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Effect of chaperone-adhesin complex on plug release by the PapC usher

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Abstract

The P pilus of uropathogenic *Escherichia coli* is a multi-subunit fiber assembled at the outer membrane in a defined sequence by a chaperone/usher secretion system, comprising a periplasmic chaperone and a beta-barrel outer membrane protein, the PapC usher. To gain insight into the pilus biogenesis mechanism, we used patch clamp electrophysiology to investigate the effect of the initiating adhesin subunit, as it is delivered to PapC in a complex with the chaperone. We show that the chaperone-adhesin complex facilitates opening of the PapC pore and appears to engage within the PapC lumen, in agreement with prior biochemical and structural data.

Key terms

E. coli; secretion; patch clamp; P pilus; outer membrane

1. Introduction

Type 1 and P pili (fimbriae) are virulence factors assembled at the cell surface of uropathogenic *E. coli* to provide attachment to the host urinary tract epithelium during colonization [1]. Specific chaperone/usher secretion systems are used for the biogenesis of these appendages [2–4]. The pili are organized in two parts: a proximal rod anchored to the outer membrane and made up of over one thousand identical subunits, and a distal tip fibrillum composed of different subunits, with the most distal subunit serving as the adhesin. All pilus subunits are first synthesized in the cytoplasm and exported through the plasma membrane via the general secretory pathway. In the periplasm, dedicated chaperone proteins bind to the subunits to ensure their proper folding and delivery to the outer membrane usher for pilus assembly and translocation to the cell surface. The association of the chaperone with the subunit involves the complementation of the incomplete Ig-like fold of the pilus subunit by a β -strand provided by the chaperone, a mechanism termed donor strand

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complementation (DSC) [5, 6]. The usher effectively catalyzes the release of the subunit from the chaperone and its polymerization into the growing fiber, by promoting the substitution of the strand contributed by the chaperone for a N-terminal extension donated by the neighboring subunit in the fiber. This process is termed donor strand exchange (DSE) [7, 8]. The pilus is built from top to bottom, with the first incorporated subunit being the tip adhesin. The proper sequence of subunit incorporation in the pilus is ensured by differential binding affinities for the usher, DSE rates between specific subunits, and the relative subunit concentrations [9].

The usher is a twin β -barrel protein embedded in the outer membrane, with an N-terminal and two C-terminal globular domains facing the periplasmic side [3]. Although the PapC usher is an oligomer of identical β -barrels, cryo-electron microscopy imaging revealed that a single pilus emerges through a single β -barrel only [10], and the contribution of the other non-translocating subunit to the overall functioning of the usher has been debated. Recent evidence suggests that the non-translocating protomer of the oligomer, and perhaps the protomers of neighboring ushers as well, contribute to pilus biogenesis, most likely by acting as additional recruiting platforms of chaperone-subunit complexes and perhaps also through modulating usher-usher interactions [11, 12]. Each kidney-shaped usher β -barrel contains 24 β -strands (Figure 1), making it one of the largest outer membrane β -barrel known to date, with luminal dimensions on the order of 45 Å by 25 Å [10]. These dimensions are large enough to allow the passage of the growing pilus, as shown by X-ray crystallography [13]. However, to prevent leakage of essential periplasmic proteins and entry of large noxious compounds, the lumens of the β -barrels are occupied by a plug domain (PD) in the inactive, non-secreting form of the usher [10]. When the usher is actively engaged in pilus secretion, the PD swings towards the periplasmic side, to a position wherein it contacts with the N-terminal globular domain [13]. The binding of the initiating chaperone-adhesin complex to the N-terminal domain triggers a conformational change leading to plug release and subsequent entry of the tip subunit into the pore. Recent experiments have identified clusters of residues involved in allosteric propagation of intramolecular changes leading to plug release, as well as two electrostatic networks of arginine residues that appear to control stabilization of the PD in the lumen of the inactive usher [14, 15] (Figure 1).

Our prior electrophysiological studies of the PapC usher demonstrated that the usher is indeed gated shut by the PD [16]: in a mutant lacking the PD, each β -barrel monomer undergoes large opening transitions of 3.5 nS or 600 pS conductance, in 1 M or 150 mM KCl, respectively [15, 16]. In wildtype (WT), very brief openings of such large size are occasionally seen, and more so as the voltage is increased [14, 16], indicating that the PD can spontaneously displace from the β -barrel lumen. The application of a membrane potential above a threshold of ~ 130 mV leads to the simultaneous displacement of both plugs from the twin β -barrels of PapC followed by a typical relaxation to lower levels of conductance, leading to a great increase in current for a duration of several seconds [15]. In the intervals between PD displacements events, the channel maintains a predominantly closed state, with transient openings of small size (50–600 pS in 150 mM KCl), which have been attributed to small water-filled conduits at the lumen-PD interface [16]. The mobility of

the plug within the lumen, and/or the partial occlusion of these conduits by dynamic N- and C-terminal domains might be responsible for these transitions of small-conductance.

Here we have used patch clamp electrophysiology to analyze the behavior of the PapC usher before and after it is exposed to the complex of the chaperone PapD and adhesin PapG (hereafter named the PapDG complex). We present data indicating that the PapDG complex facilitates displacement of the plug domain, and appears to engage in one of the barrel lumens.

2. Materials and Methods

Strains, media and chemicals

The PapC usher was expressed in the multi-porin *E. coli* mutant BL21(DE3)Omp8 for purification [17]. Bacteria were grown in LB medium containing 100 µg/ml ampicillin at 37°C with aeration, and protein expression was induced at an OD₆₀₀ of 0.6 for 2–2.5 h by addition of 0.1 % L-arabinose. The detergents used during protein purification were dodecyl-maltopyranoside (DDM) and lauryl(dimethyl)amine oxide (LDAO) and were obtained from Anatrace. Thrombin was from Novagen. Proteoliposomes were prepared for patch clamp experiments by reconstituting purified PapC with the soybean phospholipids preparation “asolectin” (L-α-Phosphatidylcholine Type II-S) from Sigma, and BioBeads (Biorad) were used for detergent removal. All other chemicals were from Sigma.

Protein Purification

The WT and mutant PapC proteins were expressed from plasmid pDG2, encoding PapC with a thrombin cleavage site and hexahistidine tag (His-tag) added to the C terminus [18]. The mutant PapC contained quadruple alanine mutations at two arginine pairs (R237A - R305A and R256A - R332A) and is subsequently referred to as the “R-pairs mutant”. Construction of the mutant has been described [15]. Purification of the PapC proteins was performed as described [12, 15]. Purification of the PapDG complex was accomplished according to published protocols [19].

Patch-Clamp Electrophysiology

Buffers used in patch clamp experiments were Buffer A (150 mM KCl, 10 µM CaCl₂, 0.1 mM EDTA, 5 mM HEPES, pH 7.2) and Buffer B (Buffer A + 20 mM MgCl₂). All buffers were filtered prior use through a 0.2 µm filter. Patch pipettes of ~10 MΩ were pulled with a Sutter Instruments puller and filled with Buffer A. Purified PapC in LDAO was reconstituted into asolectin liposomes at protein:lipid ratios of 1:2000 to 1:3000 (w:w), as described [20]. Multilamellar proteoliposomes were placed in the patch clamp chamber (bath) filled with a Mg²⁺-containing buffer (Buffer B) to promote the appearance of unilamellar blisters suitable for patch clamp [21]. Once a gigaohm seal was formed on the blister membrane, the patch was excised by brief air exposure and Buffer B replaced by magnesium-free Buffer A, thus resulting in symmetric buffer solutions on each side of the membrane. Currents were recorded under voltage clamp, with an Axopatch-1D amplifier (Axon Instruments) and a CV-4 headstage. Voltages are given as pipette voltages (bath is ground). The currents were filtered at 1KHz, digitized at 100 µs sampling intervals (ITC-18, Instrutech), and stored on a

computer using the Acquire software (Bruxton). No channel activity is observed in control experiments on protein-free liposomes. pClamp (Axon Instruments) was used for data visualization and analysis.

A typical experiment included 10-min long recordings at voltages starting at -70 mV and increased (made more negative) by 10 mV until the threshold for PD displacement was reached and noted. After this occurred, the voltage was returned to -70 mV to ensure the channel had closed, and a second attempt at plug displacement was initiated again. Subsequently, recordings in the presence of PapDG were obtained on the same patch in a similar fashion, in order to obtain the threshold voltage for plug displacement in the presence of PapDG. For this, an aliquot of the PapDG complex stock solution was introduced in the bath such that the final concentration was 325 nM. Typically, several plug displacement events were attempted in the presence of PapDG, but this varied between experiments depending on the robustness of the patch. Finally, the PapDG complex was perfused out with fresh Buffer A, and the same protocol of 10-min long recordings in 10 -mV voltage increments was implemented to assess the reversibility of the PapDG effect. Thus, a successful experiment typically lasted over an hour.

3. Results and Discussion

We used patch clamp electrophysiology to monitor the release of the plug domain from the PapC usher in real time. As shown previously [14–16], a reconstituted PapC usher dimer displays a characteristic kinetic signature in electrophysiological experiments, whereby the protein undergoes spontaneous transient openings of small sizes at all voltages, but is also capable of releasing the plug domain upon application of a threshold voltage on the order of -130 mV. Plug displacement events are witnessed as a rapid current increase to a level of large amplitude corresponding to the dimeric conductance of ~ 1200 pS, followed by a relaxation to lower conductance states with a high level of dynamic current fluctuations (Figure 2A). This relaxation is due in part to the re-entry of the plug domain inside the barrel, as well as motions of other domains (likely the N- and C-terminal domains) since it is also observed in a plugless mutant [15]. Once the channel resumes a mostly closed state with transient small openings, it is possible to re-trigger a plug displacement event by applying a transmembrane voltage at or close to the threshold voltage. This can be repeated multiple times. We have previously shown that a PapC construct with quadruple alanine mutations at two specific pairs of arginine (R237–R305 and R256–R332) has a lower threshold voltage for plug displacement [15]. This suggests that the value of the threshold voltage can be used as a read-out of the propensity of the channel to release the plug from the β -barrel domain.

The current model of P pilus biogenesis is based on the top-down assembly of the various pilus subunits, with PapG, found at the very tip of the pilus, being the first one to interact with the usher due to the uniquely high binding affinity of the PapDG complex for the usher [22, 23]. The PapDG complex is thought to initiate the release of the plug to the periplasmic side to allow for the subsequent threading of the growing pilus through the barrel [9]. Therefore, we set out to investigate the effect of a PapDG complex on PapC usher activity. We anticipated that PapDG might trigger the opening of the channel via plug displacement.

Therefore, we determined the threshold voltage for plug displacement of a single WT PapC dimer prior and after bath perfusion with 325 nM of PapDG (a value ~ 5 times higher than the K_d for PapDG binding to PapC [22, 23]). We found that in the presence of PapDG the threshold voltage for plug displacement was about 30% lower than prior to application of the chaperone-subunit complex (Fig. 3A), indicating an increased propensity for plug displacement. The effect was sustained as long as PapDG remained in the bath. Consecutive re-displacements of the plug were observed at voltages that remained about 30% lower than in the absence of PapDG. However, the PapDG effect was reversible upon washing out of PapDG from the bath, when the threshold voltages recovered to values similar to prior to PapDG application. PapDG had no effect on the spontaneous opening transitions that occurred in-between plug displacement events. It also did not lead to noticeable changes in the kinetics of channel activity during the relaxation following plug displacement, although fine details might be masked by the great amount of current fluctuations during this event. Effects on conductance have been observed, though, and are described below.

Interestingly, when we performed the same experiments on a PapC usher with a quadruple alanine mutation at two arginine pairs shown to control plug displacement (R-pairs mutant: R237A- R256A-R305A- R332A) [15], the application of PapDG to the bath was without any effect on the threshold voltage (Fig. 3B). This mutant has an intrinsically lower threshold voltage than WT (-104 mV on average, as opposed to -134 mV for WT), and is deficient in pilus biogenesis [15]. The lack of effect confirms the involvement of these two pairs of arginines in the molecular mechanism underlying the plug displacement event, and substantiates the conclusion that PapDG does indeed promote plug release. Essentially, since the mutations have already enhanced the ability of the channel to release the plug, the application of PapDG does not further contribute to plug displacement in such a mutant. These results suggest that one mechanism by which PapDG promotes plug release in the WT usher is to weaken the repulsive interactions between the arginines of the pairs R237-R305 and R256-R332, as mimicked by the alanine mutations at these sites. In other words, the arginines may play a major role in mediating the effect of PapDG which ultimately triggers plug release, and thus are necessary for the productive interaction of PapDG with the usher. The R256-R332 pair is on the periplasmic side of the usher, close to the N-terminal domain where PapDG first binds. This pair is more influential in the plug release mechanism than the R237-R305 pair, which faces the extracellular side [15]. The proximity of the R256-R332 arginine pair to a bound PapDG might further enhance its involvement in mediating productive PapDG interactions with the usher. Although the R237-R305 pair is further removed from the PapDG binding site, we have identified clusters of residues along the interface of the plug, the β -barrel, and the $\beta 5-6$ hairpin that may mediate an allosteric propagation of conformational changes eventually weakening the repulsion between the arginines of the R237-R305 pair and further contributing to plug destabilization and release [14].

Although plug displacement events still occurred for WT ushers in the presence of PapDG, we observed that the conductance of such events was significantly reduced in those conditions (Fig. 2B and Fig. 4A). The effect was fully reversible upon PapDG washout. Interestingly, the reduced conductance appeared when the plug displacement was triggered not the 1st time, but the 2nd time in the presence of PapDG, and subsequently until PapDG

was washed out. It is possible that the reduced conductance is due to a reversible block of one of the usher β -barrels by a delivered PapG subunit. A possible scenario is that the first plug displacement event occurred in both monomers of the dimer, allowing PapG to start moving inside one of the open pores, as shown in structural studies [13]. Subsequent displacements would originate from the remaining non-occupied pore, with a conductance $\sim 60\%$ of the dimeric one. It is not surprising that the conductance of the remaining pore is not exactly half of the dimeric conductance, as we have seen that the monomeric conductances of the plugless PapC [16] and *Vibrio cholerae* OmpT porin [24], for example, are also not exactly the same for all the monomers of the respective oligomers. Since the effect is fully reversible, the PapG subunit must be able to dissociate from the usher pore upon wash-out of the PapDG complex. This is consistent with the measured dissociation rates of chaperone-adhesin complexes from the usher in the absence of other subunits required for pilus assembly [23].

Since the reduced conductance is not observed in the R-pairs mutant (Fig. 2C and Fig. 4B), we infer that the mere displacement of the plug domain, which also occurs in the R-pairs mutant albeit in a non-PapDG dependent way, is not sufficient for PapG to move inside the pore and block it. This entry of PapG in the pore must require a productive association of PapDG with the usher that leads to a PapDG initiated plug release, rather than one that occurs simply because the PD has been released. This observation is consistent with the complete defect in pilus biogenesis of the R-pair mutant that was reported previously [15], as well as of plugless PapC mutant (Henderson & Thanassi, unpublished).

In our experimental setting, the displacement of the PD is triggered by a high membrane potential. We do not suggest that this is the case *in vivo*, since it is commonly believed that the outer membrane does not sustain a membrane potential. We propose that the *in vitro* membrane potential triggers conformational changes similar to those initiated *in vivo* by binding of the chaperone-adhesin complex to the usher. Indeed, our observation that the chaperone-adhesin complex has reduced the threshold voltage for plug displacement, and thus facilitates the release of the PD is in agreement with this proposal. Even in the presence of PapDG complex, a membrane potential was still needed, however. This may be due to concentration-dependent effects, or the absence of the complete physiologically relevant environment, such as the presence of lipopolysaccharides or of other pilus subunits. During pilus biogenesis in bacteria, the presence of chaperone-subunit complexes in addition to PapDG will affect the progression of engagement of the usher and pilus polymerization. Therefore, observing the return of the PD into the channel would be unlikely under conditions replicating the composition and concentrations of periplasmic chaperone-subunit complexes *in vivo*. There is evidence, however, that the PD might spontaneously come in and out of the channel *in vivo*, even in the absence of a polymerizing pilus. We have identified several PapC mutants (R305A, V327A, T331A, and F438A) that display an increased propensity at spontaneous plug displacement [14, 25]. These events are short (on the order of a few seconds), but occur more frequently than in WT PapC. As a result, cells expressing these mutated ushers have an increased sensitivity to the antibiotics erythromycin and/or vancomycin, indicating a leaky outer membrane phenotype [14, 25]. The destabilized PD in these mutant ushers tends to displace spontaneously more frequently than in WT, thus repeatedly opening up a large channel for entry of antibiotic molecules.

In conclusion, the results presented here suggest that we captured the productive associations of PapDG with PapC, and the subsequent entry of the adhesin subunit into one of the barrel lumens. The results also imply that the orientation of the usher in the patch is with the periplasmic side facing the bath, where interactions can occur between the PapDG complex and the N-terminal domain of PapC.

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References

1. Johnson JR. Virulence factors in *Escherichia coli* urinary tract infection. *Clinical microbiology reviews*. 1991; 4:80–128. [PubMed: 1672263]
2. Busch A, Waksman G. Chaperone-usher pathways: diversity and pilus assembly mechanism. *Philosophical transactions of the Royal Society of London Series B, Biological sciences*. 2012; 367:1112–22. [PubMed: 22411982]
3. Geibel S, Waksman G. The molecular dissection of the chaperone-usher pathway. *Biochimica et biophysica acta*. 2014; 1843:1559–67. [PubMed: 24140205]
4. Thanassi DG, Bliska JB, Christie PJ. Surface organelles assembled by secretion systems of Gram-negative bacteria: diversity in structure and function. *FEMS microbiology reviews*. 2012; 36:1046–82. [PubMed: 22545799]
5. Sauer FG, Futterer K, Pinkner JS, Dodson KW, Hultgren SJ, Waksman G. Structural basis of chaperone function and pilus biogenesis. *Science*. 1999; 285:1058–61. [PubMed: 10446050]
6. Choudhury D, Thompson A, Stojanoff V, Langermann S, Pinkner J, Hultgren SJ, Knight SD. X-ray structure of the FimC-FimH chaperone-adhesin complex from uropathogenic *Escherichia coli*. *Science*. 1999; 285:1061–6. [PubMed: 10446051]
7. Sauer FG, Pinkner JS, Waksman G, Hultgren SJ. Chaperone priming of pilus subunits facilitates a topological transition that drives fiber formation. *Cell*. 2002; 111:543–51. [PubMed: 12437927]
8. Zavialov AV, Berglund J, Pudney AF, Fooks LJ, Ibrahim TM, MacIntyre S, Knight SD. Structure and biogenesis of the capsular F1 antigen from *Yersinia pestis*: preserved folding energy drives fiber formation. *Cell*. 2003; 113:587–96. [PubMed: 12787500]
9. Busch A, Phan G, Waksman G. Molecular mechanism of bacterial type 1 and P pili assembly. *Philosophical transactions Series A, Mathematical, physical, and engineering sciences*. 2015:373.
10. Remaut H, Tang C, Henderson NS, Pinkner JS, Wang T, Hultgren SJ, Thanassi DG, Waksman G, Li H. Fiber formation across the bacterial outer membrane by the chaperone/usher pathway. *Cell*. 2008; 133:640–52. [PubMed: 18485872]
11. So SS, Thanassi DG. Analysis of the requirements for pilus biogenesis at the outer membrane usher and the function of the usher C-terminus. *Molecular microbiology*. 2006; 60:364–75. [PubMed: 16573686]
12. Werneburg GT, Henderson NS, Portnoy EB, Sarowar S, Hultgren SJ, Li H, Thanassi DG. The pilus usher controls protein interactions via domain masking and is functional as an oligomer. *Nature structural & molecular biology*. 2015; 22:540–6.
13. Phan G, Remaut H, Wang T, Allen WJ, Pirker KF, Lebedev A, Henderson NS, Geibel S, Volkan E, Yan J, Kunze MB, Pinkner JS, Ford B, Kay CW, Li H, Hultgren SJ, Thanassi DG, Waksman G. Crystal structure of the FimD usher bound to its cognate FimC-FimH substrate. *Nature*. 2011; 474:49–53. [PubMed: 21637253]
14. Farabella I, Pham T, Henderson NS, Geibel S, Phan G, Thanassi DG, Delcour AH, Waksman G, Topf M. Allosteric signalling in the outer membrane translocation domain of PapC usher. *eLife*. 2014:3.
15. Pham T, Henderson NS, Werneburg GT, Thanassi DG, Delcour AH. Electrostatic Networks Control Plug Stabilization in the PapC Usher. *Molecular Membrane Biology*. 2016 in press.

16. Mapingire OS, Henderson NS, Duret G, Thanassi DG, Delcour AH. Modulating effects of the plug, helix, and N- and C-terminal domains on channel properties of the PapC usher. *The Journal of biological chemistry*. 2009; 284:36324–33. [PubMed: 19850919]
17. Prilipov A, Phale PS, Van Gelder P, Rosenbusch JP, Koebnik R. Coupling site-directed mutagenesis with high-level expression: large scale production of mutant porins from *E. coli*. *FEMS microbiology letters*. 1998; 163:65–72. [PubMed: 9631547]
18. Li H, Qian L, Chen Z, Thibault D, Liu G, Liu T, Thanassi DG. The outer membrane usher forms a twin-pore secretion complex. *Journal of molecular biology*. 2004; 344:1397–407. [PubMed: 15561151]
19. Henderson NS, Thanassi DG. Purification of the outer membrane usher protein and periplasmic chaperone-subunit complexes from the P and type 1 pilus systems. *Methods in molecular biology*. 2013; 966:37–52. [PubMed: 23299727]
20. Simonet VC, Basle A, Klose KE, Delcour AH. The *Vibrio cholerae* porins OmpU and OmpT have distinct channel properties. *The Journal of biological chemistry*. 2003; 278:17539–45. [PubMed: 12606562]
21. Delcour AH, Martinac B, Adler J, Kung C. Modified reconstitution method used in patch-clamp studies of *Escherichia coli* ion channels. *Biophysical journal*. 1989; 56:631–6. [PubMed: 2477074]
22. Li Q, Ng TW, Dodson KW, So SS, Bayle KM, Pinkner JS, Scarlata S, Hultgren SJ, Thanassi DG. The differential affinity of the usher for chaperone-subunit complexes is required for assembly of complete pili. *Molecular microbiology*. 2010; 76:159–72. [PubMed: 20199591]
23. Saulino ET, Thanassi DG, Pinkner JS, Hultgren SJ. Ramifications of kinetic partitioning on usher-mediated pilus biogenesis. *The EMBO journal*. 1998; 17:2177–85. [PubMed: 9545231]
24. Duret G, Delcour AH. Deoxycholic acid blocks *Vibrio cholerae* OmpT but not OmpU porin. *The Journal of biological chemistry*. 2006; 281:19899–905. [PubMed: 16670088]
25. Volkan E, Kalas V, Pinkner JS, Dodson KW, Henderson NS, Pham T, Waksman G, Delcour AH, Thanassi DG, Hultgren SJ. Molecular basis of usher pore gating in *Escherichia coli* pilus biogenesis. *Proceedings of the National Academy of Sciences of the United States of America*. 2013; 110:20741–6. [PubMed: 24297893]

Figure 1. Molecular model of the PapC usher

A ribbon representation of the structure of a single monomeric translocation domain is rendered with the program Chimera from the PDB QV2I [10] and highlights different structural elements in the following colors: Gray – barrel; Purple – PD; Aqua – β 5–6 hairpin; Orange – α -helix. The arginine residues mutated to alanine in the R-pairs mutant are shown in a ball-and-sticks model. One electrostatic network involves two arginines located on the plug linkers (R232 and R256), and the other includes two arginines on the β 5–6 hairpin (R237) and the plug domain (R305). “EC” and “PERI” denote the extracellular and periplasmic sides, respectively.

Figure 2. Examples of plug displacement events

Patch-clamp recordings of PapC activity illustrate the sudden rapid increase in current corresponding to plug displacement in WT PapC (A and B) and the R-pairs mutant (C), in the absence (A) or the presence of 325 nM PapDG (B and C). Note that the PD is displaced only in a single monomer of WT PapC in the presence of PapDG (B) (4th measurement). The 300 msec segments denoted by the asterisk on the top trace of panels A and C are shown on an expanded time scale below the corresponding trace to better visualize the current level corresponding to the dimeric conductance. A characteristic feature of these events is the current relaxation with a high-level dynamic behavior, which eventually leads to the closure of the channel. Current levels corresponding to the closed channel, monomeric conductance and dimeric conductance are marked by C, M and D, respectively. Note that the traces are shown in conductance (pS) rather than current to allow for comparison between the recordings which were obtained at different voltages: -160 mV (panel A), -110 mV (panel B), -130 mV (panel C).

Figure 3. Effect of PapDG on threshold voltage for plug displacement

The threshold voltage for plug displacement was determined for WT PapC (A) and the R-pairs mutant (B). The graphs plot the ratio of this voltage in each condition to the threshold voltage in the initial condition prior to application of PapDG (PRE 1), with the PRE 1 voltage set to 100%. The dashed line marks the 100% level. Multiple measurements of the threshold voltage were performed, as detailed in Materials and Methods. For both graphs, the conditions were: 1st and 2nd measurements prior to PapDG application (PRE 1 and PRE 2); 1st to 5th measurement in the presence of 325 nM PapDG (DG 1 to DG 5); and 1st and 2nd measurement after perfusion of PapDG out of the bath (OUT 1 and OUT 2). The data points represent averages $\pm$ s.e.m. of 9 and 5 measurements for WT and R-pairs mutant, respectively. Note that the number of measurements taken into the average varies for each time point, because it was not always possible to maintain the patch for the whole series of measurements.

Figure 4. Effect of PapDG on conductance

The immediate peak conductance reached upon plug displacement was measured for WT PapC (A) and the R-pairs mutant (B). Multiple displacement events were triggered, as detailed in Materials and Methods. For both graphs, the conditions were: 1st and 2nd measurements prior to PapDG application (PRE 1 and PRE 2); 1st to 5th measurement in the presence of 325 nM PapDG (DG 1 to DG 5); and 1st and 2nd measurement after perfusion of PapDG out of the bath (OUT 1 and OUT 2). The data points represent averages $\pm$ s.e.m. of 7 and 5 measurements for WT and R-pairs mutant, respectively. Note that the number of measurements taken into the average varies for each time point, because it was not always possible to maintain the patch for the whole series of measurements.