Differential Effects of Allosteric M_1 Muscarinic Acetylcholine Receptor Agonists on Receptor Activation, Arrestin 3 Recruitment, and Receptor Downregulation

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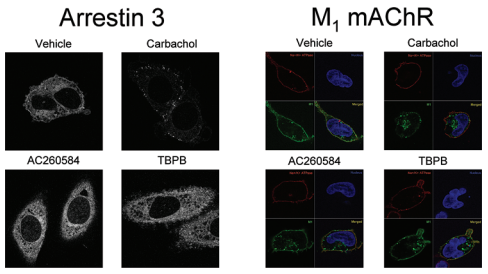
Abstract



Muscarinic acetylcholine receptors (mAChRs) are drug targets for multiple neurodegenerative and neuropsychiatric disorders, but the full therapeutic potential of mAChR-targeted drugs has not been realized, mainly because of a lack of subtype-selective agonists. Recent advances have allowed the development of highly selective agonists that bind to an allosteric site on the M_1 mAChR that is spatially distinct from the orthosteric acetylcholine binding site, but less is known about the profile of intracellular signals activated by orthosteric versus allosteric M_1 mAChR agonists. We investigated the activation and regulatory mechanisms of two structurally distinct allosteric M_1 mAChR agonists, AC260584 and TBPB. We show that allosteric agonists potently activate multiple signal transduction pathways linked to the M_1 mAChR receptor but, compared to orthosteric agonists, much less efficiently recruit arrestin 3, a protein involved in the regulation of G-protein coupled receptor signaling. Consistent with decreased arrestin recruitment, both allosteric agonists showed blunted responses in measurements of receptor desensitization, internalization, and downregulation. These results advance the understanding of mAChR biology and may shed light on unanticipated differences in the pharmacology of orthosteric versus allosteric agonists that might be capitalized upon for drug development for the treatment of CNS diseases.

Keywords: Muscarinic, acetylcholine, receptor, allosteric, arrestin, internalization

Muscarinic acetylcholine receptors (mAChRs) regulate many central nervous system (CNS) functions, including cognition, movement, and emotion. Five muscarinic receptor subtypes (M_1−M_5) have been cloned (*1*, *2*), and their distinct tissue- and cell-specific expression patterns predict their roles in modulating CNS functions (*3*, *4*). The M_1 receptor is the most abundant mAChR subtype in the brain, and its predominantly postsynaptic localization in the cortex, hippocampus, and striatum underlies its involvement in regulating neural signals that govern cognition, memory, and locomotion (*5*, *6*). Studies in M_1 mAChR knockout mice have confirmed the importance of this receptor subtype, revealing deficits in signal transduction (*7*, *8*) and in specific cognitive and locomotor tasks (*7*−*10*). Furthermore, evidence in a variety of model systems indicates that the M_1 mAChR is also a therapeutic target in several disease states, including Alzheimer's disease, epilepsy, and schizophrenia (*7*, *11*).

Because of the high degree of sequence similarity among mAChR subtypes at the acetylcholine (orthosteric) binding pocket, it has proved extremely difficult to develop ligands that are highly subtype-selective, a limitation that has hampered progress in drug development for disorders involving the cholinergic system. In recent years, however, several compounds have been developed that display unprecedented selectivity for the M_1 mAChR subtype. Using a small molecule library screening approach, Spalding and colleagues identified and characterized a series of compounds that potently activate the M_1 mAChR and show minimal activity at

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other mAChR subtypes (*12*, *13*). This improvement in selectivity is likely attributable to binding at an allosteric site that is spatially distinct from the acetylcholine binding site and more divergent among mAChR subtypes (*12*). Recently, Bridges, et al. and Miller, et al. reported the discovery and characterization of a novel series of allosteric agonists for the M_1 mAChR (*14*, *15*). In addition to being highly selective for the M_1 mAChR, these compounds are structurally distinct from existing orthosteric and allosteric mAChR agonists. TBPB, the first lead compound in this series, activates the M_1 mAChR and potentiates NMDA glutamate receptor currents in hippocampal slices (*16*). Importantly, TBPB was shown to produce antipsychotic activity in rodents and regulate nonamyloidogenic processing of the amyloid precursor protein, lending support to the concept that allosteric agonists of the M_1 mAChR are efficacious *in vivo* and highlighting their therapeutic potential for neurological and neuropsychiatric disorders.

Muscarinic receptors belong to the superfamily of G-protein coupled receptors (GPCRs), a class of seven transmembrane-spanning proteins that comprise the largest group of cell surface receptors. Following agonist binding and activation of GPCRs, a series of well characterized homeostatic mechanisms act to terminate signaling (for reviews, see refs *17* and *18*). Typically, activated receptors are rapidly phosphorylated, serving as a site of recruitment for a family of regulatory proteins called arrestins. Arrestins attenuate GPCR signaling by uncoupling the receptor from its cognate G-protein and also promote receptor internalization by facilitating interactions with the endocytic proteins clathrin and AP2. Internalized GPCRs can either be recycled back to the cell surface or, following continuous agonist stimulation, may be targeted to the lysosome for degradation. However, it is known that not all GPCR agonists activate these homeostatic mechanisms equally (*19*), and an emerging paradigm suggests that, for a given receptor, distinct agonists can have differential actions on G-protein and arrestin-linked signaling pathways, a phenomenon recently termed biased agonism (*17*, *20*).

In this study, we examined activation and regulatory mechanisms for the M_1 mAChR in response to the orthosteric agonist carbachol (CCh) and two allosteric agonists, AC260584 and TBPB. All three agonists produced robust activation of the M_1 mAChR in calcium mobilization and ERK 1/2 phosphorylation assays, but in contrast to CCh, the allosteric agonists had either a minimal effect (TBPB) or a delayed effect (AC260584) on the recruitment of arrestin 3. CCh treatment induced endocytosis and downregulation of the M_1 mAChR, but in cells exposed to AC260584 or TBPB, M_1 mAChR receptors remained on the cell surface and were spared from degradation. Finally, in contrast to carbachol, M_1 mAChR receptors pretreated with allosteric agonists remained sensitive to subsequent stimulation. Taken together, these results indicate that allosteric and orthosteric agonists may fundamentally differ in their mechanism of M_1 mAChR activation, regulation, and their effects on downstream signaling pathways. Subtype-selective allosteric agonists represent a major step forward in cholinergic pharmacology and will likely have a significant impact on the understanding of basic receptor biology and on the ability to modulate cholinergic receptors in clinical settings.

Results and Discussion

Activation of the M_1 mAChR by Orthosteric and Allosteric Agonists

As previously reported, AC260584 and TBPB are potent and highly selective M_1 mAChR agonists (*12*, *16*). In order to more extensively characterize the signal transduction pathways activated by allosteric versus orthosteric M_1 mAChR agonists, we compared functional responses in two separate assays. Phosphorylation of the extracellular signal regulated kinase ERK 1/2 is an M_1 mAChR-dependent response in neurons and plays a key role in synaptic plasticity, learning, and memory (*8*, *21*). In order to test whether allosteric agonists are capable of activating ERK 1/2, we performed concentration−response analysis in HEK293T cells expressing wild type human M_1 mAChR. CCh, AC260584, and TBPB all produced concentration dependent increases in the phosphorylation of ERK 1/2 (Figure 1A). Atropine (1 μM) completely blocked ERK 1/2 phosphorylation by all three agonists, confirming that ERK 1/2 phosphorylation by AC260584 and TBPB is mAChR-dependent (Figure 1B). However, the response was not changed by preincubation of cells with AG1478 (250 nM), a tyrosine kinase inhibitor that blocks activation of the epidermal growth factor receptor (EGFR). ERK phosphorylation has been shown to be dependent on EGFR activation in some systems, and CCh stimulation is known to induce EGFR transactivation in specific cell lines (*22*). Thus, the results of these experiments demonstrate that allosteric and orthosteric M_1 mAChR agonists activate ERK 1/2 phosphorylation in an EGFR-independent manner in HEK293T cells.

The M_1 mAChR couples to the G_q G-protein, which activates phospholipase C to initiate a series of signaling events, including the release of intracellular Ca^{2+}. Following agonist stimulation of G_q-coupled receptors, intracellular Ca^{2+} concentrations typically rise within seconds and gradually return to baseline as G_q signaling is terminated and Ca^{2+} is resequestered in intracellular stores. In M_1-CHO cells loaded with the calcium-sensitive dye

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Fluo-4, CCh (EC_{50} 99.0 nM, E_{max} 100), AC260584 (EC_{50} 146 nM, E_{max} 104), and TBPB (EC_{50} 95.8 nM, E_{max} 75.7) all caused a rapid release of intracellular calcium (Figure 2A). In order to assess the temporal profile of agonist-induced calcium signaling, we performed live-cell intracellular calcium imaging in M_1-CHO cells stimulated with a brief agonist application and subsequently washed with buffer. The CCh-evoked response (100 μM) returned to baseline within 4 to 5 min of agonist washout, while cells treated with AC260584 (320 nM) and TBPB (1 μM) showed a more prolonged response, returning to baseline within 8 to 10 min following agonist washout (Figure 2B). Hence, allosteric agonists evoke more enduring activation of M_1 than the orthosteric agonist CCh.

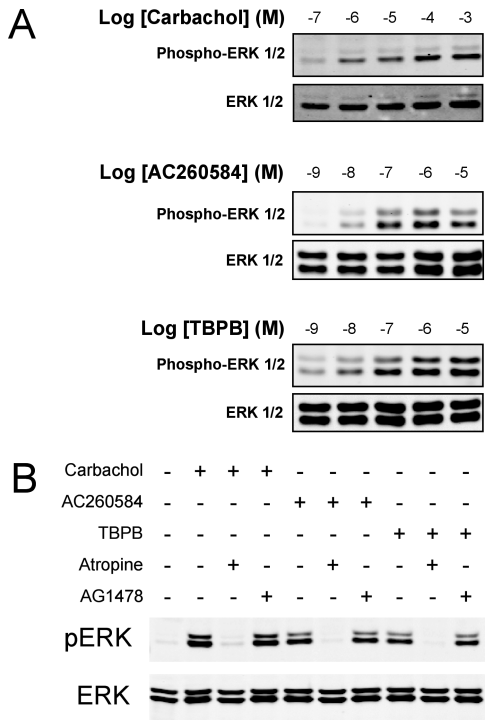

Figure 1. (A) Phosphorylation of ERK 1/2 regulated by orthosteric and allosteric M_1 mAChR agonists. In M_1-HEK cells, 5 min stimulation with CCh, AC260584, and TBPB promotes concentration-dependent increases in the phosphorylation of the mitogen activated protein kinase ERK 1/2. For each panel, detection of phospho-specific ERK 1/2 bands are shown above, and bands corresponding to total (nonphospho-specific) ERK 1/2 are shown below. (B) Specificity of ERK 1/2 signaling by orthosteric and allosteric M_1 mAChR agonists. In M_1-HEK cells, all three agonists tested induced ERK 1/2 phosphorylation that was completely blocked by atropine (1 μM). Activation was not affected by the tyrosine kinase inhibitor AG1478 (250 nM), indicating that M_1 mAChR-regulated ERK 1/2 phosphorylation is not dependent on EGFR activation in this cell type.

Differential Regulation of Arrestin 3 by Allosteric M_1 mAChR Agonists

Because GPCR signaling is commonly regulated by recruitment of members of the arrestin family (*18*), we investigated whether orthosteric and allosteric M_1 mAChR agonists differed in their ability to recruit arrestin. Of the four mammalian arrestin subtypes (for a review, see ref *23*), arrestin 1 and arrestin 4 are restricted to the visual system; thus, we focused our attention on arrestin 2 (also called β-arrestin 1) and arrestin 3 (also called β-arrestin 2). We did not observe agonist-induced recruitment of arrestin 2 in M_1-CHO cells (data not shown). When the M_1 mAChR and GFP-tagged arrestin 3 (Arr3) were coexpressed in CHO-K1 cells, CCh (100 μM) produced a striking translocation of Arr3 from a cytoplasmic reservoir to discrete puncta on or near the cell surface within 5 min. In contrast, Arr3 recruitment induced by both AC260584 (320 nM) and TBPB (1 μM) was severely blunted, with no significant change in depletion of cytoplasmic Arr3 and only occasional formation of puncta (Figure 3A). We next sought to determine the effect of prolonged exposure to agonist on Arr3 recruitment. Overnight treatment with TBPB

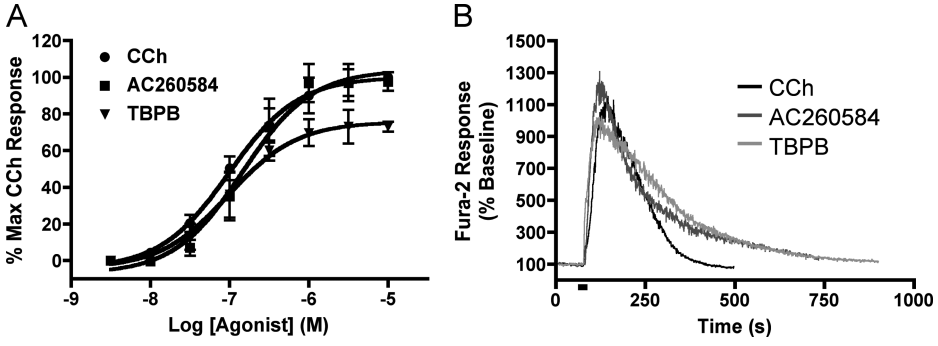

Figure 2. Intracellular calcium mobilization induced by orthosteric and allosteric M_1 mAChR agonists. (A) Concentration−response curves for intracellular calcium mobilization in M_1-CHO cells induced by orthosteric and allosteric M_1 mAChR agonists. Data are the mean ± SD of three independent experiments performed in quadruplicate. (B) Ratiometric measurement of intracellular calcium concentration. M_1-CHO cells were loaded with Fura-2 AM and perfused with loading buffer. One minute into the recording protocol, cells were stimulated with the indicated agonists (100 μM CCh, 320 nM AC260584, or 1 μM TBPB) for 15 s (the bar shown beneath the *x*-axis represents the duration of agonist application). Recording was continued in the prescence of buffer alone until calcium concentrations returned to baseline. Data are shown as the average response from 8 to 12 cells per treatment group and are representative of three independent experiments.

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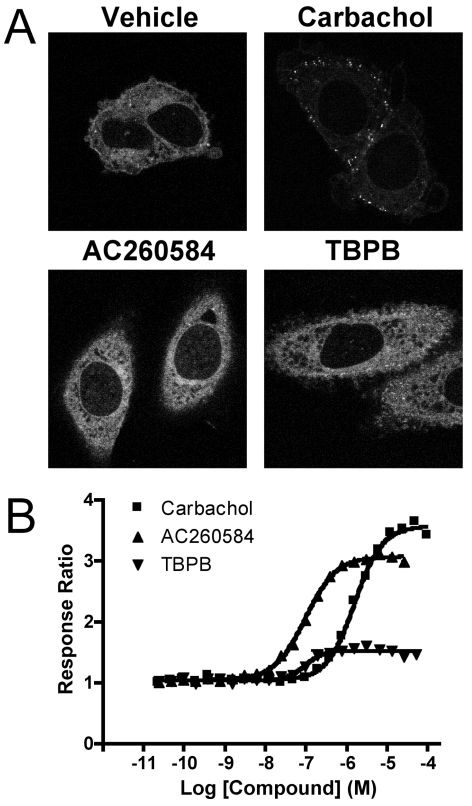

Figure 3. Differential regulation of Arr3 by allosteric M_1 mAChR agonists. (A) GFP-tagged Arr3 is localized diffusely throughout the cytoplasm at baseline and is recruited by CCh stimulation (100 μM, five minutes) to discrete puncta. In contrast, neither AC260584 (320 nM) nor TBPB (1 μM) produce a substantial shift of Arr3 from its cytoplasmic reservoir after 5 min. Images shown are representative of three independent experiments. (B) Concentration−response curve showing recruitment of Arr3 following overnight drug treatment in a recombinant cell-based assay in which Arr3 recruitment causes activation of a β-lactamase reporter gene and disruption of fluorescence resonance energy transfer (FRET) between fluorophores of the β-lactamase substrate. Data are shown as a reponse ratio (RR) of FRET fluorophores where a higher ratio indicates increased Arr3 recruitment.

resulted in modest Arr3 recruitment (~20% of CCh max), but treatment with AC260584 produced a substantial Arr3 response (~80% of CCh max) (Figure 3B). Together, these data indicate that orthosteric and allosteric M_1 mAChR agonists differ in their profile of Arr3 recruitment. Because arrestins have been shown to mediate specific signaling cascades independent of G-proteins (*24*), this finding may have additional implications for the specificity of signaling regulated by allosteric mAChR agonists.

M_1 mAChRs Exposed to Allosteric Agonists Remain on the Cell Surface

Because arrestin recruitment is tightly linked with receptor endocytosis (*25*), we next asked whether the reduced arrestin recruitment observed following acute stimulation with allosteric agonists was associated with decreased internalization of M_1 mAChRs from the cell

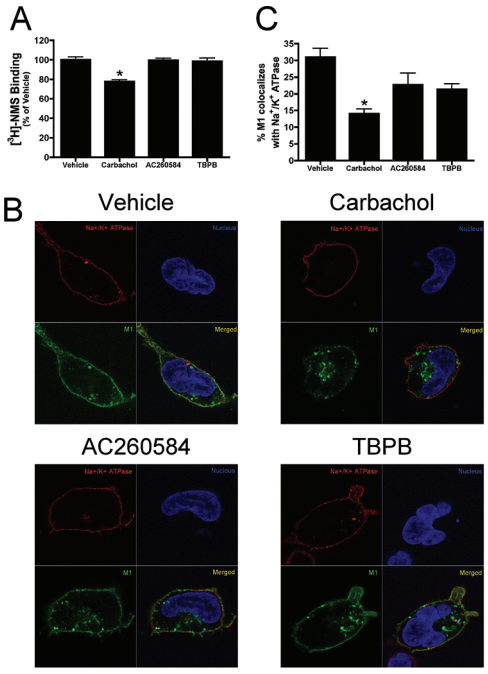

Figure 4. Measurement of agonist-induced M_1 mAChR internalization. (A) CHO-K1 cells expressing M_1 mAChR and Arr3 were treated for 60 min with CCh (100 μM), AC260584 (320 nM), or TBPB (1 μM), and surface M_1 mAChRs were quantified by membrane-impermeant [^{3}H]-NMS radioligand binding. Specific binding (determined by subtracting the background from atropine pretreated samples) is expressed as a percentage of binding in vehicle treated cells from three independent experiments performed in triplicate. Binding is significantly reduced in CCh-treated cells as compared to the vehicle (paired t test, $p < 0.0001$). (B) M_1-HEK cells were treated with the indicated drugs as in panel A for 60 min, and cells were double-labeled by immunocytochemistry for M_1 mAChR and Na^+/K^+ ATPase, a marker of the cell surface. (C) Colocalization of M_1 mAChR and Na^+/K^+ ATPase was determined from confocal microscopy images and is expressed as the percentage of specific M_1 mAChR pixels that overlap with Na^+/K^+ ATPase pixels. Data represent three independent experiments, with 10 cells imaged per experiment for each drug condition. One-way ANOVA is significant ($p = 0.0083$), and Tukey's multiple comparison post-test demonstrates a significant difference in colocalization between the vehicle and CCh-treated cells ($p < 0.01$).

surface. In the case of orthosteric agonists (e.g., CCh), initial exposure (minutes to hours) causes internalization from the plasma membrane and trafficking to endosomal compartments, from which receptors can either be recycled to the cell surface or targeted to lysosomes for degradation. In M_1-CHO cells, 60 min of CCh treatment (100 μM) induced only minimal internalization of M_1 mAChRs as measured by radioligand binding with membrane-impermeant [^{3}H]-NMS. Coexpression of Arr3 with the M_1 mAChR significantly accentuated this CCh-mediated internalization, with a ~25% reduction in [^{3}H]-NMS binding following 60 min of CCh stimulation. However, the same duration of exposure to AC260584 (320 nM) and TBPB (1 μM) failed to cause significant internalization of M_1 mAChRs (Figure 4A). In order to directly visualize agonist effects

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on M_1 mAChR internalization, we performed double label immunocytochemistry and confocal microscopy to colocalize the M_1 mAChR with Na^+/K^+ ATPase, a marker of the cell surface (Figure 4B). In M_1-HEK cells, 60 min of CCh treatment (100 μM) resulted in an ~50% decrease of colocalization between the M_1 mAChR and Na^+/K^+ ATPase, while neither AC260584 (320 nM) nor TBPB (1 μM) resulted in significant loss of M_1 mAChR colocalization with Na^+/K^+ ATPase (Figure 4C). For these experiments, drug concentrations were chosen on the basis of potency and selected to achieve maximal activation. We note that for each compound, the concentration used corresponds to maximal or near-maximal arrestin 3 recruitment, an important parameter given that receptor internalization and downregulation are likely arrestin-mediated responses. Together, these results demonstrate that M_1 mAChR activation by allosteric agonists produces significantly less internalization of the M_1 mAChR than that induced by the orthosteric agonist CCh.

M_1 mAChRs Exposed to Allosteric Agonists Are Spared from Degradation

Prolonged agonist exposure typically induces lysosomal degradation of GPCRs within hours to days (*26*). Given the blunted Arr3 recruitment and receptor internalization observed following stimulation with allosteric M_1 mAChR agonists, we predicted that these compounds would produce significantly less receptor degradation. In order to assess M_1 mAChR receptor downregulation, CHO-K1 cells expressing M_1 mAChR and Arr3 were exposed to CCh (100 μM), AC260584 (320 nM), or TBPB (1 μM) for 24 h, and total-cell receptors were measured using the lipophilic muscarinic ligand [^{3}H]-QNB. As shown in Figure 5, 24-h exposure to CCh results in the degradation of ~25% of M_1, but neither AC260584 nor TBPB caused significant loss of M_1 mAChR.

M_1 mAChR Receptors Exposed to Allosteric Agonists Remain Functionally Sensitive

The differential effects on arrestin recruitment, receptor internalization, and downregulation suggest that allosteric and orthosteric compounds may lead to important differences in functional desensitization. We directly tested the effects of AC260584 and TBPB exposure on the ability of the M_1 mAChR to respond to subsequent agonist stimulation. Pretreatment of M_1-HEK cells with CCh (100 μM) almost completely attenuated the ERK 1/2 phosphorylation response to a subsequent CCh challenge (100 μM), but cells pretreated with AC260584 (320 nM) or TBPB (1 μM) responded to subsequent CCh stimulation. These results indicate that M_1 mAChRs remain more sensitive to stimulation following exposure to allosteric agonists than the orthosteric agonist CCh (Figure 6). Furthermore, as shown in

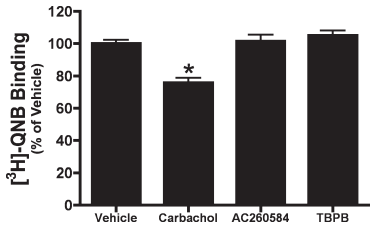

Figure 5. Regulation of M_1 mAChR downregulation by orthosteric and allosteric agonists. CHO-K1 cells expressing M_1 mAChR and Arr3 were treated for 24 h with CCh (100 μM), AC260584 (320 nM), or TBPB (1 μM), and total-cell M_1 mAChRs were measured using the lipophilic radioligand [^{3}H]-QNB. Specific binding (determined by subtracting the background from atropine controls) is shown as a percent of vehicle-treated cells from five independent experiments performed in triplicate. Binding is significantly reduced in CCh-treated cells as compared to that in the vehicle (paired t test, $p < 0.0001$).

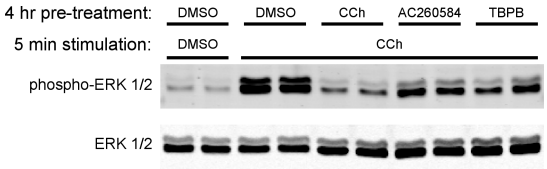

Figure 6. M_1 mAChR receptors exposed to allosteric agonists remain sensitive to CCh stimulation. In M_1-HEK cells, pretreatment (4 h) with CCh (100 μM) virtually abolishes a secondary ERK 1/2 phosphorylation response to a 5-min CCh (100 μM) stimulation. In contrast, cells pretreated with AC260584 (320 nM) and TBPB (1 μM) show a measurable, though slightly blunted, response to CCh stimulation.

Figure 2B, the time required for intracellular calcium concentrations to return to baseline following agonist stimulation was longer in AC260584- and TBPB-stimulated cells than in CCh-stimulated cells. This finding may reflect less effective termination of signaling and is consistent with the blunted recruitment of Arr3 observed following brief stimulation with AC260584 and TBPB.

Conclusions

Allosteric agonists represent a major advance in cholinergic pharmacology, allowing much greater selectivity for mAChR subtypes than is achievable with traditional orthosteric compounds. In the present study, we provide evidence that two structurally distinct allosteric M_1 mAChR agonists effectively mobilize intracellular Ca^{2+} and induce phosphorylation of ERK 1/2 but are markedly different from the orthosteric agonist CCh with much less ability to rapidly recruit Arr3 and trigger compensatory mechanisms including receptor desensitization, endocytosis, and downregulation. Our finding that allosteric M_1 mAChR agonists potently activate G_q-coupled signal transduction pathways while inducing minimal receptor endocytosis and degradation suggests that the specific receptor conformations stabilized by

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allosteric agonists may regulate distinct signaling mechanisms. This discovery has broad implications for the understanding of GPCR biology and for the application of cholinergic therapies in treating neurodegenerative and neuropsychiatric disorders.

As the understanding of GPCR signaling and regulation has been refined, much attention has focused on the role of arrestins. While originally characterized as proteins that mediate receptor desensitization and endocytosis, it is now known that arrestins can directly regulate signaling events independent of G-proteins (*24*) and participate in several cell biological processes including chemotaxis (*27*), stress fiber formation (*28*), and protein synthesis (*29*). Further investigation has shown that specific agonists for the β_2-adrenergic receptor display efficacy for arrestin-based signaling that is disproportionately higher than their efficacy for G-protein-based signaling would have predicted, leading to the coining of the term biased agonism to describe selective or preferential activation of arrestin-mediated signaling (*30*). In this study, we present data demonstrating that the allosteric M_1 mAChR agonists AC260584 and TBPB stimulate Ca^{2+} release and ERK 1/2 activation but are impaired in their ability to recruit Arr-3 following acute stimulation. We observed a more complex pattern of Arr3 recruitment following prolonged agonist treatment, with AC260584 inducing levels of Arr3 recruitment approaching levels in CCh-treated cells, whereas TBPB caused only modest Arr3 recruitment. Our results significantly extend recent studies showing that allosteric M_1 mAChR agonists induce weak recruitment of arrestin 2 (β-arrestin 1) (*31*, *32*). Additional studies will be required to determine the physiologic implications of allosteric mAChR agonist induced arrestin recruitment in native systems, particularly with regard to the temporal pattern of arrestin recruitment, but the present study suggests that structurally distinct ligands may signal through specific arrestin-linked mechanisms.

Previous reports have established a tight correlation between the intrinsic activity of a GPCR agonist and its efficacy for promoting receptor endocytosis (*33*), providing support for the model that GPCR activation is directly linked to regulatory mechanisms that attenuate signaling and lead to receptor sequestration and downregulation. While the majority of agonists display this pattern, it has also been shown that certain GPCR agonists activate receptors without promoting receptor desensitization or endocytosis (*34*), prompting revision of the model in which intrinsic activity and receptor endocytosis are fundamentally linked. Recently, Thomas et al. reported that allosteric M_1 agonists related to AC260584 fail to elicit the full pattern of M_1 mAChR internalization and downregulation observed with orthosteric M_1 mAChR agonist treatment (*35*). Here, we demonstrate that two structurally distinct allosteric agonists activate the M_1 mAChR while inducing much less compensatory receptor endocytosis and downregulation than the orthosteric agonist CCh. These differential effects suggest that allosteric agonist binding may put the M_1 mAChR in a conformation in which it interacts with certain intracellular signaling and/or scaffolding proteins but not others. Recently, Li and colleagues demonstrated that different classes of agonists induce distinct structural changes in the M_3 mAChR subtype (*36*, *37*), providing evidence for a molecular basis by which distinct agonists acting on a single receptor can differentially regulate signaling pathways. It is possible that in addition to activating signaling cascades shared by orthosteric agonists, allosteric agonists could also regulate additional pathways. Privileged signaling regulated by allosteric agonists is beginning to be explored for a variety of GPCRs including metabotropic glutamate receptors (*38*), providing an intriguing and potentially clinically useful aspect of GPCR signaling.

An alternate explanation for our observation of blunted arrestin 3 recruitment and receptor endocytosis is that the allosteric agonists evaluated in this study display a lower efficacy compared to that of CCh, rather than a bias in agonism. Because radioligands for allosteric binding sites on the M_1 mAChR do not exist, it is impossible to precisely determine the relationship between occupancy and efficacy for allosteric compounds using traditional methods of receptor affinity and competitive binding. However, careful analysis of the available data aid in the interpretation of this question. The potency of CCh at each of the responses measured in this study gives clear insight into the levels of receptor reserve for the individual assays. CCh has an affinity for the M_1 mAChR in the low micromolar range. CCh has a potency for activating M_1 coupling to ERK 1/2 phosphorylation and for activating arrestin 3 recruitment in the micromolar range. The finding that CCh potencies in these assays are the same as CCh affinity for the M_1 mAChR suggests that there is no significant receptor reserve for the activation of ERK 1/2 phosphorylation or arrestin 3 recruitment. In contrast, CCh has a potency of approximately 100 nM for the activation of Ca^{2+} mobilization. This suggests that there is significant receptor reserve when using CCh in the Ca^{2+} mobilization assay. Interestingly, both AC260584 and TBPB have potencies in the 10−100 nM range for the activation of ERK 1/2 phosphorylation and arrestin 3 recruitment. If there is little or no receptor reserve in these assays (as suggested by the CCh potency), this is likely to reflect the true efficacies of these compounds in eliciting these responses.

It is also important to note that CCh, TBPB, and AC260584 all have submicromolar potencies in the Ca^{2+} mobilization assay. While it is clear that this assay

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displays significant receptor reserve when CCh is used as the agonist, the potencies of TBPB and AC260584 are similar in all three assays. This suggests that differences in receptor reserve do not have the same influence on the potencies of these allosteric agonists as they do with CCh. The fact that the potencies of TBPB and AC260584 are constant across assays suggests that the differences in efficacy of TBPB at inducing arrestin 3 recruitment are not likely to be explained simply by differences in receptor reserve in the different assays. However, because we cannot directly assess affinities, we cannot fully evaluate the occupancy/efficacy relationships for the three compounds. Therefore, we cannot definitively rule out the possibility that a more traditional view of TBPB as a partial agonist with similar efficacies across signaling pathways could explain these results.

The fact that AC260584 and TBPB do not rapidly recruit arrestin or induce M_1 mAChR endocytosis may have important pharmacological and cell biological implications. Agonist-induced receptor endocytosis and lysosomal degradation could limit efficacy over extended periods of administration, making allosteric agonists that do not induce these compansatory changes attractive targets for chronic therapeutic applications. Indeed, studies in acetylcholinesterase knockout mice have revealed that the loss of this enzyme, which regulates attenuation of signaling at cholinergic synapses, results in significant downregulation of mAChRs, aberrant receptor trafficking, and blunted response to agonist stimulation (*39*, *40*). These perturbations in the cholinergic system serve as a model for the alterations that likely occur following chronic administration of cholinesterase inhibitors, the predominant therapy for Alzheimer's disease (AD), and may account for the limited clinical efficacy of these drugs. It is worth noting, however, that there is evidence supporting a role for arrestin-mediated endocytosis in maintaining the ability of a GPCR to respond to repeated agonist stimulation. Whistler et al. showed that morphine, an agonist at the μ-opioid receptor, fails to promote arrestin recruitment and receptor internalization, in contrast to the μ-opioid receptor agonist etorphine (*34*). Interestingly, morphine causes more physiological tolerance and dependence than etorphine, and the authors hypothesize that persistant receptor activation in the absence of desensitization, endocytosis, and recycling triggers alternative mechanisms of compensation that lead to tolerance. The effects of chronic *in vivo* administration of allosteric M_1 mAChR agonists need to be investigated directly in order to determine whether they induce functional changes *in vivo* following repeated administration. It is conceivable that drug discovery efforts could include arrestin recruitment as a key screening parameter for the development of future M_1 mAChR-based therapeutics.

Subtype-selective allosteric agonists represent a tremendous advance in cholinergic pharmacology and will likely have a major impact on cholinergic-based therapies for neurological and neuropsychiatric disorders. The findings of this study complement a growing body of literature indicating that GPCR signaling is remarkably diverse and that structurally distinct agonists differ with respect to the profiles of responses they elicit. Ongoing investigation in this exciting field should continue to enhance both the understanding of basic receptor biology and the utility of clinical pharmacotherapy.

Methods

Cell Culture and DNA Transfections

CHO-K1 cells were maintained at 37 °C and 5% CO_2 in DMEM containing 10% fetal bovine serum (FBS), 1% nonessential amino acids, 100 U/mL penicillin, and 100 μg/mL streptomycin. CHO-K1 cells stably transfected with a human M_1 mAChR cDNA (M_1-CHO) were maintained in the presence of 50 μg/mL G418 (Calbiochem, San Diego, CA). HEK293T cells were transduced with a high-titer lentivirus vector in which the human M_1 mAChR was cloned in place of GFP in the FUGW backbone (M_1-HEK). M_1-HEK cells were maintained in DMEM containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. The receptor density (Bmax) in the M_1-CHO and M_1-HEK cell lines was 170 ± 34 and 1761 ± 316 fmol/mg protein, respectively. The Arrestin 3-GFP construct was a gift from Dr. Vsevolod Gurevich (Vanderbilt University). Transient transfections were performed using FuGENE 6 transfection reagent (Roche, Basel, Switzerland) according to the manufacturers' instructions.

Calcium Mobilization Assay

M_1-CHO cells were plated on poly lysine coated glass coverslips and loaded with 5 μM Fura-2 AM in 1 μM pluronic acid (Invitrogen, Carlsbad, CA) for 1 h at 37 °C in buffer containing 150 mM NaCl, 10 mM HEPES, 3 mM KCl, 22 mM sucrose, 10 mM glucose, 1 mM $MgCl_2$, 2 mM $CaCl_2$, and 2.5 mM probenecid at pH 7.4. Coverslips were transferred to the microscope stage and perfused continuously with buffer. One minute into the recording protocol, the indicated drugs were administered for 15 s. Fura-2 emission was detected at 510 nm (following excitation at 340 and 380 nm), and ratiometric images were captured using Imaging Workbench software (INDEC Biosystems, Santa Clara, CA) in conjunction with an Olympus BX51WI microscope and a PTI IC200 intensified camera. Data are represented as the ratio of fluorescence intensity from 340 nm/380 nm excitation normalized to baseline.

ERK 1/2 Phosphorylation Assay

M_1-HEK cells were plated at a density of 50,000/cm^2 in 6-well culture dishes 3 days before use. On the day before the experiment, the culture medium was replaced with 2 mL of serum-free DMEM. Prior to beginning the experiment, cells were rinsed with 2 mL of serum-free DMEM. Atropine control conditions were pretreated for 30 min with 1 μM

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atropine sulfate. Following agonist treatment, cells were collected in PBS containing protease inhibitor cocktail (Roche), 1 mM sodium orthovanadate, and 0.1 mM ammonium molybdate. Fifty micrograms of protein per sample was separated by SDS−PAGE on 12% acrylamide gels, transferred to PVDF membranes, and probed with phospho- and total-ERK 1/2 antibodies (Cell Signaling, Danvers, MA). Following primary antibody incubation, blots were rinsed and incubated with Alexa 680 (Molecular Probes, Eugene, OR) and IR Dye 800 (Rockland, Gilbertsville, PA) conjugated secondary antibodies. Blots were scanned on an Odyssey Infrared Imager (Li Cor, Lincoln, NE).

Radioligand Binding Assays

M_1-CHO cells were treated with the drug concentrations indicated in the figure legends. All drug treatments were carried out in the presence of 20 μg/mL cycloheximide. For measurement of total M_1 mAChR receptors, cells were suspended in PBS and incubated at 37 °C for 90 min with 1 nM [^{3}H]-quinuclidinyl benzilate (QNB) and 1% bovine serum albumin (BSA). Cell suspensions were collected onto GF/B glass microfiber filters (Whatman, Piscataway, NJ) using a Brandel Harvester (Brandel, Gaithersburg, MD). For measurement of surface M_1 labeling, cells were rinsed with cold DMEM and incubated overnight at 4 °C with 1 nM [^{3}H]-*N*-methylscopolamine (NMS) and 1% BSA. The cells were then rinsed three times with cold DMEM and suspended in PBS. Radioligand binding was quantified by liquid scintillation spectroscopy. Nonspecific binding was determined using atropine.

Immunocytochemistry

Immunocytochemistry and confocal microscopy were performed as previously described (*41*). Briefly, M_1-HEK cells were treated with the indicated agonists and processed for double-label immunocytochemistry to visualize the M_1 mAChR and the cell surface marker Na^+/K^+ ATPase. Images were captured using a Zeiss LSM 510 laser scanning confocal microscope. Quantitation of colocalization was performed in a blinded fashion on unprocessed images using MetaMorph image analysis software (Molecular Devices, Sunnyvale, CA). Adobe Photoshop (San Jose, CA) was used for final image preparation.

Arrestin 3 Recruitment

Fluorescence Microscopy. CHO-K1 cells were cotransfected with Arrestin 3-GFP and M_1 mAChR and replated 1 day later onto glass coverslips coated with Matrigel (BD Biosciences, Franklin Lakes, NJ). On the day of the experiment, cells were incubated for 5 min at 37 °C with the indicated agonist, fixed with 2% paraformaldehyde, and processed by fluorescence immunocytochemistry to verify M_1 mAChR expression. Cells expressing both Arrestin 3-GFP and M_1 mAChR were imaged on a Zeiss LSM 510 confocal microscope. Adobe Photoshop was used for final image preparation.

FRET. Tango M_1-UAS-*bla* U2OS cells (Invitrogen K1861) were plated in a black walled, clear bottom 384 well plate with the indicated concentrations of Carbachol or TBPB and incubated overnight at 37 °C/5% CO_2. Cells were then loaded with the LiveBLAzer FRET-B/G substrate (Invitrogen K1096) kit according to manufacturer's instructions. Tango β-lactamase activity was determined by fluorescence measurement using a bottom reading plate reader with excitation set at 409 nm and emission set at 460 nm (blue) and 530 nm (green). Background fluorescence from a media-only well was subtracted from both 460 and 530 nm reads. Blue/Green (B/G) ratios were obtained by dividing the background subtracted 460 nm read by the background subtracted 530 nm value. The response ratio (RR) was obtained by dividing the B/G ratio of a stimulated/induced well by the B/G ratio of an unstimulated/uninduced well.

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Author Contributions

A.A.D. designed and performed experiments, analyzed data, and wrote the paper. C.J.H. performed experiments and analyzed data. A.E.B. performed experiments and analyzed data. N.R.M. performed experiments and analyzed data. M. F.-S. performed experiments and analyzed data. B.J.H. performed experiments and analyzed data. C.W.L. directed the synthesis of TBPB and analyzed data. P.J.C. designed experiments, analyzed data, and wrote the paper. J.J.L. designed experiments, analyzed data, and wrote the paper. A.I.L. designed experiments, analyzed data, and wrote the paper.

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Abbreviations

mAChR, muscarinic acetylcholine receptor; GPCR, G-protein coupled receptor; CCh, carbachol; TBPB, 1-(1-2-methylbenzyl)-1,4-bipiperidin-4-yl)-1*H*-benzo[*d*]imidazol-2(3*H*)-one; Arr3, arrestin-3; GFP, green fluorescent protein; CHO, Chinese hamster ovary; HEK, human embryonic kidney; DMEM, Dulbecco's modification of Eagle's medium; ERK, extracellular signal-regulated kinase; PBS, phosphate buffered saline; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride, QNB, quinuclidinyl benzilate, NMS, *N*-methyl scopolamine; BSA, bovine serum albumin.

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