Stimulation of $G\alpha_q$-coupled M1 muscarinic receptor causes reversible spectrin redistribution mediated by PLC, PKC and ROCK

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Accepted 4 January 2006
Journal of Cell Science 119, 1528-1536 Published by The Company of Biologists 2006
doi:10.1242/jcs.02872

Summary

Spectrin is a cytoskeletal protein that plays a role in formation of the specialized plasma membrane domains. However, little is known of the molecular mechanism that regulates responses of spectrin to extracellular stimuli, such as activation of G-protein-coupled receptor (GPCR). We have found that αII spectrin is a component of the $G\alpha_{q/11}$-associated protein complex in CHO cells stably expressing the M1 muscarinic receptor, and investigated the effect of activation of GPCR on the cellular localization of yellow-fluorescent-protein-tagged αII spectrin. Stimulation of $G\alpha_{q/11}$-coupled M1 muscarinic receptor triggered reversible redistribution of αII spectrin following a rise in intracellular Ca^{2+} concentration. This redistribution, accompanied by non-apoptotic membrane blebbing, required an intact actin cytoskeleton and was dependent on activation of phospholipase C, protein kinase C, and Rho-associated kinase ROCK. Muscarinic-agonist-induced spectrin remodeling appeared particularly active at localized domains, which is clear contrast to that caused by constitutive activation of ROCK and to global rearrangement of the spectrin lattice caused by changes in osmotic pressure. These results suggest a role for spectrin in providing a dynamic and reversible signaling platform to the specific domains of the plasma membrane in response to stimulation of GPCR.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/119/8/1528/DC1

Key words: αII spectrin, Membrane-skeleton remodeling, Gαq-coupled receptor signaling, Signaling complex, Rho-associated kinase

Introduction

Spectrin is an important component of the membrane skeleton, composed of α- and β-subunits. Its functional unit, an $(\alpha\beta)_2$ tetramer, is cross-linked at nodes by actin filaments and adaptor proteins, and elongates further to form a sub-plasmalemmal lattice responsible for the structural integrity of the plasma membrane (Bennett and Baines, 2001). Spectrins can interact with various proteins directly, or through adaptor proteins, implicated their involvement in diverse physiological processes, including protein sorting, trafficking and endocytosis (De Matteis and Morrow, 2000; Bennett and Baines, 2001).

Recent proteomic studies revealed that spectrins are components of the synaptic multiprotein complex (Husi et al., 2000; Bacamel et al., 2002) and $G\alpha_i$-containing membrane complex (Nebl et al., 2002), suggesting a role of spectrin in G-protein-coupled receptor (GPCR) signaling. Spectrin-based protein-complex formation appears to be essential for effective signaling, in particular, for cell-type specific responses, exemplified in neurons and astrocytes, where αII and βI spectrin link the plasma membrane microdomains to the underlying endoplasmic reticulum (ER) domains to form the specific Ca^{2+} signaling complexes (Lencesova et al., 2004). In addition to physical coupling, active roles of spectrin in signaling have been demonstrated in the recent studies. For example, the linkage between βI spectrin and neural cell adhesion molecule mediates the localized assembly of spectrin microdomains and recruitment of activated protein kinase C (PKC) to the microdomain and, further, translocation of activated PKC to lipid rafts in a fibroblast growth factor receptor-dependent manner (Leshchyns'ka et al., 2003). Phosphorylated embryonic liver fodrin (ELF) β-spectrin switches its binding partner from structural proteins (ankyrin and tropomyosin) to Smad proteins upon transforming growth factor-β stimulation, and these newly formed associations are crucial for microtubule-independent transport of Smad proteins to the nucleus and their target gene expression (Tang et al., 2003). Also, α spectrin in the specialized integrin-rich signaling domains participates in the activation of the Rho GTPase Rac, suggesting a pivotal role of spectrin in initiating intergrin-induced physiological and pathological events, such as development, proliferation, cell survival, wound healing and metastasis (Bialkowska et al., 2005).

Cytoskeletal components, actin and microtubules have been shown to associate with $G\alpha_{q/11}$ (Ibarrondo et al., 1995; Cote et al., 1997; Roychowdhury et al., 1999) and participate in

regulation of either activities or special organization of the molecules involved in $G\alpha_{q/11}$-coupled-receptor signaling. Tubulin translocates to the plasma membrane in response to muscarinic receptor stimulation in neuroblastoma cells, and this membrane-associated tubulin links $G\alpha_q$ to phospholipase C (PLC) β and activates PLC β through a mechanism involving direct interaction and transactivation of $G\alpha_q$ (Popova and Rasenick, 2000; Popova et al., 2002). An intact actin cytoskeleton plays a role in activation of effectors downstream of the gonadotropin-releasing receptor (Davidson et al., 2004), and in formation of the signaling microdomain in sympathetic neurons, where conformational coupling of the inositol(1,4,5)-trisphosphate receptor [Ins(1,4,5)P_3R)] with the B2 bradykinin receptor, but not with the M1 muscarinic acetylcholine receptor (M1 mAChR), achieves rapid release of Ca^{2+} from the ER (Delmas et al., 2002).

Despite those observations for the actin and microtubule cytoskeleton, little is known about responses of spectrin to stimulation of GPCR, and its role in downstream signaling. In this report, we first show that αII spectrin is a component of the $G\alpha_{q/11}$-associated protein complex in Chinese hamster ovary (CHO) cells stably expressing M1 mAChRs (M1-CHO). Second, we show how the spectrin-based membrane skeleton responds to stimulation of M1 mAChRs by monitoring yellow fluorescent protein (YFP)-tagged αII spectrin (YFP-αII spectrin) transfected into M1-CHO cells. Third, we provide evidence for the signaling mechanism linking $G\alpha_{q/11}$-coupled M1 mAChR to remodeling of the spectrin cytoskeleton, mediated by PLC, PKC and Rho-associated kinase (ROCK).

Results

αII Spectrin is a component of the $G\alpha_q$-associated protein complex

We first performed yeast two-hybrid screenining to identify potential binding partners of $G\alpha_q$. A cDNA encoding part of αII spectrin (amino acid residues 653-1181), containing the SH3 domain, the isoform insert 1 and the μ-calpain cleavage site, was one of several positive clones found in an adult rat brain cDNA library. This αII spectrin fragment (653-1181) also contains interaction sites for two actin binding proteins identified in the recent report (Rotter et al., 2005). A β-galactosidase analysis revealed that the yeast two-hybrid interaction between $G\alpha_q$ and the partial αII spectrin (653-1181) was approximately 14% of the positive control interaction (p53 and T antigen), but was 12-fold stronger than the association between $G\alpha_q$ and an empty pGADT7 vector (average of three separate analyses).

We have prepared HA-tagged αII spectrin (653-1181) that might serve as a dominant negative by interfering the association between αII spectrin and $G\alpha_q$. Co-immunoprecipitation of HA-tagged αII spectrin (653-1181) with Myc-tagged $G\alpha_q$ indicated interaction of the two proteins in co-transfected cells. However, we found that the αII spectrin (653-1181) formed aggregates in the cytosol, which does not represent localization of endogenous spectrin at the membrane skeleton. To eliminate possible mislocalization, we examined the association of endogenous αII spectrin and $G\alpha_{q/11}$ in M1-CHO cells. CHO cells have been reported to express αI spectrin, but were not tested for αII spectrin (Leshchyns'ka et al., 2003). Therefore, we first performed RT-PCR using the specific primers that distinguish between αII spectrin and αI spectrin transcripts. As shown in Fig. 1A, expression of αII spectrin was confirmed in both wild type and M1-CHO cells. We next fractionated whole lysates of M1-CHO cells using a rabbit anti-$G\alpha_{q/11}$ antibody, or a control rabbit anti-Myc antibody, and the resulting co-immunoprecipitates were probed with a goat anti-αII spectrin antibody. A band corresponding to endogenous αII spectrin (240 kDa) was detected in the $G\alpha_{q/11}$-associated protein complex, but was absent in the complex precipitated by the control antibody (Fig. 1B). A faint band (approximately 100 kDa), similar to that observed in a previous study (Nedrelow et al., 2003), was likely to be a proteolytic product of αII spectrin by residual protease activity remaining in the immunoprecipitates.

We next assessed the expression of YFP-αII spectrin prior to the live imaging study, and also examined its colocalization with $G\alpha_q$ in M1-CHO cells. A cell surface marker, CD8, was expressed at the plasma membrane (Fig. 2A, red), while YFP-αII spectrin was expressed in the cytosol (Fig. 2A, green) with enrichment at the membrane skeleton, an inner layer of the plasma membrane (Fig. 2A, merge). In agreement with expression of other classes of Gα protein (Sarma et al., 2003), both endogenous $G\alpha_{q/11}$ (Fig. 2B, red) and Myc-tagged $G\alpha_q$ (Fig. 2C, red) appeared to be distributed throughout the cytoplasm. A small population of YFP-αII spectrin colocalized with endogenous $G\alpha_{q/11}$ (Fig. 2B, merged), and this trend was same for the Myc-tagged $G\alpha_q$, although

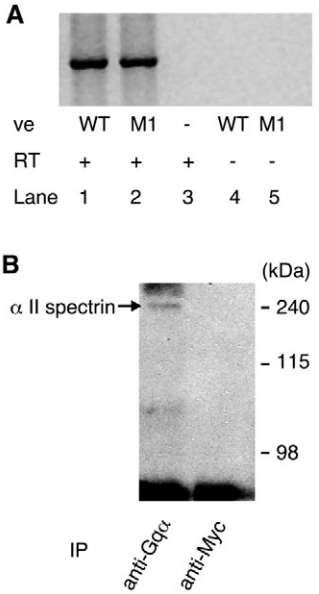

Fig. 1. Endogenous αII spectrin is a component of the $G\alpha_{q/11}$-associated protein complex. (A) RT-PCR. Both wild-type (WT, lane 1) and M1-CHO cells (M1, lane 2) express αII spectrin transcripts. No protein was detected in the samples without reverse transcription (RT: lane 4 and 5) and without vector (lane 3).
(B) Immunoprecipitation assay. Full-length αII spectrin (240 kDa) is present in the $G\alpha_{q/11}$-associated protein complex isolated from M1-CHO cells, but not in the immunocomplex incubated with the control antibody anti-Myc.

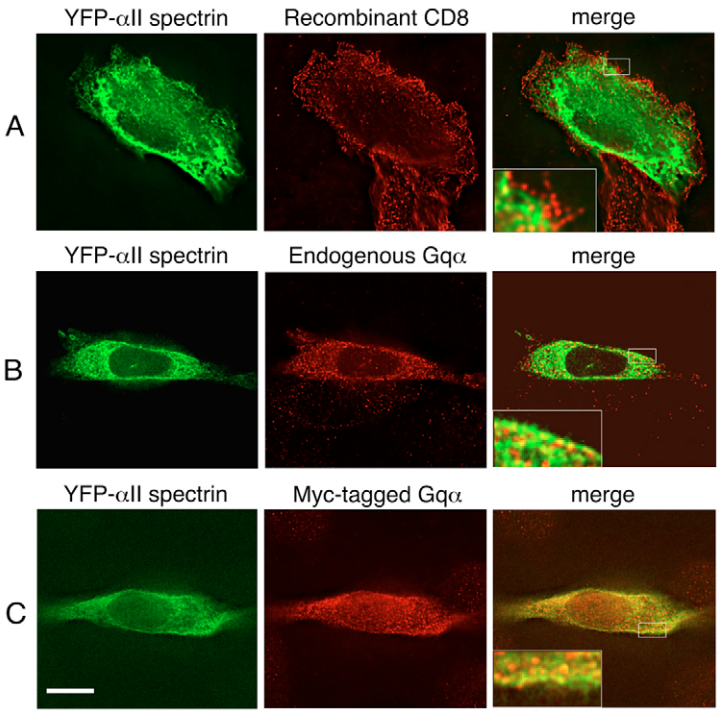

Fig. 2. αII spectrin colocalizes with $G\alpha_{q/11}$ at the periphery of M1-CHO cells. (A) Expression of the surface marker CD8 (red) is at the plasma membrane, whereas YFP-αII spectrin (green) is distributed in the cytosol with enrichment under the plasma membrane (merge). YFP-αII spectrin is not expressed in the second cell in this field because of its low transfection efficiency (maximum 30%) compared with CD8 (80-90%). (B) Endogenous $G\alpha_{q/11}$ (red) is distributed throughout the cytoplasm and colocalization with YFP-αII spectrin (green) is seen at the membrane skeleton (merge). (C) Myc-tagged $G\alpha_q$ (red) similarly colocalizes with YFP-αII spectrin (green) at the plasma membrane skeleton (merge). Insets in merged images show higher magnification views of the boxed region of the main images. Bar, 10 μm.

colocalization was clearer in the co-transfected M1-CHO cells (Fig. 2C, merged). These results corroborate the interaction observed in the yeast two-hybrid analysis and the co-immunoprecipitation study.

Activation of M1 mAChR triggers reversible αII spectrin redistribution

To address the question whether αII spectrin in the $G\alpha_{q/11}$-associated protein complex acts as a stationary 'platform' or a mobile element that responds to stimulation of M1 mAChRs, we employed a live imaging system to monitor the movements of YFP-αII spectrin transiently expressed in M1-CHO cells. CHO cells lack endogenous muscarinic receptors and, therefore, M1-CHO cells provide an ideal model system to study responses of αII spectrin induced by stimulation of the $G\alpha_{q/11}$-coupled M1 mAChRs.

In the absence of stimuli, distribution of YFP-αII spectrin, enriched under the plasma membrane, remained largely unchanged for up to 90 minutes. By contrast, application of the muscarinic agonist oxotremorine-M (Oxo-M) for 3-5 minutes caused marked morphological changes in the M1-CHO cells, and spontaneous blebs appeared on the plasma membrane (Fig. 3, supplementary material Movie 1). Redistribution of YFP-αII spectrin was equally dynamic and reversible: the dense patch of αII spectrin under the plasma membrane stretched along with cell elongation and moved towards the bleb ends (Fig. 3, arrows), and then re-established its original distribution upon withdrawal of blebs (Fig. 3, arrowheads). No vesicles containing YFP-αII spectrin were secreted during this period (supplementary material Movie 1). The first bleb appeared at 41.9±9.3 seconds after Oxo-M application (n=12), and spectrin rearrangements lasted for 369±39.9 seconds (n=12). None of the M1-CHO cells tested failed to show reversible spectrin remodeling (n=150), whereas wild-type CHO cells lacking muscarinic receptors were unresponsive to Oxo-M stimulation (n=10). Plasma membrane integrity appeared generally intact after drastic rearrangement of the membrane skeleton. Phosphatidylserine externalization of plasma membranes and nuclear condensation were not detected in those cells, and dynamic αII spectrin redistribution can be repeatedly induced by five consecutive application of Oxo-M. Thus, membrane blebbing induced by stimulation of M1 mAChRs is not related to apoptosis, and most probably represents a transient remodeling of the spectrin-based membrane skeleton.

Muscarinic agonist-induced αII spectrin redistribution is downstream of the $G\alpha_{q/11}$-PLC pathway

The linkage between M1 mAChR-$G\alpha_{q/11}$ mediated signal transduction and the PLC pathway is well established (Popova and Rasenick, 2000; Delmas et al., 2002; Popova et al., 2002). We therefore tested a PLC inhibitor, U73122, to see whether PLC activation is required for Oxo-M-induced spectrin redistribution. Preincubating M1-CHO cells with U73122 totally abolished redistribution of YFP-αII spectrin (n=4, Fig.

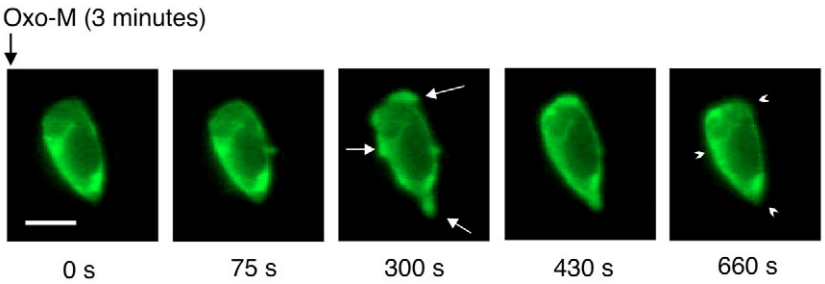

Fig. 3. Reversible redistribution of αII spectrin is a downstream event of M1 mAChR activation. Redistribution of YFP-αII spectrin starts approximately 42 seconds after Oxo-M application and lasts for 370 seconds in M1-CHO cells. αII spectrin redistributes into the bleb-ends (arrows) during blebbing and reverts to the pre-activation positions (arrowheads) when stabilized. For the live image of this cell, see supplementary material Movie 1. Bar, 10 μm.

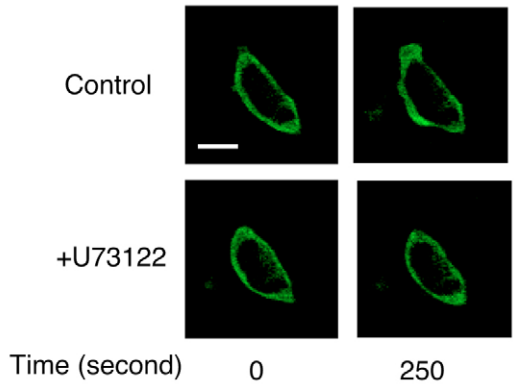

Fig. 4. PLC mediates muscarinic agonist-induced αII spectrin redistribution. Digital deconvolved images of a M1-CHO cell expressing YFP-αII spectrin (green) were taken at 0 and 250 seconds after Oxo-M application. Inhibition of PLC activity by U73122 abolishes both redistribution of YFP-αII spectrin and membrane blebbing. Bar, 10 μm.

4, Table 1), indicating that spectrin remodeling is downstream of the $G\alpha_{q/11}$-PLC pathway. U73343, an inactive analog of U73122, showed no effect (n=4, Table 1). The next question was which of the second messengers of PLC, diacylglycerol or Ins(1,4,5)P_3, mediates the signal to the spectrin membrane skeleton. To answer this, we first used 30 μM of the membrane-permeable analog of diacylglycerol 1-oleoyl-2-acetyl-*sn*-glycerol (OAG). Despite the ability of OAG to activate PKC directly, OAG-treated cells showed a delay in bleb formation (202±109 seconds, n=3 of eight experiments), or impaired bleb formation (n=5 of eight experiments). Blocking the effects of Ins(1,4,5)P_3, by preincubating cells in the modulators of Ins(1,4,5)P_3-induced Ca^{2+} release 2-aminoethoxy diphenyl borate (n=10) or Ins(1,4,5)P_3 xestospongin C (n=8), resulted in partial inhibition (30-60% inhibition; Table 1), suggesting that both diacylglycerol and Ins(1,4,5)P_3, at least in part, play roles in $G\alpha_{q/11}$-PLC mediated spectrin redistribution.

Table 1. Effects of inhibitory compounds on αII spectrin remodeling and membrane blebbing

Compound	Conc. (μM)	Inhibition (%)	Number of experiments
2-aminoethoxy diphenyl borate	80	30	10
Cytochalasin D	10	83*	6
KN-93	10	0	6
Nocodazole	32	0	7
PD98059	25	0	6
PP1	20	0	9
Staurosporine	0.1	100	6
U73122	5	100	4
U73343	5	0	4
Wortmannin	0.1	0	4
Xestospongin C	3	63	8
Y-27632	10	50†	6

Effects of inhibitory compounds on αII spectrin remodeling and membrane blebbing. M1-CHO cells expressing YFP-αII spectrin were pre-incubated in Hepes buffer containing inhibitors at the indicated concentration. Stimulation with Oxo-M was carried out in the presence of the inhibitors. Inhibitory effects were judged on the live-imaging system: Inhibition (%) = 100 × (unresponsive cell number)/(total cell numbers).

*Blebs did not revert in responsive cells; †blebbing start was delayed in responsive cells.

Spectrin remodeling occurs in specific domains of $G\alpha_{q/11}$ signaling subsequent to elevation of intracellular Ca^{2+} concentration

Activation of the $G\alpha_{q/11}$-PLC pathway leads to an increase in the intracellular Ca^{2+} concentration $[Ca^{2+}]i$ triggered by a release of Ca^{2+} through ER-associated Ins(1,4,5)P_3Rs (Felder, 1995; Delmas et al., 2002). In M1-CHO cells, $[Ca^{2+}]i$ peaked rapidly (<20 seconds) after application of Oxo-M, and then gradually decreased to control levels over a period of 200 seconds (n=90, Fig. 5). The $[Ca^{2+}]i$ peak was more than 30 seconds before the first visible spectrin redistribution, and spectrin redistribution continued after the $[Ca^{2+}]i$ had returned to basal levels (Fig. 5). We also found that a transient rise in $[Ca^{2+}]$, induced by the inhibitor of ER Ca^{2+} ATPase thapsigargin, also induced YFP-αII spectrin redistribution and membrane blebbing (n=10), suggesting that a $[Ca^{2+}]i$ rise can induce remodeling of the spectrin cytoskeleton in the absence of $G\alpha_{q/11}$ activation. This was further confirmed by use of the Ca^{2+} ionophore ionomycin (n=5, Fig. 6, supplementary material Movie 2), which causes greater increase in $[Ca^{2+}]i$ than Oxo-M. However, a noticeable difference from Oxo-M-induced spectrin redistribution was that redistributed YFP-αII spectrin (Fig. 6, arrows) did not revert to the membrane skeleton even upon prolonged washing. Instead, it diffused into the cytosol (Fig. 6, arrowheads) indicating enzymatic breakdown of αII spectrin as reported previously in apoptotic cells (Nath et al., 1996; Wang et al., 1998). These observations suggest that the non-apoptotic signaling following an elevation of $[Ca^{2+}]i$ is responsible for reversible spectrin redistribution.

We next examined whether changes in osmotic pressure can induce membrane blebbing associated with αII spectrin distribution in M1-CHO cells. Switching the perfusion buffer between the isotonic and hypotonic solution caused the global expansion and/or shrinkage of the spectrin lattice, as reported previously (Sun and Levitan, 2003), but lengthy oscillatory

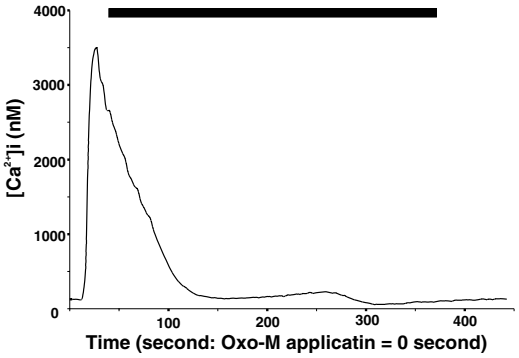

Fig. 5. Muscarinic agonist-induced spectrin remodeling occurs subsequent to a rise in $[Ca^{2+}]i$. Activation of M1 mAChRs causes a transient increase in $[Ca^{2+}]i$ in M1-CHO cells. This rapid rise in $[Ca^{2+}]i$ (<20 seconds) precedes redistribution of YFP-αII spectrin, and spectrin redistribution (solid bar on the top indicates duration) continues after $[Ca^{2+}]i$ returned to the basal levels. The graph represents a typical response of Fura-2AM-loaded M1-CHO cells, and the average duration of spectrin redistribution (n=12).

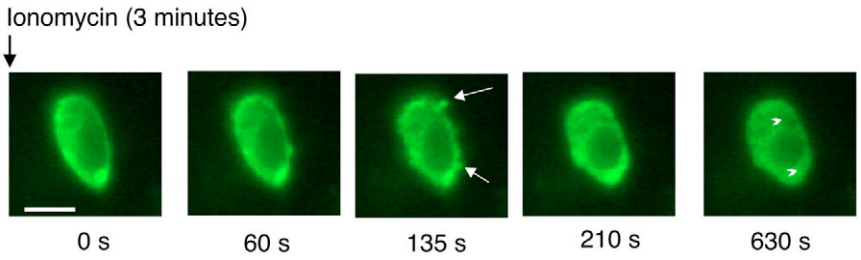

Fig. 6. Ionomycin can trigger redistribution of αII spectrin but lacks a reversible mechanism. αII spectrin redistributes into the bleb ends (arrows) upon an application of ionomycin. After ionomycin-induced membrane blebbing, αII spectrin loses its assembly at the membrane skeleton and diffuses into the cytosol (arrowheads). For the live image of this cell, see supplementary material Movie 2. Bar, 10 μm.

blebbing, characteristic of muscarinic agonist-induced spectrin redistribution, was absent (*n*=5, supplementary material Movie 3). This suggests that spectrin remodeling upon stimulation of M1 mAChR is not due to global changes in osmotic pressure, but occurs in response to activation of the $G\alpha_{q/11}$-coupled signaling domains.

Muscarinic agonist-induced αII spectrin redistribution is PKC-dependent but independent of CaM II kinase, MAP kinase and Src kinase

PKC is implicated in the $G\alpha_{q/11}$-signaling pathway (Marsh et al., 1995; Mundell et al., 2004) and in agonist-induced membrane blebbing (Torgerson and McNiven, 1998), and its activation requires diacylglycerol and Ca^{2+} (Medkova and Cho, 1999). We used a potent PKC inhibitor, staurosporine, to examine possible involvement of PKC in regulation of the Oxo-M-induced spectrin remodeling. Inhibition of PKC activities completely blocked YFP-αII spectrin redistribution and membrane blebbing (*n*=6, Table 1). Although staurosporine is known to inhibit Ca^{2+}-dependent CaM kinase, the CaM kinase II inhibitor KN-93 showed no effects (*n*=6, Table 1). This indicates that PKC, but not CaM kinase II, plays a role in agonist-induced remodeling of the plasma membrane skeleton. Activation of the mitogen-activated protein kinase pathway has been reported to be downstream of $G\alpha_{q/11}$-PLC-PKC signaling in CHO cells expressing M3 mAChRs (Budd et al., 2001); however, we found no evidence for inhibitory effects by PD98059 (*n*=6, Table 1).

The αII spectrin protein phosphorylated by Src kinase becomes resistant against cleavage by Ca^{2+}-dependent calpain, thus, αII spectrin is proposed to serve as a point of convergence between the tyrosine kinase/phosphatase and the Ca^{2+}-mediated signal transduction pathways (Nicolas et al., 2002; Nedrelow et al., 2003). Despite the fact that Oxo-M-induced redistribution of YFP-αII spectrin occurs after a rise in $[Ca^{2+}]i$, the Src kinase inhibitor PP1 neither delayed nor blocked spectrin distribution (*n*=9, Table 1). This suggests that, Src kinase activation is either not required for spectrin rearrangement or αII spectrin is already phosphorylated by Src under our culture condition, as it has been reported in other types of cells (Nicolas et al., 2002; Nedrelow et al., 2003).

ROCK links M1 mAChR signaling to redistribution of αII spectrin

Activation of ROCK appears crucial for bleb formation induced by activation of $P2X_7$-ATP receptor (Morelli et al., 2003), and constitutive activation of ROCK itself causes membrane blebbing without extracellular stimuli (Coleman et al., 2001; Sebbagh et al., 2005). We have, therefore, tested the ROCK inhibitor Y-27632, and found that Oxo-M induced redistribution of YFP-αII spectrin was completely blocked (*n*=3 of six experiments) or bleb formation was delayed (100.0±52.6 seconds, *n*=3 of six experiments) by Y-27632 (Table 1). Enzymatic cleavage of the C-terminus domain is one of the activation mechanisms of ROCK, and C-terminally cleaved ROCK is often used as an indicator for its activation status (Coleman et al., 2001; Ueda et al., 2001; Morelli et al., 2003; Sebbagh et al., 2005). We have, therefore, tried to take a quantitative approach to ROCK activation by measuring the ratio of the full length and C-terminally truncated forms of ROCK (Ueda et al., 2001). As seen in the previous reports (Ueda et al., 2001; Morelli et al., 2003; Sebbagh et al., 2005), proteolytic conversion of ROCK was detectable even in cells that had received no stimulation. The amount of active form of ROCK, however, was not elevated after application of Oxo-M for 5 minutes (Fig. 7A). An increase in cleaved ROCK became clear only after sustained stimulation with Oxo-M for 1 hour (Fig. 7A), suggesting that ROCK activation caused by a short period of muscarinic receptor stimulation is a transient and/or localized event. We next employed two types of ROCK mutants, the kinase-dead and the constitutively active ROCK-II. Transient expression of the kinase-dead ROCK-II did not cause obvious changes in spectrin redistribution in non-stimulated M1-CHO cells. However, following activation of M1 mAChRs for 3 minutes, we observed impaired agonist-induced membrane blebbing in cells expressing kinase-dead ROCK-II (*n*=10, Fig. 7B, top row) compared with mock transfected cells (*n*=10) that exhibited similar extent of membrane blebbing to that shown in Figs 3 and 4. These data support a role of ROCK in spectrin redistribution downstream of M1 mAChRs signaling. However, in good agreement with the previous studies (Sebbagh et al., 2005), overexpression of constitutively active ROCK-II resulted in membrane blebbing without activation of M1 mAChRs. Intensity of membrane blebbing caused by this ROCK mutant (*n*=10, Fig. 7B, bottom row) was much higher than that caused by Oxo-M (Figs 3 and 4). This vigorous blebbing was often accompanied by cell rounding (Fig. 7B), which was absent in muscarinic agonist-induced membrane blebbing. These observations support a view that, a short period of agonist stimulation (3-5 minutes) causes transient activation of ROCK and its effect on the membrane skeleton reorganization are subtle and highly localized, which is in clear contrast to the effect caused by constitutive activation of ROCK.

It has been shown that the RhoA-ROCK pathway links $G\alpha_{q/11}$-coupled M3 mAChR signaling to cytoskeleton responses involved in muscle-cell contraction (Murthy et al., 2003) and cell migration (Chernyavsky et al., 2004). Also, overexpression of both constitutively active ROCK-II and RhoA V14 caused cell rounding and membrane blebbing in PC12 cells (Frantz et al., 2002), suggesting that RhoA was upstream of ROCK activation. However, we did not observe significant changes in

either cell morphology or membrane blebbing in M1-CHO cells transfected with constitutively active RhoA V14 (n=10; Fig. 7C, bottom row). Further, the dominant-negative Rho N19 showed only a little impact on membrane blebbing after activation of M1 mAChRs for 3 minutes (n=10; Fig. 7C, top row) compared with mock transfected cells (n=10). These results were in clear contrast to the inhibitory effects by the kinase-dead ROCK-II (Fig. 7B, top row), and indicate a partial involvement of RhoA in ROCK activation and the downstream redistribution of the spectrin cytoskeleton.

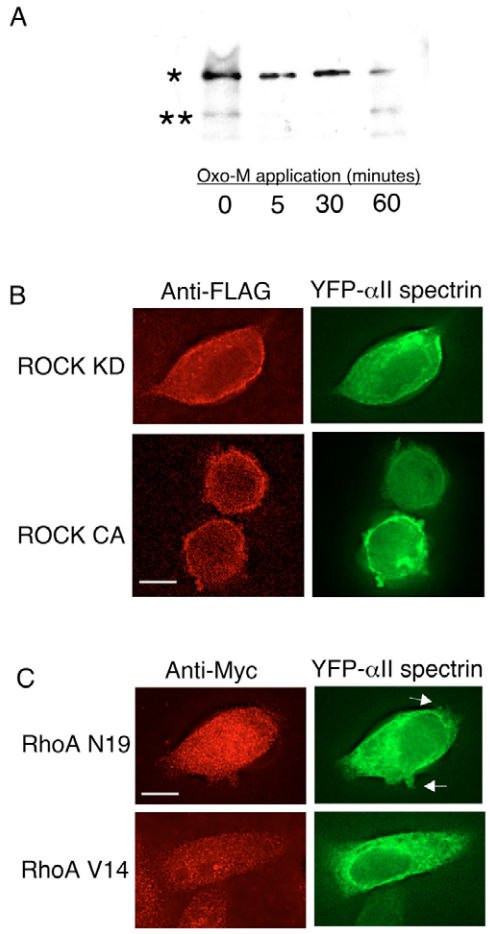

Fig. 7. Muscarinic agonist-induced spectrin redistribution depends on transient activation of ROCK. (A) Proteolytic conversion from the full-length (*) to C-terminally cleaved, active ROCK (**) is not significantly induced in M1-CHO cells stimulated with 5 μM Oxo-M for 5 minutes. (B) Transient expression of Flag-tagged kinase-dead (KD) ROCK-II (top row, red) diminishes Oxo-M-induced membrane blebbing and redistribution of YFP-αII spectrin (top row, green). Images were taken 3 minutes after activation of M1 mAChRs. Flag-tagged constitutively active (CA) ROCK-II (bottom row, red) causes intense membrane blebbing accompanied with cell rounding in the absence of M1 mAChR activation. Images were taken 0 minutes after activation of M1 mAChRs. (C) Transient expression of Myc-tagged dominant-negative Rho (RhoA N19, top row, red) has little effects on Oxo-M-induced redistribution of YFP-αII spectrin to the bleb end (top row, green, arrows). Images were taken 3 minutes after activation of M1 mAChRs. Overexpression of Myc-tagged constitutively active RhoA (Rho V14, bottom row, red) itself causes neither membrane blebbing nor cell rounding. Images were taken 0 minutes after activation of M1 mAChRs. Bar, 10 μm.

Muscarinic agonist-induced αII spectrin remodeling depends on actin but not tubulin

Spectrin is a principal component of the membrane skeleton that links to F-actin and provides a structural support for the plasmalemma. The actin cytoskeleton was reported to be an important component for both agonist-induced (Torgerson and McNiven, 1998) and ROCK-dependent membrane blebbing (Song et al., 2002). To determine whether Oxo-M-induced spectrin redistribution requires the actin cytoskeleton, we disrupted actin filaments using cytochalasin D. By this treatment, the localization of YFP-αII spectrin at the membrane skeleton was fundamentally altered to concentrated foci distributed throughout the cytoplasm (supplementary material Movie 4). These spectrin foci did not respond to the subsequent agonist stimulation (n=6, Table 1, Movie 4). Thus spectrin requires interaction with the actin cytoskeleton for its organized localization and responsiveness to extracellular stimuli. Phosphoinositide-3 kinase (PI 3-kinase) is known to play a key role in GPCR-mediated actin polymerization (Chodniewicz and Zhelev, 2003) and in inhibition of the astrocyte actin depolymerization (Perez et al., 2005). Oxo-M induced YFP-αII spectrin redistribution, however, appeared to be independent of the PI 3-regulated actin dynamics, since the PI 3-kinase inhibitor wortmannin showed negligible effects (n=4, Table 1). The muscarinic agonist promotes microtubule depolymerization and translocation of tubulin to the plasma membrane in neuroblastoma cells (Popova and Rasenick, 2000), however, microtubule disruption with nocodazole showed little effect (n=7, Table 1), suggesting that rearrangement of the spectrin membrane skeleton is independent of microtubule integrity.

Discussion

The principal finding of this study is that the cytoskeletal protein αII spectrin is a component of the $G\alpha_{q/11}$-associated protein complex, and that reversible remodeling of the spectrin cytoskeleton is a downstream event of $G\alpha_{q/11}$-coupled activation of M1 mAChR. Muscarinic agonist-induced spectrin redistribution is accompanied by membrane bleb formation, distinct from global shrinkage and/or expansion of the spectrin lattice that are induced by changes in osmotic pressure. Also, this membrane blebbing is highly localized, and not as vigorous and uniform as seen in cells expressing constitutively active ROCK. These observations suggest that spectrin redistribution occurs in the specialized domains in response to extracellular stimuli. We have provided evidence for the molecular mechanism that regulates rearrangement of the spectrin membrane skeleton.

Activation of PLC, the well-established $G\alpha_{q/11}$ effector, is essential and the two second messengers diacylglycerol and Ins(1,4,5)P_3 both play roles in spectrin redistribution. An transient elevation in $[Ca^{2+}]i$, caused either by Oxo-M-induced M1 mAChR activation or by thapsigargin-triggered store depletion, precedes rearrangement of the spectrin cytoskeleton. However, spectrin remodeling activities do not correlate directly to changes in $[Ca^{2+}]i$ (Fig. 5). Further, membrane blebbing can occur in the absence of any extracellular signals simply by overexpressing epithelial membrane proteins (Wilson et al., 2002) or constitutively active ROCK, as demonstrated here (Fig. 7B) and in previous studies (Coleman et al., 2001; Sebbagh et al., 2005). Thus, we

conclude that an elevation in [Ca^{2+}]i is not by itself sufficient. Rather, it appears to serve as a trigger of the enzymatic cascade crucial for rearrangements of the cytoskeleton. We have identified PKC and ROCK as those enzymes involved, and this agrees with previous studies demonstrating roles of both enzymes in agonist-induced modification of the cytoskeleton (Torgerson and McNiven, 1998; Strassheim et al., 1999; Murthy et al., 2003). Interestingly, both PKC and ROCK are known to modulate the balance between assembly and disassembly of the spectrin-F-actin meshwork by phosphorylating α-adducin, an adaptor protein of spectrin (Matsuoka and Bennett, 1998; Fukata et al., 1999; Barkalow et al., 2003). This might explain marked differences in cellular localizations of spectrin and actin during the course of agonist-induced membrane blebbing. Whereas actin rearrangement is restricted to the neck of the blebs (Torgerson and McNiven, 1998), spectrin appears to transiently dissociate from actin and follows formation and withdrawal of membrane blebs (Fig. 3, supplementary material Movie 1). These observations point to the role of spectrins in providing mechanical support and the protein-protein interaction platform beneath the plasma membrane.

Our live-imaging study using Y-27632 clearly indicate activation of ROCK upon a short period of muscarinic stimulation (3-5 minutes). However, we could not detect increases in the C-terminally cleaved form of ROCK under the same activation condition (Fig. 7A). This might be owing to the differences in sensitivity between the two methods used. Whereas live imaging can depict effects of local and transient enzymatic activities, immunoblotting is sensitive only to changes in ROCK proteolysis in the global levels, as seen after sustained $G\alpha_{11}$ activation (after 2-4 hours) that leads to the apoptotic pathway (Ueda et al., 2001). Also, M3 mAChR-mediated transient activation of kinases is sufficient for sustained phosphorylation of the myosin light chain and resulting contraction (Murthy et al., 2003). Taking together, our results are not necessarily contradictory, rather, support the view that spectrin redistribution is a consequence of transient activation of the localized signaling domains.

RhoA has been shown to participate in muscarinic agonist-induced reorganization of both myosin (Strassheim et al., 1999) and actin (Linseman et al., 2000). Inhibition of RhoA and ROCK causes parallel effects on M3 mAChR signaling (Chernyavsky et al., 2004; Murthy et al., 2003), whereas ROCK and RhoA activations are independent of Ins(1,4,5)P_3-induced [Ca^{2+}]i rise and PKC (Ueda et al., 2001; Vogt et al., 2003), which suggests that RhoA, but not PLC and PKC, is upstream of ROCK, linking $G\alpha_{q/11}$-coupled receptor signaling to reorganization of the cytoskeleton. However, two lines of evidence in our study argue against this activation mechanism. First, dominant-negative RhoA N19 failed to abolish ROCK-dependent membrane blebbing and spectrin redistribution (Fig. 7C). Second, effects caused by constitutively active RhoA V14 (Fig. 7C) were minimal, in clear contrast to the marked effects caused by constitutively active ROCK (Fig. 7B). Thus, muscarinic agonist-induced ROCK activation appeared to only partially depend on RhoA, supporting the view for a diversity of ROCK-activation mechanisms (Feng et al., 1999; Morelli et al., 2003; Ueda et al., 2004).

Our study has provided insights for the molecular mechanism that guide spectrin redistribution; however, the physiological meaning of this phenomenon is not yet clear. One possibility is its close relation to plasma membrane-ER complexes-based signaling. First, spectrin controls store-operated Ca^{2+} entry through membrane protein 4.1 (Wu et al., 2001), and links the plasma membrane microdomain to the ER microdomain in a cell-specific manner (Lencesova et al., 2004). The cytoskeletal components link the B_2 bradykinin receptor, but not M1 mAChR, to Ins(1,4,5)P_3R, thus, only the B_2 bradykinin receptor can activate the Ins(1,4,5)P_3R and transient receptor potential channel 1 (TRPC1) in sympathetic neurons, whereas both $G\alpha_{q/11}$-coupled receptors are able to activate Ins(1,4,5)P_3R and TRPC1 in M1-CHO cells (Delmas et al., 2002). Second, agonist-induced but not apoptotic plasma-membrane blebs contain significant amounts of ER (Torgerson and McNiven, 1998). Third, in clear contrast to internalization of receptors upon $G\alpha_{q/11}$-coupled M1 or M3 mAChR activation (Cant and Pitcher, 2005; Mundell et al., 2004; Popova and Rasenick, 2004), exocytotic insertion of TRPC6 into the plasma membrane – in which conformational coupling with Ins(1,4,5)P_3R plays a crucial role – occurs less than 1 minute after stimulation with the muscarinic agonist carbacol (Cayouette et al., 2004). This timing is similar to initiation of Oxo-M-induced spectrin redistribution demonstrated in our study and, moreover, both spectrin redistribution and insertion of TRPC6 can be induced by OAG or Ca^{2+}-store depletion. It is an intriguing issue for future studies to ascertain whether reversible spectrin redistribution represents activities of the plasma-membrane ER signaling microdomain and whether spectrin itself participates in signaling activities – as seen in Rac activation in specific integrin-rich domains (Bialkowska et al., 2005).

Association of $G\alpha_{q/11}$ with actin (Ibarrondo et al., 1995) and microtubules (Cote et al., 1997; Roychowdhury et al., 1999), and also, as shown in our study, with spectrin, strongly indicates involvement of the cytoskeleton in diverse GPCR signaling. It is possible that the cytoskeletal proteins serve initially as a scaffold upon which signaling complexes are formed, and thereby themselves become integral signaling components. The dynamics of membrane-skeleton remodeling might vary depending on the composition of the spectrin-containing signaling complex. We predict that the redistribution of YFP-αII spectrin demonstrated in this study represents, at least to some degree, the formation and recruitment of the spectrin-signaling complex to the appropriate localization in response to extracellular stimuli.

Materials and Methods

Expression plasmids and cell culture

A cDNA encoding the full-length mouse $G\alpha_q$ (GenBank NM 008139) was PCR-amplified and inserted into the pCMV.Myc and pAS2-1 vectors. The pEYFP-αII spectrin was prepared, by subcloning a cDNA-encoding full-length human αII spectrin (GenBank U83867) into the pEYFP-C1 vector. cDNA encoding a partial sequence of αII spectrin (amino acid residues 653-1181) was PCR-amplified from an adult rat brain cDNA library (BD Bioscience) and cloned into pCMV.HA and pGADT7. All vectors were from BD Bioscience. The plasmid containing CD8 cDNA has been described elsewhere (Selyanko et al., 1999). Constructs for Myc-tagged constitutively active RhoA V14 and dominant-negative RhoA N19 (Frantz et al., 2002) were kind gifts of Romano Regazzi (University of Lausanne, Switzerland), and Flag-tagged constitutively active and kinase dead mutant ROCK-II (Leung et al., 1996) were kindly provided by Thomas Leung (Institute of Molecular and Cell Biology, Singapore).

CHO cells stably transfected with cDNA encoding human M1 mAChR (Buckley et al., 1989) were cultured in 5% CO_2 at 37°C in α-MEM supplemented with 10% fetal calf serum and 1% L-glutamine. The expression level of M1 mAChR is approximately 1 pmol/mg membrane proteins (Buckley et al., 1989).

RT-PCR and yeast two-hybrid screening and analysis

Total RNA was prepared from wild-type and M1-CHO cells by using TRI Reagent (Sigma-Aldrich). For first-strand cDNA synthesis whith H-M-MLV reverse transcriptase (Promega), 3 μg of total RNA were used; cDNA was amplified with Taq polymerase (Promega). Intron-spanning primers specific for rat αII spectrin were used for PCR.

Yeast two-hybrid screening was performed with the pAS2-1.Gα_q as bait to screen an adult rat brain cDNA library fused into pGAD10. Positive cDNA clones were isolated from transformants, subcloned into pGADT7 and reintroduced with the bait construct into yeast AH109 to confirm the two-hybrid interaction under selective conditions. Yeast Y187 strain was similarly co-transformed and the relative strength of two-hybrid interaction of transformants was analyzed by using a luminescent β-galactosidase detection kit (BD Bioscience).

Co-immunoprecipitation and western blotting

M1-CHO cells (80-90% confluent) were lysed in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40) under gentle rocking for 30 minutes on ice. The lysate was treated with a normal rabbit IgG (Sigma-Aldrich) and a mixture of protein-G- and protein-A-Sepharose (Sigma-Aldrich). The resulting pre-cleared fractions were incubated with a rabbit anti-G$\alpha_{q/11}$ antibody (Sigma-Aldrich) or a rabbit anti-Myc antibody (A14; Santa Cruz Biotechnology) for 4 hours, followed by incubation with protein-A-Sepharose beads overnight. All procedures were carried out at 4°C in the presence of Complete protease inhibitor cocktail (Roche Applied Science). After washing the Sepharose-beads extensively with PBS, the immune complexes were eluted from the beads with Laemmli loading buffer, and subjected to SDS-PAGE. The separated proteins were transferred to Hybond-P membrane (Amersham Bioscience) and probed with a goat anti-αII spectrin antibody (C-20; Santa Cruz Biotechnology). Bands were detected with a peroxidase-conjugated donkey antibody against goat IgG (Santa Cruz Biotechnology), and visualized using the enhanced chemiluminescence (ECL) system (Amersham Bioscience).

For detection of ROCK, M1-CHO cells grown in 6-well culture dishes (80-90% confluent) were washed, stimulated with 10 μM oxotremorine-M (Oxo-M, RBI) and directly lysed in 50 μl of 2×SDS buffer, and 20 μl of each samples were applied to SDS-PAGE. Protein was transferred to Hybond-P membrane and probed with a mouse anti-ROCK antibody (clone 46 or 21, BD Biosciences), that recognizes both the full-length and the C-terminally cleaved form of ROCK.

Immunofluorescence

M1-CHO cells, grown on poly-L-lysine-coated 10 mm coverslips, were transiently cotransfected with a pEYFP-αII spectrin and a pCMV.Myc.Gα_q construct, or a plasmid containing CD8 by using FuGene 6 (Roche Applied Science). After 24 hours, cells were washed with PBS, fixed in 4% paraformaldehyde, permeabilized in PBS/0.1% Triton X-100, then incubated in a rabbit anti-Myc antibody or a mouse anti-CD8 antibody (UCHT-4, Sigma-Aldrich) for 4 hours. For CD8 staining, the permeabilization step was omitted. Following washes with PBS, cells were incubated in a Cy3-conjugated donkey antibody against rabbit IgG, or against mouse IgG for 2 hours. M1-CHO cells transiently expressing Flag-tagged constitutively active ROCK-II or Myc-tagged RhoA V14 mutants were fixed immediately after washes with PBS without stimulation of M1 mAChRs. M1-CHO cells transiently expressing Flag-tagged kinase-dead ROCK-II or Myc-tagged RhoA N19 mutants were washed and stimulated similarly to the conditions used for live imaging, then fixed immediately after stimulation. Cells were incubated with mouse antibodies against the Flag tag (M2, Sigma-Aldrich) or the Myc tag (9E10, Santa Cruz Biotechnology), followed by incubation with a Cy3-conjugated donkey antibody against mouse IgG. All conjugates were from Jackson ImmunoResearch. After washing with PBS, coverslips were mounted with Vectashield (Vector Laboratories), and fluorescence was examined with an Olympus IX-70 inverted microscope (60× oil immersion lens). Three dimensional data sets of representative cells were collected by using Delta Vision System (Applied Precision), subsequently deconvolved and projected onto a single plate.

Live imaging and $[Ca^{2+}]$i imaging

M1-CHO cells were grown on coverslips adapted as 500 μl perfusion chambers, and transiently transfected with a pEYFP-αII spectrin using FuGene 6. After 24 hours, cells in the chamber were placed on the stage of Nikon Diaphot inverted fluorescent microscope (40× oil immersion lens), and washed in the Hepes buffer pH 7.4 (10 mM Hepes, 130 mM NaCl, 2.5 mM $CaCl_2$, 3 mM KCl, 1.5 mM $MgCl_2$ and 11 mM glucose) until stabilized. Cells were then stimulated with 10 μM Oxo-M for 3-5 minutes. Alternatively, cells were exposed to 5 μM ionomycin (Sigma-Aldrich), 0.1 μM thapsigargin and 30 μM 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) for 3 minutes, 3-5 minutes and 13 minutes, respectively. All procedures were performed at a flow rate of 10 ml/minute at room temperature. Fluorescence images were captured and analyzed with OpenLab software 3.09 (Improvision), which also controlled a Hamamatsu C4880 CCD greyscale camera, a monochromator (TILL photonics) and optical shutters. Some images were subjected to a nearest-neighbor digital deconvolution-algorithm to remove optical bluring.

For inhibition studies, cells were preincubated in Hepes buffer containing the following compounds: 2-aminoethyl diphenylborinate (80 μM), cytochalasin D (10 μM), KN-93 (10 μM), nocodazole (32 μM), PD98059 (25 μM), PP1 (20 μM), staurosporine (0.1 μM), U73122 and U73343 (5 μM, Affinity Research Products), wortmannin (0.1 μM), xestospongin C (3 μM), Y-27632 (10 μM). None of compounds at the indicated concentration caused redistribution of spectrin within a period of 90 minutes. Stimulation of cells with Oxo-M was carried out in the presence of the inhibitor. Concentrations of inhibitors and preincubation time (15-30 minutes, depending on the compound) tested here are in a range used in the studies for the GPCR signaling (Torgerson and McNiven, 1998; Delmas et al., 2002; Chodniewicz and Zhelev, 2003; Davidson et al., 2004; Mundell et al., 2004) or the Ins(1,4,5)P_3R signaling pathway (Bishara et al., 2002). All compounds were purchased from Calbiochem or Sigma-Aldrich unless indicated otherwise. Hypotonic and isotonic solutions were based on Hepes buffer (5 mM Hepes pH 7.4, 50 mM NaCl, 2.5 mM $CaCl_2$, 6 mM KCl, 1.5 mM $MgCl_2$, and 10 mM glucose) containing 20 mM and 170 mM mannitol, respectively.

For $[Ca^{2+}]$i imaging, cells were loaded with 5 μM Fura-2AM for 60 minutes at room temperature, and incubated in Hepes buffer until stabilized. Fura-2-loaded cells were excited alternately at 340 nm and 380 nm and ratios of the resulting images (340/380 nm) were captured every 5 seconds. Fluorescence emission was monitored at 510 nm using a Hamamatsu C4880 CCD greyscale camera, and analyzed using OpenLab software 3.09. Free $[Ca^{2+}]$i was calculated from the ratio of 340 nm to 380 nm fluorescence following the method of Grynkiewicz (Grynkiewicz et al., 1985).

We thank Thomas Leung and Romano Regazzi for providing constructs used in this study. This work was supported by Wellcome Trust Programme grant 038170, and by grants from the National Institutes of Health (J.M.S.).

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