The calcium-sensing receptor changes cell shape via a β-arrestin-1–ARNO–ARF6–ELMO protein network

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Summary

G-protein-coupled receptors (GPCRs) transduce the binding of extracellular stimuli into intracellular signalling cascades that can lead to morphological changes. Here, we demonstrate that stimulation of the calcium-sensing receptor (CaSR), a GPCR that promotes chemotaxis by detecting increases in extracellular calcium, triggers plasma membrane (PM) ruffling via a pathway that involves β-arrestin 1, Arf nucleotide binding site opener (ARNO), ADP-ribosylating factor 6 (ARF6) and engulfment and cell motility protein (ELMO). Expression of dominant negative β-arrestin 1 or its knockdown with siRNA impaired the CaSR-induced PM ruffling response. Expression of a catalytically inactive ARNO also reduced CaSR-induced PM ruffling. Furthermore, β-arrestin 1 co-immunoprecipitated with the CaSR and ARNO under resting conditions. Agonist treatment did not markedly alter β-arrestin 1 binding to the CaSR or to ARNO but it did elicit the translocation and colocalisation of the CaSR, β-arrestin 1 and ARNO to membrane protrusions. Furthermore, ARF6 and ELMO, two proteins known to couple ARNO to the cytoskeleton, were required for CaSR-dependent morphological changes and translocated to the PM ruffles. These data suggest that cells ruffle upon CaSR stimulation via a mechanism that involves translocation of β-arrestin 1 pre-assembled with the CaSR or ARNO, and that ELMO plays an essential role in this CaSR-signalling-induced cytoskeletal reorganisation.

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Introduction

The calcium-sensing receptor (CaSR) is a G-protein-coupled receptor (GPCR) that detects variations in the extracellular calcium concentration ($[Ca^{2+}]_o$) and plays a fundamental role in the maintenance of calcium homeostasis (Bouschet and Henley, 2005; Brown and MacLeod, 2001; Hofer and Brown, 2003). In addition, CaSR activation can lead to stem cells homing and chemotaxis (Adams et al., 2006; Godwin and Soltoff, 1997; Olszak et al., 2000). These locomotive events presumably depend on the ability of CaSR expressing cells to detect a gradient in environmental (extracellular) calcium and induce changes of shape that results in movement towards a calcium gradient.

Chemotaxis involves the dynamic formation and retraction of cytoplasmic protrusions enriched in polymerised filamentous actin (F-actin) at the cell leading edge (Small et al., 2002). This process, known as plasma membrane (PM) ruffling, is essential to locomotion (Ridley et al., 2003). Recent evidence has suggested that, in addition to their well-characterised involvement in GPCR desensitisation (Lefkowitz and Whalen, 2004; Lohse et al., 1990), β-arrestins play a key role in GPCR-mediated PM ruffling (Bhattacharya et al., 2002; Scott et al., 2006) and chemotaxis (Fong et al., 2002; Ge et al., 2003; Ge et al., 2004; Walker et al., 2003). However, the

protein partners coupling β-arrestin to cytoskeleton reorganisation remain to be determined.

ARNO (Arf nucleotide binding site opener, also called cytohesin 2) is a guanine nucleotide exchange factor (GEF) that activates GTP binding on the small G protein ARF6 (ADP-ribosylating factor 6). ARNO interacts with β-arrestins to regulate GPCR-desensitisation (Claing et al., 2001; Mukherjee et al., 2000). Furthermore, exogenous expression of ARNO in Madin-Darby canine kidney (MDCK) epithelial cells results in the formation of broad lamellipodia and cell migration (Santy and Casanova, 2001). These processes of morphological adaptation are impaired when an ARF6 point mutant defective in GTP binding (ARF6T27N) is co-expressed with ARNO (Santy and Casanova, 2001). It has also been reported recently that ARF6 couples to the cytoskeleton via ELMO (engulfment and cell motility protein) (Santy et al., 2005). Overall, these data suggest that an ARNO-ARF6-ELMO signalling module could connect extracellular signals, potentially those acting through GPCR and β-arrestin, to the cytoskeleton.

In this study we test this hypothesis and demonstrate that increasing $[Ca^{2+}]_o$ generates PM ruffles in HEK cells transiently or stably expressing the CaSR. We show that β-arrestin 1 and ARNO are crucial for this CaSR-induced

cytoskeletal reorganisation and that the two ARNO effector proteins ARF6 and ELMO are also required for CaSR-induced PM. These results therefore demonstrate for the first time the involvement of ARNO and its effector ELMO in GPCR-induced cytoskeletal reorganisation.

Results

### CaSR stimulation induces plasma membrane ruffling

We recently reported the characterisation of a CaSR tagged with the pH sensitive GFP molecule super ecliptic pHluorin (SEP). This construct named SEP-CaSR allowed us to track cell-surface expressed CaSR (Bouschet et al., 2005). One major advantage of tagging receptors with SEP is that it permits monitoring of the real-time dynamics of SEP-tagged receptors from or to the plasma membrane (PM) (Ashby et al., 2004; Yudowski et al., 2006). Our initial intention was therefore to determine the fate of SEP-CaSR expressed in HEK cells following agonist stimulation. Intriguingly, increasing the extracellular calcium concentration ($[Ca^{2+}]_o$) in cultures of cells transfected with SEP-CaSR resulted in intense

Fig. 1. (A) CaSR stimulation generates PM ruffling. HEK cells were transfected with SEP-CaSR. 48 hours post-transfection, cells were challenged (bottom) or not (top) with 5 mM $CaCl_2$ for 10 minutes, fixed and stained for SEP-CaSR (green) and F-actin (Alexa-Fluor-568–phalloidin, red). Colocalisation areas appear in yellow in the merge panels. Cells exhibiting PM ruffles are shown by empty arrowheads while cells that did not change shape are indicated by filled arrowheads. Bars, 10 μm. (B) Representative micrographs of CaSR-agonist-induced cytoskeleton reorganisation in SEP-CaSR-expressing HEK cells before (untreated) and after 5 mM $CaCl_2$ or 10 μM NPS R-467 stimulation for 10 minutes. Colocalisation of SEP-CaSR (green) and F-actin (red) appears in yellow in the merge panels. The right panels show fourfold magnified views of the boxed regions. Note the presence of SEP-CaSR at the edge of protrusions. The PM ruffles are shown by arrowheads. Bars, 5 μm. Pictures shown are representative of six independent experiments. (C) Quantification of the effects of CaSR agonists on PM ruffling of cells. These results are the mean±s.e.m. of three to six independent experiments. *$P \leq 0.001$ compared with controls. (D) Extracellular-calcium-mediated PM ruffling is CaSR-dependent. Representative confocal images of F-actin staining of wild-type (WT) or stably expressing CaSR HEK cells challenged with 5 mM $CaCl_2$ for 10 minutes, in the presence (+) or absence (–) of CaSR antagonist (NPS 89636 at 1 μM, pre-incubated for 10 minutes). Ruffling cells are indicated by arrowheads. Bars: 10 μm. (E) Quantification of cells exhibiting PM ruffling in the conditions described in (D). The results shown are the mean± s.e.m. of three independent experiments. *$P \leq 0.001$ compared with untreated. (F) Time course of CaSR-induced PM ruffling. The percentage of SEP-CaSR-expressing cells exhibiting PM ruffling after different times of $CaCl_2$ stimulation was determined. All time points of stimulation were significantly different ($P \leq 0.001$) from the untreated (time 0 minutes) condition.
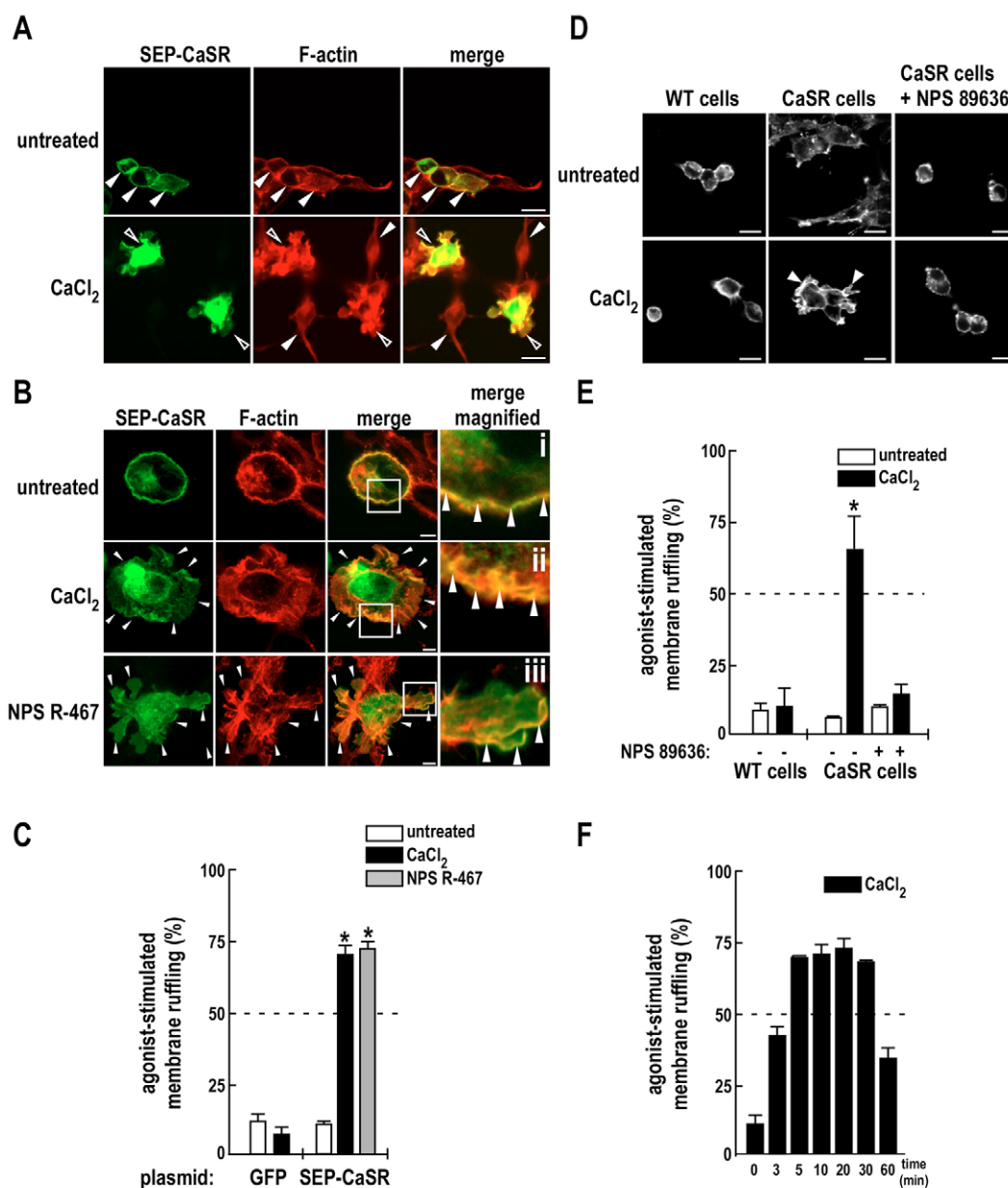
cytoplasmic protrusions that dynamically extended and retracted (see supplementary material Movie 1). This process of PM ruffling is central to cell locomotion (Ridley et al., 2003; Small et al., 2002) and, although chemotactic actions of CaSR have been reported (Godwin and Soltoff, 1997; Olszak et al., 2000), this is to our knowledge the first report of CaSR-induced PM ruffling.

Fig. 1A illustrates that an increase in $[Ca^{2+}]_o$ from 1 to 5 mM resulted in a marked actin polymerisation (filamentous actin stained with Alexa-Fluor-568–phalloidin) at the extending cell surface of HEK cells expressing SEP-CaSR but not in neighbouring untransfected HEK cells that do not express endogenous CaSR (Bouschet et al., 2005). The shape of HEK cells expressing SEP-CaSR was also dramatically changed by addition of the selective allosteric CaSR activator NPS R-467 (10 μM) but not by the S-enantiomer NPS S-467, which is 10-100-fold less potent in activating CaSR (not shown). In all cases the CaSR was present at the edge of the protrusions (Fig. 1B).

Calcium- and NPS R-467-induced morphological changes occurred in the majority of SEP-CaSR cells (70.4±3.5% and 72.5±3.1%, respectively, after 10 minutes, $P<0.001$ compared with the untreated cells) (Fig. 1C). Increased $[Ca^{2+}]_o$ had no effect in neighbouring untransfected or GFP-only-expressing HEK cells (Fig. 1A,C). To confirm these effects are CaSR-dependent, wild-type (WT) or stably expressing CaSR HEK

cells (cell line characterised in supplementary material Fig. S1) were challenged with $CaCl_2$ in the presence or absence of a CaSR antagonist (NPS 89636, 1 μM). As shown in Fig. 1D, $CaCl_2$ elicited robust PM ruffling in CaSR-HEK cells (from 6±1.2% to 74.7±13.3%, $P<0.001$; see also supplementary material Movie 2 and Movie 3) but not in control HEK cells (10.7±7.7% following calcium treatment, not significantly different from the 8.7±3.3% of the untreated cells, $P>0.05$) (Fig. 1D,E). Pre-incubation with the CaSR antagonist NPS 89636 prevented the ruffling response induced by calcium (15±4.4% in the presence of antagonist versus 74.7±13.3% in DMSO control solution, $P<0.001$) (Fig. 1D,E). Cytoplasmic protrusions developed rapidly following agonist treatment (within 3 minutes) and the number of cells bearing protrusions reached a plateau within 5-10 minutes after agonist addition (Fig. 1F). The extent of ruffling diminished after 30 minutes but was still significantly raised above basal level 1 hour after $CaCl_2$ stimulation (Fig. 1F).

β-arrestin 1 is required for CaSR-induced PM ruffling

We next investigated the molecular mechanisms underlying CaSR-induced ruffling. We reasoned that β-arrestins represent particularly attractive candidates because they are involved in GPCR-induced migration (Ge et al., 2003; Ge et al., 2004; Sun et al., 2002) and in CaSR desensitisation (Pi et al., 2005). We therefore co-expressed SEP-CaSR with a dominant negative

Fig. 2. β-arrestin 1 is required for CaSR-induced PM ruffling. (A) Expression of a dominant negative form of β-arrestin 1 impairs CaSR-induced PM ruffling. Representative pictures of HEK cells expressing SEP-CaSR in combination with dominant negative β-arrestin 1 (319-418) or wild-type (WT) β-arrestin 1. HEK cells were co-tranfected with SEP-CaSR (1 μg) and excess (4 μg) dominant negative β-arrestin 1 (319-418), wild-type (WT) β-arrestin 1 or CAT (as a control plasmid). Cells were stimulated with CaSR agonists (5 mM $CaCl_2$, 10 μM NPS R-467) for 10 minutes, fixed and stained for SEP-CaSR (green), and F-actin (red). Arrowheads indicate ruffles. The micrographs are representative of three independent experiments. (B) Histogram (expressed as mean±s.e.m.) of CaSR agonist-induced PM ruffling in the conditions presented in A. *$P\leq0.001$ compared with control plasmid-expressing cells stimulated with agonists ($n=3$). (C) siRNA-mediated silencing of β-arrestin 1 impairs CaSR-induced PM ruffling. HEK cells were transfected with a siRNA duplex targeting β-arrestin 1 (or a siRNA control) together with SEP-CaSR. 50 hours post-transfection, cells were stimulated with agonists, imaged and scored. Arrows indicate transfected cells whose morphology was unaffected and arrowheads point to the ruffles. (D) Quantification of data represented in C. *$P\leq0.001$ compared with control siRNA-expressing cells stimulated with agonists. ($n=3$). (E) Protein samples prepared in parallel to the ruffling experiments from cells transfected simultaneously with CaSR and siRNA, described in C and D, were probed with CaSR (top panel) and pan β-arrestin (A1CT, bottom panel) antibodies, respectively. (F) Pan β-arrestin (A1CT) blot of cells transfected with control or β-arrestin 1 siRNA alone showing ~60% knockdown of β-arrestin 1 (see text for details).
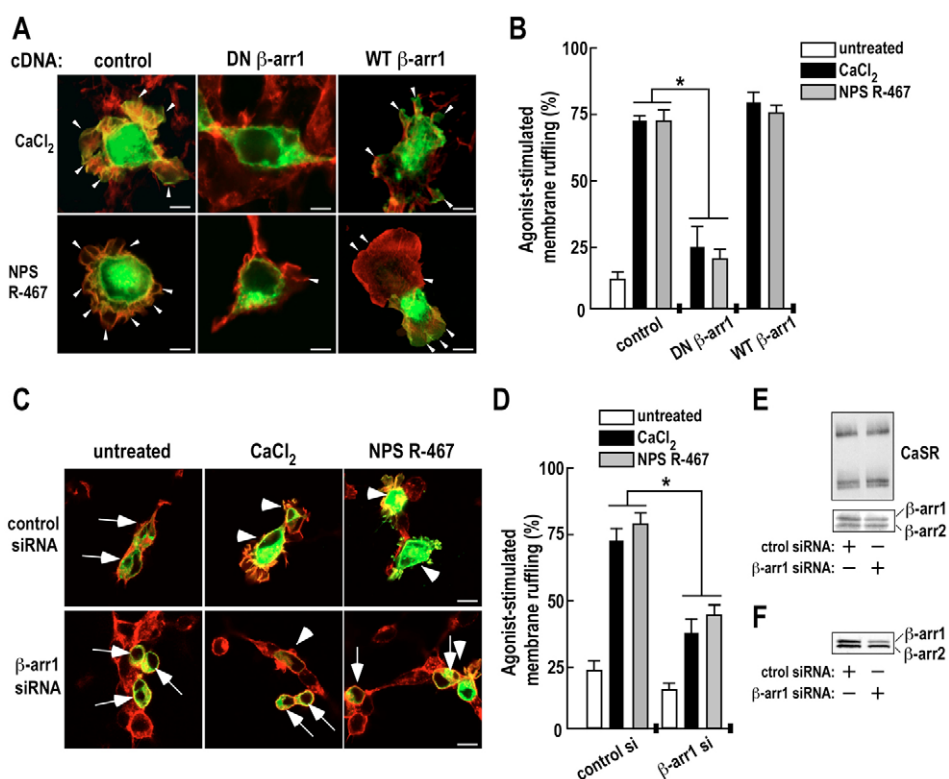
(DN) form of β-arrestin 1 (Krupnick et al., 1997). The DN, but not WT, β-arrestin 1 caused a marked decrease in CaSR-induced PM ruffling (Fig. 2A,B). Similarly, when the GPCR was co-expressed with a siRNA targeting β-arrestin 1 (Ahn et al., 2003), we observed a significant reduction in ruffling induced by calcium (72.2±5.6% for control siRNA reduced to 37±5.6% for β-arrestin 1 siRNA, $P<0.001$) or NPS R-467 (79.3±4.1% for control siRNA versus 43.7±4.3% for β-arrestin 1 siRNA, $P<0.001$) (Fig. 2C,D). Note that control or β-arrestin 1 siRNA were co-transfected with the plasmid encoding SEP-CaSR resulting in a specific ~30% decrease in β-arrestin 1 levels (Fig. 2E). When siRNA alone was transfected there was a ~60% decrease in β-arrestin 1 consistent with previous reports (Ahn et al., 2003; Ge et al., 2004) (Fig. 2F).

Intracellular molecules mediating cell migration exert part of their function by relocating to the cell leading edge (Kraynov et al., 2000; Ridley et al., 2003). Application of calcium or NPS-R467 resulted in the rapid translocation of GFP-β-arrestin 1 from the cytosol to PM ruffles (Fig. 3A; see supplementary material Movies 4 and 5), where there was partial colocalisation with F-actin (Fig. 3B). The current model of β-arrestin coupling to GPCR is that agonist stimulation drives the recruitment of β-arrestin which, in turn, recruits associated signalling and/or internalisation molecules (Lefkowitz and Whalen, 2004). We therefore co-transfected CaSR with Flag-β-arrestin 1 and measured their association by co-immmunoprecipitation. Consistent with a recent report of direct binding of β-arrestin 1 to the receptor (Pi et al., 2005), β-arrestin 1 specifically co-immunoprecipated with the CaSR under basal non-stimulated conditions (Fig. 3C). Interestingly, CaCl$_2$ stimulation did not detectably alter the extent of CaSR–β-arrestin 1 association, which suggests that this interaction is not dynamically regulated by agonist stimulation (Fig. 3C).

Expression of catalytically inactive ARNO impairs CaSR-induced PM ruffling

To explore which proteins link β-arrestin 1 to the cytoskeleton we first tested for the involvement of ARNO, a β-arrestin 1 interactor (Claing et al., 2001) that activates ARF6 and generates PM ruffling when overexpressed in MDCK cells (Santy and Casanova, 2001). Although ARNO is known to participate in GPCR desensitisation (Claing et al., 2001; Mukherjee et al., 2000), its role in GPCR-dependent PM ruffling has not been established. CaSR-HEK cells were transfected with either ARNO(E156K), a catalytically inactive point mutant, WT-ARNO or GFP alone. ARNO(E156K), but not WT-ARNO, expressing CaSR-HEK cells exhibit significantly lower PM ruffling than control GFP-expressing cells (Fig. 4A,B; 59.2±3.4% for GFP calcium, 37.7±3.9% for ARNO(E156K) calcium, $P<0.01$ compared with GFP calcium, 53±2.5% for WT-ARNO calcium, $P>0.05$ compared with GFP calcium). Furthermore, following CaSR activation, Myc-ARNO was redistributed to the ruffles (Fig. 4C).

We next co-transfected CaSR-HEK cells with Flag-β-arrestin 1 and Myc-ARNO and co-immunoprecipitated using anti-Flag beads. Consistent with a previous report (Claing et al., 2001), Myc-ARNO (or GFP-ARNO, not shown) specifically associated with Flag-β-arrestin 1 in resting conditions (Fig. 5A). Myc-ARNO(E156K), which blocked PM ruffling (Fig. 4), also bound to β-arrestin 1 (Fig. 5B). Interestingly, agonist stimulation did not substantially modify
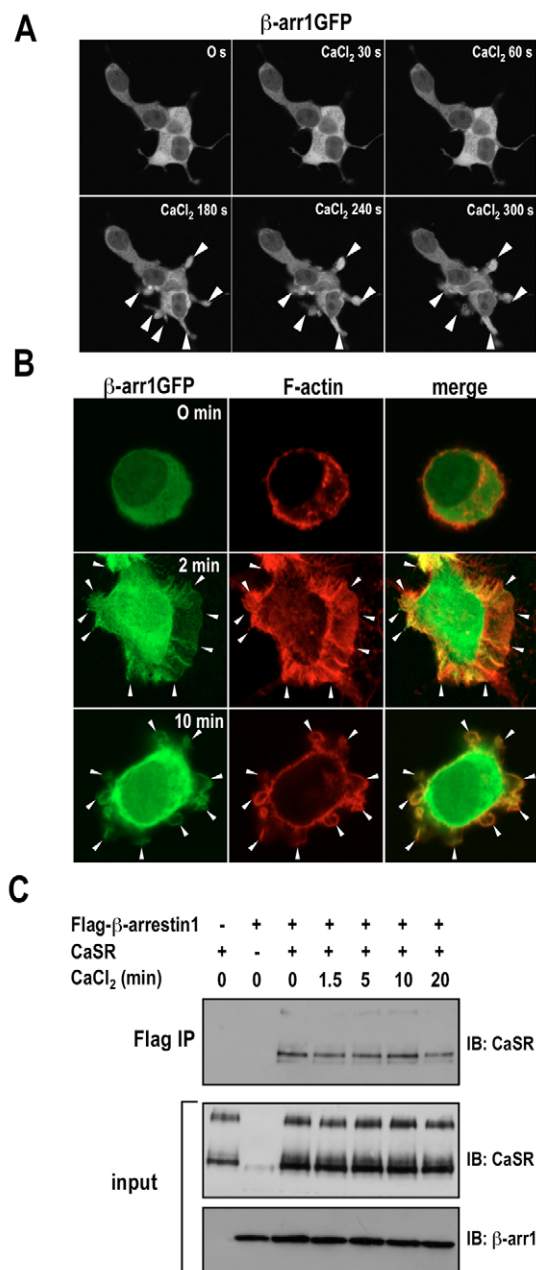

Fig. 3. Effect of CaSR stimulation on β-arrestin 1.
(A) Representative frames from live confocal microscopy experiments showing the subcellular redistribution of GFP-β-arrestin 1 in HEK cells co-expressing CaSR before (0 seconds) and after (30-300 seconds) activation by 5 mM CaCl$_2$. (B) HEK cells co-expressing GFP-β-arrestin 1 and CaSR (1 and 4 µg, respectively) were stimulated with 5 mM CaCl$_2$ for 2 or 10 minutes, fixed and stained for F-actin. Colocalisation of GFP-β-arrestin 1 (green) and F-actin (red) appears in yellow. Arrows indicate ruffles. Bar, 5 µm. Data are representative of four independent experiments. (C) The CaSR is constitutively associated with β-arrestin 1. HEK293 cells were transfected with SEP-CaSR and Flag-β-arrestin 1 in the indicated combinations. 48 hours post-transfection, cells were stimulated with 5 mM CaCl$_2$ for different periods of time and lysed. 400 µg of resulting proteins were subjected to immunoprecipitation (IP) using sepharose beads coupled to Flag-M2 antibodies. CaSR present in the immunoprecipitate and total fractions of CaSR and β-arrestin 1 (input) were assessed by immunoblotting (IB).

the association between β-arrestin 1 and ARNO (Fig. 5C) but the proteins colocalised together at nascent and mature ruffles (obtained after a CaSR stimulation of 1 and 10 minutes, respectively (Fig. 5D), consistent with translocation, rather than formation of the complex, being a key factor. Interestingly, we saw triple colocalisation of CaSR, β-arrestin 1 and ARNO in the ruffles (Fig. 5E), which suggests they could associate to form a signalling unit in this structure.

ARF6 and ELMO, two ARNO effectors, are also required for CaSR-induced PM ruffling

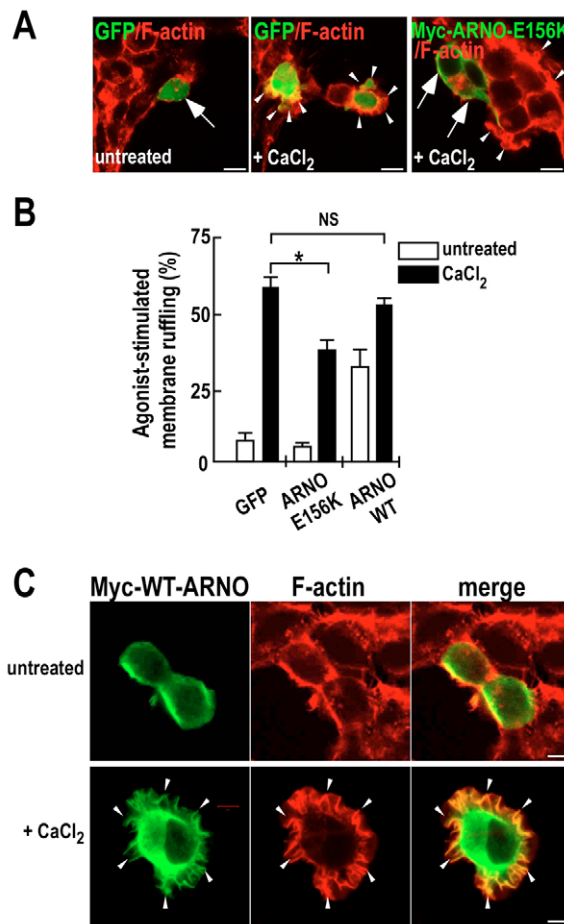In MDCK cells, expression of ARNO exerts its effect on cell

Fig. 4. Role of ARNO in CaSR-induced PM ruffling.
(A) ARNO(E156K) impairs CaSR-induced PM ruffling. CaSR-HEK cells expressing GFP, Myc-ARNO(E156K) or Myc-WT-ARNO (cells shown in C) were stimulated with 5 mM CaCl$_2$, for 10 minutes, fixed and stained for Myc-ARNO (using 9E10 anti-Myc monoclonal antibody followed by a Cy2-labelled anti-mouse Ab) (green), and F-actin (red). Arrows indicate the cells that did not change shape. Arrows point to transfected cells whose morphology was unaffected and arrowheads point to ruffles. The micrographs are representative of three independent experiments. Bar, 10 μm. (B) Quantification of CaSR-induced PM ruffling illustrated in A. *$P \leq 0.001$. NS, no significant difference between tested conditions ($P > 0.05$); $n = 3$.
(C) Myc-ARNO-expressing CaSR-HEK cells were challenged or not with 5 mM CaCl$_2$ for 10 minutes, fixed and stained for Myc-ARNO and F-actin as described in A. Bar, 5 μm. Note ARNO relocation to protrusions following CaSR stimulation.

shape and migration via ARF6 (Santy and Casanova, 2001). Therefore, we tested whether ARF6 has a role in mediating the ARNO effect on CaSR-induced PM ruffling in HEK cells. Expression of GFP-ARF6(T27N), a dominant negative mutant that is defective in GTP binding, but not of WT-ARF6, significantly antagonized the CaSR effect on cell shape (Fig. 6A,B). In addition, endogenous ARF6 translocated to CaSR-induced ruffles (Fig. 6C). Finally, we tested whether ELMO, a protein whose ortholog CED-12 is involved in the engulfment of apoptotic cells in *C. elegans* (Gumienny et al., 2001) and that was recently identified as coupling ARNO-ARF6 to cytoskeletal reorganisation (Santy et al., 2005), has a role in connecting the CaSR–β-arrestin-1–ARNO–ARF6 signalling unit to morphological change. Expression of ELMOT629, a truncated ELMO lacking the DOCK180-binding domain, was sufficient to reduce significantly the CaSR-induced effect on the cell shape of CaSR-HEK cells (from 59±3.4% for GFP calcium to 25±6.4% for GFP-ELMOT629 calcium, $P < 0.001$) (Fig. 7A,B). By contrast, WT-ELMO had no significant effect on ruffling (57±2.9%, $P > 0.05$ compared with GFP calcium) and was abundant in the PM ruffles (Fig. 7A-C), where it colocalised with Flag-CaSR (Fig. 7C).

Discussion

Migrating cells generate PM ruffles to move towards chemotactic factors (Ridley et al., 2003; Small et al., 2002) and here we show that CaSR stimulation leads to PM ruffling that likely underlie the reported chemotactic functions of extracellular calcium on CaSR-expressing cells (Godwin and Soltoff, 1997; Olszak et al., 2000). Indeed, using a Boyden-related migration assay (supplementary material Fig. S2) we demonstrate that CaSR-expressing HEK cells move up a calcium gradient.

β-arrestin 1, ARNO, ARF6 and ELMO are targeted to membrane protrusions and they are all required for CaSR-induced PM ruffling. This is the first report that ARNO and ELMO are involved in GPCR-dependent changes in cell shape and, based on these data, we propose that the β-arrestin–ARNO–ARF6–ELMO module may provide a general molecular mechanism to couple GPCR activation to PM ruffling.

Here, we show for the first time that a rise in extracellular calcium triggers CaSR-dependent ruffling (Fig. 1). Since we have shown previously that SEP-CaSRs have indistinguishable biophysical properties from untagged CaSRs (Bouschet et al., 2005), the presence of SEP fluorescence at the edge of the protrusions (Fig. 1) indicates that the CaSR localises at the surface of ruffles. This may allow CaSR-expressing cells to constantly monitor the direction of a calcium gradient in vivo and to orientate the direction of migration. Significantly in this context, CaSR stimulation also leads to cofilin phosphorylation on serine 3, which is essential for the lamellipodia extension/retraction cycle (Giannone et al., 2004) (T.B. and J.M.H., unpublished). CaSR stimulation also induces stress fibre formation (Davies et al., 2006), a process that parallels PM ruffling during cell migration (Ridley et al., 2003). Furthermore, this is consistent with the extracellular calcium-induced increase in chemotaxis we observed (supplementary material Fig. S2). In our system the increase in chemotaxis was relatively modest (~20%), possibly reflecting the fact that, in our chemotaxis assay, extracellular calcium in the basal

Fig. 5. β-arrestin 1 interacts and colocalises with ARNO. (A) Myc-WT-ARNO co-immunoprecipitates with Flag-β-arrestin 1. CaSR-HEK cells were transfected with plasmids encoding Myc-WT-ARNO ± Flag-β-arrestin 1. 48 hours post-transfection, cells lysates were prepared and subjected to immnoprecipitation using Flag beads (see Materials and Methods). Retained proteins were eluted with Laemmli's buffer and immunoblotted using anti-β-arrestins and ARNO antibodies. A total of the lysate (input) was run in parallel. (B) Catalytically inactive Myc-ARNO(E156K) co-immunoprecipitates with Flag-β-arrestin 1. (C) CaSR stimulation does not affect ARNO association with Flag-β-arrestin 1. Cells co-expressing WT-ARNO and Flag-β-arrestin 1 were stimulated with CaCl₂ for the indicated periods of time and Flag immunoprecipitation was performed as in A. Total ARNO and ARNO bound to Flag-β-arrestin 1 are shown.

(D) Colocalisation of Myc-WT-ARNO and GFP–β-arrestin 1 in protrusions. Transfected CaSR-HEK cells were challenged with 5 mM CaCl₂ for 1 or 10 minutes, fixed and stained for GFP-β-arrestin 1 (green), Myc-ARNO (using 9E10 Ab followed by a Cy5-coupled anti-mouse Ab, shown in blue) and F-actin (Alexa-Fluor-568–phalloidin, red). An inset showing a magnified view for the 1 minute stimulation highlights β-arrestin 1 and ARNO colocalisation in protrusions. Bar, 5 μm. (E) GFP-ARNO, RFP-β-arrestin 1 and Flag-CaSR colocalise in protrusions. Transfected HEK cells were challenged with 5 mM CaCl₂ for 10 minutes, fixed and stained for GFP-ARNO (green), mRFP-β-arrestin 1 (red) and Flag-CaSR (detected by mouse anti-Flag Ab followed by a Cy5-coupled anti-mouse Ab, blue). A magnified view of the PM ruffling area is shown in the bottom panels. Merge areas appear in white. Bar, 5 μm.
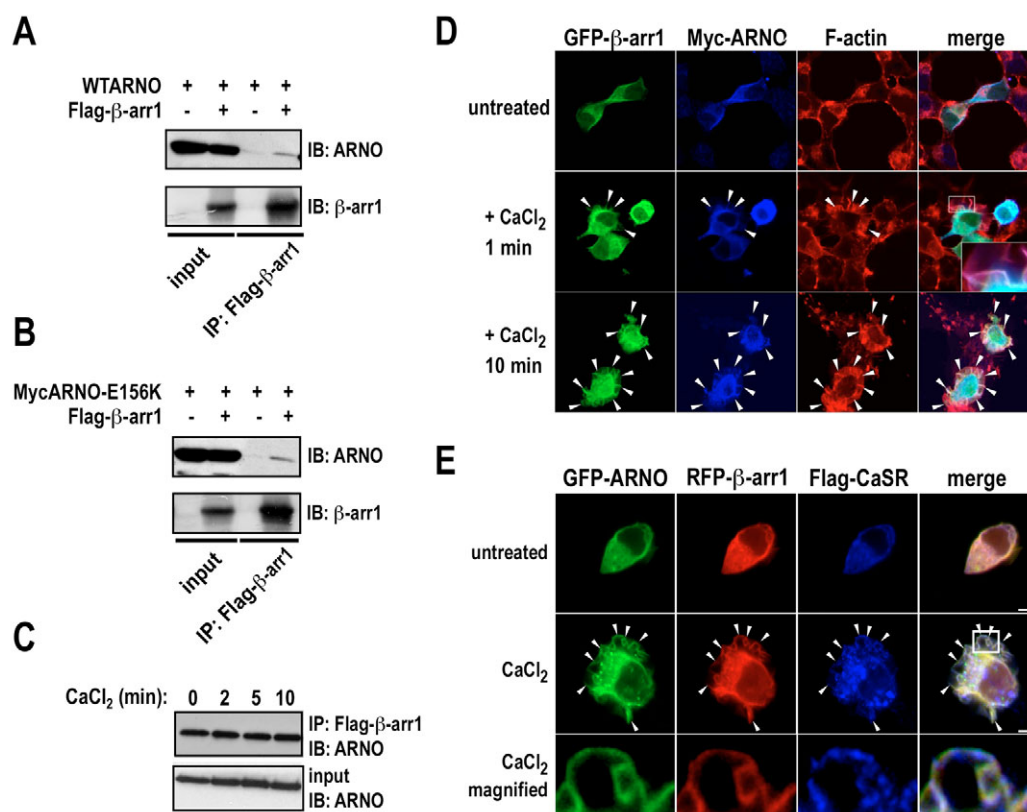
medium is at a concentration sufficient to activate the CaSR (1.8 mM CaCl₂). Several reports have implicated β-arrestins in cell migration mediated by GPCR (Bhattacharya et al., 2002; Fong et al., 2002; Ge et al., 2003). Here, we show that a DN form of β-arrestin 1, or its knockdown by RNA interference, strongly affect CaSR-induced morphological changes, consistent with it playing a role in CaSR-induced ruffling (Fig. 2). Given that the β-arrestin 1 knockdown was not 100% effective and that the DN form of β-arrestin 1 could also interfere with the closely related β-arrestin 2, we cannot completely rule out the possibility that β-arrestin 2 also participates in CaSR-induced ruffles. A widespread model for the actions of β-arrestins is that they bind to phosphorylated GPCRs, thereby recruiting downstream effector proteins (Lefkowitz and Whalen, 2004). For CaSR-induced PM ruffling, however, this appears not to be the case, since we observe a constitutive binding of β-arrestin 1 to the CaSR that is not markedly altered by receptor stimulation (Fig. 3). Interestingly, a similar result was reported for the association of β-arrestin 1 with mGluR (Dale et al., 2001), a receptor belonging to the same GPCR family as the CaSR (class C GPCRs). Thus, although β-arrestin 1 is required for CaSR-induced ruffling, there is no apparent recruitment of β-arrestin 1 to the receptor and the transduction complex is already

assembled. What could then be the mechanism of β-arrestin 1 action? By analogy with active Rac1 forming a gradient culminating at the cell edge (Kraynov et al., 2000), β-arrestin 1 may ensure its function by relocating to the forming ruffles to recruit specific machinery (Fig. 3). Indeed, β-arrestin 1 becomes enriched in pseudopodia, where it sequesters ERK1/2 necessary for PAR-2-induced chemotaxis (Ge et al., 2003). In addition to ERK1/2, several other proteins have been reported to participate in β-arrestin-1-mediated PM ruffling. For example, fMLP-receptor-induced PM ruffling depends on the dissociation of RalGDS from β-arrestin 1 and subsequent RalA activation (Bhattacharya et al., 2002). Activation of the Ral pathway (and therefore subsequent PM ruffling) does not require endocytic function of β-arrestin 1 (Bhattacharya et al., 2002). Recent evidence has suggested that β-arrestin 1 does not modulate CaSR endocytosis in HEK cells (Lorenz et al., 2007).

We also show that ARNO plays a key role in GPCR-induced cell-shape change and that, in common with CaSR and β-arrestin 1, it translocates to PM ruffles (Fig. 4). Since the inactive mutant ARNO(E156K) binds to β-arrestin 1 (Fig. 5), it is likely that overexpression of this catalytically inactive mutant competitively inhibits binding of endogenous ARNO, thereby blocking the CaSR transduction cascade. No marked
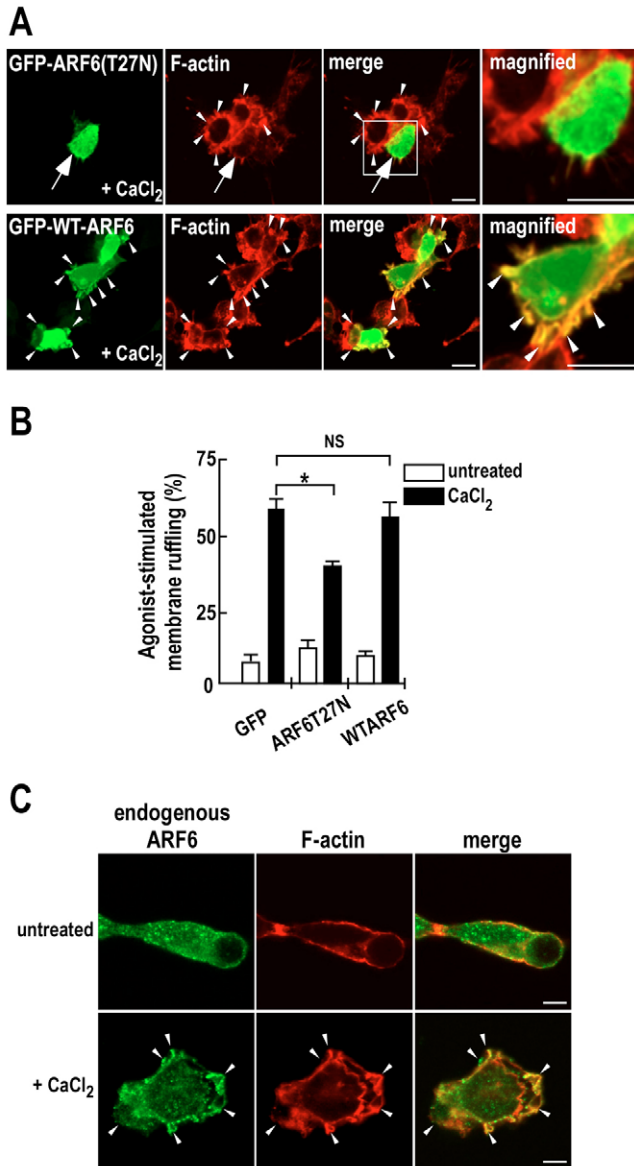
Fig. 6. ARF6 is involved in CaSR-induced PM ruffling. (A) ARF6(T27N) impairs CaSR-induced PM ruffling. CaSR-HEK cells transfected with GFP, GFP-ARF6T27N or GFP-ARF6WT were treated with 5 mM CaCl$_2$ for 10 minutes, fixed and stained for F-actin (red). The micrographs are representative of three independent experiments. Arrowheads indicate cells exhibiting ruffles and the arrow indicates the cell that did not ruffle. Bars, 10 μm. Right panels are a magnified view of inserts. (B) Quantification of CaSR-induced PM ruffling in cells presented in (A). *$P \leq 0.001$. NS, no significant difference ($P > 0.05$, $n=3$). (C) Endogenous ARF6 is targeted to PM ruffles. CaSR-HEK cells were challenged with 5 mM CaCl$_2$ for 10 minutes, fixed and stained for endogenous ARF6 (using a rabbit anti-ARF6 Ab followed by a Cy2-coupled anti-rabbit Ab, green) and for F-actin (red). Ruffles are indicated by arrowheads. Colocalisation areas appear in yellow. Bars, 5 μm.

Fig. 7. ELMO is involved in CaSR-induced PM ruffling. (A) Expression of GFP-ELMO(T629) but not GFP-WT-ELMO impairs CaSR-induced PM ruffling. CaSR-HEK cells transfected with GFP-ELMO(T629), GFP-WT-ELMO or GFP were stimulated with 5 mM CaCl$_2$ for 10 minutes, fixed and stained for GFP (green) and F-actin (red). The micrographs are representative of three independent experiments. Arrowheads indicate cells exhibiting ruffles and arrows indicate cells not ruffling. Right panels are a magnified view of the boxed regions in the merge panels. (B) Quantification of cells presented in (A). *$P \leq 0.001$. NS, not significantly different ($P > 0.05$) ($n=3$). (C) GFP-ELMO and Flag-CaSR colocalise in protrusions. HEK cells co-expressing the two plasmids indicated above were challenged with 5 mM CaCl$_2$ for 2 or 10 minutes, fixed and stained for GFP-ELMO (green) and Flag-CaSR (red). Arrowheads show protrusions, and colocalisation areas appear in yellow. Bar, 5 μm.

changes in the levels of ARNO or CaSR association with β-arrestin 1 were detected on calcium stimulation (Fig. 5), which suggests that either an, as yet unknown, additional partner is required for their activation or that the complex relocates to ruffles (Fig. 5E) is sufficient for their signalling function.
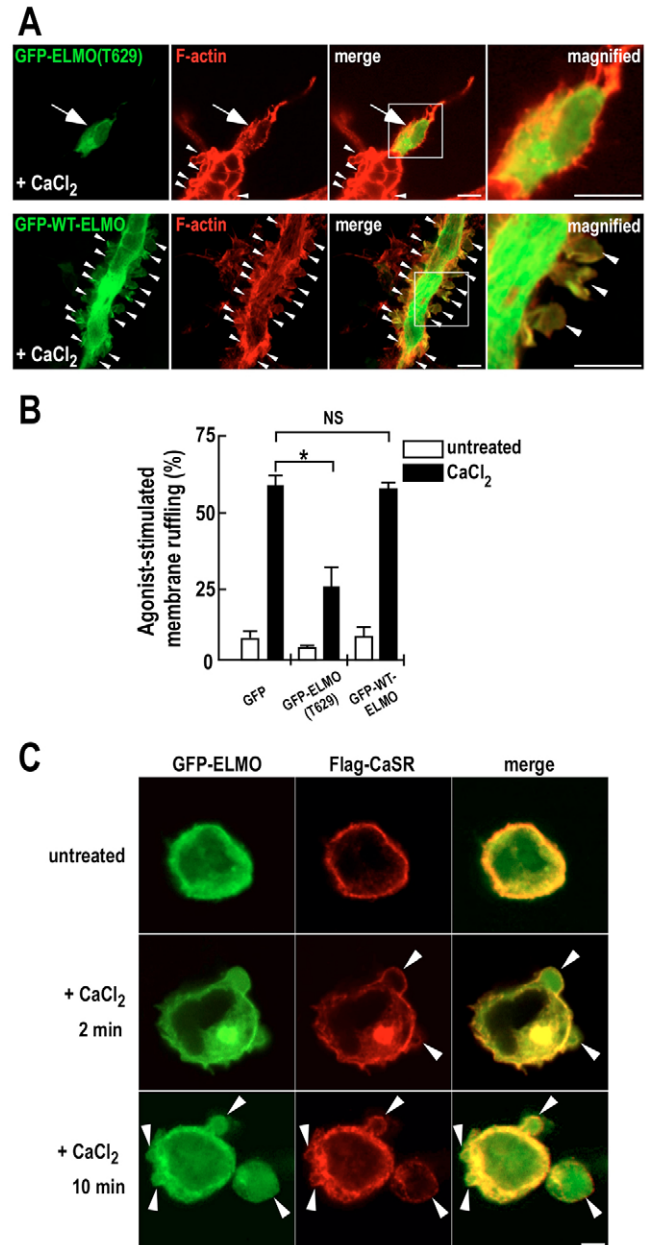
ARF6 and ELMO are also associated with the CaSR–β-arrestin-1–ARNO signalling unit since dominant negative

mutants of these ARNO effector proteins (Santy and Casanova, 2001; Santy et al., 2005) impaired CaSR ruffling (Figs 6, 7). To our knowledge, this is the first report describing a role for ARNO in GPCR-mediated ruffling and for ELMO in any form of GPCR signalling.

In conclusion, here we demonstrate that, in common with some other GPCRs, CaSRs induce cytoskeletal reorganisation and PM ruffling. Further, we elucidate key components of the signalling cascade that allows CaSR activation to mediate PM ruffling, a prerequisite for cell migration. These results provide a framework for future investigations to determine whether this is a general signalling pathway for other GPCRs. Furthermore, our findings make accessible a series of potential targets for molecular manipulation to allow better understanding of the mechanisms involved in ruffling and possible sites for intervention, with a view to regulating these processes experimentally in normal and pathological conditions.

Materials and Methods

Materials
The CaSR agonist NPS R-467 and antagonist NPS 89636 were kindly provided by Edward Nemeth and Alan Mueller (NPS Pharmaceuticals, Salt Lake, UT). Rabbit anti-β-arrestins (A1CT) and anti-ARF6 antibodies were provided by Robert Lefkowitz (Duke University, Durham, NC) and Julie Donaldson (NIH, Bethesda, MD), respectively. Rabbit anti-ARNO antibody was kindly provided by Sylvain Bourgoin (Université Laval, Québec, Canada). Mouse monoclonal anti-CaSR and anti-Myc (9E10) antibodies were from Affinity Bioreagents and Roche, respectively. Mouse monoclonal anti-MAP kinase activated (diphosphorylated ERK-1 and 2) and β-tubulin antibodies were from Sigma. Peroxydase-linked secondary antibodies were from Amersham. Minimal interspecies cross-reaction secondary antibodies coupled to Cy2, Cy3 and Cy5 fluorophores were from Jackson Immunoresearch Laboratories. Alexa-Fluor-568–phalloidin was from Molecular Probes.

Cell culture, transfection and expression plasmids
Human embryonic kidney (HEK) cells were grown and transfected as previously described (Bouschet et al., 2005). The following plasmids were used: pRK5-CaSR (Ruat et al., 1995) and pRK5-SEPCaSR (Bouschet et al., 2005). Flag-CaSR was kindly provided by Gerda Breitwieser (Syracuse University, NY). GFP, WT and dominant negative β-arrestin 1 (Krupnick et al., 1997) were generously provided by Jeffrey Benovic (Thomas Jefferson University, Philadelphia, PA). mRFP-β-arrestin 1 was a kind gift from Eric Prossnitz (University of New Mexico, Albuquerque, NM). Flag-β-arrestin 1 was obtained from Robert Lefkowitz. GFP-ARNO was previously described (Venkateswarlu et al., 1998). GFP-WT-ARF6 and GFP-ARF6(T27N) were generated by PCR amplification of HA-WT-ARF6 and HA-ARF6(T27N), respectively, subsequent digestion with *Bam*HI/*Eco*RI, and cloned into *Bgl*II/*Eco*RI of peGFP-N1 (Clontech). Myc-ARNO, Myc-ARNO(E156K) (Santy and Casanova, 2001), GFP-ELMO and GFP-ELMO(T629) (Santy et al., 2005) were kindly provided by Lorraine Santy (Pennsylvania State University, University Park, PA). As an internal control, cells were transfected with Chloramphenicol Acetyl Transferase (CAT) cDNA to adjust the cDNA levels and the translation efficiency between the different conditions.

HEK cells stably expressing rat CaSR were generated by co-transfecting pRK5-CaSR with pSV2-BSR (encoding the blasticidin-resistance gene). Selection was achieved in media supplemented with 600 μg/ml blasticidin and positive clones were selected on three criteria: purity (100% of cells expressing the receptor), surface expression of the receptor and functionality of the receptor (positive coupling to ERK1 and 2) (see supplementary material Fig. S1).

siRNA synthesis and transfection
Chemically synthesised, duplex siRNAs were purchased from Qiagen. We used an effective sequence targeting human β-arrestin 1, AGCCTTCTGCGCGGAGAAT, previously reported (Ahn et al., 2003). One unrelated sequence, TTCTCCGAACGTGTCACGT, showing no significant homology to any known human gene (analysed using a BLAST search) was used for the control siRNA. siRNAs were transfected – in combination or not with SEP-CaSR – using Transit-KO according to the manufacturers' instructions (Mirus).

Confocal microscopy
All samples were analyzed by confocal laser-scanning microscopy using a Zeiss LSM510 META confocal system as described (Bouschet et al., 2005).

Plasma membrane ruffling
Cells seeded on 6 cm dishes were transfected as described in the figure legends. Cells were transferred the following day onto collagen-coated coverslips and used after 48-72 hours expression. Cells were rinsed in Earle's low calcium media (140 mM NaCl, 5 mM KCl, 1 mM CaCl$_2$, 0.8 mM MgCl$_2$, 25 mM HEPES pH 7.4, 0.9 g D-glucose) to reduce CaSR activity. After a 10 minute incubation at 37°C, cells were treated as indicated in the figure legends, rinsed with PBS and fixed for 20 minutes in a 4% paraformaldehyde solution. Cells were then incubated with primary antibodies for 1 hour and antibodies were revealed with secondary antibodies coupled to Cy2 or Cy5. F-actin was subsequently stained with Alexa-Fluor-568-conjugated phalloidin (Molecular Probes) in a 2% BSA solution (in PBS). Membrane ruffling was visualised by confocal microscopy. A cell was scored as ruffling when meeting one of these three criteria: (1) more than three ruffles; (2) a circular ruffling, or (3) more than a doubling in size. Cells present in the centre of clusters were not analysed since they are difficult to score. For each condition, the percentage of ruffling cells averaged over 5 separate randomly chosen microscope fields was determined and at least 50 cells were counted. All PM ruffling experiments were replicated at least three times.

Live-cell confocal imaging
Live-cell confocal imaging was performed on the heated stage (set at 37°C) using a 63× water-immersion objective, and cells were continually perfused at 2 ml/minute with solutions pre-heated to 37°C.

Immunoprecipitation
Cells were harvested in a lysis buffer consisting of 20 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol, 0.5% NP40, 2 mM EDTA 0.5% Triton X-100, 1 mM PMSF, and a proteases inhibitors cocktail. Lysates were clarified by centrifugation at 12,000 *g* for 10 minutes. 300-400 μg of resulting proteins were subjected to immunoprecipitation using 15 μl of Flag-M2 beads (Sigma). After an overnight incubation on a rotating wheel, beads were washed three times with 1 ml of the corresponding lysis buffer and resuspended in Laemmli's buffer.

Western-blotting
Cell lysates resuspended in Laemmli's sample buffer were heated at 95°C for 5 minutes (37°C for 15 minutes when probed for CaSR), and proteins were resolved by SDS-PAGE, blotted onto nitrocellulose membrane, and probed with the indicated antibodies. The nitrocellulose was then incubated with anti-rabbit or anti-mouse/horseradish peroxidase conjugate (1:5000; Amersham Biosciences) for 60 minutes and developed using the west pico chemiluminescence's kit (Pierce).

Chemotaxis assays
The assays were conducted in serum-free DMEM (containing 1.8 mM CaCl$_2$) using Transwell chambers of 24-well inserts with 8 μm pore membranes. DMEM solution supplemented or not with high calcium (5 mM) was placed in the lower chamber and 10^5 HEK CaSR cells were placed in the upper chamber and incubated for 5 hours at 37°C and 5% CO$_2$. Membranes were then fixed and cells stained with DAPI. Cells were removed from the upper chamber, leaving only those that migrated through the membrane pores. The membrane was then excised, mounted on slides, and cells were counted on five separate fields on a fluorescence microscope (see supplementary material Fig. S2).

Data analysis
Statistical analyses were calculated using Graph Pad Prism 4 (Graph Pad Software). Data are expressed as mean±s.e.m. Statistical significances were assessed by an unpaired *t*-test or one way ANOVA test followed by a Newman-Keuls multiple comparison test when required.

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