2c). The results from the present study show that the $P2X_1$ K367A mutant has a dominant negative effect on the properties of co-expressed $P2X_2$ receptor subunits and supports the heteromeric assembly of the $P2X_1$ and $P2X_2$ receptor subunits.

Discussion

The majority of individual point mutations of positively charged amino acids in the intracellular amino and carboxy terminus had no effect on $P2X_1$ receptor channel properties indicating that they do not play an essential role in channel function. The lack of effect on channel time-course for K369A in the $P2X_1$ receptor contrasts with the marked speeding effect of mutation of the equivalent conserved residue at the $P2X_4$ receptor (K373, but a negatively charged residue for $P2X_{2,3,5-7}$ receptors) that resulted in a change from ~30% decrease in current amplitude over a 4s application to >90% desensitisation [15]. These differences may suggest that for $P2X_4$ receptors it is the interaction of this lysine residue with other variant residues in the intracellular domain that regulates the time-course of desensitisation and that this interaction is absent in the $P2X_1$ receptor and that is why the K369A mutant has no effect on time-course.

The H355A mutant showed a reduction in peak current amplitude, this most likely results from the decrease in expression of the receptor at the cell surface. In addition the H355A mutation has an effect on the voltage dependence of channel properties; resulting in an increase in channel rectification and removal of the voltage dependence of the time-course of receptor desensitisation. Histidine has a pK_a of 6, given that the intracellular pH of oocytes is reported to be ~7.2 [21] only a small proportion of the H355 residues would be positively charged under normal conditions. H355 is close to the second transmembrane segment that forms part of the ion conducting pore of the channel [22,23] and our results suggests that this amino acid normally contributes to the voltage dependence of the channel in terms of both ionic permeation (the rectification index) and the time-course of channel desensitisation.

There is a YXXXK motif in the intracellular carboxy terminus that is conserved throughout the P2X receptors and the lysine residue has been shown to be involved in

receptor stabilisation at the cell surface for $P2X_{2-6}$ receptors and mutation leads to up to a 70% decrease in surface expression [13]. In the present study the equivalent K367A mutation at $P2X_1$ receptors did not appear to affect surface receptor expression suggesting that the YXXXK motif plays less of a role in trafficking of the $P2X_1$ receptor. The >99% decrease in peak current amplitude and a speeding in the time-course of the K367A $P2X_1$ mutant response to ATP clearly indicates that this residue contributes to regulation of $P2X_1$ receptor currents. This is consistent with studies on other P2X receptors where positive charged residues in the intracellular carboxy terminus have been show to regulate channel properties [11,12,24]. It is interesting that the $P2X_1$ receptor mutants T18A [18] and K367A have similar phenotypes and that both of these residues are conserved throughout the P2X receptor family. When these mutants were co-expressed there was partial rescue in the amplitude of responses but no effect on the time-course of currents. In a heterotrimeric channel with T18A and K367A mutant subunits there would be either one WT amino and two WT carboxy termini (Fig. 2a) or two WT amino and one WT carboxy termini however in both cases their would be one pair of WT amino and carboxy termini from adjacent subunits. The T18 and K367 are both ~12 amino acid residues away from the predicted transmembrane segments and it is tempting to speculate that the amino and carboxy termini from adjacent subunits normally interact to control channel function. The partial rescue of current amplitude on co-expression of T18A and K367A could thus result from the normal interaction of amino and carboxy termini from adjacent subunits at one of the three subunit interfaces.

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