A₁ adenosine receptor–stimulated exocytosis in bladder umbrella cells requires phosphorylation of ADAM17 Ser-811 and EGF receptor transactivation

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ABSTRACT Despite the importance of ADAM17-dependent cleavage in normal biology and disease, the physiological cues that trigger its activity, the effector pathways that promote its function, and the mechanisms that control its activity, particularly the role of phosphorylation, remain unresolved. Using native bladder epithelium, in some cases transduced with adenoviruses encoding small interfering RNA, we observe that stimulation of apically localized A₁ adenosine receptors (A₁ARs) triggers a Gᵢₒ-phospholipase C-protein kinase C (PKC) cascade that promotes ADAM17-dependent HB-EGF cleavage, EGFR transactivation, and apical exocytosis. We further show that the cytoplasmic tail of rat ADAM17 contains a conserved serine residue at position 811, which resides in a canonical PKC phosphorylation site, and is phosphorylated in response to A₁AR activation. Preventing this phosphorylation event by expression of a nonphosphorylatable ADAM17S811A mutant or expression of a tail-minus construct inhibits A₁AR-stimulated, ADAM17-dependent HB-EGF cleavage. Furthermore, expression of ADAM17S811A in bladder tissues impairs A₁AR-induced apical exocytosis. We conclude that adenosine-stimulated exocytosis requires PKC- and ADAM17-dependent EGFR transactivation and that the function of ADAM17 in this pathway depends on the phosphorylation state of Ser-811 in its cytoplasmic domain.

INTRODUCTION Protein ectodomain shedding, a process regulated by proteolysis, is a fundamental mechanism for the release of cytokines, growth factors, and cell adhesion molecules (Reiss and Saftig, 2009) and is altered in cancer, autoimmune and inflammatory diseases, cardiovascular disease, and neurodegeneration (Murphy, 2008). The best-understood sheddases include the “a disintegrin and a metalloproteinase” (ADAM) family members ADAM10 and ADAM17 (also known as TACE), both of which shed a variety of substrates, including the transmembrane ligands for the epidermal growth factor receptor (EGFR). Whereas ADAM10 targets betacellulin, EGF, and neuregulin, ADAM17 is the principal sheddase for transforming growth factor (TGF) α, amphiregulin, epiregulin, epigen, and heparin-binding (HB) EGF (Jackson et al., 2003; Sahin et al., 2004; Sahin and Blobel, 2007; Blobel, 2005; Horiuchi et al., 2005; Sternlicht et al., 2005; Hassemer et al., 2010; Luo et al., 2011). The physiological cues that trigger shedding remain to be specified; however, ADAM17-elicted shedding occurs in response to some G protein-coupled receptor (GPCR) ligands as well as treatment with ionomycin or phorbol-12-myristate-13-acetate (PMA), a diacylglycerol (DAG) mimic and activator of classical protein kinase C (PKC) isoforms (Horiuchi et al., 2007; Le Gall et al., 2009; Dang et al., 2011).
In contrast, ADAM10-dependent shedding responds to Ca\textsuperscript{2+} ionophores (e.g., ionomycin) but generally not PMA (Horiiuchi et al., 2007; Le Gall et al., 2009; Dang et al., 2011). In addition, our understanding of the signaling and associated effector pathways that act downstream of these stimuli remains incomplete. The extracellular signal-regulated kinase (ERK), as well as the related p38 mitogen-activated protein kinase (MAPK), PKC\textalpha, PKC\textbeta, and the PKC-regulated protein phosphatase inhibitor 14D, has also been linked to ADAM17-dependent shedding, and PKC\textbeta is required for shedding of neutregulin (Bell and Gooz, 2010; Killick and Ioveti, 2010; Dang et al., 2011, 2013; Hall and Blobel, 2012).

An additional unresolved question is how these effectors promote ADAM-dependent shedding of EGFR ligands. Data indicate that they work through multiple mechanisms and perhaps in a cell-and stimulus-dependent manner (Fan et al., 2003; Mifune et al., 2005; Xu and Derynck, 2010). Potential regulatory steps include membrane trafficking of the ADAM or its ligands (Soond et al., 2005), effects on the ADAM17 dimer–monomer equilibrium, and association with tissue inhibitor of metalloproteinases (TIMP) 3 (Xu et al., 2012) or changes in the redox potential of the extracellular protein disulfide isomerase (Willems et al., 2010), which is hypothesized to alter ADAM17 activity by rearrangement of its disulfide bonds. An additional regulatory mechanism may be phosphorylation of EGFR receptor substrates (Dang et al., 2013) or the ADAM metalloproteinase itself. For example, phosphorylation of ADAM17 cytoplasmic residues Tyr-702, Ser-791, Ser-819, and Thr-735 have been reported (Fan et al., 2003; Hall and Blobel, 2012; Niu et al., 2013). Tyr-702 is reportedly phosphorylated by the Src kinase when myogenic precursor cells are stretched (Niu et al., 2013). In contrast, Ser-791 is phosphorylated before stimulation, and mutations of Ser-819 do not appear to affect shedding (Fan et al., 2003). In the case of Thr-735, very high doses of PMA (1 \mu M) are reported to promote ERK-dependent phosphorylation of this residue (Soond et al., 2005). However, others report that phosphorylation of this residue is mediated by the p38 MAPK and is required for ADAM17-dependent shedding in response to various forms of stress but not PMA (Xu and Derynck, 2010). A significant argument against the role for phosphorylation relies on reports that show that stimulus-evoked shedding of ADAM17 occurs in cells expressing truncated versions of this metalloproteinase that lack a C-terminus (Reddy et al., 2000; Le Gall et al., 2010; Hall and Blobel, 2012).

A potentially useful and physiologically relevant model system in which to study the signals, effector pathways, and mode of ADAM activation is the uroepithelium, a tissue that can be studied both ex vivo and in vivo (Khandelwal et al., 2008, 2010, 2013). Bladder filling triggers the exocytosis of an abundant subapical pool of discoidal- and/or fusiform-shaped vesicle (DFVs) in the outer umbrella cell layer (Wang et al., 2003a,b). Stretch-induced exocytosis progresses in two phases. “Early-phase” exocytosis occurs during bladder filling, as the epithelium is bowing outward, and is triggered by apical Ca\textsuperscript{2+} entry, likely conducted by a nonelective cation channel (Yu et al., 2009). In contrast, “late-phase” exocytosis is initiated once the tissue is maximally bowed outward (i.e., in response to a full bladder) and requires metalloproteinase-dependent cleavage of HB-EGF, leading to “transactivation” of apical EGFRs (Balestreire and Apodaca, 2007). The latter initiates a downstream ERK pathway that culminates in protein synthesis and exocytosis (Balestreire and Apodaca, 2007). We now report that adenosine, which is released from the epithelium in response to stress (Prakasam et al., 2012), spurs a late phase–like response in umbrella cells. We also find that a G\textsubscript{q/11}, G\textsubscript{\beta\gamma}, phospholipase C (PLC), and PKC-dependent effector cascade is initiated downstream of the adenosine A\textsubscript{1} receptor (A\textsubscript{1}AR). Finally, PKC likely promotes the phosphorylation of a previously unreported canonical PKC site centered at Ser-811 in the cytoplasmic domain of rat ADAM17. This phosphorylation event is required for adenosine-induced and ADAM17-dependent HB-EGF cleavage, EGFR transactivation, and exocytosis. These studies lend support to the hypothesis that posttranslational modification of ADAM17—phosphorylation in particular—can control its activity in a physiologically relevant setting.

**RESULTS**

A\textsubscript{1}AR-stimulated apical exocytosis occurs through transactivation of the EGFR

Late-phase exocytosis is triggered when uroepithelial tissue is maximally stretched (Balestreire and Apodaca, 2007; Yu et al., 2009), but it is unknown whether other stimuli promote a similar response. Because adenosine release is significantly increased as the bladder reaches its capacity (Prakasam et al., 2012), and because extracellular adenosine also triggers exocytosis (Yu et al., 2006), we tested the hypothesis that adenosine stimulates exocytosis by way of EGFR transactivation. Adenosine can stimulate exocytosis when added to either the serosal or mucosal surfaces of isolated uroepithelium; however, in this study, we focused our attention on the mucosal events, as these are primarily mediated by the A\textsubscript{1}AR, and EGFR transactivation occurs at this surface (Yu et al., 2006; Balestreire and Apodaca, 2007). Consistent with our previous reports (Yu et al., 2011; Prakasam et al., 2012), the A\textsubscript{1}AR was localized to the apical pole of rat umbrella cells (as well as in the underlying lamina propria; Figure 1A). When adenosine was added to the mucosal surface of isolated rabbit tissue, it stimulated increased tissue capacitance (C\textsubscript{T}; 1 \mu F = 1 cm\textsuperscript{2}; Figure 1B), which in this tissue correlates well with other measures of apical exocytosis (Truschel et al., 2002; Wang et al., 2003a). Similar results were obtained when the highly selective and high-affinity A\textsubscript{1}AR agonist Z-chloro-N6-cyclopentyladenosine (CCPA) was used. Unlike adenosine, CCPA is not rapidly converted to inosine or to AMP (Manjunath and Sakhare, 2009; Prakasam et al., 2012) and was thus used in our subsequent studies. We next determined whether A\textsubscript{1}AR-stimulated exocytosis, like that observed in response to excess stretch, is dependent on protein synthesis or secretion. Indeed, treatment with cycloheximide or brefeldin A significantly blocked CCPA-mediated exocytosis (Figure 1C). The effect of the latter was particularly pronounced.

We also assessed whether CCPA triggered apical exocytosis by transactivating the EGFR. We first tested the effect of treating the mucosal surface of the tissue with CRM197, a mutant version of diphtheria toxin that binds selectively and with high affinity to membrane-bound HB-EGF and prevents its cleavage (Uchida et al., 1973). Indeed, we observed that CRM197 almost completely blocked CCPA-mediated apical exocytosis (Figure 2A). Next we pretreated tissue with AG1478, a small-molecule inhibitor of EGFR, which also significantly blocked CCPA-mediated apical exocytosis (Figure 2B). As further evidence that the EGFR was transactivated, we generated lysates from CCPA-treated epithelium and then probed Western blots with an antibody that detects phosphorylation of the EGFR at Tyr-1173. This residue promotes assembly of a MAPK signaling cascade downstream of other GPCRs (Balestreire and Apodaca, 2007). Phosphorylation of Tyr-1173 was maximally stimulated 10 min after continuous treatment with CCPA, decreased at 30 min posttreatment, and returned to control levels by 60 min (Figure 2C and D). The Tyr-1173 phosphorylation was prevented when the tissue was pretreated with the EGFR inhibitor AG1478 before CCPA treatment (Figure 2C and D), confirming that phosphorylation of Tyr-1173 likely results from autophosphorylation.
Finally, we observed that U0126, an inhibitor of MEK activity, also caused a significant decrease in CCPA-stimulated changes in $C_T$ (Figure 2A).

Taken together, these data indicate that, like the previously described late-phase response (Balestreire and Apodaca, 2007), $A_1$AR activation stimulates apical exocytosis by promoting transactivation of the EGFR downstream of HB-EGF cleavage, leading to MEK/ERK activation and protein synthesis.

$A_1$AR is localized to the apical surface of umbrella cells, where it stimulates CCPA-induced EGFR transactivation and exocytosis

In vivo studies implicate ADAM17 as the physiologically relevant sheddase in HB-EGF-mediated transactivation (Jackson et al., 2003; Sahin et al., 2004). Consistent with these observations, we observed that ADAM17 was localized to small vesicular elements under the apical surface of the rat umbrella cells (Figure 3A), the likely site of HB-EGF cleavage and EGFR transactivation during the late-phase response (Balestreire and Apodaca, 2007). Rat tissues were used in these experiments because our antibody was produced in rabbits. ADAM17 was also detected in the intermediate and basal cell layers of the uroepithelium, as well as in cells in the underlying lamina propria (Figure 3A, A and C). The signal for ADAM17 was diminished when the anti-ADAM17 antibody was preincubated with immunizing peptide, confirming the specificity of the antibody (Figure 3B).

ADAM17 showed a high degree of colocalization with uroplakin 3a (Manders coefficient of colocalization, 0.92); this is associated with exocytosis in the umbrella cells, and expression of ADAM17 in the underling cell layers was largely undisturbed (Figure 4C).

Next we used ADAM17-specific shRNAs to test whether ADAM17 was required for CCPA-stimulated EGFR transactivation and exocytosis in rat epithelium. Treatment with ADAM17-shRNA, but not scrambled shRNA, decreased EGFR phosphorylation (Figure 4, D and E). We show later in Figure 6, F and G, that the effects of the ADAM17-shRNA are specific, and ADAM17 function is restored when the shRNA is coexpressed with shRNA-resistant variant of wild-type ADAM17. We also attempted to measure changes in $C_T$ in the rat bladders treated with apical CPA; however, we did not observe a response. Because $C_T$ is dependent on the rates of membrane addition and removal, we reasoned that one possible explanation was that CCPA stimulated both exocytosis and endocytosis in rat epithelium, thus obviating any change in apparent $C_T$. Indeed, we observed that wheat germ agglutinin–fluorescein isothiocyanate (WGA-FITC), added to the apical hemichamber of mounted rat bladders, was endocytosed in CCPA-treated tissue but much less so in control, untreated tissue (Figure 4F). To circumvent the effects of endocytosis, we instead measured release of exogenously expressed human growth hormone (hGH), which we and others previously showed is endocytosed. Compared to control bladders, CCPA stimulated a large increase in the mucosal release of hGH from the tissue (Figure 4, G and H). Moreover, expression of ADAM17-specific shRNA, but

DFVs and the apical surface of umbrella cells (Figure 3C, Apodaca, 2004). Consistent with a role for ADAM17 in DFV trafficking, we observed that the broad-spectrum metalloproteinase inhibitor GM6001 and the ADAM17-selective inhibitor Tapi-2 (Balestreire and Apodaca, 2007; Kveiborg et al., 2011) both significantly inhibited the CCPA-mediated increases in $C_T$ (Figure 3D). Moreover, Tapi-2 blocked CCPA-mediated phosphorylation of EGFR Tyr-1173 (Figure 2, C and D).

To provide further evidence that ADAM17 is required for EGFR transactivation, we exploited our previously described in situ viral transduction approach (Khandelwal et al., 2008, 2010), in this case to express ADAM17-specific short hairpin RNAs (shRNAs) or scrambled shRNAs in the rat bladder uroepithelium. We used rat bladders in these studies because the volume capacity of the bladder, and therefore the number of virus particles needed, was relatively small (~500 μl) compared with the volumes required to fill the rabbit bladder (~60–100 ml). This technique targets the umbrella cell layer and achieves transduction efficiencies of 70–95% (Khandelwal et al., 2008, 2010). Indeed, we were able to achieve >90% knockdown of ADAM17 expression (Figure 4, A and B). On examining the expression and distribution of ADAM17 in cross sections of uroepithelium, we observed that knockdown of ADAM17 was largely complete.
not scrambled shRNA, caused a large inhibition (>90%) in mucosal hGH release (Figure 4, G and H).

Finally, because stretch-mediated, late-phase exocytosis is dependent on EGFR transactivation and sensitive to metalloproteinase inhibitors (Balestreire and Apodaca, 2007), we determined whether ADAM17 also played a role in stretch-mediated exocytosis. We observed that shRNA-mediated ADAM17 knockdown significantly reduced the stretch-induced apical release of hGH by ~80% compared with tissues transduced with scrambled shRNA (Figure 4, G and H). Taken together, our results provide strong evidence that in rat tissues, ADAM17 is critical for CCPA- and stretch-induced EGFR transactivation and exocytosis.

**Gβ, PLC, and PKC act upstream of ADAM17 and HB-EGF to promote A1AR-mediated EGFR transactivation**

We next addressed how ADAM17 activity is coupled to A1AR activation. Previous studies showed that A1AR signals through Gq to inhibit the activity of adenyl cyclase, whereas the βγ subunits of Gq increase the activity of phospholipase C-β (PLCβ), which hydrolyzes phosphatidylinositol 4,5-bisphosphate to generate inositol trisphosphate (IP3) and diacylglycerol (Freund et al., 1994; Buehler and Linden, 2004; Chang et al., 2008). The latter stimulates the activity of PKC, a well-known regulatory kinase that was previously implicated in ADAM17 activation (Dang et al., 2011; Kveiborg et al., 2011; Lemjabbar-Alaoui et al., 2011). We found that pertussis toxin–mediated inhibition of Gq or inhibition of Gβγ subunit activity by the inhibitor M119K (Kirui et al., 2010) significantly impaired CCPA-mediated apical exocytosis in rabbit bladder umbrella cells (Figure 5A). Furthermore, the PLC-selective antagonist U73122 caused marked inhibition of CCPA-induced changes in Cyt (Figure 5A). Thus ADAM17 activation downstream of A1AR likely occurs by way of a classical Gq-stimulated signaling cascade involving Gβγ and PLCβ.

Next we examined whether PKC is important for A1AR-dependent EGFR transactivation and exocytosis. Strikingly, the PKC inhibitor calphostin C caused a marked decrease in CCPA-stimulated changes in Cyt and EGFR transactivation (Figures 2, C and D, and 5B). In contrast, treatment with PMA, an activator of classical PKCs (Nishizuka, 1992), caused robust stimulation of exocytosis in the absence of CCPA (Figure 5B). Of interest, the kinetics of PMA-mediated exocytosis were faster than those mediated by CCPA, particularly during the first 30 min, and then appeared to increase at a similar rate to CCPA-treated tissue. This may indicate that PKC stimulates not only late-phase-like responses, but perhaps early-phase ones as well. To confirm that PMA acted by way of ADAM17, we treated the tissue with Tapi-2, which significantly inhibited PMA-mediated apical exocytosis (Figure 5C). We also observed that AG1478 impaired PMA-mediated exocytosis (Figure 5C). Our data pointed to the possibility that PKC acted upstream of ADAM17, which acted before HB-EGF cleavage. We reasoned, therefore, that the calphostin C–or TAPI-2–mediated inhibition of Cyt should be relieved by addition of HB-EGF. To test this possibility, we pretreated...
requires Gi, Gβγ, PLC, PKC, and ADAM17, and these effectors likely act upstream of HB-EGF release and EGFR transactivation.

CCPA-stimulated HB-EGF shedding and exocytosis are dependent on phosphorylation of ADAM17 Ser-811
To explore how PKC might act to stimulate ADAM17 activity, we compared the amino acid sequences from multiple vertebrate species using the proteomics tool Scansite and identified a conserved, canonical PKC phosphorylation motif (X-R/K-X-S/T-X-R/K; Pearson and Kemp, 1991; Nishikawa et al., 1997) in the cytoplasmic tail of the protein centered at Ser-811 (equivalent to Ser-808 in the human protein; Figure 6A). To our knowledge, a functional role for this residue was not explored previously. We first confirmed that ADAM17 was phosphorylated in response to CCPA by expressing the A1AR in combination with epitope-tagged ADAM17-HA (this variant of ADAM17 is resistant to shRNA; see Figure 6F) in 32P-orthophosphate-labeled HEK cells (Figure 6B). HEK cells were used in these assays because these experiments were difficult to perform in whole tissues. On addition of CCPA, we observed that phosphorylation of both the “immature” proform of ADAM17-HA (∼120 kDa) and the “mature” cleaved form of the enzyme (∼93 kDa) was stimulated approximately threefold (Figure 6, B and C). In contrast, CCPA had no significant effect when wild-type ADAM17-HA was substituted with a nonphosphorylatable mutant of ADAM17 in which Ser-811 was changed to an Ala residue (ADAM17S811A-HA; Figure 6, B and C). These results indicate that in response to A1AR activation, Ser-811 may be a major site of ADAM17 phosphorylation.

Because the physiological role of ADAM17 phosphorylation is a matter of some controversy (Reddy et al., 2000; Horiuchi et al., 2007; Le Gall et al., 2010; Dang et al., 2013), we next determined whether phosphorylation of Ser-811 was biologically relevant. First, we measured shedding of a chimeric protein in which alkaline phosphatase (AP) was fused to the extracellular domain of HB-EGF (HB-EGF-AP; Sahin et al., 2004; Uttarwar et al., 2011), an ADAM17 substrate that was coexpressed in HEK cells along with the A1AR and ADAM17-HA (Figure 6D). In response to CCPA, we observed a significant ∼70% increase in HB-EGF-AP release. In contrast, when we substituted ADAM17-HA with ADAM17S811A-HA, there was no significant increase in CCPA-stimulated HB-EGF-AP release. We also tested a mutant in which Ser-811 was mutated to an Asp residue (ADAM17S811D-HA). In the absence of CCPA, this variant did not significantly affect constitutive cells with calphostin C or TAPI-2, added CCPA at t = 0, and then added HB-EGF 2 h later (Figure 5, D and E). In both cases, we observed that HB-EGF significantly stimulated exocytosis, even in the presence of the PKC or ADAM17 inhibitor. Together our data are consistent with a model in which A1AR-mediated transactivation EGFR-AP release. In contrast, when we substituted ADAM17-HA with ADAM17S811A-HA, there was no significant increase in CCPA-stimulated HB-EGF-AP release. We also tested a mutant in which Ser-811 was mutated to an Asp residue (ADAM17S811D-HA). In the absence of CCPA, this variant did not significantly affect constitutive

FIGURE 3: ADAM17 expression in the uroepithelium. (A–C) Cryosections of rat bladder uroepithelium were reacted with antibodies specific for ADAM17 (A), a mixture of antibody and inhibitory peptide (B), or antibodies specific for ADAM17 and uroplakin 3a (C). After incubation with fluorophore-labeled secondary antibodies, the samples were analyzed using confocal microscopy. Where indicated, actin was labeled with phalloidin and nuclei were labeled with TOPRO-3. The umbrella cells are marked with the asterisk in the merged images and the apicolateral junction is indicated by arrowheads; scale bar, 10 μm. (D) Rabbit uroepithelium was pretreated with Tapi-2 (15 μM) or GM6001 (15 μM) for 90 min, CCPA (500 nM) was added, and C_T was recorded. Control CCPA data are reproduced from Figure 1B. Data are mean ± SEM (n ≥ 3), and values significantly different (p < 0.05) from CCPA alone are marked with an asterisk.
**FIGURE 4:** A1AR-stimulated exocytosis is dependent on ADAM17. (A) Uroepithelial lysates obtained from rat tissues transduced in situ with scrambled shRNA or ADAM17 shRNA were resolved by SDS–PAGE and Western blots probed with rabbit-anti ADAM17 antibody (top) or mouse anti-β-actin (bottom). (B) Quantification of ADAM17 expression in rat tissue treated with scrambled shRNA or ADAM17 shRNA. Data are mean ± SEM (n ≥ 4). The means of the two treatment groups are significantly different (p < 0.05). (C) Immunolocalization of ADAM17 expression (red) in rat tissue treated with scrambled or ADAM17-specific shRNAs. Nuclei (blue) are labeled with TOPRO-3. Scale bar, 13 μm. The cell junctions are marked with arrows and the umbrella cells with an asterisk. (D) CCPA (500 nM) was added to rat tissue treated with scrambled shRNA or ADAM17-specific shRNA. Data are mean ± SEM (n ≥ 4). The two treatment groups were significantly different (p < 0.05). (E) Quantification of effects of scrambled or ADAM17-specific shRNA treatment on EGFR activation. Data are mean ± SEM (n = 4). The two treatment groups were significantly different (p < 0.05). (F) WGA-FITC (50 μg/ml) was added to the mucosal hemichamber of untreated rat tissue (control) or that treated with 500 nM CCPA. After 120 min, the tissue was incubated at 4°C with N-acetylglucosamine to remove surface lectin, fixed, and then processed for immunofluorescence. In these whole-mounted tissues, internalized WGA-FITC is shown in green, phalloidin-labeled actin in red, and TOPRO-3-labeled nuclei in blue. Bar, 15 μm. Quantification of the fluorescence intensity of WGA-FITC below the apical membrane. Data are mean ± SEM (n ≥ 8). The two treatment groups were significantly different (p < 0.05). (G) Rat tissues were transduced with hGH alone (control) or transduced with hGH and either scrambled or ADAM17 shRNAs. The excised bladder tissue was mounted in an Ussing stretch chamber and left untreated (control), treated with CCPA (500 nM), or stretched by filling the mucosal hemichamber. The mucosal fluid was collected and concentrated, the tissues were lysed, and hGH was detected in the secreted fraction (1/20 of total) or tissue lysates (1/13 of total) using Western blot. (H) Quantification of effects of ADAM17-specific shRNAs on hGH secretion. Data are mean ± SEM (n ≥ 3). Statistically significant effects (p < 0.05) are indicated with an asterisk.
DISCUSSION

ADAM17 is expressed in a variety of tissues, including heart, lungs, brain, kidney, skeletal muscles, and the bladder (Gooz, 2010), and there exists a large body of evidence that implicates it in normal biological phenomena (e.g., migration, adhesion, differentiation), as well as in numerous pathologies (e.g., inflammation, multiple sclerosis, diabetes, and kidney disorders; Plumb et al., 2006; Shah and Catt, 2006; Serino et al., 2007; Arribas and Esselens, 2009). None-etheless, we still lack sufficient insight into the physiological cues that stimulate ADAM17 activity, the upstream signaling pathways that promote ADAM17 activation, or the mechanisms that control ADAM17-dependent sheddase activity. Our studies show that 1) like stretch, the A<sub>1</sub>AR stimulates umbrella cell exocytosis by way of EGFR transactivation and ADAM17 is the physiologically relevant protease; 2) an A<sub>1</sub>AR → G<sub>αi</sub> → G<sub>βγ</sub> → PLC → PKC signaling cascade likely acts upstream of ADAM17 to promote HB-EGF cleavage; and 3) phosphorylation of Ser-811 in the cytoplasmic domain of ADAM17 appears to be required for A<sub>1</sub>AR-stimulated HB-EGF shedding and exocytosis.

A<sub>1</sub>AR-mediated exocytosis requires ADAM17 and EGFR transactivation

Similar to the effects of maximal stretch (Balestreire and Apodaca, 2007), we found that activation of the A<sub>1</sub>AR stimulated a slow and gradual increase in apical exocytosis, which required metalloproteinase-dependent HB-EGF cleavage, EGFR transactivation, protein synthesis, and secretion. Although these two stimuli appear dissimilar at first blush, adenosine is released from the uroepithelium in response to maximal stretch (Yu et al., 2006; Prakasam et al., 2012) and can function as a stress hormone in some settings (Fredholm et al., 2001; Hasko et al., 2008; Wilson and Mustafa, 2009). Thus EGFR transactivation may be a common pathway the uroepithelium uses to cope with stressful stimuli. A critical step in transactivation is the cleavage of EGFR substrates by a metalloproteinase, whose identity in umbrella cells was previously unknown. We focused our studies on ADAM17 in part because it is the major sheddase for several EGFR ligands, including HB-EGF (Sahin et al., 2004; Blobel, 2005; Hassemer et al., 2010). In addition, we found that in umbrella cells, ADAM17 was localized to uroplakin 3a–positive DFVs, the major apically directed vesicle population in these cells. Of importance, these vesicles position ADAM17 at or near the apical surface of the umbrella cells, which we previously showed is a primary site for EGFR substrates and HB-EGF-dependent receptor transactivation (Balestreire and Apodaca, 2007). Furthermore, our use of adenovirally mediated expression of shRNAs allowed us to significantly decrease ADAM17 expression in the uroepithelium, which caused >90% decrease in CCPA-induced hGH release. Although we cannot rule out a role for other ADAMs in A<sub>1</sub>AR-mediated exocytosis, the knockdown of ADAM17 in rat umbrella cells was sufficient to block the majority of A<sub>1</sub>AR- or stretch-stimulated exocytosis in rat uroepithelium.
PKC may promote HB-EGF cleavage and apical exocytosis by phosphorylation of Ser-811 in the cytoplasmic domain of ADAM17.

An important but unresolved question concerns the mechanism(s) by which upstream stimuli promote ADAM17-dependent function. One possible mechanism is phosphorylation, and several cytoplasmic targets have been described, including Tyr-702, Ser-791, Ser-829, and Thr-735 (Fan et al., 2003; Soond et al., 2005; Niu et al., 2013). However, other reports show that ADAM17 lacking its cytoplasmic domain can still trigger stimulus-evoked shedding and EGFR transactivation (Doedens and Black, 2000; Black et al., 2003; Doedens et al., 2003; Le Gall et al., 2010), a finding seemingly at odds with any role for phosphorylation in ADAM17 activation.

We observed that ADAM17 contains a canonical PKC phosphorylation site (which includes Ser-811) that was phosphorylated in response to A1AR activation. Although we cannot completely rule out that PKC acts upstream of a downstream kinase, as reported for ERK-dependent phosphorylation of Thr-735 (Diaz-Rodriguez et al., 2002), the most straightforward interpretation of our data is that PKC phosphorylates the residue directly. We further showed that the nonphosphorylatable mutant ADAM17S811A blocked CCPA-induced HB-EGF shedding and hGH secretion. In contrast, ADAM17S811D (which also cannot be phosphorylated at Ser-811) was able to promote CCPA-stimulated HB-EGF cleavage and hGH release. However, its inability to stimulate constitutive HB-EGF cleavage likely indicates that phosphorylation of Ser-811 is necessary but not sufficient to promote A1AR-induced events. Finally, we observed that a “tail-minus” construct was unable to stimulate CCPA-induced HB-EGF-AP release. Thus our results are inconsistent with the hypothesis that ADAM17 functions independently of its cytoplasmic domain and phosphorylation. However, it is possible that the mechanism(s) of ADAM17 activation may depend on the stimulus, cell type, or growth conditions. Furthermore, some stimuli may primarily work by directly affecting ADAM17 activity, whereas other stimuli may work through other mechanisms that affect the traffic and/or “activation” of ADAM17 ligands (e.g., Dang et al., 2013).

If phosphorylation of Ser-811 is physiologically relevant, then how might it act? One possibility is that phosphorylation of this residue alters the conformation of ADAM17, thus promoting its activity. This could be a direct effect on ADAM17 or might be indirect; for example, phosphorylation could act by modifying the association of this proteinase with TIMP3, which may modulate ADAM17 activity (Amour et al., 1998; Kwak et al., 2009; Xu et al., 2012). An additional possibility is that phosphorylation or Ser-811 forms a docking site for other proteins to bind and trigger ADAM17 activation or the association of ADAM17 with its substrates. Potential interacting proteins include MAD-2, a component of mitotic spindle assembly (Nelson et al., 1999), the protein tyrosine phosphatase-H1 (PTPH1), and SAP9. These last two interact with the COOH terminal of ADAM17 and negatively regulate its function (Zheng et al., 2002; Peiretti et al., 2003). Other potential interacting partners include Eve-1 (Tanaka et al., 2004) and the N-arginine dibasic convertase (nardilysin), which interacts with both HB-EGF and ADAM17 and regulates cleavage of the latter (Nishi et al., 2006). Obviously, more work is needed to understand the mechanisms by which ADAM17 activity is regulated.

In summary, we propose the following model for the role of ADAM17 and the role of Ser-811 phosphorylation in A1AR-stimulated exocytosis in umbrella cells. In the quiescent state, before adenosine stimulation (or stretch, in the case of rat umbrella cells), ADAM17 at the apical surface may be kept in its inactive, dimeric state by the inhibitory effects of TIMP3 (Figure 7A; Xu et al., 2012). As a result of its increased production (or decreased turnover), adenosine binds to the A1AR, triggering a Gβγ → PLC → DAG → PKC pathway, which leads to phosphorylation at Ser-811 in the cytoplasmic domain of ADAM17 (Figure 7B). This leads to activation of ADAM17, possibly by altering its conformation and/or its association with regulatory proteins (e.g., TIMP3) and/or its substrates. Although not shown, signaling pathways downstream of the A1AR are also likely to function by regulating other processes (e.g., “activation” of HB-EGF). This could explain why ADAM17S811D-HA was unable to stimulate HB-EGF release in the absence of CCPA stimulation. Finally, in its activated state, ADAM17 cleaves and releases HB-EGF, which binds to the EGFR, promoting autoporphosylation of EGFR Y1773, ultimately leading to downstream ERK1/2 activation, protein synthesis, and exocytosis (Figure 7B).

MATERIALS AND METHODS

Reagents and antibodies

Unless otherwise specified, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO) and were of reagent grade or better. Adenosine was freshly prepared and dissolved in Krebs buffer (110 mM NaCl, 5.8 mM KCl, 25 mM NaHCO3, 1.2 mM KH2PO4, 2.0 mM CaCl2, 1.2 mM MgSO4, 11.1 mM glucose, pH 7.4). The following stock solutions were prepared in dimethyl sulfoxide: AG1478 (10 mM), calphostin C (250 μM), CCPA (10 mM), GM6001 (15 mM), SB203580 (1 mM), Tapi-2 (15 mM; Tocris, Bristol, United Kingdom), and U0126 (10 mM). The following stocks were made in dimethyl sulfoxide: 11.1 mM glucose, pH 7.4. These following stock solutions were prepared in dimethyl sulfoxide: AG1478 (10 mM), calphostin C (250 μM), CCPA (10 mM), GM6001 (15 mM), SB203580 (1 mM), Tapi-2 (15 mM; Tocris, Bristol, United Kingdom), and U0126 (10 mM). The following stocks were made in dimethyl sulfoxide: AG1478 (10 mM), calphostin C (250 μM), CCPA (10 mM), GM6001 (15 mM), SB203580 (1 mM), Tapi-2 (15 mM; Tocris, Bristol, United Kingdom), and U0126 (10 mM). The following stocks were made in dimethyl sulfoxide: AG1478 (10 mM), calphostin C (250 μM), CCPA (10 mM), GM6001 (15 mM), SB203580 (1 mM), Tapi-2 (15 mM; Tocris, Bristol, United Kingdom), and U0126 (10 mM). The following stocks were made in dimethyl sulfoxide: AG1478 (10 mM), calphostin C (250 μM), CCPA (10 mM), GM6001 (15 mM), SB203580 (1 mM), Tapi-2 (15 mM; Tocris, Bristol, United Kingdom), and U0126 (10 mM).
FIGURE 6: Effect of Ser-811 phosphorylation on the function of ADAM17. (A) Top, alignment of the C-termini of ADAM17 from different species. Numbers indicate the amino acids involved. The canonical PKC phosphorylation site is shaded, and the position of the conserved Ser residue (Ser-811 in rat) is indicated in red. Bottom, domain structure of ADAM17. CR, cysteine-rich domain; C-tail, cytoplasmic domain; Dis, disintegrin domain; EL, EGF-like; MP, metalloproteinase domain; Pro, propeptide; TM, transmembrane domain. The consensus PKC phosphorylation motif is shown in an expanded view. Ser-811 is shaded, and the critical, flanking basic residues at the −3 and + 2 positions are marked with arrows. (B) HEK-293FT cells expressing the A1-AR in combination with ADAM17-HA or ADAM17S811A-HA were labeled with 32P-orthophosphate and then left untreated or treated with CCPA (500 nM). HA-tagged ADAM17
polyclonal anti-A1AR rabbit antibody was obtained from Abcam (ab82477; Cambridge, MA), anti-ADAM17 rabbit polyclonal antibody from EMD-Millipore (Billerica, MA), anti-EGFR and anti–EGFR-phospho-Y-1173 rabbit polyclonal antibodies from Cell Signaling Technology (Danvers, MA), anti-hemagglutinin (HA) rabbit polyclonal antibody from Covance (Princeton, NJ), and anti–HA-horseradish peroxidase (HRP) rabbit monoclonal antibody from Roche (Mannheim, Germany); fluorophore- or HRP-conjugated secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA), and tetramethylrhodamine isothiocyanate–labeled phalloidin and TO-PRO3 were from Molecular Probes/Invitrogen (Grand Island, NY). Mouse monoclonal anti–uroplakin 3a antibody was described previously (Truschel et al., 1999).

Animals
Animals used in this study were female New Zealand white rabbits (3–4 kg; Covance) and female Sprague Dawley rats (250–300 g; Harlan Laboratories, Indianapolis, IN). Rabbits were killed by intravenous injection of 300 mg of Buthensia D into the ear vein after the area was numbed using topical lidocaine ointment. After death, the bladders were rapidly excised and processed as described later. Rats were sedated by inhalation of isoflurane and kept under sedation during the adenoviral transduction procedure by constant inhalation of isoflurane. At the end of the procedure, the rats were allowed to revive. At 24 h after infection, the rats were killed by inhalation of 100% CO2, a thoracotomy was performed, and the bladder was excised. All animal studies were carried out with the approval of the University of Pittsburgh Animal Care and Use Committee.

Cell culture
HEK293FT cells were obtained from Invitrogen. A HEK cell variant that has flattened morphology and increased viral transduction, HEK293A cells (Invitrogen), was used to prepare ADAM17- or scrambled-shRNA viruses. The cells were grown in DMEM purchased from Corning Cellgro (Corning, NY) supplemented with 10% (vol/vol) fetal bovine serum (GE Healthcare Life Sciences, Pittsburgh, PA), 1% (vol/vol) MEM nonessential amino acids (Life Technologies, Grand Island, NY), 2 mM glutamate (Sigma-Aldrich), and 1% (vol/vol) penicillin/streptomycin (Lonza, Walkersville, MD). Cre8 cells were grown in DMEM purchased from Sigma-Aldrich and supplemented with 10% (vol/vol) defined fetal bovine serum and 1% (vol/vol) penicillin/streptomycin (Lonza, Walkersville, MD). HEK293 cells were grown in DMEM supplemented with 10% (vol/vol) fetal bovine serum and 1% (vol/vol) penicillin/streptomycin. When producing viruses, cells constructs were immunoprecipitated and Western blots probed with an anti–HA-HRP-conjugated secondary antibody to detect total ADAM17 (middle) and subsequently exposed to a PhosphorImager screen to detect 32P-labeled ADAM17 (top). Top A1AR was detected by Western blot (bottom). (C) Data (mean ± SEM; n = 3) were quantified, and values significantly different from ADAM17-HA+ CCPA are indicated with an asterisk. (D) HEK-293FT cells were cotransfected with the A1AR, HB-EGF-AP, and ADAM17-HA, ADAM17S811A-HA, or ADAM17S811D-HA. Fold stimulation of HB-EGF-AP release in CCPA-treated cells vs. control, untreated cells. Data are mean ± SEM, n = 5. Data significantly different from HB-EGF-AP alone are indicated with an asterisk. (E) HEK-293FT cells were cotransfected with the A1AR, HB-EGF-AP, and ADAM17-HA. Cells were pretreated with calphostin C (500 nM) for 60 min, and the fold stimulation of HB-EGF-AP release in CCPA-treated cells vs. control, untreated cells is reported. Data are mean ± SEM, n = 7. Values significantly different from the control, as assessed by ANOVA, are indicated (*p < 0.05). (F) Rat tissues were transduced in situ with hGH and ADAM17 shRNA alone or in combination with ADAM17-HA, ADAM17S811A-HA, or ADAM17S811D-HA. The excised bladder tissue was mounted in an Ussing stretch chamber, and CCPA (500 nM) was added to the mucosal hemichamber. After 60 min, the mucosal fluid was collected and concentrated, the tissues were lysed, and hGH detected using Western blot. (G) Quantification of hGH secretion. Data are mean ± SEM (n = 5). Values that are statistically different from ADAM17 shRNA alone (p < 0.05) are indicated with an asterisk.
were grown in their respective media but without penicillin/ streptomycin.

Immunofluorescence labeling, image acquisition, and colocalization analysis
Bladder tissue was fixed and processed as described previously (Truschel et al., 1999; Khandelwal et al., 2008). Briefly, tissues fixed with 4% PFA fixative (4% paraformaldehyde in 100 mM sodium cacodylate buffer, pH 7.4) were embedded in Optimal Cutting Temperature (OCT; Sakura Finetek, Torrance, CA) medium and frozen and stored at −80°C. Frozen sections, 4 μm in thickness, were obtained using a Leica CM1950 cryostat and adhered to Fisher brand Superfrost slides. The sections were washed three times for 5 min each with phosphate-buffered saline (PBS), and the fixation was stopped with quench buffer (20 mM glycine, 75 mM NH₄Cl, dissolved in PBS) for 10 min. When staining for ADAM17, the sections were quenched for 7 min and subsequently treated with quench buffer supplemented with 0.05% (wt/vol) SDS for an additional 3 min. After quenching, the tissue was then washed with PBS and transferred to block buffer (7% [wt/vol] fish gelatin, 0.25% saponin [vol/vol], and 0.05% Na₃ [wt/vol] made in PBS) for 1 h at room temperature. A 1:200 dilution of the antibodies was made in the block buffer, applied to the sections, and incubated at room temperature for either 2 h (in the case of ADAM17 antibody) or overnight at 4°C for the A1aR or UP3a antibodies. The primary antibody was removed by three 5-min washes with PBS and detected using Alexa 488- or Cy3–labeled secondary antibodies diluted in block buffer. Additional washes with PBS were performed. Actin and nuclei were stained with either rhodamine or fluorescein phalloidin and TO-PRO3, respectively. After staining, the samples were post-fixed for 10 min with 4% PFA fixative and then mounted in medium containing 1% (wt/vol) phenylendiamine, 90% (vol/vol) glycerol, and 20 mM Tris, pH 8.0.

Images were captured using a 63×/1.2 numerical aperture glycerol objective and the appropriate laser lines of a Leica TCS SP5 CW-STEM confocal microscope (in normal confocal mode). The photomultipliers were set at 900–1200 V, and images were collected using an average of six line scans. Serial 0.25-μm z-sections were acquired. The images were imported into Volocity 4-D software (PerkinElmer, Waltham, MA) and, after image reconstruction and contrast correction, exported as TIFF files. Composite images were prepared in Illustrator CS5 (Adobe, San Jose, CA). Colocalization analysis and measurements of fluorescence intensity were performed as described previously, using a fixed threshold of 40 (Khandelwal et al., 2008, 2010).

Mounting rabbit uroepithelium or rat bladders in Ussing stretch chambers and measurement of Cᵽ
Isolation of rabbit uroepithelium from the underlying muscle layers and mounting in Ussing stretch chambers was performed as described earlier (Wang et al., 2003a). Briefly, rabbit bladders were excised, slit open vertically along one of the lateral veins, and spread on a custom-made Teflon rack with the uroepithelium facing down. The muscles were carefully removed with the pair of tweezers and sharp scissors. The remaining tissue containing the intact uroepithelium was mounted on the pins at the outer edges of a plastic ring with an opening of 2 cm². The rings were locked between two Ussing stretch hemichambers, which were clamped into position on a Teflon base. Warm Krebs buffer was simultaneously added to the mucosal (apical facing) and serosal (muscle facing) chambers. The tissue was gassed with 95% air/5% CO₂ during a 30- to 45-min equilibration period and treated with the indicated drug added to the mucosal and serosal hemichambers bathing the tissue. However, CCPA, CRM197, or PMA was added only to the mucosal hemichamber. The Cᵽ and transepithelial resistance were measured as described previously for a period up to 300 min (Wang et al., 2003a).

Rat bladders were mounted in Ussing stretch chambers as described earlier (Khandelwal et al., 2008, 2010). Briefly, the bladders were excised, cut open along one of the lateral veins and then carefully spread and pinned out on rubber dissection mats. The dissected bladders were then mounted on the pins of a plastic ring with an opening of 0.75 cm² and the rings clamped between two Ussing stretch hemichambers. The chambers were filled with Krebs buffer and equilibrated. To stretch the tissue, buffer was added to the mucosal hemichamber via Luer ports at a rate of 35 μl/min using a syringe pump (New Era Pump Systems, Farmingdale, NY). Once the chamber was filled, an additional 250 μl was then pumped into the chamber to stretch the tissue.

Preparation of cell lysates and Western blot analysis
To prepare lysates, bladder tissue was placed on a rubber dissection mat with the mucosal surface facing up. The tissue was held in place by pinning it at the four corners using 20-gauge ¾-inch needles (BD Biosciences). An aliquot (35–50 μl) of SDS lysis buffer (50 mM triethanolamine, pH 8.6, 100 mM NaCl, 5 mM EDTA, 0.2% [wt/vol] Na₃, 0.5% [wt/vol] SDS) containing a protease and phosphatase inhibitor cocktail (Cell Signaling Technologies, Boston, MA), as well as 10 mM 1,10-phenanthroline, was added to the mucosal surface and the outer uroepithelium recovered by gently scraping the cells using a rubber cell scraper (Sarstedt, Newton, NC). The cell lysate was transferred to a 1.5-ml Eppendorf tube, vortex shaken at 4°C for 10 min using a model 5432 vortex mixer (Eppendorf, Hauppauge, NY), and centrifuged at 13,000 rpm for 10 min at 4°C in a table-top model 5415D microcentrifuge (Eppendorf). The clear supernatant was collected, flash frozen, and stored at −80°C. Before use, the protein concentration was quantified using the bichinchoninic acid assay (Pierce, Rockford, IL). Equal amounts of protein from the bladder lysates were resolved by SDS–PAGE on 4–15% polyacrylamide gradient gels (Bio-Rad, Hercules, CA) at 200 V and constant current for 30 min. For ADAM17 and hGH, proteins were transferred to Immobilon P membranes (Millipore) at 375 mA constant current using a 100 mM 3-[cylohexy lamino]-1-propanesulfonic acid (CAPS), pH 11.0, transfer buffer. In the case of the EGFR, proteins were transferred to nitrocellulose membranes (GE Healthcare Life Sciences) at 375 mA constant current using a Tris-glycine running buffer (25 mM Tris, 190 mM glycine) containing 0.01% (wt/vol) SDS for 2 h. In either case, the membrane was blocked for 30 min with 5% (wt/vol) nonfat milk made in TBST buffer (Tris-buffered saline + 0.1% Tween 20). After the blocking step, the membrane was incubated with primary antibodies overnight at 4°C. After several washes with TBST buffer, the membrane was incubated with goat anti-rabbit-HRP or goat anti-mouse-HRP secondary antibodies for 1 h at room temperature and washed with TBST buffer. The bands were detected by incubating the membrane with enhanced chemiluminescence (ECL) solution for 2 min (Pierce), followed by film capture on Carestream Kodak Biomax films (Carestream, Rochester, NY). Data were quantified using QuantityOne quantification software (Bio-Rad).

Generation of adenoviruses encoding ADAM17 shRNA
The iRNAi software (Nucleobiotics.com) was used to search the Rat ADAM17 cDNA (PubMed accession number NM_020306) for
In situ adenoviral transduction and measurement of hGH release

In situ transduction of rat bladder uroepithelium and measurement of hGH release were performed as described (Kandelwal et al., 2008). Briefly, rats were sedated with isoflurane, and a Jenico IV catheter (Smith Medical, Southington, CT) was introduced into the bladder via the urethra. The bladder was rinsed with PBS, and then washed with 400 μl of PBS containing adenoviruses encoding hGH alone or in combination with adenoviruses encoding scrambled-shRNA, ADAM17-shRNA, or the ADAM17-HA constructs described earlier (2.5 × 10^9 infectious virus particles, typically in a volume of 2–10 μl). The bladder was then closed. After 30 min, the clamp was removed, and the virus solution was allowed to void. The bladder was rinsed with PBS, anesthesia was discontinued, and the rats were allowed to revive. At 30 h postinfection, the animals were killed by inhalation of CO₂, and the bladder was immediately excised, slit open, and mounted on tissue rings as described. After 90 min of equilibration, the buffer bathing the apical surface was isosmotically replaced with fresh buffer and then treated or not with 500 nM CCPA. At 60 min later, the apical buffer was removed and concentrated using a 10K molecular weight cutoff Amicon Centricon (Millipore) to a volume of 250 μl. The corresponding tissue was unmounted from its tissue ring, and a lysate was prepared before Western blot analysis to detect hGH. The fraction of hGH secreted was calculated as secreted hGH/secreted hGH + cell-associated hGH in lysate).

Endocytosis of WGA-FITC

Rat bladders were mounted in Ussing chambers, and after equilibration, they were incubated with 25 μg/ml WGA-FITC for 2 h with or without 500 nM CCPA added to the mucosal hemichamber. The tissues were then unmounted from the chambers, washed with ice-cold 100 mM N-acetyl-d-glucosamine four times for 20 min, and then washed with ice-cold PBS three times for 15 min and then fixed with 4% PFA for 30 min at 37°C. The tissue was stained, and the images were captured and processed as described. Because some WGA-FITC remained bound to the apical surface of the umbrella cells even after washing with glycogen, the apicalmost sections (~1–1.5 μm) in the confocal stacks were excluded from the quantitation.

32P-orthophosphate labeling of ADAM17

HEK293FT cells were cotransfected with 3 μg of pcDNA-Ar-AR and 3 μg of either pADLOX-ADAM17-HA or pADLOX-ADAM17^5811A, HA using Lipofectamine 2000 reagent. At 48 h posttransfection, the cells were washed twice with PO efflux buffer (140 mM NaCl, 2 mM KCl, 1 mM MgSO₄, 1 mM CaCl₂, 10 mM glucose, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, titrated to pH 7.4.
using 1 M Tris-base) and then incubated with PO₄ efflux buffer supplemented with 300 μCi of [³²P]-orthophosphate (5 mCi/ml; MP Biomedicals; Santa Ana, CA) for 2 h at 37°C. During the last 15 min of the incubation, the cells were treated or not with 500 nM CCPA. Subsequently, the efflux buffer was aspirated, the cells were washed two times with Tris-buffered saline (pH 7.5), and the cells were dissolved in 500 μl of RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 50 mM NaF, 0.1% [vol/vol] SDS, 1% [wt/vol] sodium deoxycholate, 1% [vol/vol] Triton X-100, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM orthovanadate, 0.1 mg/ml aprotinin, 1 mM ethylene glycol tetraacetic acid, and 10 mM 1,10-phenanthroline). The cell lysate was collected, placed in 1.5-ml Eppendorf tubes, and incubated on ice for 30 min to complete the cell lysis. The lysate was centrifuged at 16,000 × g at 4°C using a Marathon 16km table-top centrifuge (Fisher Scientific, Waltham, MA) for 1 h, and the resulting supernatant was collected in a fresh tube. The volume in each tube was brought up to 900 μl with RIPA lysis buffer, and then 7 μl of rabbit-anti-HA antibody (Covance) and 40 μl of 10% SDS were added to each sample. After a 1-h incubation at 4°C with constant rotation, 100 μl of a 20% (wt/vol) slurry of protein G-Sepharose beads (GE Healthcare) was added to each sample and incubated at 4°C overnight with constant rotation. The beads were washed with RIPA lysis buffer three times, resuspended in 40 μl of 2x Laemmli sample buffer, heated for 15 min at 65°C, and centrifuged at 16,000 × g for 10 min, and then the proteins in the supernatant were resolved by SDS–PAGE. The proteins were transferred to nitrocellulose membranes as described and the immobilized HA-labeled proteins reacted with mouse monoclonal anti-HA antibody conjugated to HRP (Roche, Mannheim, Germany) overnight at 4°C. The membrane was then washed three times with TBST buffer and then incubated with ECL chemiluminescence reagent for 5 min (GE Healthcare). The ECL signal was captured using a VersaDoc molecular imager (Bio-Rad). The same membrane was then exposed on a phospho-screen (GE Healthcare) to capture the phosphorylation signal. The phosphorylation signal on the phospho-screen was visualized using a Personal Molecular Imager system (Bio-Rad), and the image was analyzed using Quantity One quantification software (Bio-Rad).

Expression of HB-EGF-AP and monitoring of its activity

An HB-EGF release assay was performed using the pRC/CMV-HB-EGF-AP cDNA construct, kindly provided by J. C. Krepinsky (McMaster University, Hamilton, Canada). HEK293FT cells, seeded in 12-well plates, were transfected with 1.5 μg of pRC/CMV-HB-EGF-AP cDNA construct alone or in combination with pcDNA-Ar and pADLOX-ADAM17-HA', pADLOX-ADAM17S181A-HA', pADLOX-ADAM17S181D-HA', or pADLOX-ADAM17-DCT-HA' using Lipofectamine 2000 as described. At 48 posttransfection, the cells were washed two times with phenol red– and serum-free DMEM (Invitrogen), which was replaced after 1 h by fresh medium with or without 500 nM CCPA. After 1 h of treatment, the culture supernatant was collected and the cells lysed to quantify total and released AP activity using the Sensolyte pNPP Alkaline Phosphatase Assay Kit (AnaSpec, Freemont, CA) according to the manufacturer’s protocols. Briefly, 10 μl of culture supernatant (1 ml total) and 2 μl of cell lysate (500 μl total; prepared in 1x assay buffer containing 0.2% [vol/vol] TX-100) were incubated with the AP substrate (p-nitrophenyl phosphate) for 20 min in the dark at 25°C. After the addition of 50 μl of stop solution, the AP activity was measured at an absorbance of 405 nm. The percentage of HB-EGF-AP release was calculated by dividing the total AP activity in the supernatant by the sum of the total activity in the supernatant and the cell lysate. Next the percentage of HB-EGF-AP released in the presence of CCPA was normalized to reactions in which CCPA treatment was omitted. Each experiment was repeated at least five times. No AP activity was present in the supernatant of nontransfected cells.

Statistical analysis

Statistical significance between means was determined by Student’s t test or, in the case of multiple comparisons, by analysis of variance (ANOVA). If a significant difference in the means was detected by ANOVA, multiple comparisons were performed using Dunnett’s posttest correction. Statistical analyses were performed using Prism 5 software (GraphPad, La Jolla, CA). In the figure legends, n refers to the number of individual animals or experiments.

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