Corrections

PHARMACOLOGY

The authors note that the author name Matthew Seager should have appeared as Matthew A. Seager. The online version appears incorrectly. This error does not affect the conclusions of the article. The corrected author line, affiliation line, and a related footnote appear below.

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Author contributions: L.M., M.A.S., and M.W. contributed equally to this work.

ANTHROPOLOGY

The authors note that, due to a printer’s error, on page 15227, right column, the equation on lines 14 and 15 of the first full paragraph appeared incorrectly. This error does not affect the conclusions of the article. The corrected equation appears below.

\[ E = \int_{0}^{t_m} GV(t)/S \, dt \]

APPPLIED PHYSICAL SCIENCES

The authors note that, due to a printer’s error, on page 15109, right column, the equation on lines 14 and 15 of the first full paragraph appeared incorrectly. This error does not affect the conclusions of the article. The corrected equation appears below.

\[ E = \int_{0}^{t_m} GV(t)/S \, dt \]
Selective activation of the M1 muscarinic acetylcholine receptor achieved by allosteric potentiation


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Edited by Robert J. Lefkowitz, Duke University Medical Center, Durham, NC, and approved July 22, 2009 (received for review February 17, 2009)

The forebrain cholinergic system promotes higher brain function in part by signaling through the M1 muscarinic acetylcholine receptor (mAChR). During Alzheimer’s disease (AD), this cholinergic neurons degenerate, therefore selectively activating M1 receptors could improve cognitive function in these patients while avoiding unwanted peripheral responses associated with non-selective muscarinic agonists. We describe here benzyl quinolone carboxylic acid (BQCA), a highly selective allosteric potentiator of the M1 mAChR. BQCA reduces the concentration of ACh required to activate M1 up to 129-fold with an inflection point value of 845 nM. No potentiation, agonism, or antagonism activity on other mAChRs is observed up to 100 μM. Furthermore studies in M1 knockout mice demonstrates that BQCA requires M1 to promote inositol phosphate turnover in primary neurons and to increase c-fos and arc RNA expression and ERK phosphorylation in the brain. Radioligand-binding assays, molecular modeling, and site-directed mutagenesis experiments indicate that BQCA acts at an allosteric site involving residues Y179 and W400. BQCA reverses scopolamine-induced memory deficits in contextual fear conditioning, increases blood flow to the cerebral cortex, and increases wakefulness while reducing delta sleep. In contrast to M1 allosteric agonists, which do not improve memory in scopolamine-challenged mice in contextual fear conditioning, BQCA induces β-arrestin recruitment to M1, suggesting a role for this signal transduction mechanism in the cholinergic modulation of memory. In summary, BQCA exploits an allosteric potentiation mechanism to provide selectivity for the M1 receptor and represents a promising therapeutic strategy for cognitive disorders.

Basal forebrain cholinergic neurons innervate information processing centers in the hippocampus and cortex to promote attention and memory. During AD, these neurons profoundly degenerate, contributing to cognitive impairment (1). While cholinesterase inhibitors demonstrate the therapeutic potential for boosting cholinergic function in AD, they are limited by tolerability and provide modest benefit, thus there remains a tremendous need for improved therapies (2). Selectively targeting the ACh receptors involved in memory, while sparing receptors involved in other physiological processes, could provide additional efficacy, a widely pursued approach that has yet to lead to new medicines.

ACh signals by activating ligand-gated ion channels (nicotinic receptors) and metabotropic (muscarinic) G protein-coupled receptors (GPCRs) designated M1–M5. Among the mAChRs, M1 is most abundantly expressed in the hippocampus, cortex, and striatum, and localizes to postsynaptic membranes (3), where it signals via Gq/11 G-proteins to phospholipase C and through other G-proteins to additional signaling systems (4, 5). M1 regulates several ion channels including KCNO inwardly rectifying K+ currents, voltage-gated calcium channels, and NMDA receptors (4–9). Thus M1 could mediate much of the cognitive effects of ACh. Supporting this hypothesis xanomeline, an M1/M4 preferring agonist, improved cognition and behavior in AD patients but was not tolerated due to unwanted cholinergic effects (10). Additional studies suggest that M1 activation could slow AD progression by reducing Aβ42 peptides (11). Thus a drug that activates M1 could potentially improve cognition while over time slowing the progression of the disease. Unfortunately, conservation of the ACh binding site has precluded the discovery of selective agonists.

Many GPCRs, including mAChRs (12), have allosteric binding sites by small molecules that activate the receptor in the absence of ligand (allosteric agonist) or enhance the response to native ligand (positive allosteric modulator) (13). As allosteric sites are theoretically under less evolutionary constraint, targeting them affords opportunities for selectivity. This concept was demonstrated by the M1 allosteric agonist TBPA (14) and a collection of relatively selective positive allosteric modulators (15). Here we describe BQCA [(4-methoxybenzyl)-4-oxo-1,4-dihydroquinoline-3-carboxylic acid], an orally available drug-like molecule that regulates memory and brain function by potentiating M1.

Results

Identification of BQCA. To identify M1 potentiators a screen was conducted on >1,000,000 compounds (see Materials and Methods). CHO cells stably expressing human M1 and an NFAT-responsive β-lactamase reporter to monitor Gq activation were treated with the EC50 of acetylcholine (15 nM) plus 6.23 μM of test compound. Compounds that increased signal more than 3 standard deviations from the mean, but had no effect in the absence of ACh, were selected for confirmation. This strategy yielded BQCA (Fig. L4), which is unrelated to other muscarinic ligands. BQCA alone had no effect on calcium mobilization up to 10 μM but increased ACh potency 128.8 ± 20.1-fold at 100 μM (n = 12) (Fig. 1B), with an inflection point (IP) value when potentiating 3 nM ACh of 845 ± 27 nM (n = 225) (Fig. 1C). Similar potentiation was observed in CHO cells stably expressing rhesus, dog, rat, or mouse M1 [IP = 300 ± 30 (n = 25), 300 ± 23 (n = 20), 330 ± 24 (n = 26), 210 ± 11(n = 32), respectively]. At 100 μM BQCA activated M1 in the absence of ACh to an approximate 50% maximal response (Fig. 1C). BQCA was then tested up to 100 μM on the other human mAChRs. For M2 and M3, CHO cells stably expressed the mutant


Conflict of interest statement: All authors were employed by Merck and Company, Inc. at the time of this study.

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G protein Gqi5 (16), which allows these Gi-coupled receptors to activate phospholipase C, permitting direct comparison between receptor subtypes. No effect was detected on M2–M5, indicating >100-fold selectivity (Fig. 24). To rule out the possibility that selectivity was caused by differences in M1 expression, [3H]-NMS binding to membrane preparations was measured and revealed that levels were comparable (see Materials and Methods). Next we addressed the possibility that selectivity over M2 and M4 was due to enzyme assays at 10 μM phosphate (IP3) metabolism in neurons by activating endogenous M1, BQCA is a drug-like molecule that potentiates M1 with brain concentrations approximating its potency (Fig. S3). Thus carboxylic acid that when dosed orally at 10 mg/kg in rats achieves S2). BQCA is a low molecular weight (331.3 Da) quinolone (Fig. S1). BQCA had no activity in pH9262 confirmng selectivity for M1. Stiction in vivo. To evaluate the effects of BQCA on c-fos and arc mRNA induction, markers of neuronal activation, mice were treated orally with 15 mg/kg BQCA and brains were collected 90 min later.

Selectivity on Native Receptors. ACh increases inositol triphosphate (IP3) metabolism in neurons by activating endogenous M1, or in the presence of BQCA at the indicated concentrations (colored lines). BQCA does not change the maximal response but causes a leftward shift in the ACh dose-response curve. Mean values from four replicate wells are plotted; data are representative of 12 independent experiments. (C) BQCA effects on calcium mobilization in hM1-CHO cells as measured by FLIPR when added at the indicated concentrations alone (bottom line) or in the presence of 3 nM ACh (top line). Effect of 3 nM ACh alone is plotted as single point, lower left corner. Each point is the mean of eight replicate determinations ± SEM and is representative of 225 independent experiments.

Selectivity in Vivo. To evaluate the effects of BQCA on c-fos and arc RNA induction, markers of neuronal activation, mice were treated orally with 15 mg/kg BQCA and brains were collected 90 min later.
reduced the concentration of ACh required to displace \(^{3}H\)NMS 45-fold at 10 µM (Fig. 3C). Using Schild analysis, BQCA decreased the concentration of ACh required to displace \(^{3}H\)NMS across all concentrations tested, but did not change the dose ratio, indicating that it does not exhibit cooperativity with ACh under equilibrium binding conditions (Fig. S5). Therefore BQCA binds an allosteric site to enhance the binding and efficacy of ACh.

**Mechanism of Potentiation.** We next determined if BQCA interacted with the ACh binding site. In radioligand competition assays 100 µM BQCA had no effect on the binding of the ACh-site directed antagonist \(^{3}H\)NMS to hM₁-CHO membranes (Fig. 3A). Instead BQCA reduced the concentration of ACh required to displace \(^{3}H\)NMS 45-fold at 10 µM (Fig. 3B), and enhanced \(^{35}S\)-GTPγS binding to hM₁-CHO membranes in response to ACh 32-fold at 10 µM (Fig. 3C). Using Schild analysis, BQCA decreased the concentration of ACh required to displace \(^{3}H\)NMS across all concentrations tested, but did not change the dose ratio, indicating that it does not exhibit cooperativity with ACh under equilibrium binding conditions (Fig. S5). Therefore BQCA binds an allosteric site to enhance the binding and efficacy of ACh.

**Interaction with the Extracellular Domain.** Molecular modeling was used to dock BQCA into human M₁. A potential extracellular binding site was identified near amino acids Y179 and W400. These two residues and their immediately adjacent amino acids are 100% conserved in rhesus, dog, rat, and mouse M₁. In contrast Y179 is not well conserved in other human mAChRs, and although whereas W400 is, E401 is not conserved and only M₁ possesses a negatively charged residue at this position (Fig. 3D). We mutated Y179 and W400 to alanine; both mutations abrogated the effect of BQCA without affecting ACh (Fig. 3E). Thus sequence diversity in the regions near Y179 and W400 could underlie BQCA selectivity.

for in situ hybridization. In wild-type mice, BQCA induced c-fos and arc RNA in the cortex, hippocampus, and cerebellum; arc was also elevated in the striatum (Fig. 2C). In contrast BQCA had no effect in M₁⁻/⁻ mice. We then assessed the phosphorylation of ERK in wild-type and M₁⁻/⁻ mouse cortex and hippocampus. ERK phosphorylation is a common downstream signaling event that functions in synaptic plasticity and memory (17). In wild-type mice, 15 mg/kg BOCA dosed orally increased the ratio of phosphoERK (pERK) to total ERK as revealed by western blot (28% increase, 15 mg/kg BOCA dosed orally increased the ratio of phosphoERK (pERK) to total ERK levels or cortical pERK. The increase in pERK was absent in M₁⁻/⁻ mice.

**Mechanism of Potentiation.** We next determined if BQCA interacted with the ACh binding site. In radioligand competition assays 100 µM BQCA had no effect on the binding of the ACh-site directed antagonist \(^{3}H\)NMS to hM₁-CHO membranes (Fig. 3A). Instead BQCA reduced the concentration of ACh required to displace \(^{3}H\)NMS 45-fold at 10 µM (Fig. 3B), and enhanced \(^{35}S\)-GTPγS binding to hM₁-CHO membranes in response to ACh 32-fold at 10 µM (Fig. 3C). Using Schild analysis, BQCA decreased the concentration of ACh required to displace \(^{3}H\)NMS across all concentrations tested, but did not change the dose ratio, indicating that it does not exhibit cooperativity with ACh under equilibrium binding conditions (Fig. S5). Therefore BQCA binds an allosteric site to enhance the binding and efficacy of ACh.
Physiological effects of BQCA. (A) Contextual fear conditioning. On day one animals received 0.3 mg/kg scopolamine + BQCA administered IP at the indicated doses before being introduced to a novel environment and receiving two foot shocks. Twenty-four hours later animals (n = 12–16/group) were reintroduced to the environment and freezing measured by automated detection equipment. Shown is mean percent of time freezing (± SEM). Data are representative of four experiments. *, different from vehicle; #, different from scopolamine + vehicle (P < 0.05, Dunnet test). (B) Increased cerebral blood flow in anesthetized rats in response to BQCA. Data were averaged over 1 min at the indicated time points (A–C) and is expressed as mean percent change from baseline ± SEM. (n = 4 animals). *, significantly different from baseline, P < 0.01, repeated measures ANOVA, t test.

Contextual Fear Conditioning. To examine the role of M1 in memory we measured contextual fear conditioning (CFC) in mice. This task requires the hippocampus (18) where M1 is highly expressed. Subjects were introduced to a novel environment where they received an aversive stimulus (foot shock). The next day the animal exhibited freezing behavior, indicative of fear, if it associated the aversive stimulus with the environment. Scopolamine, a nonselective muscarinic antagonist, was dosed 30 min before introduction to the novel environment to block formation of this association (19) (Fig. 4A). BQCA was co-dosed at 5, 10, 15, and 20 mg/kg IP with scopolamine on training day and had no effect on freezing behavior at that time. When animals were reintroduced into the test chamber those given scopolamine previously showed marked reduction in freezing compared to controls. However, animals that had been co-dosed with BQCA at 15 or 20 mg/kg had no scopolamine deficit (P < 0.05, ANOVA, Dunnett test). This experiment was repeated with structurally distinct derivatives of BQCA that are also >100-fold selective for M1 with similar results. Thus BQCA prevents scopolamine-induced memory deficits in CFC.

Cerebral Blood Flow. ACh promotes cerebral blood flow (CBF) (20) and M1 is expressed in cortical neurons and endothelial cells (21), however a role for M1 in CBF has not been defined. BQCA, but not vehicle, intravenously infused into anesthetized rats at 10 mg/kg caused a sustained increase in CBF by 20.5 ± 0.9% as measured by laser Doppler flowmetry (F12, 207) = 14.1; P = 0.005) (Fig. 4B). BQCA had no effect on arterial pressure (F12, 243) = 0.51; P = 0.63) and caused a brief but significant 11.0 ± 2.1% increase in heart rate (F12, 70) = 6.1; P = 0.036). Thus BQCA promotes CBF in rats.

Effects on Sleep. The cholinergic system modulates wakefulness and the onset of REM sleep (22, 23). To assess the role of M1 in sleep, rats were dosed with BQCA at 10 mpk IP 30 min before the beginning of the light (inactive) cycle for seven days. Electrooculogram and electromyogram activities were recorded and the time spent in active wake, light sleep, REM, and delta sleep were averaged in 30 min epochs across 7 days of testing. Over the first 90 min of the light phase BQCA increased the time spent in active wake and light sleep while concomitantly decreasing delta sleep (P < 0.001, Student’s t test) (Fig. 5B). By 120-min sleep patterns between BQCA-treated and control animals were similar and no lasting effects were observed.

Allosteric Agonists Exhibit Differential Efficacy. We then compared the activities of BQCA to TBPB, an M1-selective allosteric agonist that has anti-psychotic-like activity in rats (14). Like TBPB, BQCA at 10 and 30 mg/kg IP repressed amphetamine-induced locomotion in mice (Fig. 7A). However TBPB did not reverse the scopolamine deficit in CFC at 10 or 30 mg/kg IP (Fig. 5A); and similarly the allosteric agonist AC-42 was without effect at 3, 10, and 30 mg/kg (12) (Fig. 7B). To explore this difference we measured recruitment of β-arrestin, which mediates GPCR internalization and second messenger signaling (24). CHO cells expressing hM1 fused to a portion of β-galactosidase and β-arrestin fused to a complementary fragment of β-galactosidase (25) were treated with TBPB, AC-42, or ACh ± BQCA or eight BQCA analogs. Recruitment of
the β-galactosidase/β-arrestin fusion protein to M₁ restores β-galactosidase activity. BQCA and its analogs potentiated ACh-induced β-arrestin recruitment with EC₅₀ values correlated with potency in calcium mobilization (Fig. 5B). Neither TBPB nor AC-42 induced β-arrestin recruitment beyond ~20% of maximal activity up to 10 μM, thus no EC₅₀ was measurable (Fig. 5B and Fig. S7). Together these data indicate that BQCA diverges mechanistically from these allosteric agonists.

Discussion

Despite the therapeutic potential of M₁, high selectivity against other mAChRs has not been achieved. Here we describe BQCA, which sensitizes M₁ to ACh up to 100-fold (Fig. 1). It has little effect on M₂-M₃ or other class A GPCRs (Fig. 2 and Fig. S3), or in >300 other assays. In the absence of M₁, BQCA does not enhance ACh-mediated inositol phosphate metabolism or induce c-fos or arc RNA or ERK phosphorylation in the brain (Fig. 2 and Fig. S4). BQCA does not interact with the ACh site but instead enhances ACh activity from an allosteric pocket (Fig. 3). Molecular modeling combined with site-directed mutagenesis identified a potential extracellular interaction site conserved in M₁ that diverges in other mAChRs (Fig. 3). Interestingly Y179, one of the residues required for BQCA activity, corresponds to Y177 in M₂, which is in a common site critical for multiple allosteric agonists (26). Thus probing this region further could improve our understanding of allosteric modulation.

We used BQCA to explore the function of M₁ in brain function. BQCA promotes the learned association of a novel environment with an aversive stimulus, a task dependent on the hippocampus and sensitive to the anti-muscarinic scopolamine (Fig. 4). Selectively sensitizing M₁ with BQCA fully overcomes the amnestic effects of scopolamine in this model. These results contrast with data in M₁⁻/⁻ mice (27) and with an M₁ selective antagonist, VX0255035 (28). In the former study the M₁⁻/⁻ mice showed no deficit in CFC, and in the latter study VX0255035 reduced pilocarpine-induced seizures in rats, which requires M₁ (29), but did not interfere with CFC. Together these data suggest that deletion or inhibition of M₁ does not interfere with this form of memory. However our data indicate that M₁ activation during acquisition promotes memory, perhaps indicating that M₁ is not required for memory formation in CFC, but rather reinforces it by stimulating other receptor signaling systems such as the NMDA receptor (6).

Based on prior work (30) M₁ expressed in CA3 hippocampal neurons may be specifically involved in this positive modulation of memory.

We found that BQCA enhanced CBF (Fig. 4), a function of ACh attributed to M₃ based on receptor localization and knockout mouse experiments (31, 32). Our data indicate that M₁ also regulates CBF perhaps via neurovascular coupling. The cholinergic system also modulates sleep; ACh levels are elevated in the cortex and hippocampus during wakefulness and REM sleep relative to slow wave delta sleep (33). When dosed before the onset of sleep BQCA increased wakefulness and inhibited delta sleep without significant lasting effects (Fig. S7). This observation is consistent with M₁ promoting arousal and suggests that ACh levels decline during slow wave sleep in part to reduce M₁ activity.

Since allosteric ligands of GPCRs can selectively modulate some, but not all, available second messenger signaling systems (34) we compared BQCA to the allosteric agonists TBPB and AC-42. Both BQCA and TBPB inhibit amphetamine-induced locomotion in mice (14 and Fig. S7), suggesting that they modulate striatal dopaminergic activity. In contrast neither TBPB nor AC-42 reversed scopolamine deficits in CFC (Fig. 5 and Fig. S7). A potential reason for this difference is that these compounds do not efficiently induce β-arrestin recruitment as measured by enzyme complementation (Fig. 5). Other studies show that AC-42 does not activate all G-proteins coupled to M₁ (35), together suggesting that these allosteric agonists may exhibit ligand bias (36), the propensity to activate a subset of signaling pathways presumably by stabilizing unique receptor conformations. Since β-arrestins couple M₁ to diacyl glycerol kinases (24) and likely play other signaling functions, it will be interesting to identify β-arrestin-dependent responses triggered by ACh via BQCA.

In summary we identified a highly selective positive allosteric modulator of the M₁ muscarinic receptor. This compound will allow for further understanding of allostery at GPCRs, insight into the function of M₁, and potentially a class of therapies for diseases involving impaired function of the central cholinergic system.

Materials and Methods

Materials. Reagents were from Sigma unless noted, and animals were from Taconic Farms. Procedures were approved by the Institutional Animal Care and Use committee (IACUC) in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Chrm1⁻/⁻ animals were provided by Dr. Neil Nathanson (37).

Data Presentation. Experimental values are the mean of n independent determinations ± SEM unless otherwise indicated.

High-Throughput Screening. Using an automated screen for Gq activation by GPCRs (38), 2 × 10⁶ cells/well of CHO cells expressing human M₁, and an NFAT-responsive β-lactamase reporter were plated into 3,456 well plates and treated with 15 nM ACh plus 6.25 μM BQCA (or compound for 4 h before adding CCΦ4-AM (Invitrogen), to detect β-lactamase activity.

Fluorometric Imaging Plate Reader (FLIPR). CHO-NFAT cells expressing human mAChRs or rhesus, dog, mouse, or rat M₁ (in CHO-K1, ATCC) were plated (25,000 cells/well) in clear-bottomed polyd-lysine (PDL)-coated 384-well plates in growth medium using Labsystems Multidrop. Receptor expression levels for the human lines were measured by radioligand binding and were M₁ (0.70 ± 0.03), M₂ (3.22 ± 0.08), M₃ (0.53 ± 0.04), M₄ (1.61 ± 0.09), and M₅ (0.80 ± 0.07) pmol/mg protein (n = 3). Cells were grown overnight 37 °C at 5% CO₂. Washed 100 μL assay buffer (Hanks’ balanced salt solution/10 mM HEPES/2.5 mM probenecid/0.1% BSA) then incubated with 1 μM Fluo-4.3 mM (Invitrogen) 1 h at 37 °C. Extracellular dye was removed and Ca²⁺ was measured with a FLIPR384 fluorometric imaging plate reader (Molecular Devices) during incubation with compound 4 min followed by 4 min with the EC₂₀ of ACh.

IP₁. Cortical hippocampal neurons from embryonic day 15–16 wild-type or Chrm1⁻/⁻ mice were plated (70,000 cells/well) in PDL 96-well white plates and maintained days 9. IP₁ levels were measured using HTRF (Cisbio Biosysys) using Envision (Perkin-Elmer) following 10-min incubation with BQCA then 30 min with ACh. Ten mM LiCl was added to block degradation of inositol phosphates.

β-Arrestin. The hM₁AchR PathHunter CHO cells (DiscoveRx) stably expressing two fragments of β-galactosidase, one fused to M₁ and the other to β-arrestin, were treated with ligand and β-galactosidase complementation was monitored by PathHunter Detection reagent and read on a ViewLux (Perkin-Elmer). Compounds were assayed in 384-well plates ± ACh EC₅₀.

Binding Assays. Competition binding reactions used 25 μg human M₁ CHO membrane protein (Perkin-Elmer), test compounds or vehicle, and 0.15 nM [³²P]NMS in 96-well deep-well plates. Binding reactions (30 °C for 2–3 h) were terminated by rapid filtration. Nonspecific binding was determined by adding 10 μM atropine. Filter plates were washed 4× with ice-cold 20 mM HEPES, 100 mM NaCl, and 5 mM MgCl₂, pH 7.4 using a 96-well Perkin-Elmer harvester. Plates were dried and radioactivity counted with a TopCount NXT microplate scintillation counter (Perkin-Elmer). Counts were normalized to maximal specific binding with vehicle. The GTP-γ-S assay reactions used 25 μg CHO membrane protein, 2 μM GDP, and 0.1 nM [³²P]GTP-γ-S in a total volume of 500 μL; samples were incubated in a 96-well deep-well plate (0.5 h at 30 °C). The binding assay buffer, reaction termination, and counting were as above.

Counterscreens. BQCA was tested in duplicate (10 μM) for activity or radioligand displacement on >300 enzymes and receptors using commercially available assays (MDS Pharma). For potentiation of class A GPCRs, BQCA (37.5 μM) was analyzed ± agonist by FLIPR in CHO cells stably expressing the receptors using GPCRprofiler, details are available from the manufacturer (Millipore).

Molecular Modeling. Mutations were made by site-directed mutagenesis and sequence confirmed before transient transfection into CHO cells. Maximal bind-
immediate early gene induction. wild-type or chrm1 (m1) -/- mice were individually housed and desensitized by handling 5 times before the experiment. mice (n = 5/group) received 15 mg/kg BQCA in 5% beta-cyclodextrin/saline IP. after 1.5 h animals were euthanized in situ hybridization has been described in detail [41].

ERK phosphorylation. Adult (4-6 week) male mice (n = 6) were dosed with 15 mg/kg PO BQCA in sterile water or with vehicle alone and 1 h later cortical and hippocampal samples were collected. Homogenization was performed on ice in 0.3 M sucrose/1 mM HEPEs/1 mM MgCl2/1 mM EDTA/1 mM NaHCO3 (pH 7.4) supplemented with protease and phosphatase inhibitors. Thirty μg total protein was resolved on 10% SDs gels and probed with rabbit polyclonal anti-p44/p42 MAPK (T202/Y204) or mouse monoclonal anti-p42 MAPK (3A7) (Cell Signal Technologies). Immunoblots were quantitated on a Li-Cor Odyssey infrared imager (Li-Cor Biosciences).

contextual fear conditioning. on day one 10-week-old experimentally naive male B6SJL mice (n = 12-16/group) were dosed IP with BQCA in 5% beta-cyclodextrin and/or 0.3 mg/kg scopolamine in 0.9% saline 30 min before placement into a chamber (MED-VFC-M, Med Associates) for 2 min before 2 tone-footshock pairings (3 kHz, 85 dB) for 30 s co-terminated with a 0.5 mA, 1 s shock) 2 min apart. mice were removed to their home cage 30 s after the last pairing. Twenty-four hours later mice were placed back into the same chamber and freezing was measured by Video Freeze (Med Associates).

cerebral blood flow. Six- to eight-week-old male Sprague-Dawley rats were anesthetized with urethane (1 g/kg IP). Depth of anesthesia was monitored by toe pinch; supplemental urethane (10% of initial dose) was given to achieve deep anesthesia. Body temperature was kept at 37 °C via a heating pad and rectal temperature feedback probe. A femoral artery was cannulated with P50 tubing to record arterial pressure and heart rate via a pressure transducer (ADInstruments); the femoral vein was cannulated for drug infusion (20 μL/min). animals were placed in a stereotactic frame and a midline incision exposed the skull. A buffer hole was drilled over the frontal cortex (1.0 mm anterior and 4.0 mm lateral to bregma) leaving the dura intact. A laser Doppler probe connected to a data acquisition system (PowerLab/8/30, ADInstruments) was placed over the hole. CBF recordings began after MAP, HR, and CBF were stable for 15–20 min. Data are presented as percent increase over resting CBF.

sleep electroencephalography (EEG). sleep was evaluated in adult male Sprague-Dawley rats (age 3-8 months) chronically implanted with telemetric physiological monitors recording electrocorticogram and electromyogram activities as described [42]. Dosing was 30 min before lights on for 7 days in a crossover experiment.

amphetamine-induced locomotor activity. Six week old male B6SJL mice (n = 12) were dosed IP with the indicated dose of BQCA and placed into standard open field. motor activity was measured for 60 min. data were then given 2.5 mg/kg amphetamine then returned to the open field box and tracked for additional 40 minutes.

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Supporting Information

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SI Text

Molecular Modeling. Residues L225-K353 were deleted to facilitate modeling. One thousand models were built using MOE 2005.06 (CCG) and the 10 lowest energy models were refined with Maestro’s Prime module (Shrodinger), while maintaining the C98/C178 bond. Rotamer sampling was allowed during all phases. Ligand docking used the induced fit protocol in Maestro using GlideXP with enlarged inner and outer boxes and a distance-dependent dielectric constant (ε = 4). Ligand poses within 25 kcal/mol of the global minima were flexibly refined using MMFF94s [Halgren T (1999) MMFF VI. MMFF94s option for energy minimization studies. J Comp Chem 20:720–729] in our in-house modeling in UNIX system. Protein side chains within 8 Å and the backbone of the extracellular loops were allowed mobility. Next ligand poses within the lowest 10 kcal/mol of their global in vacuo minima were inspected and the partner protein conformer from each was examined for side-chain/backbone geometries within allowed Ramachandran space. Those with many Ramachandran violations were rejected. Individual sidechain rotamers were sampled using PyMol (DeLano Scientific) and optimized as above.
Fig. S1.  G protein activation in membranes from CHO cells expressing hM2 or hM4. [35S]-GTPyS recruitment to membranes following treatment with the indicated concentrations of ACh + either vehicle (black squares), 0.1 μM BQCA (red triangle), 1 μM BQCA (green inverted triangle), or 10 μM BQCA (blue diamond). The EC50 values for each condition are given on the right. Plotted are means of four replicate wells ± SEM.
Fig. S2. Effect of BQCA on the response of eight class A GPCRs to their respective agonists. BQCA (37.5 μM, tan line) or vehicle (DMSO, gray line) was added to a dose-response curve study of orthosteric agonists for the indicated human GPCR, each stably expressed in CHO cells. Each point is the mean of four wells ± SEM. The chart (bottom) shows the measured EC50s in the presence and absence of BQCA; no significant effects were observed.
Fig. S3. Brain and plasma BQCA concentrations after a single oral dose in sterile water to 4- to 5-week-old male Sprague-Dawley rats ($n = 3$ per time point). Compound was measured in plasma or brain homogenates using standard LC/MS pharmacokinetic methodology and concentration determined by comparing to a standard curve generated in plasma or brain homogenate from untreated animals spiked with known concentrations of BQCA. Plotted is the mean of the three values (note the log scale for concentration). Brain concentrations at the 16- and 24-h time points were below the limit of quantitation and thus are not plotted.
Fig. S4. BQCA induced ERK phosphorylation in the hippocampus in wild-type and M1/−/− mice. Adult (4–6 week) male mice (n = 6) were dosed with 15 mg/kg PO of BQCA in sterile water or with vehicle alone and 1 h later cortical and hippocampal samples were collected. Thirty μg total protein was resolved using 10% SDS gels and probed with rabbit polyclonal anti-p44/42 MAPK or mouse monoclonal anti-p42 MAP Kinase and normalized to β-actin band intensity. Data are expressed as the mean of six animals ± SEM. *, P < 0.05 different from all other groups, one-way ANOVA.
Fig. S5. Effect of BQCA on antagonist binding to hM1-CHO membranes. The apparent binding affinity, measured as EC$_{50}$ of $[3H]$-ACh was measured in the presence of N-methylscopolamine (NMS) ± 10 µM BQCA at the indicated concentrations. (A) BQCA decreased the EC$_{50}$ of $[3H]$-ACh 10-fold at each dose of NMS. (B) Schild plot analysis showing that BQCA does not alter the dose ratio as a function of NMS concentration, indicating that it does not exhibit cooperativity. Mean values from four replicates are plotted.
Fig. S6. Effects of BQCA on sleep architecture. Sleep was monitored by electrocorticogram and electromyogram in \( n = 8 \) chronically telemetered adult (4- to 12-month-old) male rats over 7 days each for vehicle and 10 mg/kg IP BQCA for a total of 16 measurements over 7 days for each condition. These data were used to determine the amount of time spent in each of the four phases (awake, light sleep, delta sleep, and REM sleep) per 30 min epoch. Data across non-overlapping 30 min time bins was averaged across days into a single value for each animal, then group means were calculated across all days to generate these means. ● - \( P \) value < 0.05, ○○○ - \( P \) value < 0.01, and ○○○○ - \( P \) value less than 0.001, student’s \( t \) test.
Fig. S7. Comparison between BQCA and allosteric agonists on amphetamine-induced locomotor activity and β-arrestin recruitment. (A) Mice (n = 12/group) were dosed with the indicated dose of BQCA or with vehicle alone for 20 min and activity monitored in an open field cage equipped with infrared motion detectors. Amphetamine (2.5 mg/kg) was then dosed IP at time 0 and total distance traveled was determined for the next 40 min. BQCA had no effect on motion before amphetamine dosing, but decreased amphetamine-induced locomotor activity dose-dependently with effects significant (P < 0.05) at 10 and 30 mg/kg (Dunnet test). Shown are means for each group ± SEM. from a representative of three experiments. (B) Effect of the allosteric agonist AC-42 on scopolamine deficits in contextual fear conditioning in mice. Data were collected, processed, and graphed as in Fig. 5. Note the lack of effect of AC-42 at any dose tested. (C) Enzyme complementation assay measuring β-arrestin recruitment to hM1 in CHO cells. The indicated concentrations of BQCA or TBPB were added alone or to the EC15 of ACh, which was empirically determined for each cell line and for each experiment. Values plotted are the mean of four replicate determinations. Note the increased enzyme complementation signal produced by BQCA + ACh and lack of pronounced effect of TBPB or BQCA alone.