Mapping physiological G protein-coupled receptor signaling pathways reveals a role for receptor phosphorylation in airway contraction

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G protein-coupled receptors (GPCRs) are known to initiate a plethora of signaling pathways in vitro. However, it is unclear which of these pathways are engaged to mediate physiological responses. Here, we examine the distinct roles of $G_{q/11}$-dependent signaling and receptor phosphorylation-dependent signaling in bronchial airway contraction and lung function regulated through the M3-muscarinic acetylcholine receptor (M3-mAChR). By using a genetically engineered mouse expressing a G protein-biased M3-mAChR mutant, we reveal the first evidence, to our knowledge, of a role for M3-mAChR phosphorylation in bronchial smooth muscle contraction in health and in a disease state with relevance to human asthma. Furthermore, this mouse model can be used to distinguish the physiological responses that are regulated by M3-mAChR phosphorylation (which include control of lung function) from those responses that are downstream of G protein signaling. In this way, we present an approach by which to predict the physiological/therapeutic outcome of M3-mAChR–biased ligands with important implications for drug discovery.

G protein-coupled receptor | asthma | muscarinic | ligand bias | signaling

Airflow in the lung is limited by vagally derived ACh that mediates airway smooth muscle (ASM) contraction primarily via activation of M3-muscarinic acetylcholine receptor (M3-mAChRs) (1). In both asthma and chronic obstructive pulmonary disease, this activity can be up-regulated, leading to bronchoconstriction and impaired lung function (1), thus explaining the therapeutic benefits provided by anticholinergics, such as tiotropium bromide (2).

M3-mAChRs can mediate ASM contraction through both calcium-dependent and calcium-independent mechanisms (1). The calcium-dependent mechanism is centered on $G_{q/11}$-mediated phospholipase C activation generating inositol 1,4,5-trisphosphate, which mobilizes calcium from the sarcoplasmic reticulum together with promoting extracellular calcium entry that ultimately results in a rise in intracellular calcium, activation of myosin-light chain kinase, and the phosphorylation of myosin leading to contraction (3). In contrast, the mechanism by which the M3-mAChR mediates calcium-independent ASM contraction is via activation of the small GTPase RhoA, which, in turn, activates Rho-kinase. The phosphorylation of myosin light chain (MLC) phosphatase by Rho-kinase decreases enzymatic activity, which results in higher levels of phosphorylated myosin and smooth muscle contraction (3–5). Although G protein-coupled receptor (GPCR) activation of RhoA is generally considered to be via $G_{q/11}$ and $G_{12/13}$ heterotrimeric G proteins (6), the mechanism by which the M3-mAChR activates the RhoA pathway is unclear, with some researchers indicating that activation might proceed in a G protein-independent manner and possibly involve a direct interaction between the receptor and RhoA (7). The prominence of the calcium-independent pathway in the excessive airway narrowing (i.e., bronchial hyperresponsiveness) previously observed in animal models of allergic bronchial asthma has prompted the suggestion that inhibiting the M3-mAChR pathways leading to RhoA activation might be of therapeutic potential (8, 9).

GPCRs become rapidly phosphorylated following agonist stimulation, and this phosphorylation results in the recruitment of adaptor arrestin proteins that (*i*) uncouple receptors from their cognate G proteins and (*ii*) act as signaling scaffolds to drive G protein-independent signaling (10). The activation of RhoA signaling has been identified as one of those pathways regulated by receptor phosphorylation/arrestin-dependent signaling (11, 12). Because we have shown previously that the M3-mAChR is multiply phosphorylated following agonist stimulation (13) and

Significance

Studies in transfected cells have established that G protein-coupled receptors (GPCRs) activate a number of intracellular signaling pathways; however, which of these pathways are physiologically important is unclear. Here, we use a genetically engineered mouse to demonstrate a novel role for M3-muscarinic acetylcholine receptor (M3-mAChR) phosphorylation in airway constriction, with implications for human respiratory disease, including asthma and chronic obstructive pulmonary disease. Combining this finding with other M3-mAChR physiological responses, we generate a map of responses that are downstream of G protein-dependent signaling or receptor phosphorylation-dependent signaling. Such a map predicts the outcome of biased GPCR drugs designed to drive receptor signaling preferentially toward pathways that improve therapeutic efficacy while minimizing toxic/adverse outcomes and provides a fundamental approach to the rational design of next-generation GPCR-based therapies.

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that removal of the phosphorylation sites on the receptor results in the uncoupling of the M3-mAChR from arrestin, as well as arrestin-dependent processes such as receptor internalization (14, 15), we wanted to determine if M3-mAChR phosphorylation might play a role in calcium-independent ASM contraction and regulate lung function and airway hyperresponsiveness through this mechanism.

In addition, we aimed to establish an experimental system whereby we might distinguish between GPCR-mediated physiological responses that are downstream of G protein-dependent signaling and those responses that are downstream of receptor phosphorylation/arrestin-dependent signaling processes (16, 17). In this way, we might develop an approach by which to predict the physiological action of biased GPCR ligands, with important implications for drug discovery.

To achieve these dual aims, we use here a mouse model in which the M3-mAChR gene locus has been targeted to express a form of the M3-mAChR that is mutated in its phosphorylation sites (14, 15). This mutant receptor is uncoupled from phosphorylation-dependent pathways, including the recruitment of arrestin (14), but maintains coupling to $G_{q/11}$-dependent signaling (14, 15). In this regard, this mutant receptor can be considered as G protein-biased. Using this model, we determined a role for M3-mAChR phosphorylation in the regulation of lung function and in driving allergen-induced airway hyperresponsiveness. In addition, we were able to include this analysis in a map of M3-mAChR physiological responses that lay downstream of receptor phosphorylation/arrestin-dependent signaling and, in this way, provide a predictive model for the action of biased ligands to the M3-mAChR.

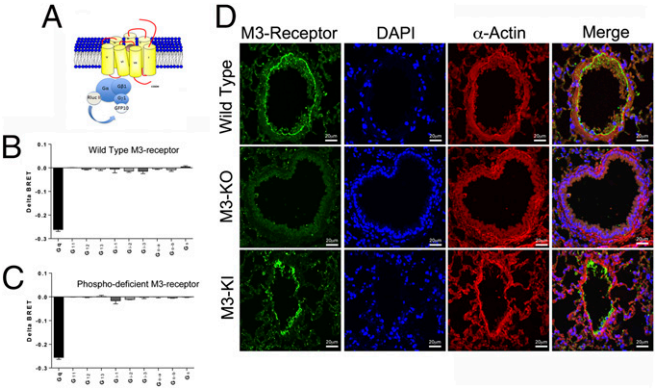

Fig. 1. Characterization of M3-mAChR expression and G protein coupling in the airways of WT and M3-KI mice. (*A*) Illustration of the BRET-based biosensor to measure G protein coupling, where various donor Rluc II Gα-subunits can be transfected with an acceptor GFP_{10}-Gγ1 subunit together with the M3-mAChR. (*B* and *C*) Cells were transfected with the BRET biosensor consisting of various Rluc Gα-subunits. The change in BRET signal following receptor stimulation with carbachol (100 μM) in cells expressing the WT (*B*) or phosphodeficient M3-mAChR (*C*) is shown. Data represent the mean ± SEM of three independent experiments. (*D*) M3-mAChR (green) and smooth muscle α-actin immunoreactivity (red) in the airways of WT, M3-KO, and M3-KI mice. DAPI (blue) was used to stain nuclei. (*Right*) Overlaid images are shown.

Results

Phosphorylation-Deficient M3-mAChR Mutant Is Expressed in ASM and Coupled Normally to G_q Signaling. We have previously established that a mutant of the M3-mAChR, where 15 Ser phosphoacceptor sites in the third intracellular loop were mutated to Ala, showed reduced levels of agonist-mediated phosphorylation and significantly attenuated coupling to arrestin and arrestin-dependent signaling while maintaining near-normal coupling to $G_{q/11}$-dependent signaling (14, 15). Here, we present further evidence that G protein coupling of the phosphodeficient M3-mAChR mutant is normal by evaluating the coupling of both WT and mutant receptors to 10 different Gα-subunits in a bioluminescence resonance energy transfer (BRET)-based live cell assay (18, 19) (Fig. 1*A*). These experiments demonstrated that the profile of G protein activation between the WT M3-mAChR and the phosphodeficient mutant was very similar, with both receptor types predominantly coupling to G_q (Fig. 1 *B* and *C*). Importantly, there appeared to be no significant coupling of the M3-mAChR to $G_{12/13}$ proteins (Fig. 1 *B* and *C*). Control experiments in which the receptor was not transfected showed no significant activation of G proteins, including G_q (Fig. S1 *A* and *B*).

A mutant mouse strain, termed M3-knockin (M3-KI), was generated, where the M3-mAChR gene locus was modified to express the phosphodeficient M3-mAChR mutant in place of the WT receptor (14, 15). The muscarinic receptor expression levels in lungs from M3-KI mice (determined by radioligand binding) were not significantly different from the muscarinic receptor expression levels in lungs from WT mice (Fig. S2 *A* and *B*). Furthermore, the levels of the mutant receptor transcript in the lungs from M3-KI mice were similar to the levels of the WT receptor in C57BL/6J mice as determined by RT-PCR (Fig. S2*C*). Using an in-house Ab to the M3-mAChR, we found a normal expression profile of the mutant M3-mAChR in ASM of M3-KI mice, as indicated by colocalization of immunostaining of the receptor and α-actin in airways (Fig. 1*E*).

M3-mAChR Mediates ASM Contraction in a Receptor Phosphorylation-Dependent Manner. To determine the impact of mutating phosphorylation sites on the M3-mAChR in the lung, we used precision cut lung slices (PCLS) to monitor bronchoconstriction (20). In agreement with previous reports (21), we showed that muscarinic receptor stimulation of PCLS from WT mice resulted in a concentration-dependent narrowing of the airways [half-maximum effective concentration (pEC_{50}) = 5.47 ± 0.06, maximum drug effect (Emax) = 82.47 ± 5.82] (Fig. 2 *A*–*D*). In comparison, the responsiveness of PCLS from M3-KI mice was significantly reduced in both maximal response and potency (pEC_{50} = 4.01 ± 0.06, Emax = 40.79 ± 5.73) (Fig. 2 *A*–*D* and Movies S1 and S2.). Importantly, the coupling of the phosphodeficient M3-mAChR mutant to G_q/calcium signaling in PCLS derived from M3-KI mice was seen to be very similar to the coupling observed in WT PCLS cultures (Fig. 2 *E*–*G*). Thus, despite normal coupling to G_q/calcium mobilization, the mutant receptor expressed in M3-KI mouse lung showed reduced airway contraction, indicating an important role for receptor phosphorylation-dependent signaling in M3-mAChR–mediated ASM contraction.

Because previous studies had established that the RhoA pathway mediates calcium-independent ASM contraction (3–5), we investigated the role of RhoA signaling downstream of M3-mAChR phosphorylation. In vitro analysis of RhoA activation using a FRET-based biosensor (Fig. 3*A*) revealed that the phosphodeficient M3-mAChR mutant coupled with >200-fold lower potency to the RhoA pathway (pEC_{50} = 5.41 ± 0.63) than the WT receptor (pEC_{50} = 7.74 ± 0.17) (Fig. 3*B*). Activation of the RhoA pathway ultimately results in the phosphorylation of MLC and smooth muscle contraction (3–5). Staining for phosphorylation of MLC on Ser-19 showed pronounced MLC phosphorylation in the ASM in PCLS from WT animals in both the basal state and following carbachol stimulation (Fig. 3*C*). In contrast, ASM in PCLS from M3-KI mice showed substantially less MLC phosphorylation (Fig. 3*D*), a result consistent with reduced activation of RhoA signaling in M3-KI airways. Furthermore, the RhoA-kinase inhibitor H1152 reduced the maximal M3-mAChR contractile response in PCLS from WT mice by 43% and the potency of ACh-mediated contraction by >10-fold (pEC_{50} was reduced from 5.07 ± 0.15 to 3.98 ± 0.13 following H1152 treatment) (Fig. 3 *E*–*G*). These data indicate that a significant component of the M3-mAChR–mediated ASM contractile response was independent of G_q/calcium signaling but was dependent on M3-mAChR phosphorylation and the activation of the RhoA pathway.

Consistent with this notion was the fact that inhibition of M3-mAChR–mediated G_q/calcium mobilization in PCLS from WT mice using the recently characterized G_q inhibitor FR900359 (22) (Fig. 3*H* and Fig. S3 *A* and *B*) did not affect the M3-mAChR–mediated contractile response (Fig. 3 *H* and *J*). The concentration of FR900359 used in these experiments (30 nM) completely prevented the carbachol-mediated calcium response in PCLS

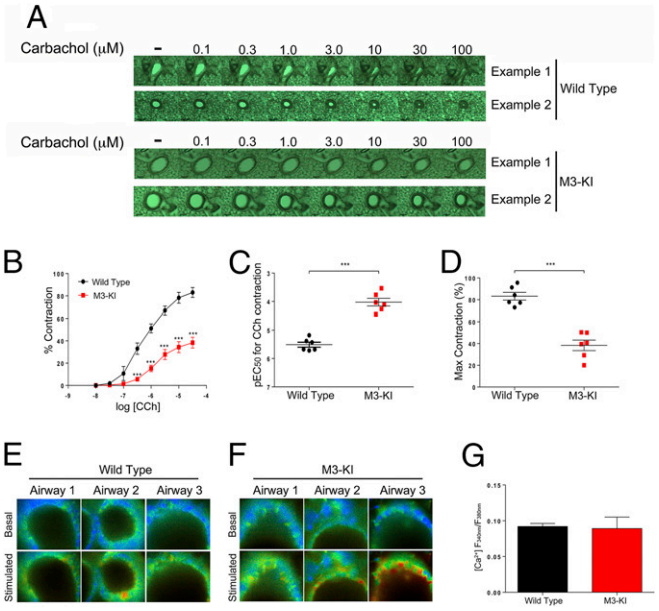

Fig. 2. M3-mAChR–mediated ASM contraction is dependent on receptor phosphorylation-dependent signaling. (*A*) Four representative experiments, two from WT controls and two from M3-KI mice, showing the bronchoconstriction responses to increasing concentrations of carbachol in PCLS. (*B–D*) Contractile responses in PCLS derived from WT and M3-KI mice. (*B*) Mean concentration–response curves to carbachol (CCh) in WT and M3-KI PCLS. Mean pEC_{50} (*C*) and mean Emax (*D*) values calculated from the above PCLS concentration–response curves ($n = 6$ in each group). (*E* and *F*) In PCLS from WT and M3-KI mice, calcium responses following ACh (100 μM) stimulation were evaluated. Shown are three representative airway responses from PCLS derived from WT mice (*E*) and three representative airway responses from PCLS derived from M3-KI mice (*F*). (*G*) Average (±SEM) peak calcium responses from representative airways shown in *E* and *F* (all responsive single cells from each airway were included in the analysis). Data show the mean ± SEM and were analyzed using an unpaired *t* test (***$P < 0.001$).

(Fig. 3*H*) but had no significant effect on carbachol-mediated smooth muscle contraction (Fig. 3 *I* and *J*). Importantly, inhibition of G_q/calcium signaling with FR900359 did not significantly affect the small contractile response seen in PCLS from M3-KI mice in response to carbachol, (Fig. S4 *A* and *B*) and FR900359 did not appear to affect the level of MLC phosphorylation in PCLS from WT mice stimulated with carbachol (Fig. 3*K*). These data point to RhoA-mediated signaling downstream of receptor phosphorylation having a prominent role in M3 receptor-mediated ASM contraction.

Lung Function and Allergen-Associated Airway Hyperresponsiveness Are Dependent on M3-mAChR Phosphorylation. To test if the reduction in the contractile response of PCLS obtained from M3-KI mice translated to changes in in vivo lung function, lung resistance in response to ACh challenge in WT mice and M3-KI mice was monitored. These studies revealed a rightward shift in the potency and a decrease in the maximal response of the lung resistance induced by administration of ACh in M3-KI mice (Fig. 4*A*). The decrease in lung resistance in response to ACh in the M3-KI mice was also reflected in a significant increase in the concentration of ACh required to elevate lung resistance by 100% ($-\log PC_{100}$) (Fig. 4*B*).

M3-mAChR signaling through RhoA in ASM has been closely linked with experimentally induced airway hyperresponsiveness in different studies using murine models of allergy (23, 24). We tested here if the reduced coupling of the phosphodeficient M3-mAChR mutant to RhoA signaling and the subsequent reduction in ASM contraction observed in M3-KI mice had an impact on allergen-induced airway hyperresponsiveness using a murine model of allergic inflammatory airway disease (25). In these experiments, WT mice sensitized with ovalbumin and challenged with saline (0.9% NaCl) showed an increase in lung resistance to ACh that was significantly augmented in mice sensitized with ovalbumin and challenged with ovalbumin (Fig. 4*C*), a response defined as classical airway hyperresponsiveness (24, 26, 27). In contrast, M3-KI mice sensitized with ovalbumin and challenged with ovalbumin showed no hyperresponsiveness in response to ACh administration (Fig. 4*C*). The lack of hyperresponsiveness following ovalbumin sensitization/challenge in M3-KI mice was also reflected by the lack of change in the $-\log PC_{100}$ value in M3-KI mice following ovalbumin sensitization/challenge (Fig. 4*D*). Hence, the $-\log PC_{100}$ value fell from 2.38 ± 0.02 in WT mice sensitized with ovalbumin and challenged with saline to 1.68 ± 0.06 after ovalbumin sensitization and ovalbumin challenge, indicative of airway hyperresponsiveness (Fig. 4*D*). In contrast, the $-\log PC_{100}$ value for the M3-KI mice that were ovalbumin-sensitized and saline-challenged (2.64 ± 0.17) was not significantly different from the $-\log PC_{100}$ value for M3-KI mice that had been ovalbumin-sensitized and ovalbumin-challenged (2.59 ± 0.06) (Fig. 4*D*). These data indicate that M3-mAChR phosphorylation was required in the development of allergen-induced airway hyperresponsiveness.

The acute ovalbumin-sensitization model used here is associated with an inflammatory response, as indicated by changes in cytokine levels and infiltration of immune cells into the lung. Analysis of cell infiltration by assessing total cells in the bronchoalveolar lavage (BAL) determined that the accumulation of inflammatory cells into the lung following ovalbumin sensitization and ovalbumin challenge was not significantly different between WT and M3-KI mice (Fig. S5*A*). Analysis of different inflammatory cell types in the BAL was carried out. Whereas the macrophage levels (Fig. S5*B*) were not seen to change following ovalbumin sensitization/challenge of WT and M3-KI mice, the numbers of eosinophils (Fig. S5*C*), neutrophils (Fig. S5*D*), and lymphocytes (Fig. S5*E*) were significantly increased, by the same extent, in WT and M3-KI mice sensitized with ovalbumin and challenged with ovalbumin.

Phosphorylation of the M3-mAChR Regulates Specific Physiological Responses. We show here how the G protein-biased M3-mAChR mutant receptor expressed in M3-KI mice can be used to define the role of receptor phosphorylation-dependent signaling in bronchial ASM. We reasoned that this approach could be extended to include other M3-mAChR–mediated responses and that a map of the physiological responses that lay downstream of the two fundamental signaling arms of the M3-mAChR, namely, G protein-dependent and phosphorylation/arrestin-dependent signaling, could be generated. To test this hypothesis, we compared the M3-mAChR–mediated contraction of bronchial smooth muscle with other M3-mAChR–mediated physiological responses, namely, salivary secretion and weight gain.

Previous gene KO studies established that salivary secretion in response to low doses of the muscarinic partial agonist pilocarpine was almost completely dependent on M3-mAChR (28). The results of these studies were confirmed here, where salivary secretion in response to pilocarpine was significantly reduced in M3-mAChR KO mice (Fig. 5*A*). In contrast, salivary secretion in response to pilocarpine in M3-KI mice was significantly enhanced compared with WT animals (Fig. 5*A*). These results indicated that in contrast to M3-mAChR–mediated contraction of ASM, M3-mAChR–mediated secretion of saliva was independent of receptor phosphorylation signaling but was dependent on signaling through G_q pathways. The slight increase in salivary secretion observed in M3-KI mice may reflect the fact that phosphorylation-dependent mechanisms, which are traditionally considered to desensitize G protein responses, are not in operation in M3-KI mice.

M3-mAChR KO mice have also previously been reported to show reduced food intake and decreased body fat (29). This phenotype has been associated with the action of the M3-mAChR on the hypothalamic leptin/melanocortin system (29). In addition, the M3-mAChR KO mice showed an increase in both resting and total energy expenditure (oxygen consumption) as well as an increase in the rate of oxidation of fatty acids (30),

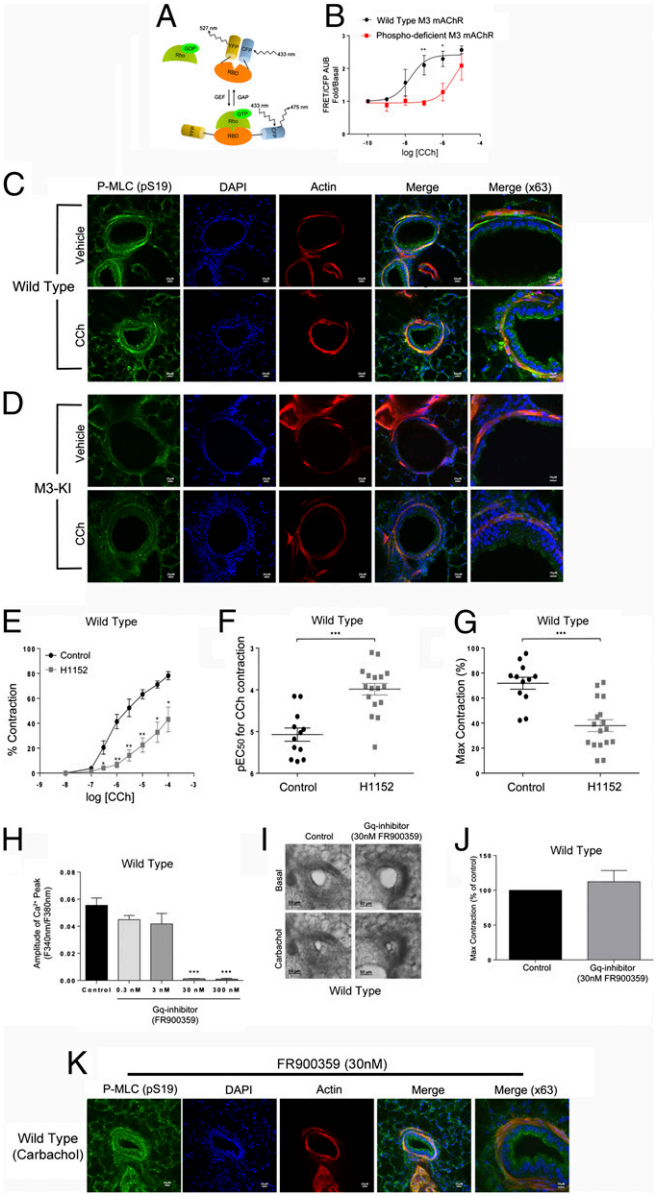

Fig. 3. M3-mAChR–mediated ASM contraction is dependent on receptor phosphorylation-dependent coupling to RhoA signaling. (*A*) Illustration of the FRET-based biosensor used to detect activated RhoA (GTP-bound). (*B*) Rho activity in CHO cells expressing the WT or the phosphodeficient M3-mAChR mutant in response to CCh. Data represent the mean ± SEM ($n = 4$). Data were analyzed using two-way ANOVA (*$P < 0.05$; **$P < 0.01$). Phospho-MLC2 (Ser-19, green) and smooth muscle α-actin (red) immunoreactivity is shown in the airways of WT (*C*) or M3-KI (*D*) mice stimulated with vehicle or CCh (100 μM,10 min). DAPI (blue) was used to stain nuclei. (*Right*) Overlaid images and images of the same airway using a 63× objective are shown. (*E–G*) Effect of Rho-kinase inhibitor H1152 on contractile responses in WT PCLS. (*E*) Mean concentration–response curves to CCh in WT PCLS pretreated with vehicle or H1152 (100 nM, 45 min of preincubation). Mean pEC50 (*F*) and mean Emax (*G*) values calculated from the above PCLS CCh concentration–response curves in the presence and absence of H1152 ($n = 12$ in vehicle-treated PCLS and $n = 17$ in H1152-treated PCLS) are shown. Data in *E–G* were analyzed using an unpaired *t* test (***$P < 0.001$). (*H–J*) Effects of the G_q inhibitor FR900359 on CCh (100 μM)-stimulated calcium responses and bronchoconstriction in PCLS from WT mice. (*H*) Average (±SEM) peak calcium responses stimulated by CCh after preincubation with increasing concentrations of FR900359 (0.3–300 nM, 30 min). (*I*) Representative examples of CCh-induced bronchoconstriction in PCLS from WT mice under control conditions (*Left*) or following preincubation with 30 nM FR900359 (*Right*). (*J*) Mean contractile response to CCh in WT PCLS following preincubation with FR900359 (30 nM, 30 min), expressed as a percentage of CCh-stimulated maximum contraction in control slices. Max, Maximum. (*K*) Phospho-MLC2 (Ser-19, green) and smooth muscle α-actin (red) immunoreactivity in PCLS from WT mice stimulated with CCh (100 μM, 10 min) after preincubation with 30 nM FR900359. DAPI (blue) was used to stain nuclei. (*Right*) Overlaid image and an image of the same airway using a 63× objective are shown.

phenotypes that have been linked with M3-mAChR regulation of sympathetic flow (30). Disruption of both of these physiological processes in M3-mAChR KO mice combines to generate an overall lean phenotype, where the mice show reduced total body weight (29, 31). We confirm these data here by demonstrating that M3-mAChR KO mice have reduced body weight compared with WT controls (Fig. 5 *B* and *C*). In contrast to the M3-mAChR KO mice, M3-KI mice were slightly, but significantly, heavier than the WT controls (Fig. 5 *B* and *D*). These data suggest that M3-mAChR signaling via G_q protein is the primary signaling pathway that regulates M3-mAChR metabolic and feeding responses responsible for weight gain.

Combining these data with previously published results (*Discussion*), a map of the physiological pathways downstream of phosphorylation/arrestin signaling and G protein-dependent signaling can be generated (Fig. 5*E*). This map can be used to predict the physiological/therapeutic outcome of biased M3-mAChR ligands (Fig. 5*F*).

Discussion

Our study demonstrates a previously unidentified role for M3-mAChR phosphorylation in the regulation of lung function and in driving airway hyperresponsiveness, one of the key features of allergic inflammatory airway disease. Our unique observation also highlights the importance of receptor phosphorylation in mediating M3-mAChR–induced ASM contraction via the activation of the RhoA pathway.

Because previous studies had demonstrated a role for RhoA signaling in calcium-independent ASM contraction (3–5), we used a genetically engineered mouse that expresses a phosphorylation-deficient variant of the M3-mAChR (M3-KI) to investigate the possibility that phosphorylation of the M3-mAChR was involved in coupling the receptor to RhoA signaling and, through this mechanism, to ASM contraction. That phosphorylation of the M3-mAChR plays a role in coupling to RhoA was indicated by (*i*) data showing that pharmacological inhibition of Rho-kinase in WT PCLS phenocopied the reduced airway contraction response observed in PCLS from M3-KI mice, (*ii*) that the phosphodeficient M3-mAChR showed markedly reduced coupling to RhoA, and (*iii*) that phosphorylation of MLC was significantly reduced in ASM of M3-KI mice. In addition, we used the recently characterized G_q inhibitor FR900359 (22) to demonstrate that M3-mAChR–induced calcium mobilization was inhibited by FR900359, whereas M3-induced airway contraction was not. Because our experimental design measured the overall calcium changes in whole PCLS, we cannot completely exclude a role of compartmentalized changes in G_q-driven calcium signals in ASM in the regulation of airway contraction, as shown by others (32, 33). Nonetheless, our data are consistent with a recent report demonstrating that muscarinic-mediated contractile responses in human PCLS were less sensitive to FR900359, and therefore less dependent on calcium, than contraction mediated by histamine receptors (34). Furthermore, knocking down β-arrestin-1 has recently been shown to impair muscarinic-mediated bronchoconstriction (33), demonstrating the complexity surrounding the procontractile pathways coupled to M3-mAChRs in ASM. These studies, coupled with our data, support the hypothesis that M3-mAChR phosphorylation mediates activation of RhoA signaling (possibly by arrestin recruitment) that results in increased MLC phosphorylation and ASM contraction. This phosphorylation-dependent calcium sensitization process plays a prominent role in M3-mAChR–dependent ASM contraction, with a minor role played by calcium transient-dependent processes.

It is known that vagally derived ACh limits airflow by bronchoconstriction mediated by the activation of the M3-mAChR on ASM

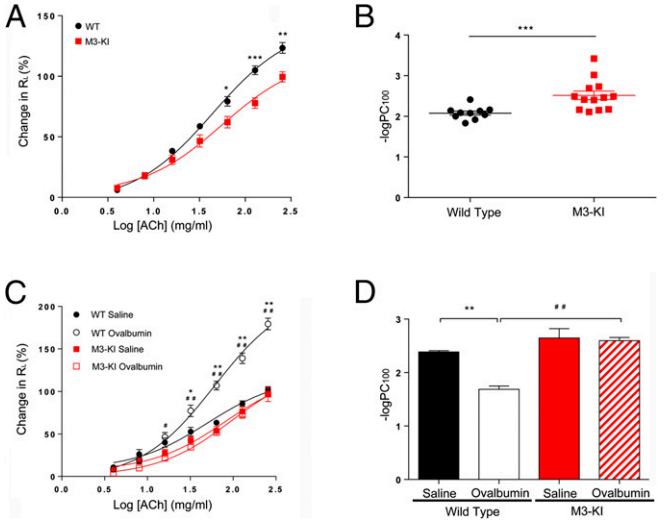

Fig. 4. Lung function and airway hyperresponsiveness are regulated by M3-mAChR phosphorylation/arrestin signaling. (*A*) Lung resistance (R_L) was measured in WT and M3-KI mice at various ACh concentrations. Data represent the mean ± SEM (WT, $n = 10$; M3-KI, $n = 13$). (*B*) $-LogPC_{100}$ was determined for WT and M3-KI mice. Data presented in *A* and *B* are the mean ± SEM (WT, $n = 10$; M3-KI, $n = 13$). Data were analyzed using Kruskal–Wallis and Mann–Whitney tests (WT vs. M3-KI: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$). (*C*) Airway hyperresponsiveness was induced using ovalbumin sensitization followed by ovalbumin challenge in WT and M3-KI mice. Controls were ovalbumin sensitization followed by saline challenge. R_L was determined at various ACh concentrations. Data represent the mean ± SEM (WT, $n = 6$; M3-KI, $n = 6$). Data were analyzed using Kruskal–Wallis and Mann–Whitney tests (WT saline vs. WT ovalbumin: $^{*}P < 0.05$; $^{**}P < 0.01$ and WT ovalbumin vs. M3-KI ovalbumin: $^{\#}P < 0.05$; $^{\#\#}P < 0.01$). (*D*) $-LogPC_{100}$ was determined for WT and M3-KI mice sensitized with ovalbumin and challenged with either control saline or ovalbumin. Data present the mean ± SEM (WT, $n = 6$, M3-KI, $n = 6$). Data were analyzed using Kruskal–Wallis and Mann–Whitney tests (WT saline vs. WT ovalbumin: $^{**}P < 0.01$ and WT ovalbumin vs. M_3-KI ovalbumin: $^{\#\#}P < 0.01$).

(1). If the role for M3-mAChR phosphorylation in ASM contraction identified here is of physiological relevance, then it might be expected that the lung function of M3-KI mice would be different from the lung function of WT mice. We found (by assessing lung resistance) that airway obstruction in response to ACh was significantly reduced in M3-KI mice compared with WT mice, an observation consistent with the notion that the phosphorylation status of the M3-mAChR is important in regulating baseline cholinergic ASM tone. This observation is in line with studies conducted in healthy volunteers showing a significant improvement in lung function by anticholinergic drugs (2). Furthermore, in an acute murine model of allergic inflammatory airway disease, which mimics several of the central hallmarks of human bronchial asthma (35), including airway hyperresponsiveness and immune cell lung infiltration, we show that the development of allergen-induced airway hyperresponsiveness to ACh was dependent on M3-mAChR phosphorylation-dependent pathways because sensitized M3-KI mice failed to demonstrate the typically enhanced airway hyperresponsiveness. Thus, both normal and pathophysiological lung function in M3-KI mice was significantly different from WT mice, indicating an important role for M3-mAChR phosphorylation in the regulation of airway narrowing in health and disease states.

The mechanisms by which allergen-exposed M3-KI mice were protected against the development of abnormal bronchial responsiveness appeared to be unrelated to the number of inflammatory cells (eosinophils, lymphocytes, and neutrophils) recruited within the airways. This dissociation between airway inflammation and the degree of airway hyperresponsiveness has been previously reported in various studies, including observations in asthmatic patients (36) as well as in animal models of allergic inflammatory airway disease (34, 37, 38). Rather, we propose that in the lung, phosphorylation of the M3-mAChR is important in driving key asthmatic features, such as airway hyperresponsiveness, in part, via M3-mAChR phosphorylation and RhoA-dependent signaling.

Because our M3-KI mouse line expressing G protein-biased M3-mAChR mutant defined a previously unidentified role for M3-mAChR phosphorylation-dependent signaling in ASM contraction, we reasoned that this mouse line could be used to investigate other M3-mAChR–mediated physiological responses and establish whether these responses were dependent on G protein- or receptor phosphorylation/arrestin-dependent signaling. We therefore examined M3-mAChR–mediated salivary secretion and weight gain and established that both of these responses were slightly up-regulated in M3-KI mice, indicating that they were not dependent on receptor phosphorylation/arrestin signaling, but more likely dependent on G protein coupling. The loss of the phosphorylation-dependent desensitization of G protein-mediated signaling may

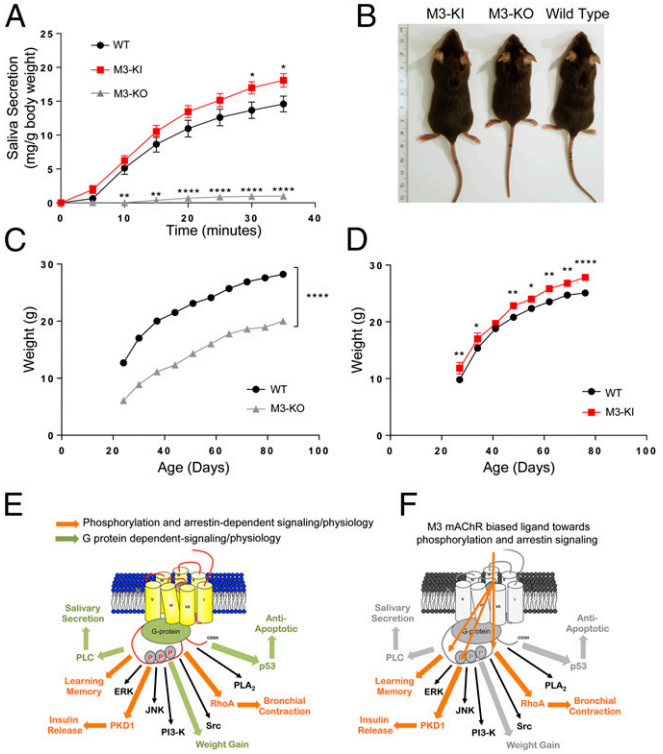

Fig. 5. Mapping M3-mAChR physiological responses mediated by G protein- and receptor phosphorylation/arrestin-dependent signaling. (*A*) Salivary secretion in response to pilocarpine (1 mg/kg) administration was measured in WT, M3-KO, and M3-KI mice. The data represent the mean ± SEM of three to six mice. (*B*) Representative images of M3-KI, M3-KO, and WT mice demonstrating differences in weight. (*C*) Weight gain in WT and M3-KO mice. (*D*) Weight gain in WT and M3-KI mice. The data in *A*, *C*, and *D* represent the mean ± SEM of six to 11 mice and were analyzed using two-way ANOVA ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{****}P < 0.0001$). (*E*) Illustration of the physiologically relevant signaling pathways downstream of the M3-mAChR. Some of the pathways activated by the M3-mAChR in heterologous systems are illustrated (i.e., PLC, ERK, PKD, JNK, PI3-K, Src, RhoA, PLA_2, and p53). P13-K, phosphoinositide 3-kinase; PKD, protein kinase D; PLA2, phospholipase A2; PLC, phospholipase C. In this study, we used a mutant mouse strain (M3-KI) expressing a G protein-biased variant of the M3-mAChR to assign those physiological responses that were downstream of either G protein signaling (green arrows) or receptor phosphorylation and arrestin signaling (orange arrows). (*F*) Such a map also allows for the rational design of biased ligands because our studies present a model by which the physiological/therapeutic outcome of biased ligands can be predicted. Thus, for the M3-mAChR, a ligand that is biased toward receptor phosphorylation and arrestin signaling would preferentially affect insulin release, learning and memory, and bronchial contraction (orange arrows), although having potentially little impact on weight gain, salivary secretion, and cell death pathways (gray arrows).

be responsible for the modest but significant increase in weight and salivary secretion observed in M3-KI mice.

These physiological responses could be added to our previous studies demonstrating a predominant role for M3-mAChR phosphorylation/arrestin coupling in the regulation of insulin secretion (14) and learning and memory (15), as well as in G protein coupling in M3-mAChR regulation of p53 subcellular localization and apoptosis (39, 40). By combining all these studies, we have generated a map of the physiological responses mediated by the M3-mAChR through either G protein- or receptor phosphorylation/arrestin-dependent signaling (Fig. 5*E*).

This map has allowed us, for the first time to our knowledge, to make a prediction of the likely physiological outcomes of a biased GPCR ligand. Thus, an M3-mAChR drug-like ligand showing stimulus bias toward receptor phosphorylation and arrestin signaling would preferentially engage physiological signaling pathways that would promote changes in insulin secretion, have an impact on learning and memory, and regulate bronchoconstriction (Fig. 5*F*). This same ligand would not be expected to have a substantial impact on salivary secretion, weight gain, and mechanisms associated with cell survival (Fig. 5*F*). This analysis has considerable potential application in drug discovery, where biased ligands can be designed to direct signaling toward those pathways that result in clinical efficacy and away from pathways that lead to adverse/toxic outcomes. Hence, by using a genetically modified mouse expressing a G protein-biased receptor, we are not only able to define the physiologically relevant signaling pathways acting downstream of a particular GPCR (in this case, the M3-mAChR) but also to determine what type of signaling bias to design into ligands targeting that receptor to promote a particular physiological/therapeutic outcome.

Experimental Procedures

Information on materials and methods used in this study is provided in *SI Experimental Procedures*. Animal experiments were conducted under the approval of the UK government home office licence PPL 802353 following ethic review by the University of Leicester.

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