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Kinetics of conformational changes revealed by voltage clamp fluorometry give insight to desensitization at ATP-gated human P2X1 receptors.

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Running title: Conformational changes at P2X1 receptors for ATP

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Abstract

ATP acts as an extracellular signalling molecule at cell surface P2X receptors mediating a variety of important physiological and pathophysiological roles. Homomeric P2X1 receptors open on binding ATP and then transition to an ATP bound closed desensitized state that requires an agonist free washout period to recover. Voltage clamp fluorometry (VCF) was used to simultaneously record ion channel activity and conformational changes at defined positions in the extracellular loop of the human P2X1 receptor during not only agonist binding and desensitization, but also recovery. ATP evoked distinct conformational changes adjacent to the agonist binding pocket in response to channel activation and desensitization. The speed of recovery of the conformational change on agonist washout was state dependent with a faster time constant from the open (5 s) compared to the desensitized (75 s) form of the channel. The ability of ATP to evoke channel activity on washout following desensitization was not dependent on the degree of conformational re-arrangement in the extracellular loop, and desensitization was faster from the partially recovered state. An intracellular mutation in the carboxyl terminus that slowed recovery of P2X1 receptor currents (7 fold less recovery at 30s) had no effect on the time-course of the extracellular conformational rearrangements. The study highlights that the intracellular portion of the receptor can regulate recovery, and shows for the first time that this is by a mechanism independent of changes in the extracellular domain suggesting the existence of a distinct desensitization gate in this novel class of ligand gated ion channels.

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Introduction

P2X receptors comprise a family of ATP gated ion channels. The seven mammalian receptor subunits (P2X1-7) assemble to form a variety of homo- and hetero-trimeric receptors that are expressed widely in the body. They have a range of physiological and pathophysiological roles; for example P2X1 receptors underlie a component of neurogenic smooth muscle contraction, and on platelets they contribute to thrombosis (for reviews see (Burnstock, 2012; Kaczmarek-Hajek et al., 2012; Surprenant and North, 2009)). The crystallization of a zebrafish P2X4 receptor in both resting and ATP-bound open-states (Hattori and Gouaux, 2012; Kawate et al., 2009) was a major advance and demonstrated extensive conformational changes in the receptor associated with agonist binding and channel gating. This is consistent with mutagenesis studies that identified residues important in agonist action, the inter-subunit nature of the binding site, the location of the channel gate and interactions between the transmembrane regions (Browne et al., 2010; Heymann et al., 2013; Jiang et al., 2013; Kaczmarek-Hajek et al., 2012; Kracun et al., 2010). Individual P2X receptor subunits have been described by analogy to a dolphin, with the ATP binding site formed predominantly from residues in the upper and lower body regions of adjacent subunits (Hattori and Gouaux, 2012; Kawate et al., 2009). Agonist binding induces movement of the dorsal fin, left flipper and the cysteine rich head regions closing the ATP binding pocket. This movement is translated through the body region to the fluke (transmembrane regions) and results in opening of the channel gate (Hattori and Gouaux, 2012).

For some P2X receptors, for example homomeric P2X1 receptors, the integral ion channel closes (desensitizes) during the continued presence of ATP and an agonist-free washout period is required for the receptor to recover (Valera et al., 1994). Transition to a desensitized state is associated with additional conformational changes, some of which have been identified with cysteine mutants introduced in the extracellular domain, studied by biochemical methods (Roberts et al., 2012), or with voltage clamp fluorometry (VCF) to simultaneously record agonist evoked currents and fluorescence of labelled cysteine residues (Lorinczi et al., 2012). With VCF changes in the local environment of the introduced cysteine-reactive flurophore can be detected as changes in the fluorescent emission to give a real-time measure of conformational rearrangements. At the rat P2X1

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receptor VCF studies showed that movement of the cysteine rich head region was associated with desensitization (Lorinczi et al., 2012). The VCF technique can report electrically silent changes and therefore has the potential to measure conformational rearrangements associated with recovery from the desensitized state following agonist washout. However due to the long washout times (>20 minutes) required at the rat P2X1 receptor (Lorinczi et al., 2012) the relationship between conformational changes in the extracellular loop and recovery of the channel from desensitization could not be determined. At the human P2X1 (hP2X1) receptor an agonist-free washout period of only 5 minutes was required to evoke reproducible currents to ATP (Allsopp et al., 2013) raising the possibility that the relationship between conformational changes in the extracellular domain and recovery from desensitization can be studied directly in real-time at this receptor.

We have shown previously that introduction of pairs of cysteine residues at the subunit interface between the cysteine rich head region and the lower body (K138C-E181C), the left flipper and the lower body region (N284C-K190C) and at the top/apex of the extracellular vestibule/lateral portal (D320C-I62C or D320C-P196C) results in disulphide bond formation between subunits, restricting conformational rearrangement, that inhibited ATP-evoked currents (Roberts et al., 2012). These are consistent with the agonist evoked movements identified in structural studies (Hattori and Gouaux, 2012). We have now used VCF to characterize ATP-evoked conformational changes at the individual cysteine mutants identified by "disulphide-locking" in the extracellular loop. This approach has provided new insight into the temporal characteristics of conformational changes during agonist binding, desensitization and recovery. The work shows directly for the first time (i) the temporal characteristics of conformational changes in the left flipper and lower body region to ATP, (ii) the kinetics of conformational changes after agonist washout were state dependent, (iii) full reversal of the conformational change in the extracellular domain associated with desensitization was not required for recovery of P2X1 receptor channel activation/opening, and (iv) the intracellular region can dominate recovery from desensitization independently of rearrangements in the extracellular loop, suggesting the existence of a distinct desensitization gate in this structurally novel class of ligand gated ion channel.

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Materials and Methods

Site-directed mutagenesis. Cysteine point mutations were introduced via the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) using a human P2X1 receptor plasmid as the template, as described previously (Ennion et al., 2000). Production of the correct mutations and absence of coding errors in the P2X1 receptor mutant constructs was verified by DNA sequencing (Automated ABI Sequencing Service, University of Leicester).

Expression in *Xenopus laevis* oocytes. Wild-type (WT) and mutant constructs were transcribed to produce sense-strand cRNA (mMessage mMachine, Ambion, Austin, TX) as described previously (Ennion et al., 2000). Manually defolliculated stage V *Xenopus laevis* oocytes were injected with 50 nl (50 ng) of cRNA using an Inject+Matic microinjector (J. A. Gaby, InjectMatic, Geneva, Switzerland) and stored at 16°C in ND96 buffer (in mM: 96 NaCl, 2 KCl, 1.8 CaCl2, 1 MgCl2, 5 sodium pyruvate, and 5 HEPES, pH 7.6). The medium was changed daily before recording 3–7 d later.

Protein labelling and SDS-PAGE. To fluorescently label plasma membrane proteins, the prepared *Xenopus* oocytes were incubated in labelling solution (110 mM NaCl, 0.5 mM $CaCl_2$, 20 mM sodium phosphate buffer, pH 8.5) containing 50 µM Cy5 N-hydroxysuccinimide ester Cy5 NHS ester (Cy5 NHS ester) (amine reactive to label membrane proteins, GE Healthcare, Little Chalfont, UK) or 5 µM 2-((5(6)-Tetramethyl-rhodamine)carboxylamino)ethyl Methanethiosulfonate (MTS-TAMRA, thiol reactive, and membrane impermeant, to label free cysteine residues, Toronto Research Chemicals, Canada) for 30 min and 15 min, respectively, on ice in the dark. Both fluorophores are not membrane permeable and labelled only proteins expressed at the oocyte surface. Oocytes were washed through multiple changes of labelling solution or ND96 (no Cy5 NHS ester or MTS-TAMRA), lysed in buffer H (100 mM NaCl, 20 mM Tris-Cl, pH 7.4, 1% Triton X-100, and 10 µl/ml protease inhibitor mixture (P8340; Sigma); 20 µl per oocyte), and centrifuged for 2 min at 16,000 g. Supernatant (85 µl) was removed, and 0.9 µg of anti-P2X1 antibody (Alomone Labs, Jerusalem, Israel) was added and

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incubated for 1 h on ice before 75 µl of protein A-Sepharose beads (30 mg/ml; Amersham Biosciences, Buckinghamshire, UK) were added and incubated overnight at 4°C with constant rolling. The Sepharose/antibody/protein complex was centrifuged for 2 min, and the beads were washed four times with buffer H. After the final wash, the Sepharose pellet was resuspended in SDS sample buffer, heated to 95°C for 5 min then centrifuged for 5 min. The supernatant containing eluted P2X1 receptors was loaded onto a 10% SDS-PAGE gel (gel solutions were filtered to remove autofluorescent particles) and electrophoresed at 120 V for 95 min. The glass plates containing the SDS-PAGE gels were thoroughly cleaned with ethanol then scanned using a Typhoon trio plus and Image Quant TL software (GE Healthcare), with further analysis conducted using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Electrophysiological recordings/Voltage clamp fluorometry. Two-electrode voltage clamp recordings (at a holding potential of -60 mV) were conducted on cRNA injected oocytes bathed in ND96 using an Axoclamp 900A amplifier with a Digidata 1440A analog-to-digital converter and pClamp 10.2 acquisition software (Molecular Devices, Menlo Park, CA, USA). VCF recordings were performed using a custom organ bath, designed to apply drug solutions directly to the underside of the oocyte (Pless and Lynch, 2009). To ensure rapid solution exchange, drugs were applied using a ValveLink 8 perfusion system (AutoMate Scientific, Berkeley, CA, USA). Oocytes were imaged using a Nikon Diaphot 200 inverted microscope equipped with HQ545/30 exciter, Q565LP dichroic and HQ572LP emitter filter set, with OptoLED lite light source (Cairn Research, Faversham, UK). Fluorescence was detected by a photomultiplier tube (Cairn Research) installed to the side port of the microscope, with data recorded using pClamp 10.2. Oocytes were labelled with MTS-TAMRA for 60 s, washed as above and stored on ice in the dark prior to VCF recordings. During VCF recordings, the baseline fluorescence output of the oocyte was recorded for 10 s before drug application. Any detected change in fluorescence was calculated as the percentage change from the pre-application level. Individual normalized concentration responses for ATP evoked currents (each oocyte was tested with multiple concentrations) for control and MTS-TAMRA treated occytes were generated. Data from individual oocytes were pooled and fitted with the Hill equation (variable slope) with

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GraphPad Prism 6. The concentration response relationship of changes in MTS-TAMRA fluorescence (% change in fluorescence) was determined from single applications of ATP to individual oocytes, results were pooled and fitted with the Hill equation (variable slope) with GraphPad Prism 6.

The fluorescence recovery from receptor desensitization was analysed by normalising the fluorescence output during agonist washout to a time point directly after the end of the ATP application (termed -100% from baseline), with any difference calculated as a percentage change (for clarity only the data points collected each second were presented here). The normalised data was then pooled and fitted with either one phase or two phase decay curves (as appropriate) with GraphPad Prism 6. For recovery from desensitization protocols responses to a 30s application of ATP were determined with either 30 s or 5 min wash intervals, individual oocytes were tested with either the 30 s or 5 min wash protocols.

Statistical analysis. All data are shown as mean ± SEM and any differences were between the means was determined by either Student's t test or one-way ANOVA with Bonferroni's post-test as appropriate. Unless stated, $n \geq 4$ for all average data.

Results

MTS-TAMRA fluorescent labelling of introduced cysteine residues in the extracellular loop of the human P2X1 receptor.

The background auto-fluorescence of unlabelled non-injected oocytes, measured as the PMT output, was 0.06 ± 0.01 V. Following labelling with the cysteine reactive flurophore MTS-TAMRA this increased to 0.24 ± 0.07 V ($p=0.05$), representing labelling of free accessible cysteine residues on the oocyte surface. Fluorescence levels of MTS-TAMRA labelled hP2X1 WT injected oocytes were indistinguishable from non-injected ooytes (0.21 ± 0.03 V, $p=0.6$) consistent with the ten native cysteine residues in the extracellular loop of the P2X1 receptor forming five disulphide bonds (Ennion and Evans, 2002) which would be inaccessible to labelling. MTS-TAMRA fluorescence >1.5 fold above background levels was detected for 6 out of the 7 hP2X1 receptor cysteine mutants (Figure 1A), with levels ≥ 2 fold higher for N284C and K190C mutants compared to K138C, E181C, D320C and I62C (Figure 1B) highlighting that introduced cysteine residues can be labelled fluorescently.

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The differences in fluorescence intensity do not reflect variations in surface expression levels as peak currents evoked by a maximal concentration of ATP were equivalent for WT and all the mutant receptors (Figure 1E).

The level of MTS-TAMRA fluorescence is governed by two factors; (i) the accessibility of the cysteine residues and (ii) the local environment of the cysteine residues in the intact receptor that determines fluorescent yield. Visualising the purified receptor on a denaturing gel removes structural/environmental factors and allows direct determination of labelling levels (Figure 1C). Following MTS-TAMRA treatment, P2X1 receptors were purified by immunoprecipitation, denatured and run on a gel. Labelling was not detected for the WT P2X1 receptor, but robust equivalent levels of labelling were seen for the seven cysteine mutants (Figure 1C,D). This demonstrates that the cysteine mutants can be labelled and used for VCF studies and that the differences in fluorescence levels in intact oocytes results predominantly from variations in the local environment of the intact receptor having an effect on quantum yield. The most striking difference was between P196C and N284C. These showed equivalent labelling on the gel, however there was an ~ 5 fold difference in fluorescence *in situ*, with the P196C mutant indistinguishable from background, indicating that the local environment quenched fluorescence to background levels at this mutant.

Agonist-evoked changes in MTS-TAMRA fluorescence.

At WT and cysteine mutant hP2X1 receptors labelled with MTS-TAMRA, ATP (100 μM) evoked currents that desensitized during the continued presence of agonist (MTS-TAMRA treatment had no effect on current amplitude, Figure 1E). For WT, and the E181C, D320C, I62C and P196C mutants, ATP application had no effect on MTS-TAMRA fluorescence (Figure 2). However at the K138C, N284C and K190C mutants ATP application produced a reduction in MTS-TAMRA fluorescence (-5.4 ± 0.7, -8.1 ± 2.0 and -18.7 ± 1.1 % respectively, Figure 2). The fluorescence change at K138C is consistent with the only previous VCF study on P2X receptors showing ATP evoked movement in the head region of the receptor (Lorinczi et al., 2012). Our work extends this to show in addition real-time ATP dependent conformational changes in the left flipper and lower body regions. For the three mutants MTS-TAMRA labelling had no effect on ATP potency

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(Supplementary Figure 1). The decrease in fluorescence does not result from direct ATP quenching as; (i) adding 10,000 fold excess of ATP (10 mM) to MTS-TAMRA (1 μM) in solution had no effect on fluorescence, (ii) introduction of a mutation in the ligand binding pocket essential for agonist binding (K68A, (Ennion et al., 2000)) had no effect on MTS-TAMRA labelling (K190C-K68A background fluorescence 4.4 fold above WT oocytes) but abolished both ATP evoked currents and changes in fluorescence (Figure 3A) and (iii) K138C and E181C mutants had similar levels of fluorescence but only K138C showed an ATP-evoked reduction in MTS-TAMRA fluorescence consistent with ATP binding to the receptor causing the fluorescence decrease.

In contrast to ATP-evoked currents, that peaked (10-90% rise time 105.4 ± 11.7ms) and then desensitized, the MTS-TAMRA fluorescence continued to decrease after the peak current was recorded for K138C, K190C and N284C mutants, reaching a level that was then sustained during continued agonist application (Figure 2). The differences between current and fluorescence changes do not result from sub-optimal solution exchange as (i) control experiments with application of the dye fast green to unlabelled oocytes showed a 10-90% rise time of fluorescence of 14.1 ± 4.9 ms (compared to rise time of ~100 ms for P2X1 receptor currents, Supplementary Figure 2), and (ii) the rise time of agonist evoked changes in fluorescence and current at MTS-TAMRA labelled mutant glycine receptors were superimposable (Figure 2) as reported previously (Pless and Lynch, 2009). To characterize the difference in time-course of the current and fluorescence changes the percentage of maximal change in fluorescence at peak current (%Fmax at Imax) was determined. This was ~40% for the K138C and K190C mutants (40.1 ± 5.7 and 40.1 ± 3.3%, respectively) and 70.7 ± 7.1% for the N284C hP2X1 receptor (Figure 2F). This suggests that the fluorescent changes at the hP2X1 K138C, K190C and N284C receptors report conformational changes associated with both ligand binding/channel opening (initial rise) and the development of desensitization (secondary post current peak phase).

VCF reports agonist binding and channel desensitization at K190C.

The K190C mutant showed the largest changes in fluorescence and became the focus of the remainder of our studies. The ATP-evoked currents and changes in fluorescence were concentration

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dependent with the same agonist sensitivity (pEC_{50} of 5.5 ± 0.08, and 5.7 ± 0.07 for currents and fluorescence respectively, Figure 2G, Supplementary Figure 1D for example traces).

The slower secondary decrease in fluorescence that occurs after the peak P2X1 receptor current may correspond directly to the channel entering the desensitized state. If this hypothesis is correct a mutant that changed the rate of channel desensitization would have no effect on the %Fmax at Imax, but would influence the time-course of the second component of the fluorescence change. We have previously shown that mutations in the intracellular termini modify channel desensitization (Wen and Evans, 2009), so we tested mutations that either decreased (D17C) or increased (P2X1 with residues 360-364 replaced by those from the hP2X2 receptor – P2X1-2(NVKYS) (Allsopp and Evans, 2011)) the rate of channel desensitization. Introducing the D17C mutant to K190C (K190C-D17C) had no effect on the peak current amplitude (-15 ± 0.7μA), MTS-TAMRA labelling (7 fold above WT), rise time of the current (10-90% 109.6 ± 7.3ms and 99.7 ± 14.4ms for K190C-D17C and K190C respectively) or %Fmax at Imax (39.9 ± 1.8%) confirming no effect on receptor expression or agonist binding/channel opening. However the K190C-D17C mutant desensitized more slowly during the continued presence of agonist (21.7 ± 1.7 and 4.2 ± 1.5 % remaining current at end of 30 s pulse for K190C-D17C and K190C, respectively, $p<0.001$) and the time to peak decrease in fluorescence was also increased to 17.5 ± 1.9 s (compared to K190C 5.1 ± 3.3 s, $p=0.015$, Figure 3B). When the fluorescent changes for K190C-D17C were re-scaled to allow comparison with the time-course of the current changes the rise of the initial fluorescence change was superimposable with the rise in current, and the time-course of the secondary slower decrease in fluorescence follows exactly with the current desensitization (Figure 3C).

Combination of the C terminal mutation NVKYS (360-364) with K190C resulted in ATP evoked rapidly desensitizing currents at the P2X1-K190C-2(NVKYS) mutant (time to 50% decay 0.3 ± 0.06s compared to K190C 1.6 ± 0.2s, $p=0.009$, mean current amplitude -407.2 ± 56.9nA, labelling 3 fold above WT). The %Fmax at Imax of 40.8 ± 4.1 % for the K190C-2(NVKYS) mutant was equivalent to that of both K190C and K190C-D17C mutants (Figure 3E). The post peak current secondary decrease in fluorescence was more rapid than at the K190C mutant receptor (50-90%

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fluorescence change of 2.7 ± 0.6s and 5.1 ± 3.3s for K190C-2(NVKYS) and K190C respectively, p=0.049) and equivalent to the rate of channel desensitization (Figure 3D).

These results show that mutants that changed the rate of current desensitization were mirrored by equivalent changes on the rate of secondary (post peak current) changes in fluorescence (with no effect on the %Fmax at Imax). This demonstrates that VCF can be used to measure conformational changes associated with the P2X1 receptor entering the desensitized state. Our studies show that MTS-TAMRA can report real time conformational changes in the P2X1 receptor associated with ligand binding, and highlight that desensitization (closure of the ionic permeation pathway in the continued presence of agonist) is co-incident with conformational changes close to the ligand binding site >45 Å away from the transmembrane spanning pore forming region.

Rate of recovery of fluorescence change following ATP removal is state dependent.

Following desensitization an agonist free period is required for recovery, however the molecular basis of this is unclear. The advantage of the VCF technique is it allows electrically silent conformational changes in the P2X1 receptors to be measured in real time. The recovery of fluorescence at the K190C mutant on washout of ATP (Figure 4A,C) had a time constant of 73 s (and was concentration independent, Supplementary Figure 2). This is ~ 120 fold slower than the solution exchange (time constant for fast green washout 0.6s, recovery of fluorescent changes for glycine receptor time constant 0.7s, Supplementary Figure 2). The rate of fluorescence recovery was unaffected by blocking receptor trafficking with brefeldin A (10 µg/mL incubated for 48 hours, Supplementary Figure 2) demonstrating it reports directly conformational rearrangement and not insertion of recycled labelled receptors to the cell surface. In these studies recovery was determined from the desensitized state. To determine whether the rate of conformational change associated with ATP washout was dependent on the state of the receptor we applied a shorter pulse of ATP (1s) to the K190C mutant so that ~80% of the peak current was present at agonist washout i.e. a greater proportion of the receptors were in the agonist bound open state of the receptor. Recovery of fluorescence to this short application (Figure 4B,C) now showed 2 components with a fast phase (25% overall, time constant 5 s) and a slower phase (time constant 74 s). This shows directly, for the

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first time, that there are state-dependent effects in the recovery of conformational changes in the extracellular loop with entry to the desensitized state slowing recovery.

Recovery of P2X1 receptor currents from desensitization is not dependent on full recovery of conformational changes in the extracellular ligand binding loop.

We have previously shown that a 5 minute interval between ATP applications is sufficient to allow full recovery of WT hP2X1 receptors from desensitization. This is also the case for the K190C MTS-TAMRA labelled receptor with peak current amplitude to a second application of ATP following a 5 minute wash period 98.5 ± 5.1 % (n=10) of the initial response. The molecular mechanisms of this recovery from desensitization were examined using the VCF technique to test the hypothesis that recovery from desensitization is dependent on reversal of the agonist-induced conformational changes in the extracellular loop. We used a double pulse ATP application protocol and measured the extent of current recovery following 30 seconds washout (after a 30 second ATP application) when fluorescence had recovered by 42.5± 3.7% (Figure 5). If recovery of the P2X1 receptor current from desensitization was dependent on the conformational change in the extracellular loop returning to pre-stimulation levels we would predict that the current would have recovered by ~ 40%. The second application of ATP (following 30 s washout) evoked a decrease in fluorescence to levels equivalent to that of the first application. Interestingly we found the peak ATP-evoked current had recovered to 74.5 ± 6.0% of the first response ($p=0.007$ vs. 5 minute washout). In addition the time-course of the second application following 30s washout was accelerated, not only did the ATP-evoked current show faster desensitization (time for current decay to 50 % 1.8 ± 0.1 and 0.8 ± 0.6 s for initial and second pulse, $p<0.001$) but also the change in fluorescence was faster with (%Fmax at Imax doubled for the second pulse at 30s). This contrasts with the reproducibility of time-course of both currents (time to 50% current decay 1.6 ± 0.1 and 1.6 ± 0.2 s for first and second response 5 minutes later respectively) and fluorescence changes (Figure 5F) following a 5 minute washout period. These results show significantly greater recovery in P2X1 receptor current compared to the fluorescence change and demonstrates that the agonist induced conformational change in the receptor does not need to fully reverse to allow receptor re-activation. In addition, the more rapid time-course

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of the response when the conformational change showed intermediate recovery demonstrates the novel gating properties of this partially recovered state. The underlying mechanism for these variations in time-course remains to be determined. However, one possibility is that differences in the number/conformation of occupied ATP binding sites may contribute.

To test further the relationship between extracellular conformation and current recovery we determined the effects of the C terminal NKVYS mutation, that in addition to producing rapid desensitization also required longer for recovery (Allsopp et al., 2013). The recovery rate of the fluorescence change of P2X1 K190C-2(NVKYS) mutant on washout of ATP was equivalent to that of the K190C mutant (Figure 5B). After a 30 second wash the change in fluorescence had recovered by 36.2 ± 1.8 % and a second application of ATP reduced fluorescence to the same level as the first application demonstrating an equivalent level of conformational change in the extracellular loop. However in contrast to the recovery in fluorescence the peak current only recovered to 9.7 ± 1.2 % of the initial peak response ($p<0.0001$ vs. K190C 5 minute and 30s washout). Therefore a mutation that prolongs the recovery of current to subsequent ATP application had no effect on the time-course of conformational recovery in the extracellular loop, or the ability of ATP to evoke the same overall conformational change, however the current evoked is considerably reduced reflecting incomplete recovery from desensitization.

These results highlight that the recovery of P2X1 receptor current to repeated applications of ATP is not dependent on conformational recovery of the extracellular domain and suggest that the intracellular regions (incorporating the NVKYS mutation) play a dominant role in regulation of recovery from desensitization independent of conformational changes in the extracellular loop.

Discussion.

Changes in MTS-TAMRA fluorescence were used to record real time conformational rearrangements in the head, left flipper and lower body regions, corresponding to the binding/opening of the hP2X1 receptor channel and the desensitized state. The initial change in fluorescence was consistent with structures demonstrating the agonist induced closure of the ligand binding pocket underlying channel activation (Hattori and Gouaux, 2012; Lorinczi et al., 2012). The secondary

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changes in the conformation, time-locked with desensitization, are consistent with previous observations in the cysteine rich head region of the rP2X1 receptor (Lorinczi et al., 2012), and these are now extended to show that desensitization also results in co-incident movements in the lower body and left flipper. Work on point mutants, splice variants and chimeric P2X receptors showed that it was the intracellular regions, and not the extracellular ligand binding loop, that were important for imparting the desensitizing phenotype (Allsopp and Evans, 2011; Boue-Grabot et al., 2000; Brandle et al., 1997; Simon et al., 1997; Werner et al., 1996). We now show for the first time that intracellular mutations have a direct dominant effect on conformational changes >70 Å away in the extracellular domain; with mutations that speed (NVKYS) or slow (D17C) the rate of desensitization having equivalent effects on the time-course of secondary conformational changes in the extracellular loop (Figure 3). This suggests that following channel opening the intracellular regions induce desensitization and coupling through the transmembrane (TM) domains to drive the concomitant secondary conformational changes in the extracellular ligand binding region.

Agonist induced fluorescence changes at K190C (lower body) reversed on washout for the hP2X1 receptor. For the fully desensitized receptor this was a slow process with a time constant of ~ 73s. Equivalent recovery (data not shown) was also seen at cysteine mutants in the head (K138C) and left flipper (N284C) regions suggesting a co-ordinated structural re-arrangement around the ligand binding pocket on agonist washout. The conformational recovery of hP2X1 receptors in the current study contrasts to the previous report by Nicke's group on rP2X1 receptors where no recovery of fluorescence was reported in the cysteine rich head region in the 50s period studied (Lorinczi et al., 2012). This reflects the greater washout time between ATP applications required to evoke reproducible currents between the rat (>20 mins) and human (~ 5mins) forms of the P2X1 receptor. Further support for distinct conformations associated with the open and desensitized receptor comes from analysis of the time for fluorescence recovery on ATP washout. For short pulses of ATP where more of the channels were in the open state a faster component of recovery (~ 5 s time constant) was recorded. This is similar to that shown for the cysteine rich head region of the rat P2X2 receptor (Lorinczi et al., 2012), but considerably slower than conformational recovery in the Glycine receptor (Supplementary Figure 2)(Pless and Lynch, 2009). The speed of conformational recovery of the

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hP2X1 receptor from the open state is likely to reflect a combination of the mean open time of the channel and agonist unbinding.

The intracellular regions of P2X receptors influence not only channel time-course but also the speed of recovery from desensitization (e.g. (Boue-Grabot et al., 2000; Brandle et al., 1997; Simon et al., 1997)). Analysis of the recovery from desensitization at P2X1 receptors has to date been difficult to study as it corresponds to a non-conducting/electrophysiologically silent state and has relied on indirect measurements characterizing the re-appearance of channel activity. The VCF approach allows for the first time recovery of electrically silent conformational changes in the extracellular loop to be correlated with recovery of P2X1 receptor currents to agonist application (however whether conformational changes in the extracellular loop that do not result in a change in fluorescence are required for recovery cannot be determined). Our data show that following 30 s agonist washout ATP evoked P2X1 receptor currents recovered to 75% of control response in contrast the conformational change showed only ~40% recovery. This highlights that recovery of current does not require full conformational rearrangement in the extracellular loop. This is further supported by the carboxyl terminal mutant K190C-2(NVKYS) P2X1 receptor that had no effect on conformational recovery following ATP washout (~ 40% at 30 s equivalent to K190C), however currents recovered by only ~10% to a second application of agonist. These results provide two new insights into desensitization and recovery. Firstly, the rate of conformational rearrangement in the extracellular loop is not the limiting factor in determining current recovery, and secondly that the intracellular carboxyl terminal of the receptor plays an important role in channel recovery that is independent of changes in the extracellular loop.

The crystal structure of the zfP2X4 receptor highlighted how agonist binding leads to conformational change around the ATP binding pocket that is transmitted through the body region of the receptor to the enlargement of the lateral portal in the extracellular domain and opening of the channel (Hattori and Gouaux, 2012). VCF (this study)(Lorinczi et al., 2012) and biochemical studies show that for P2X1 receptors there is an additional conformational change around the ATP binding pocket (Roberts et al., 2012). However it is unclear what happens to close the ion conducting pathway in the continued presence of ligand (desensitization). It is hard to visualise, given the

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coupling of the agonist binding region to the outer vestibule and TMs, how the further movement around the closed agonist binding pocket associated with desensitization would lead to a movement of the lateral portal or TM regions towards a more resting closed state. This is supported by cryo-electron microscopy studies that showed the increase in the lateral portal associated with channel activation at agonist bound purified desensitizing hP2X1 receptors (Roberts et al., 2012). In addition P2X2 and P2X3 receptor channels with cysteine mutants at the extracellular ends of TMs can be directly activated in the absence of agonist by either lipophilic MTS compounds (Rothwell et al., 2014), or photoisomerization of cysteine reactive compounds (Browne et al., 2014; Lemoine et al., 2013), that induce movement/separation of the ends of the TMs equivalent to that driven by agonist binding. Interestingly, the MTS reagent modification of P2X2 receptors, and photoactivation of P2X3 receptors, induced currents that desensitized; i.e. the channels closed even though the extracellular portions of the TMs were expected to be splayed (that would lead to opening of the channel gate). This suggests an additional conformational change in the TMs/ionic conduction pathway is required to mediate desensitization; i.e a separate desensitization gate.

A two gate model with distinct activation and desensitization gates has been suggested for cysteine loop ligand gated ion channels (Auerbach and Akk, 1998; Keramidas and Lynch, 2013). One possibility for P2X receptors is that the agonist-induced movement of the TMs triggers a conformational change in the intracellular regions of the receptor that blocks ionic permeation through the channel. The intracellular domains of the zebrafish P2X4 receptor were truncated to aid crystallization so there is no structural information on the intracellular regions (Hattori and Gouaux, 2012; Kawate et al., 2009). The results from the present work show for the first time that the intracellular regions can control recovery from desensitization independently of conformational changes associated with the extracellular domain suggesting that they regulate a distinct desensitization gate. Previous studies have demonstrated agonist evoked movement at the ends of the intracellular domains of P2X receptors (Chaumont and Khakh, 2008; Fisher et al., 2004), and re-arrangement of the intracellular region of hP2X1 receptors on agonist binding (Roberts et al., 2012). In addition, MTS modification of cysteine mutants in the amino terminus of P2X1 and P2X2 receptors inhibits ATP evoked responses (Jiang et al., 2001; Wen and Evans, 2009) suggesting this

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region contributes to gating/ion permeation. Taking these lines of evidence together we speculate that desensitization results from the closure of an intracellular vestibule formed from the interactions of the amino and carboxyl termini and that relaxation of this (that is delayed in the NVKYS mutant) is required for recovery from desensitization.

In summary the VCF technique has allowed measurement of the conformational changes associated with desensitization and recovery of ATP gated P2X1 receptors. These studies support the importance of the intracellular domains in regulation of P2X receptor channel gating and show for the first time that recovery from desensitization is not dependent on conformational changes in the extracellular ligand binding domain. This provides new insight into channel closure/desensitization in this distinct class of ligand-gated ion channels.

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Authorship contributions.

Participated in research design: Fryatt, A.G. and Evans R.J.

Conducted experiments and performed data analysis: Fryatt, A.G.

Wrote the manuscript: Fryatt, A.G. and Evans R.J.

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Figure legends.

Figure 1. Position, background fluorescence and labelling accessibility of cysteine point mutants. (A) hP2X1 homology model (Roberts et al. 2012) highlighting the positions of the single cysteine point mutants tested along the subunit interface (blue and red spheres indicate that the residues are on different subunits). (B) Histogram of the background fluorescence measurement (voltage output of the PMT) of MTS-TAMRA incubated oocytes expressing WT or cysteine point mutant P2X1 receptors (n=10 oocytes). (C) SDS-PAGE electrophoresis of purified P2X1 receptors labelled with Cy5 NHS ester (labels free cell surface amine groups and is a measure of surface expression, green) and MTS-TAMRA (labels free thiol groups, red) shows that all receptors were expressed at the surface of the oocytes and that only the introduced cysteine residues were accessible for MTS-TAMRA (n=4). (D) Quantification of MTS-TAMRA labelling in (C) expressed relative to Cy5 band intensity. (E) Average peak current to ATP (100 μM) at WT and mutant P2X1 receptors for unlabelled (open bars) and MTS-TAMRA treated (red bars) oocytes (n=12-16 oocytes).

Figure 2. VCF reports changes in fluorescence at three P2X1 receptor cysteine point mutants labelled with MTS-TAMRA. (A) Example control VCF traces from oocytes expressing (i) WT hP2X1 receptors and (ii) the L127C mutant glycine receptor (100μM ATP, 30mM glycine, indicated by bar, black trace=current recording, red trace=fluorescence). ATP evoked current was recorded from WT hP2X1 receptors with no change in fluorescence while at the L127C Glycine receptor glycine evoked currents and a large change in fluorescence (glycine evoked an increase in fluorescence but the trace has been inverted to show the same rise-time as the current). (B) P2X1 E181C expressing oocytes showed ATP evoked current but no detectible change in fluorescence. Decreases in fluorescence were detected from oocytes expressing (C) N284C and (D) K190C which were sustained during ATP application. The inset panels show the distinct time course of fluorescence change compared to the peak current. (E) Average change in fluorescence for the P2X1 receptor point mutants with only K138C, N284C and K190C showing significant decrease in fluorescence (n=10 oocytes). (F) Analysis of the percentage of the maximum change in fluorescence at peak current

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(%Fmax at Imax) shows that change in fluorescence is slower than the current generation for P2X1 receptor point mutants compared to Glycine receptor L127C. (G) Concentration-response curves for ATP evoked currents (black) and changes in fluorescence (red) at K190C mutant P2X1 receptors (n=4). Significant differences indicated as ** ($P \leq 0.01$) and **** ($P \leq 0.0001$).

Figure 3. Altering P2X1 receptor properties changes fluorescence time-course. (A) ATP (100μM, indicated by bar) has no effect on current or fluorescence at the P2X1 K190C-K68A mutant. (B) Example VCF recording from P2X1 K190C-D17C, a mutation in the amino terminus that shows slower current desensitization than P2X1 K190C. The fluorescence decrease shows two phases, which when rescaled can be superimposed (C) with the time-course of the rise (red) and decay (yellow, trace inverted) of the ATP current. (D) Example VCF recording from the mutant incorporating K190C and NKVYS (residues 360-364, P2X1-2(NKVYS)) in the carboxyl terminus which shows rapid desensitisation. (E) Histogram of the %Fmax at Imax shows that ~40% of the total fluorescence change occurs with the peak current for the K190C mutants (n=16 oocytes).

Figure 4. Distinct state dependent kinetics of P2X1 K190C fluorescence recovery. (A) Current and fluorescence changes at the P2X1 K190C mutant with 30s application of ATP (100μM, indicated by bar), showing the fluorescence returning to baseline levels following agonist wash out. When the application duration was reduced to 1s (B) the rate of recovery was initially faster, compared to the 30s application.. (C) Normalised fluorescence recovery on agonist washout taken from the end of the ATP application shows the faster initial fluorescence recovery for 1s ATP application and slower secondary recovery similar to that following 30s application of ATP to K190C mutant P2X1 receptors (n=6-9 oocytes). (D) Histogram showing the ATP evoked current remaining at the end of the agonist application for either 1s or 30s application (expressed as % of peak current) (n=6-9 oocytes). Significant differences indicated as ** ($P \leq 0.01$) and **** ($P \leq 0.0001$).

Figure 5. Differential recovery of fluorescence and channel activity from desensitization. (A) Example VCF recording from oocytes expressing K190C P2X1 receptors to two 30s ATP

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applications (100μM, indicated by bar) with 30s washout between. A sustained decrease in fluorescence was observed during the initial application of agonist that partially recovered during washout. The second application of ATP evoked a slightly reduced current and a decrease in fluorescence to a similar level to the initial application. (B) In comparison to K190C the P2X1 K190C-2(NKVYS) mutant showed faster desensitization in response to ATP but the same rate of fluorescence recovery on washout. The second application of ATP evoked a similar decrease in the level of fluorescence compared to the initial application but the current only recovered by ~10%. (C) Graph showing the percentage of the initial peak current generated during the second ATP application following 30s wash, and the level to which the fluorescence recovered before the second application of agonist for P2X1 K190C and K190C-2(NKVYS) receptors (n=10). Expanded time scale showing consecutive ATP applications with 5 minute wash between applications from the same oocyte shows the rate of fluorescence decrease was reproducible (scales normalized to compare time courses) (D). (E) When a second application of ATP was given after a 30 s wash period the fluorescence change had a faster time to peak similar to the rise-time of the second current (scales normalized to compare time courses). (F) Average %Fmax at Imax for the first and second responses with 5 minute and 30s washout periods showing the more rapid change in fluorescence when the interval between agonist applications was reduced. Significant differences indicated as *** ($P \leq 0.001$) and **** ($P \leq 0.0001$).

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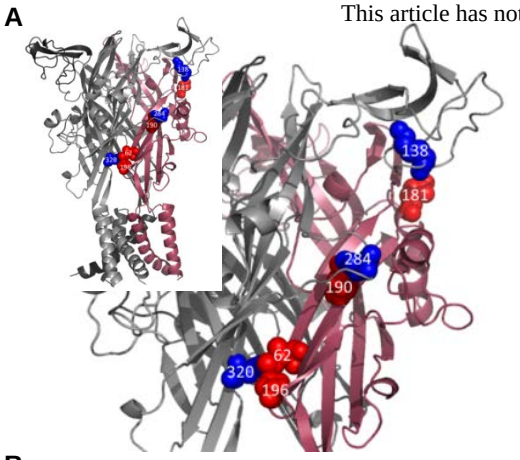
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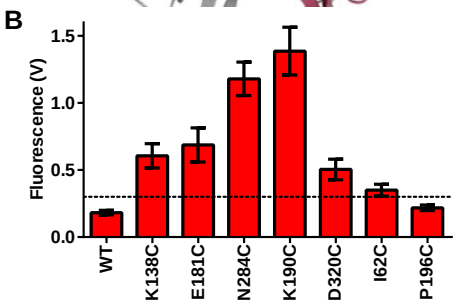
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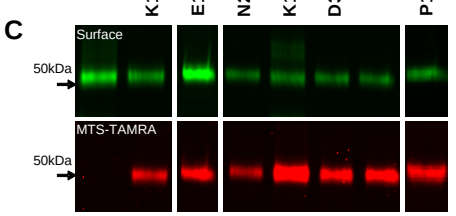
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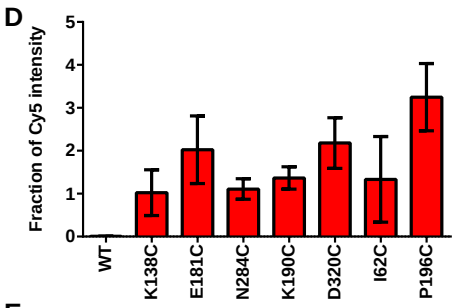
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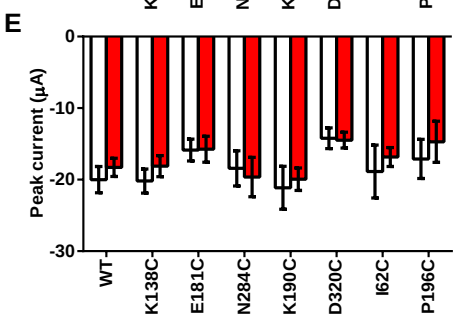
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Figure 1

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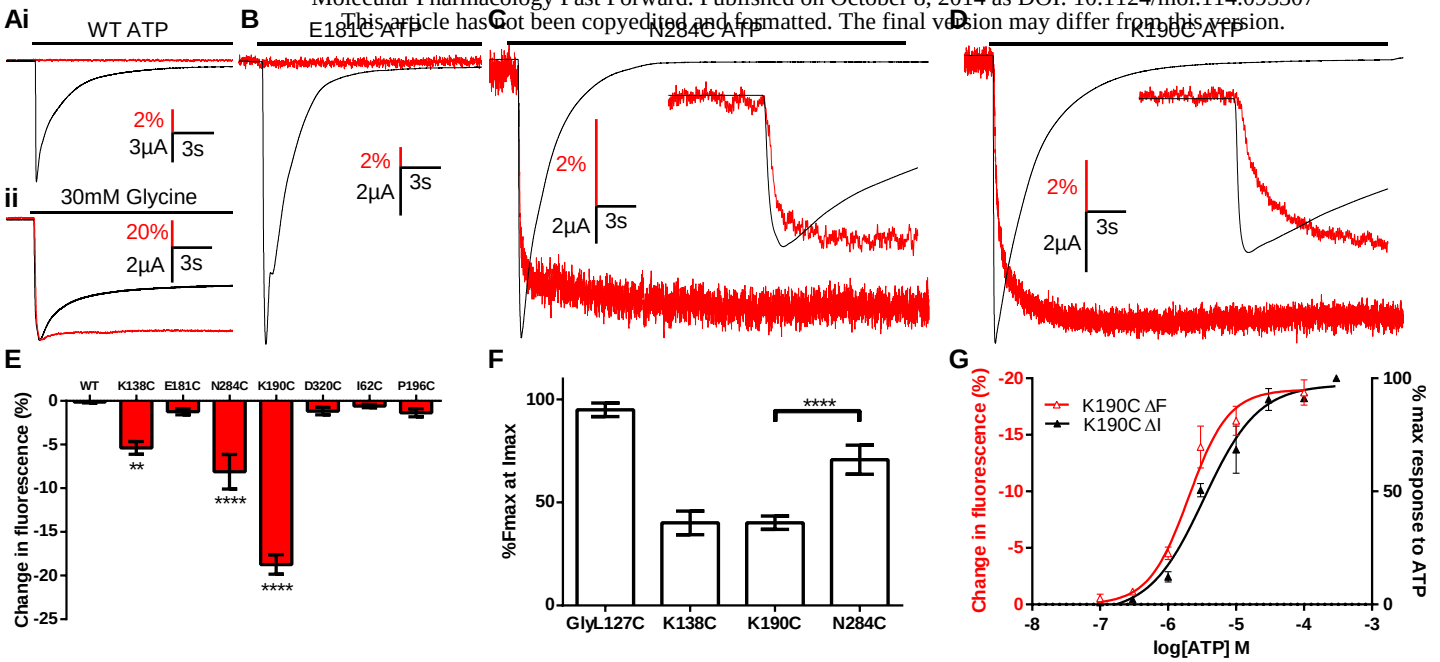

Figure 2

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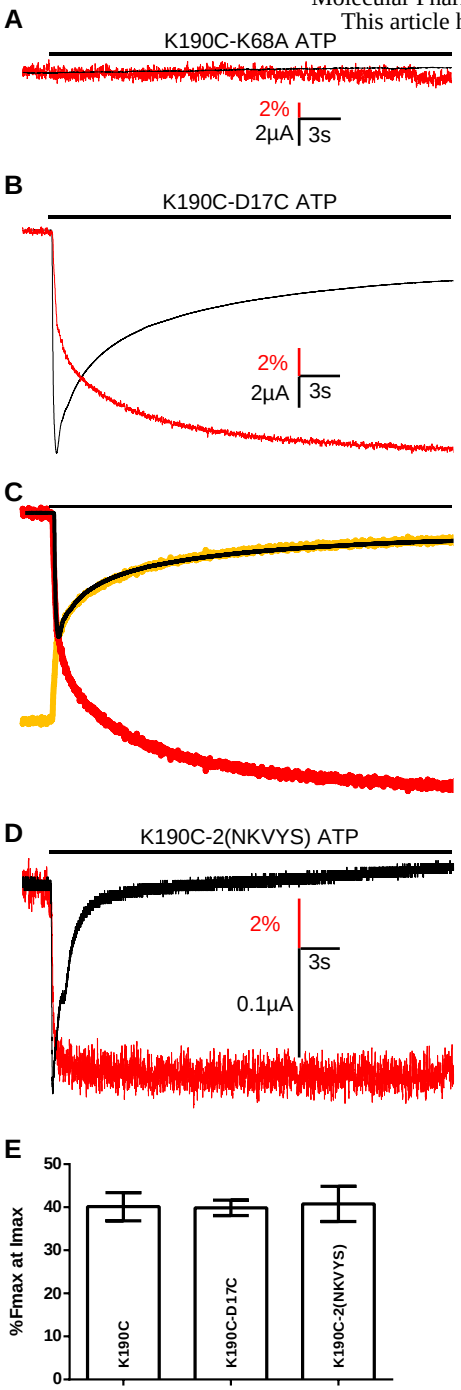

Figure 3

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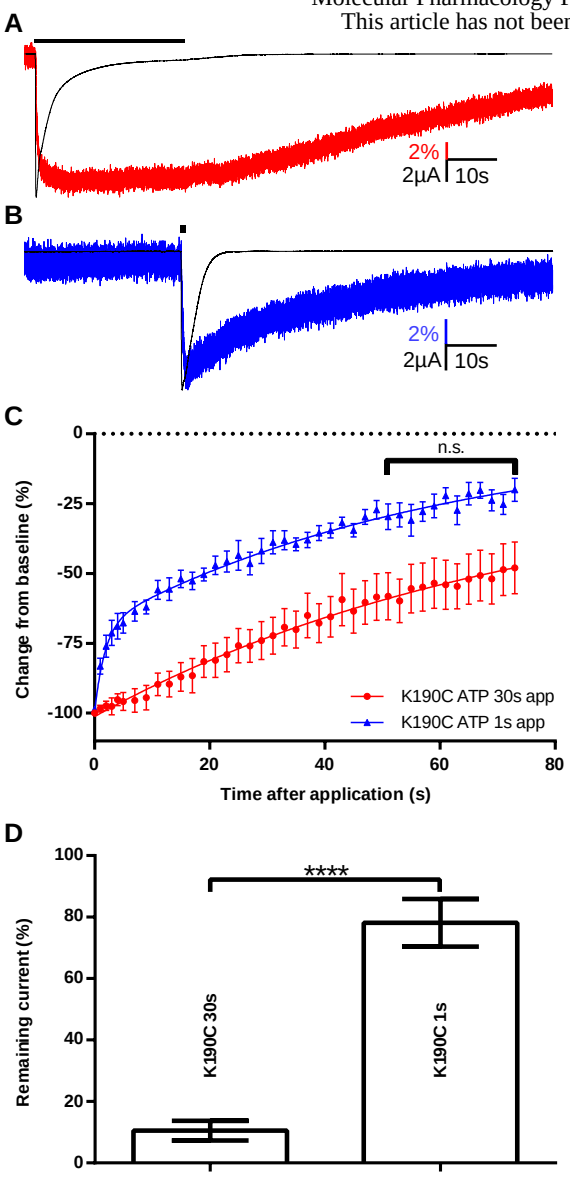

Figure 4

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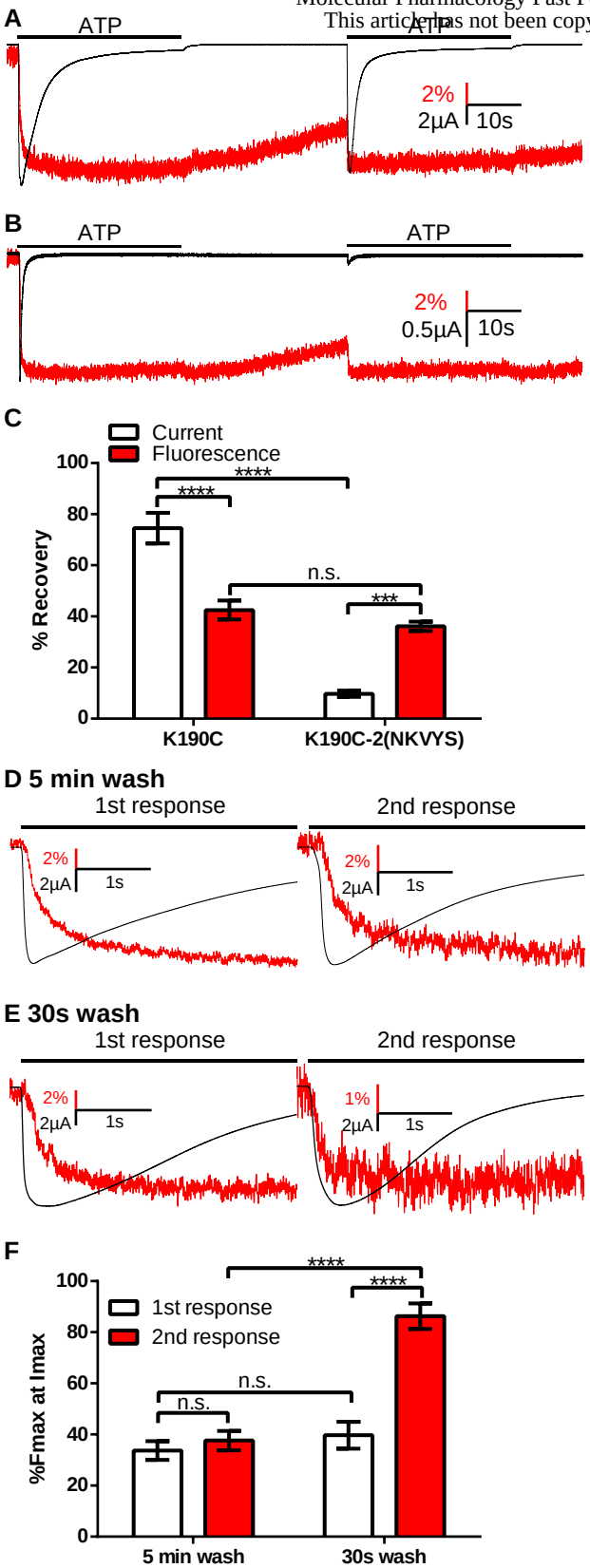

Figure 5