Exploration of the Orthosteric/Allosteric Interface in Human M1 Muscarinic Receptors by Bitopic Fluorescent Ligands

Sandra B. D'aval, Esther Kellenberger, Dominique Bonnet, Valerie Utard, Jean-Luc Galzi, and Brigitte Ilien

ABSTRACT
Bitopic binding properties apply to a variety of muscarinic compounds that span and simultaneously bind to both the orthosteric and allosteric receptor sites. We provide evidence that fluorescent pirenzepine derivatives, with the M1 antagonist fused to the boron-dipyrromethene [Bodipy (558/568)] fluorophore via spacers of varying lengths, exhibit orthosteric/allosteric binding properties at muscarinic M1 receptors. This behavior was inferred from a combination of functional, radio-ligand, and fluorescence resonance energy transfer binding experiments performed under equilibrium and kinetic conditions on enhanced green fluorescent protein–fused M1 receptors. Although displaying a common orthosteric component, the fluorescent compounds inherit bitopic properties from a linker-guided positioning of their Bodipy moiety within the M1 allosteric vestibule. Depending on linker length, the fluorophore is allowed to reach neighboring allosteric domains, overlapping or not with the classic gallamine site, but distinct from the allosteric indolocarbazole “WIN” site. Site-directed mutagenesis, as well as molecular modeling and ligand docking studies based on recently solved muscarinic receptor structures, further support the definition of two groups of Bodipy-pirenzepine derivatives exhibiting distinct allosteric binding poses. Thus, the linker may dictate pharmacological outcomes for bitopic molecules that are hardly predictable from the properties of individual orthosteric and allosteric building blocks. Our findings also demonstrate that the fusion of a fluorophore to an orthosteric ligand is not neutral, as it may confer, unless carefully controlled, unexpected properties to the resultant fluorescent tracer. Altogether, this study illustrates the importance of a “multifaceted” experimental approach to unravel and validate bitopic ligand binding mechanisms.

Introduction
The five muscarinic cholinergic receptor subtypes (mAChRs; M1–M5) play prominent roles in the central and parasympathetic nervous systems, and are potential therapeutic targets (Eglen, 2012). Decades of research have enriched a wide collection of high-affinity and efficacious molecules competing with the neurotransmitter acetylcholine (ACh) for binding to muscarinic receptors. Unfortunately, most of these agonists and antagonists lack true subtype selectivity, a drawback related to the conservation of the orthosteric binding site across mAChRs. Thus, drug discovery programs shifted to the development of allosteric compounds targeting topographically distinct and less conserved binding sites (May et al., 2007; De Amici et al., 2010).

A number of allosteric modulators and agonists now provide useful tools to finely tune orthosteric ligand affinity and efficacy at muscarinic receptors, to select subsets of signaling or regulatory pathways, and to mediate subtype-selective functional outcomes (Birdsall and Lazareno, 2005; Gregory et al., 2007; Conn et al., 2009; Digby et al., 2010). The molecular interpretation of functional versatility and its exploitation in drug discovery are still very challenging, as one has to consider the ability of every compound, or combination of allosteric partners, to stabilize discrete conformational and functional receptor states within a given cell context (Deupi and Kobilka, 2010; Kenakin and Miller, 2010; Gregory et al., 2012). The recent resolution of M2 (Haga et al., 2012) and M3 (Kruse et al., 2012) receptor structures confirmed the close proximity of allosteric and orthosteric sites and the possibility for orthosteric antagonists to pause at an allosteric site while associating with and dissociating from the receptors (Jakubik et al., 2000; Redka et al., 2008; Ilien et al., 2009). Such features may explain the difficulty in defining the molecular

Funding was provided by the Centre National de la Recherche Scientifique; the Institut National de la Sante et de la Recherche Medecine; and Universite de Strasbourg.

S.B.D. was the recipient of a grant from the Association Nationale de la Recherche et de la Technologie (Convention Industrielle de Formation par la Recherche 564/2005 with Prestwick Chemical).

Current affiliation: Quintiles, Par d’Innovation, Illkirch, France. dx.doi.org/10.1124/mol.113.085670.

This article has supplemental material available at molpharm.aspetjournals.org.

ABBREVIATIONS: ACh, acetylcholine; Bo, Bodipy; Bodipy, boron-dipyrromethene; BoPz, Bodipy pirenzepine; ecl, extracellular loop; EGFP, enhanced green fluorescent protein; HEK, human embryonic kidney; hM1, human M1 muscarinic receptor; FRET, fluorescence resonance energy transfer; mAChR, muscarinic cholinergic receptor; NMS, N-methylscopolamine; PDB, Protein Data Bank; ONB, 3-quiniucidinyl benzilate; TM, transmembrane domain; WIN 51,708, 17-β-hydroxy-17-α-ethynyl-5-α-androstano[3,2-b]pyrimido[1,2-a]benzimidazole.
mechanisms (allosteric transition versus steric hindrance) underlying the effects of muscarinic allosteric compounds (Proska and Tucek, 1994; Canals et al., 2012) and the persistent observation of hybrid properties for several molecules. Indeed, orthosteric antagonists, such as pirenzepine derivatives (Tränkle et al., 1998; Ellis and Seidenberg, 1999; Lanzafame et al., 2001) and methoctramine (Giraldo et al., 1988), as well as “allosteric” agonists such as McN-A-343 (Valant et al., 2008), xanomeline (Jakubik et al., 2002), and AC-42 derivatives (Avlani et al., 2010; Gregory et al., 2010; Daval et al., 2012) most probably inherit subtype-dependent affinity or efficacy patterns from mixed orthosteric/allosteric binding properties.

Hybrid, dualsteric, or bitopic compounds form an emerging class of molecules aimed at major improvements in affinity, efficacy, and functional selectivity through the combination (via a spacer arm) of orthosteric and allosteric building blocks (Mohr et al., 2010; Valant et al., 2012). Proof of concept of the bitopic strategy has been done by dissecting the partial muscarinic agonist McN-A-343 into an orthosteric and an allosteric fragment whose combination allowed the recapitulation of the pharmacology of the parent molecule (Valant et al., 2008). Pioneering de novo design of dualsteric molecules has been accomplished on the M2 receptor, too, with the description of molecules combining a nonselective orthosteric antagonist (Steinfeld et al., 2007) or agonist (Disingrini et al., 2006; Antony et al., 2009; Bock et al., 2012) with M2-preferring allosteric moieties. Binding and functional experiments indicated that such hybrids may indeed display better subtype selectivity, improved affinity (agonist hybrids), or stimulus-biased (agonist hybrids) signaling properties.

We previously reported on the synthesis and similar nanomolar affinity at muscarinic M1 receptors of a family (BoPz) of Bodipy pirenzepine derivatives, differing in length (10–22 atoms) of the linker connecting the Bodipy (558/568) fluorophore to the M1-selective antagonist (Tahtaoui et al., 2004). Fluorescence resonance energy transfer (FRET) studies performed on enhanced green fluorescent protein (EGFP)–fused M1 muscarinic receptors suggested the existence of two groups of molecules on the basis of linker length, donor (EGFP)–acceptor (Bodipy) distance in ligand-receptor complexes, and allosteric modulation. We proposed a bitopic binding mode for the Bo(15–22)Pz series of ligands, with their fluorophore and pirenzepine moieties exploring allosteric and orthosteric receptor domains, respectively.

In the present work, we undertook a systematic characterization of all BoPz members to gain a better knowledge on the orthostERIC and allosterIC partners (receptor sites and ligand moieties) involved in their interaction with M1 receptors. To validate true bitopic binding mechanisms, these ligands were taken as “competitors” (functional and [3H]N-methylscopolamine [NMS] assays) and as fluorescent tracers (FRET studies) and compared for their behavior under equilibrium and kinetic conditions. Topographical information on the receptor domains recruited by these fluorescent derivatives was essentially from the examination of M1W400 (7.35) and M1W405 (7.40) receptor mutants and molecular modeling.

Materials and Methods

Materials. [3H]NMS (65 Ci/mmol) was from PerkinElmer Life and Analytical Sciences (Courtaboeuf, France). Atropine sulfate, NMS bromide, pirenzepine dihydrochloride, acetylcholine chloride, carbachol chloride, gallamine triethiodide, brucine sulfate, staurosporine, and WIN 51,708 [17-β-hydroxy-17-α-ethyl-I-5-α-androstano[3,2-b]pyrimido[1,2-α]benzimidazole] hydrate were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Bodipy (558/568) pirenzepine hydrochloride [referred to as Bo(10)Pz in this paper] and Indo-1 acetoxymethyl ester were from Molecular Probes (Invitrogen, Cergy Pontoise, France).

Chemistry. Bo(12)Pz, Bo(15)Pz, and Bo(22)Pz fluorescent pirenzepine derivatives, together with the Bo(5) compound, were synthesized as reported (Tahtaoui et al., 2004). Their purity was checked by analytical reversed-phase high-performance liquid chromatography. Absorbance and fluorescence spectroscopic properties were examined using a Varian Cary 1E (SpectraLab Scientific, Markham, ON, Canada) photometer and a Spex Fluorolog 2 (Horiba Jobin-Yvon, Longjumeau, France) fluorimeter. The chemical structures of fluorescent derivatives and of other compounds used throughout this study are presented in Fig. 1.

EGFP-Fused Muscarinic M1 Receptors, Mutagenesis, and Cell Expression. The human M1 muscarinic receptor (hM1) with a truncated N terminus (deletion of 17 amino acids) fused to EGFP is the construct of reference, defined as EGFP(Δ17)hM1 (Ilien et al., 2003). The corresponding cDNA served as a template in polymerase chain reaction to get single-point-mutated M1 receptors at tryptophan residues 400 and 405. Using the QuikChange site-directed mutagenesis kit (Stratagene, Amsterdam, The Netherlands), Ala or Phe residues were inserted in place of Trp by addition of synthetic oligonucleotide primers containing the required triplet changes. Following subcloning into the pCEP4 expression vector (Invitrogen) and extraction of the plasmids using the Qiagen Plasmid Midi kit (Qiagen, Courtaboeuf, France), the mutations were finally confirmed by sequencing.

Human embryonic kidney (HEK) cells were transfected with wildtype [EGFP(Δ17)hM1] or mutant (referred to as EGFP-W400A/F and EGFP-W405A/F) plasmids using the Qiagen Plasmid Midi kit (Qiagen, Courtaboeuf, France). The human embryonic kidney (HEK) cells were transfected with wildtype [EGFP(Δ17)hM1] or mutant (referred to as EGFP-W400A/F and EGFP-W405A/F) plasmids using the Qiagen Plasmid Midi kit (Qiagen, Courtaboeuf, France). The human embryonic kidney (HEK) cells were transfected with wildtype [EGFP(Δ17)hM1] or mutant (referred to as EGFP-W400A/F and EGFP-W405A/F) plasmids using the Qiagen Plasmid Midi kit (Qiagen, Courtaboeuf, France). The human embryonic kidney (HEK) cells were transfected with wildtype [EGFP(Δ17)hM1] or mutant (referred to as EGFP-W400A/F and EGFP-W405A/F) plasmids using the Qiagen Plasmid Midi kit (Qiagen, Courtaboeuf, France). The human embryonic kidney (HEK) cells were transfected with wildtype [EGFP(Δ17)hM1] or mutant (referred to as EGFP-W400A/F and EGFP-W405A/F) plasmids using the Qiagen Plasmid Midi kit (Qiagen, Courtaboeuf, France).
EGFP-W405A/F) plasmids by calcium phosphate precipitation, and selected with 2 mg/ml hygromycin-B (Eurobio, Courtaboeuf, France) for stable receptor expression.

Cell Culture. IMR 32 human neuroblastoma cells and HEK293 cells (American Type Culture Collection, Manassas, VA; LGC Promochem, Molsheim, France) were grown at 37°C in a 5% CO₂ humidified atmosphere in Dulbecco’s modified Eagle’s medium and in minimal essential medium (with 2 mM glutamine), respectively. Both media ( Gibco/Fisher Scientific, Illkirch, France) were supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 µg/ml). Nearly confluent cells were harvested by mild 0.05% trypsin/0.02% EDTA (w/v) or Versene (phosphate-buffered saline with 5 mM EDTA) treatment, counted, and washed twice by centrifugation before final resuspension in physiologic Hepes buffer (10 mM Hepes, 0.4 mM NaH₂PO₄, 137.5 mM NaCl, 1.25 mM MgCl₂, 1.25 mM CaCl₂, 6 mM KCl, 10 mM glucose, and 1 mM bovine serum albumin, pH 7.4). Cell suspensions were kept on ice until further use in binding assays.

Calcium Mobilization Assays. Adherent cells (either nontransfected or stably expressing various M1 receptor constructs) were loaded with 5 µM Indo-1 acetoxymethyl ester for 40 minutes at 37°C, harvested by rapid trypsin/EDTA treatment, washed, and finally suspended (10⁶ cells/ml) in Hepes buffer. Agonist-evoked increases in intracellular calcium were recorded over time at 20°C through fluorescence emission at 400 nm (excitation at 338 nm). Cells were preincubated for 10 minutes with antagonists before agonist challenge. Peak amplitudes were normalized to basal and maximal (cells permeabilized with 20 µM digitonin) fluorescence levels and expressed as a percentage of the maximal control response.

Radioligand Binding Assays. [³H]NMS binding assays were conducted in Hepes buffer containing 10 mM glucose, and 1 mg/ml bovine serum albumin, pH 7.4). Tubes and filters were rinsed three times with 3-ml ice-cold 25 mM Tris-HCl buffer (pH 7.4). Radioactivity on filters was counted by liquid scintillation spectrometry, and the resulting complex was refined by energy minimization. The AMBER99 force field was used for all energy calculations.

Docking of Ligands into the M1 Receptor. The three-dimensional structures of brucine, gallamine, pirenzepine, and its fluorescent BoPz derivatives were generated using Corina 3.1 (Molecular Network GmbH, Erlangen, Germany). An ensemble of 63 conformers was generated for pirenzepine using Omega 2.4.3 (OpenEye, Inc., Santa Fe, CA). Each conformer was compared with the tiotropium-bound M3 structure (PDB ID 4daj, chain A) using ROCS 3.2.1 (OpenEye, Inc.). The best matched conformer, which yielded a Combo score of 1.27, was pasted into the M1 model, and the resulting complex was refined by energy minimization using MOE.

FRET Monitoring of BoPz Binding to EGFP-Fused M1 Receptors. Fluorescence data were acquired from living cells suspended in Hepes buffer (1–3 10⁶ cells/ml, depending on receptor expression) and kept at 20°C in a thermostated quartz cuvette under magnetic stirring. The interaction of BoPz tracers with chimeric M1 receptors was followed as a variation in cell fluorescence intensity (recorded at 510 nm) due to resonance energy transfer from the EGFP donor (excited at 470 nm) toward the ligand Bodipy acceptor species (Ilien et al., 2003, 2009).

Equilibrium binding studies, which require the parallel treatment of large series of samples, were conducted in a manner very similar to radioligand binding experiments. Cells were incubated in test tubes for 22 hours at 20°C with the fluorescent tracer and unlabeled drugs (1-mI final volume). As FRET assays do not require separation of free from bound tracer, binding levels were quantified through simple fluorescence readouts at 510 nm (excitation at 470 nm).

Association and dissociation kinetics were followed in real time as described (Tahtaoui et al., 2004; Ilien et al., 2009). Briefly, recordings of fluorescence intensity started with the addition of 4 µl of fluorescent ligand (250-fold–concentrated dimethylsulfoxide stock) to the 1-mI cell suspension (preincubated or not at 20°C with various compounds) and lasted until binding equilibrium was confirmed by visual inspection of the stability of the fluorescence trace. Therefore, dissociation of ligand-receptor complexes was initiated by adding 10 µM atropine (or any combination of unlabeled drugs; “isotopic” dilution protocol) to the incubation medium, and fluorescence was recorded over time until full recovery. An alternate “volumetric” dilution protocol was applied as follows: the 1-mI incubation was cooled down at 4°C and centrifuged for 20 seconds at 1500g, and the supernatant was carefully removed. The tiny cell pellet was rapidly resuspended in 1 ml of Hepes buffer (20°C; supplemented or not with 10 µM atropine) and immediately monitored for fluorescence recovery over time.

Specific binding of BoPz derivatives to EGFP-fused hM1 receptors was defined, under equilibrium binding conditions, as the difference in fluorescence intensity of cells incubated with the tracer, in the absence or presence of a saturating concentration of atropine. Under kinetic conditions, specific binding was derived from the amplitude of tracer-induced fluorescence extinction (association step) or of atropine-promoted fluorescence recovery (dissociation step). Theoretically, both determinations should define an identical FRET signal amplitude. When using high tracer concentrations or colored compounds, samples were systematically checked (and corrected) for possible interferences with cell fluorescence measurements.
a 2.5 Å sphere centered on the mass center defined by the 6-membered rings of Tyr82, Trp101, and Tyr404. BoPz poses not reproducing the reference binding mode of pirenzepine were filtered using the in-house Interaction Fingerprint program (Marcou and Rognan, 2007). Bodipy positioning in the M1 7 transmembrane domain (7TM) core was then explored by manually modifying the rotatable bonds in the linker using Sybyl-X1.3 (Tripos, Inc., St. Louis, MO).

Data Analyses. Nonlinear regression analyses of functional and binding data were performed using Kaleidagraph 4.0 (Synergy Software, Reading, PA).

Occupancy curves were generated by plotting the signal amplitude Y, as a function of agonist or tracer concentration X, and analyzed according to the empirical Hill equation:

\[ Y = \frac{Y_{\text{max}}}{1 + \left( \frac{L_{0.5}}{[X]^h} \right)^n} \]  

where \( Y_{\text{max}} \) is the maximal response (\( E_{\text{max}} \)) for an agonist, the maximal number (\( B_{\text{max}} \)) of binding sites for a radioactive tracer, or the maximal amplitude of fluorescence extinction (\( F_{\text{max}} \)) at saturating concentrations of a fluorescent tracer; \( L_{0.5} \) is the agonist concentration (\( EC_{50} \)) leading to half-maximal response or the apparent equilibrium dissociation constant (\( K_a \)) for tracer binding; and \( n_H \) is the midpoint slope.

Antagonist-induced rightward shifts of occupancy curves were checked for competitive interaction using the Lew and Angus (1995) equation:

\[ pL_{0.5} = \log \left( \frac{[B]}{[B] + 10^{-pK}} \right) - \log c \]  

where \( pL_{0.5} \) is the negative logarithm of agonist \( EC_{50,\text{obs}} \) or tracer \( K_{\text{app}} \) values measured at each antagonist concentration \( [B] \), \( pK \) and \( log c \) are fitting constants, and \( s \) is the Schild slope.

Curve shifts were also analyzed according to the Arunlakshana and Schild (1959) equation:

\[ \log(\text{DR}-1) = s \times \log[B] - \log K \]  

where dose-ratios (DR) (\( EC_{50,\text{obs}}/EC_{50,\text{control}} \) or \( K_{\text{app}}/K_{\text{diss}} \)) serve to quantify midpoint shifts at each antagonist concentration \( [B] \). \( K \) is an estimate of antagonist potency, and \( s \) is the Schild slope. If not significantly deviating from unity, the \( s \) value was constrained as such in eqs. 2 and 3, and \( K \) represented the affinity constant \( K_a \) for a competitive antagonist. Otherwise, a \( pK_s \) value was estimated via the \( pA_2 = pK/s \) relationship. Functional antagonism associated with depression of maximal responses was also analyzed, as reported (Christopoulos et al., 1999), using eqnaffactive agonist \( EC_{50} \) values (leading to a constant level of response equal to 25% of the maximal control response) as the dependent variable in eqs. 2 or 3.

Data from competition-type binding experiments were expressed as \( B/B_0 \) ratios, with \( B_0 \) and \( B \) referring to specific tracer binding at equilibrium, in the absence and presence of an unlabeled competitor, respectively. Fractional receptor occupancy \( B/B_0 \) was plotted against the concentration of competitor \( [X] \) and analyzed using the mass-action equation:

\[ B/B_0 = \text{Bottom} + \left[ \frac{\text{Top} - \text{Bottom}}{1 + [IC_{50}/[X]^h]} \right] \]  

where “Top” and “Bottom” parameters refer, respectively, to the upper and lower plateau values of the curve; \( IC_{50} \) denotes the \( X \) value at the injection point; and \( n_H \) is the slope factor.

\( IC_{50} \) values of compounds that led to “full displacement” at high concentrations (bottom and slope values nonsignificantly different from 0 and 1, respectively), whatever the tracer concentration, were converted into equilibrium dissociation constants \( K_a \) using the Cheng and Prusoff relationship for a competitive interaction. In case of submaximal inhibition (bottom > 0; \( B/B_0 \leq 1 \)) or binding potentiation (bottom = 1; \( B/B_0 \geq 1 \)), data were fitted to the allosteric ternary complex model (Ehlert 1988; Lazareno and Birdsell, 1995):

\[ B/B_0 = \frac{([L] + K_a)/([L] + K_a + (1 + [X]/K_a)/(1 + [X]/\alpha \times K_a))} \]  

where \([L] \) and \([X] \) are the concentrations of tracer and allosteric agent, respectively. \( K_a \) and \( K_d \) denote the equilibrium dissociation constants of the tracer and the alloster at the free receptor, respectively. The cooperativity factor \( \alpha \) denotes the magnitude by which the equilibrium dissociation constants of either ligand to its site is modified (\( \alpha > 1 \): negative cooperativity; \( \alpha < 1 \): positive cooperativity) by the concomitant presence of the other ligand. In case of nearly neutral cooperativity (\( \alpha \) close to 1), an allosteric ligand weakly impacts tracer binding at equilibrium, and curve fitting using eq. 5 does not work. Assuming the equivalence of \( \alpha \cdot K_a \) and \( EC_{50,\text{diss}} \) (see eq. 8) parameters to define the affinity of an allosteric agent for tracer-occupied receptors (Ransch et al., 2002), \( \alpha \) was replaced by \( EC_{50,\text{diss}}/K_a \) in eq. 5, and the remaining variable \( K_a \) was obtained from curve fit. The cooperativity factor \( \alpha \) was then derived from the \( EC_{50,\text{diss}}/K_a \) ratio.

Biphasic binding kinetics, as afforded from real-time monitoring of the association of fluorescent ligands to EGFP-fused M1 receptors or of the dissociation of Bo(10)Pz from the EGFP-W405A mutant, were analyzed by fitting individual traces to a two-exponential model:

\[ F(t) = A_0 + A_1 \exp^{-k_{a1}t} + A_2 \exp^{-k_{a2}t} \]  

where \( A_1 \) and \( A_2 \) are the amplitudes of fluorescence changes associated with the rapid and slow components, and \( A_0 \) is the fluorescence intensity at infinite time \( t \). Depending on the experimental paradigm, \( k_{a1} \) and \( k_{a2} \) denote either apparent association rate constants or off-rate constants measured in the presence or absence of an allosteric agent as a function of \([X]\), the alloster concentration. Fitting to eq. 8:

\[ k_{\text{off,obs}}/k_{\text{off,control}} = \text{Bottom} + \left[ \frac{1 - \text{Bottom}}{1 + \left( EC_{50,\text{diss}}/[X] \right)^{n_H}} \right] \]  

allowed the determination of the alloster concentration \( EC_{50,\text{diss}} \) leading to half-maximal reduction of control tracer off rate, the amplitude \( E_{\text{max,dis}} \) (1 - bottom value) of the retardation effect, and the slope factor \( n_H \) of the curve. If not significantly different from unity, \( n_H \) was constrained as such, and the \( EC_{50,\text{diss}} \) parameter was taken as an estimate of the equilibrium affinity constant of the allosteric modulator at tracer-occupied receptors.

Results

The members of the BoPz family (Fig. 1) display two typical building blocks, i.e., the Pz pharmacophore and the Bo (558/568) fluorophore, connected via spacers of varying length (10–22 atoms) and nature (isopeptidic or polyethylene glycol type). The Bo(5) compound, with a short aliphatic chain, lacks the pirenzepine moiety.

Along with their first description (Tahtaoui et al., 2004), BoPz derivatives have been suspected to divide into two separate Bo(10,12)Pz and Bo(15,22)Pz groups. To further explore the properties of all these ligands, a number of
experiments have been conducted and will be illustrated with select members of each putative group.

**Functional Antagonism by BoPz Derivatives of M1-Mediated Calcium Mobilization.** We first examined the ability of EGFPΔ17hM1-expressing HEK cells to adequately report on allosteric modulation of M1-mediated responses to ACh. Brucine and gallamine were taken as compounds of reference (Fig. 1), as they are known to positively and negatively tune, respectively, ACh affinity and potency at M1 receptors (Birdsall et al., 1999; Birdsall and Lazareno, 2005). The presence of endogenous muscarinic M3 receptors in HEK cells was found to introduce bias in the modulation of ACh-induced calcium signals in EGFPΔ17hM1-expressing HEK cells (Supplemental Fig. 1). In contrast, IMR 32 neuroblastoma cells, which are deprived of M3 sites and elicit a clear M1-mediated calcium response to muscarinic agonists (Heikkilä et al., 1991), allowed the observation of brucine and gallamine properties in agreement with their modulatory roles in M1 receptor function. Therefore, IMR 32 cells were selected as a useful companion system to EGFPΔ17hM1 cells to examine, from a functional point of view, the nature of Bo(10)Pz, Bo(15)Pz, and pirenzepine interaction with M1 receptors.

As shown in Fig. 2, increasing concentrations (nanomolar to micromolar range) of all three compounds promotes a dextral shift of dose-response curves together with a profound depression in agonist maximal response, both in EGFPΔ17hM1 (top panel) and in IMR 32 (lower panel) cells. Often taken as a hallmark for noncompetitive inhibitors, insurmountable antagonism for orthosteric antagonists is widespread across a range of receptor systems and assays (Kenakin et al., 2006), including pirenzepine effects on muscarinic responses in various cell lines (Christopoulos et al., 1999 and references therein).

A common explanation is to consider that a slowly dissociating antagonist, the agonist, and the receptor cannot come to proper equilibrium in fast responding systems, thereby limiting maximal response amplitude. Such kinetic artifacts most probably apply to pirenzepine, Bo(10)Pz, and Bo(15)Pz inhibition of transient calcium responses in both cell lines. Indeed, they slowly dissociate from M1 receptors with off-rate constants (sec$^{-1}$) close to 5 for pirenzepine (Potter et al., 1989; Mohr and Trankle, 1994; Christopoulos et al., 1999) and in the range of 5–15 for BoPz derivatives (Table 5). One should also add that BoPz compounds, at the lowest concentrations which were tested, probably did not reach true equilibrium when preincubated with cells for 10 minutes.

Potency estimates for insurmountable antagonists can be obtained with no prior knowledge of molecular mechanism through pA$_2$ measurements (Kenakin et al., 2006). Lew and Angus (1995) as well as Arunlakshana and Schild (1959) analyses (Fig. 2, right) favored the hypothesis of a competitive interplay of the antagonists with ACh (slope factors not significantly different than 1). The pA$_2$ values for Bo(10)Pz ($7.98 \pm 0.29$ and $8.15 \pm 0.22$), Bo(15)Pz ($8.10 \pm 0.21$ and $7.95 \pm 0.23$), and pirenzepine ($7.89 \pm 0.10$ and $7.77 \pm 0.25$) antagonism in EGFPΔ17hM1 and IMR 32 cells, respectively, were consistent with their binding affinity constants determined through FRET and [3H]NMS studies (Tables 1 and 2). Regression analyses using equieffective agonist concentrations (EC$_{50}$ values) instead of standard EC$_{50}$ values provided similar potency estimates (not shown). Thus, these functional experiments aimed

![Fig. 2](http://bitopicposes.oflows.astrojournals.org/)
at defining the competitive or noncompetitive interplay of BoPz antagonists with ACh remained nondecisive.

**FRET Studies: Kinetic Insights into BoPz Binding at EGFP(Δ17)hM1 Receptors.** Given the ideal acceptor property of the Bodipy (558/568) fluorophore for energy transfer from excited EGFP, BoPz compounds have proven valuable FRET probes to dissect ligand binding mechanisms at EGFP(Δ17)hM1 receptors (Ilien et al., 2003, 2009; Tahtaoui et al., 2004). Figure 3 depicts real-time recordings of association and dissociation processes for BoPz tracers and the impact of allosteric modulators (brucine and gallamine) on their kinetics. Top and bottom panels refer to BoPz probes with short and long linkers, respectively.

Preincubation of EGFP(Δ17)hM1 cells with increasing concentrations of brucine (Fig. 3A) led to a dose-dependent slowing down of Bo(12)Pz association ($k_{app}$ values for the fast binding step vary from 0.034, 0.016 to 0.0096 seconds$^{-1}$ in the absence and presence of 100 or 500 μM brucine, respectively). Interestingly, brucine slightly increased the amplitude of EGFP fluorescence extinction at Bo(12)Pz binding equilibrium. Such a small potentiation might be related to the use of an almost saturating tracer concentration (200 nM). Brucine modulated Bo(10)Pz binding very similarly, whereas gallamine reduced both the association rate and the amplitude of Bo(10)Pz and Bo(12)Pz binding at equilibrium (not shown).

As shown in Fig. 3B, and in agreement with previous findings on Bo(10)Pz binding properties (Tahtaoui et al., 2004), brucine (in the presence of a saturating concentration of atropine) induced a profound deceleration of Bo(12)Pz dissociation. Brucine alone (up to 2.5 mM) did not promote any dissociation of ligand-receptor complexes (not shown). Brucine had a greater impact on dissociation than on association kinetics (at 100 μM, on and off rates were reduced by 2- and 5-fold, respectively), another indication in favor of a positive modulation of Bo(12)Pz affinity. Bo(10)Pz and Bo(12)Pz dissociation from EGFP(Δ17)hM1 receptors were dose dependently retarded by brucine (Fig. 3C) and by gallamine (Fig. 3D), with half-maximal Brucine alone (up to 2.5 mM) did not promote any dissociation induced a profound deceleration of Bo(12)Pz dissociation. Gallamine reduced both the association rate and the dissociation rate and for all tracers (Table 1), as previously reported (Tahtaoui et al., 2004).

Competition-type experiments were undertaken using typical orthosteric (atropine and pirenzepine) and allosteric (gallamine and brucine) muscarinic receptor ligands (Fig. 4A). Atropine and pirenzepine fully displaced Bo(12)Pz binding according to a competitive mode of interaction at the orthosteric receptor site. Submaximal inhibition by gallamine was consistent with saturable negative binding cooperativity. Brucine clearly potentiated Bo(12)Pz binding (taken here at a concentration below its $K_d$ value). Analyses according to the allosteric ternary complex model (Ehler, 1988) yielded estimates of their equilibrium dissociation constant $K_d$ for free EGFP(Δ17)hM1 receptors and of the cooperativity factor α as a measure of their impact on Bo(12)Pz affinity (Table 1).

**TABLE 1**

<table>
<thead>
<tr>
<th>Tracer</th>
<th>$pK_{i,x}$</th>
<th>$pEC_{50,diss}$</th>
<th>$pK_{i,x}$</th>
<th>$pEC_{50,diss}$</th>
<th>$pK_d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bo(10)Pz</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tracer</td>
<td>7.91 ± 0.04</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atropine</td>
<td>8.89 ± 0.06</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pirenzepine</td>
<td>7.70 ± 0.06</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gallamine</td>
<td>4.79 ± 0.03</td>
<td>3.44 ± 0.05</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brucine</td>
<td>-1.04 ± 0.06</td>
<td>(80 ± 4)</td>
<td>4.45 ± 0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.25 ± 0.06</td>
<td></td>
<td>4.32 ± 0.08</td>
<td>5.04 ± 0.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.20 ± 0.05</td>
<td></td>
<td>0.68 ± 0.05</td>
<td>(94 ± 2)</td>
<td></td>
</tr>
<tr>
<td>Bo(12)Pz</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bo(15)Pz</td>
<td>7.67 ± 0.01</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atropine</td>
<td>9.02 ± 0.04</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pirenzepine</td>
<td>7.96 ± 0.06</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gallamine</td>
<td>4.82 ± 0.05</td>
<td>3.47 ± 0.04</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brucine</td>
<td>-1.09 ± 0.07</td>
<td>(92 ± 5)</td>
<td>4.68 ± 0.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4.27 ± 0.07</td>
<td></td>
<td>4.21 ± 0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bo(22)Pz</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Values are from Ilien et al., 2003.

**Materials and Methods; Data Analyses.**

* Cooperativity factor α (negative logarithm).

Because of nearly neutral cooperativity, $EC_{50,diss}$ was used for curve fitting according to eq. 5 as described in *Data Analyses.*
When using Bo(15)Pz as the tracer (Fig. 4B), all compounds, including brucine and gallamine, displayed a competitive-like binding behavior. Bo(22)Pz, again, shared Bo(15)Pz properties. Figure 4C illustrates Bo(22)Pz saturation experiments performed in the absence or presence of brucine (50, 200, and 500 \(\text{mM}\)). Brucine promoted a homogenous dextral shift of occupancy curves, with a decrease in Bo(22)Pz apparent affinity (\(K_{\text{app}}\) values) which was submitted to Lew and Angus analysis (Fig. 4C inset). With a Schild slope factor not significantly different from 1 (1.03 \(\pm\) 0.04), the hypothesis for a competitive interplay between brucine and Bo(22)Pz binding was clearly privileged. Moreover, the affinity constant for brucine (40.7 \(\pm\) 2.9 \(\mu\)M) determined here was comparable to that (44.9 \(\pm\) 6.1 \(\mu\)M) defined from similar saturation experiments performed with Bo(15)Pz, and was in overall agreement with \(K_x\) and \(K_i\) values derived from competition experiments (Table 1).

### TABLE 2

Summary of drug binding parameters at EGFP(Δ17)hM1 receptors as assessed from equilibrium and off-rate \([3H]NMS\) binding assays

<table>
<thead>
<tr>
<th>Affinity constants</th>
<th>(pK_d)</th>
<th>(pK_i)</th>
<th>(pK_x)</th>
<th>(pK_a)</th>
<th>(pEC_{50,\text{diss}})</th>
<th>(E_{\text{max,\text{diss}}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>NMS</td>
<td>10.02 (\pm) 0.03</td>
<td>9.96 (\pm) 0.04</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atropine</td>
<td>9.19 (\pm) 0.03</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pirenzepine</td>
<td>8.03 (\pm) 0.08</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carbachol</td>
<td>4.89 (\pm) 0.14</td>
<td>5.04 (\pm) 0.06</td>
<td>-1.12 (\pm) 0.02</td>
<td>3.94 (\pm) 0.07</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Brucine</td>
<td>4.59 (\pm) 0.02</td>
<td>4.39 (\pm) 0.02</td>
<td>-0.10 (\pm) 0.02</td>
<td>4.49 (\pm) 0.02</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Bo(10)Pz</td>
<td>6.40 (\pm) 0.05</td>
<td>8.02 (\pm) 0.04</td>
<td>-0.40 (\pm) 0.02</td>
<td>N.A.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bo(12)Pz</td>
<td>7.51 (\pm) 0.01</td>
<td>7.77 (\pm) 0.05</td>
<td></td>
<td></td>
<td>N.D.</td>
<td></td>
</tr>
<tr>
<td>Bo(15)Pz</td>
<td>7.74 (\pm) 0.07</td>
<td>7.74 (\pm) 0.07</td>
<td></td>
<td></td>
<td>5.45 (\pm) 0.04</td>
<td>72 \pm 3</td>
</tr>
<tr>
<td>Bo(22)Pz</td>
<td>7.74 (\pm) 0.07</td>
<td>N.D.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N.A., not applicable because of insufficient data points (Fig. 5D); N.D., not determined.

a \(EC_{50,\text{diss}}\) was used for curve fitting according to eq. 5 because of nearly neutral cooperativity, as described in Data Analyses.

**Fig. 3.** Real-time FRET monitoring of BoPz binding to EGFP(Δ17)hM1 receptors: impact of brucine and gallamine. Association step (A and E): EGFP(Δ17)hM1 cells were preincubated for 10 minutes at 20°C with vehicle (black) or brucine (100 \(\mu\)M: light gray; 500 \(\mu\)M: dark gray). Following the addition of Bo(12)Pz (A) or Bo(15)Pz (E) (time 0; 200 nM final concentration), association was monitored over time as a decrease in fluorescence intensity. Amplitudes for fluorescence extinction (percentage) at binding equilibrium in control, 100 and 500 \(\mu\)M-brucine treated cells were, respectively, 36, 40, and 45 [Bo(12)Pz] and 34, 32, and 27 [Bo(15)Pz]. Dissociation step (B and F): Cells were first equilibrated for 20 minutes at 20°C with 200 nM Bo(12)Pz or Bo(15)Pz. Bo(12)Pz dissociation (B) started with the addition (time 0) of 5 \(\mu\)M atropine alone (black) or combined with various concentrations of brucine (3, 10, 30, 100, and 200 \(\mu\)M). Bo(15)Pz dissociation (F) proceeded similarly except that it was initiated either with 5 \(\mu\)M atropine alone (black) or combined with 500 \(\mu\)M brucine (light gray), or with 2 mM brucine alone (light gray). Tracer dissociation was monitored as a recovery in fluorescence intensity over time and expressed as a percentage of the fluorescence extinction amplitude at binding equilibrium. All traces followed a monoexponential decay for fluorescence, and dissociation rate constants were derived from fitting to eq. 7. Off-rate values \(sec^{-1} \cdot 10^{-4}\) for Bo(15)Pz ranged from 9 (atropine) and 11 (brucine) to 12 (atropine plus brucine). Dose dependency for the allosteric delay of Bo(10)Pz (\(\bullet\)) and Bo(12)Pz (\(\bigcirc\)) dissociation (C and D): Off-rate constants for the tracers in the presence of modulator \(k_{\text{off,obs}}\), relative to control \(5.8 \text{ and } 15.8 \text{ sec}^{-1} \cdot 10^{-4}\) for Bo(10)Pz and Bo(12)Pz, respectively, were plotted as a function of brucine (C) or gallamine (D) concentration. Data are the means \(\pm\) S.E. for three independent experiments. Application of eq. 8 allowed the determination of \(EC_{50,\text{diss}}\) and \(E_{\text{max,\text{diss}}}\) parameters listed in Table 1.
Table 1 summarizes drug binding parameters at EGF(Δ17)hM1 receptors as afforded from FRET experiments using each of the four fluorescent pirenzepine derivatives as an individual tracer. As expected, drug affinity constants at the free receptor do not significantly vary with tracer type or underlying binding mechanism. They are in overall agreement with affinity values reported in the literature ([3H]NMS binding, M1-enriched membrane preparations) for atropine and pirenzepine (Caulfield and Birdsell, 1998), gallamine (Matsui et al., 1995; Lazareno et al., 2000; Fruchart-Gaillard et al., 2006), and brucine (Gharagozloo et al., 1999; Fruchart-Gaillard et al., 2006). The equivalence of EC_{50,dis} and α · K_a parameters for gallamine and brucine suggests that variations in their affinity for free and Bo(10,12)Pz-occupied receptors are driven by binding cooperativity within a ternary allosteric complex. Thus, kinetic and equilibrium FRET studies provided robust information on BoPz binding properties at M1 receptors, with a clear-cut distinction between Bo(10,12)Pz and Bo(15,22)Pz series of ligands regarding the recruitment of the gallamine allosteric site.

An Orthosteric [3H]NMS Point of View on BoPz Binding Properties. A series of experiments were performed using EGF(Δ17)hM1 cells, [3H]NMS as the radioactive orthosteric tracer, and unchanged incubation conditions (physiologic buffer, 22-hour incubation time at 20°C to ensure equilibrium). Displacement experiments were performed at two [3H]NMS concentrations (1 × K_d and 10 × K_d) to better distinguish competitive from negatively cooperative modes of interaction (Birdsall and Lazareno, 2005; May et al., 2007). Slope factors for all inhibition curves did not statistically differ from 1.

As shown in Fig. 5A, atropine fully displaced specific [3H]NMS binding at low and high tracer concentrations, as expected for competition at the orthosteric receptor site. Gallamine displayed a typical submaximal inhibition (with plateau levels depending on [3H]NMS concentration) consistent with negative binding cooperativity. Brucine was a very partial displacer, featuring almost neutral binding cooperativity with [3H]NMS. Analyses according to the allosteric ternary complex model yielded affinity (K_a) and cooperativity factor (α) estimates (Table 2) in agreement with literature reports for gallamine (Matsui et al., 1995; Lazareno et al., 2000; Fruchart-Gaillard et al., 2006) and brucine (Gharagozloo et al., 1999; Fruchart-Gaillard et al., 2006).

Bo(10)Pz and Bo(15)Pz displayed an apparent competitive mode of interaction (Fig. 5B), as did the two other derivatives. Their K_a values listed in Table 2, closely match K_d values determined under FRET conditions (Table 1). Interesting information (Fig. 5B) was provided by the Bo(5) compound, which lacks the pirenzepine moiety (Fig. 1) and exhibits negative binding cooperativity with [3H]NMS in the low micromolar range. Thus, the Bodipy fluorophore may be able, per se, to interact with an M1 allosteric site.

This prompted us to test Bo(5), Bo(10)Pz, and Bo(15)Pz derivatives in the presence of 10 μM atropine to prevent [3H]NMS reassociation for their ability to alter [3H]NMS off rate from EGF(Δ17)hM1 receptors (Fig. 5C). All kinetics followed a monoeponential time course. As expected for a competitive interplay at the orthosteric site, the addition of atropine or pirenzepine (alone or in combination) led to superimposable dissociation traces (control k_{off} = 0.043 ± 0.001 minutes^{-1}; n = 6). Gallamine and brucine, taken here as references at 200 μM, strongly impacted the [3H]NMS dissociation rate. Bo(5) and Bo(10,15)Pz compounds, at 10 μM (i.e., 25- and 1000-fold their respective equilibrium affinity constants), significantly retarded [3H]NMS dissociation. The possibility for Bo(10)Pz to bind to [3H]NMS-occupied receptors was totally unexpected, as previous FRET (Table 1) and equilibrium [3H]NMS (Table 2) studies privileged a conventional orthosteric nature for this derivative.

Figure 5D depicts the concentration dependence of the retardation effects of the allosteric modulators gallamine and...
brucine and of the three fluorescent derivatives on [3H]NMS dissociation from EGFP(D17)hM1 receptors. In agreement with M1 literature, brucine (Gharagozloo et al., 1999) and gallamine (Matsui et al., 1995) fully inhibited [3H]NMS dissociation, with EC50,dis values close to 30 and 110 μM, respectively, reflecting their affinity for [3H]NMS–occupied receptors (Table 2). Fluorescent derivatives could not be tested at concentrations above 30 μM because of stock and solubility limitations. Nevertheless, their retardation effect on [3H]NMS dissociation manifested a net concentration dependency. Bo(10)Pz and Bo(15)Pz curves were almost superimposable, and the Bo(15)Pz effect (EC50,dis 3.5 ± 0.4 μM; n = 3) was probably partial (Rmax,dis 72.5% ± 2.5%; n = 3).

Rationale Behind the Selection of M1 W400 and W405 Receptor Mutants. Accumulating evidence supports the bitopic character of the BoPz derivatives: they recognize epitopes within both orthosteric (competitive character when tested against orthosteric ligands in functional and equilibrium binding assays) and allosteric (retardation of [3H]NMS dissociation) sites. There is no doubt that the Bo(15,22)Pz compounds may bridge the orthosteric and the gallamine sites (kinetic and equilibrium FRET experiments), but the nature and position of the allosteric domain recruited by Bo(10,12)Pz are still unclear.

To address this question, we considered an ensemble of observations pointing to the M1 W400 and W405 residues as interesting mutagenesis probes: 1) the Bodipy fluorophore may bind to an allosteric site (Bo(5) data; Fig. 5B); 2) the aromatic Bodipy moiety is well suited to elicit a π–π stacking with the indole ring of a Trp residue; 3) the EGFP (donor)–Bodipy (acceptor) distances for the Bo(10,12)Pz and Bo(15,22)Pz subgroups, as determined from FRET efficacy (Tahtoui et al., 2004), only differ by 3–5 Å; 4) the conserved W400 (7.35) and W405 (7.40) residues [numbering according to Ballesteros and Weinstein (1995) convention indicated in parentheses] are spatially close, one α-helical turn apart on top of TM7; and, most importantly, 5) the conserved Trp at position 7.35 is regarded as a key component of the prototypical gallamine allosteric site of mAChRs (Matsui et al., 1995; Prilla et al., 2006).

Impact of Mutations on Orthosteric and Allosteric Ligand Binding Properties. The properties of the EGFP(D17)hM1 receptor, with its W400 or W405 residues replaced by Ala or Phe (to preserve the aromatic character), were examined through [3H]NMS binding experiments. Results are shown in Fig. 6A and summarized in Table 3.

[3H]NMS saturation studies indicated a modest impact of the mutations on receptor expression levels, with maximal 2-fold variations in Bmax values (W405F, increase; W400A, decrease) as compared with wild type. High [3H]NMS affinity was preserved, as reported for the M1 W400 mutant (Matsui et al., 1995) and for homologous W400 (M2W422, M5W477) and W405 (M2W427, M5W482) mutations in M2 and M5 receptors (Prilla et al., 2006).

Competition experiments pointed to an unchanged atropine affinity, a discrete alteration in pirenzepine affinity related to the suppression of the aromatic nature of the residues (as shown for the M1 W400 mutant; Matsui et al., 1995), and an increase in carbachol affinity for the EGFP-W405 construct. An elevation in agonist affinity and constitutive activity has already been noticed for this M1 mutant (Matsui et al., 1995; Lu et al., 2001) and interpreted as a consequence of the destabilization of a ground-state intramolecular contact network.

[3H]NMS equilibrium and kinetic studies pointed to a 2-fold reduction in gallamine affinity at free EGFP-W405A/F receptors, and to a slight reduction in its binding cooperativity with NMS. In contrast, the W400A/F mutations led to a more than 10-fold decrease in gallamine affinity at free- and [3H]NMS–occupied receptors, with little impact on binding cooperativity. These data are in agreement with previous reports on gallamine properties at the W400A/F M1 mutants (Matsui et al., 1995) and at the alanine mutants of the homologous W400 and W405 residues in M2 and M5 receptors.
for [3H]NMS receptors, at variance with the prominent role of the W400 residue, which discriminates between EGFP-400A/F mutants and wild-type sensitivity to the mutation of the W405 residue but did not

bo(10,12)pz compounds displayed a similar and moderate affinity binding properties of the fluorescent derivatives.

exceptions.

Trp for Phe is less well tolerated than Ala insertion [bo(15)Pz of 2 again pointed to the crucial importance of the W400 residue. At variance with gallamine, the affinity of brucine (EC50, diss value) was thus best apprehended from dissociation kinetics, which agreement, with the exception of bo(12)Pz and bo(15)Pz profiles of boPz ligands at EGFP-W400F and W405A/F receptor mutants were examined through competitive (taking [3H]NMS as the tracer) and FRET saturation (taking each boPz derivative as a fluorescent tracer) studies. As shown in Table 4, Kd and Kd,i values are in remarkable agreement, with the exception of bo(15)Pz and bo(15)Pz binding at the W400 mutants are noticeable, but significantly increased, as compared with wild type. All of these controls confirm that the fusion of EGFP to an hM1 receptor with a truncated N terminus does not modify orthosteric and allosteric binding.

TABLE 3

Drug binding properties at the EGFPΔ17 hM1 receptor and its mutants as assessed from equilibrium and kinetic [3H]NMS binding.

<table>
<thead>
<tr>
<th>Drug</th>
<th>EGFP-Wt hM1</th>
<th>EGFP-W405A</th>
<th>EGFP-W405F</th>
<th>EGFP-W400A</th>
<th>EGFP-W400F</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H]NMS</td>
<td>10.02 ± 0.03</td>
<td>10.27 ± 0.04</td>
<td>10.28 ± 0.03</td>
<td>10.04 ± 0.01</td>
<td>10.04 ± 0.04</td>
</tr>
<tr>
<td>Bmax</td>
<td>445 ± 35°</td>
<td>400 ± 75°</td>
<td>910 ± 125°</td>
<td>195 ± 15°</td>
<td>395 ± 45°</td>
</tr>
<tr>
<td>Atropine</td>
<td>9.19 ± 0.03</td>
<td>9.30 ± 0.08</td>
<td>9.37 ± 0.01</td>
<td>9.35 ± 0.03</td>
<td>9.03 ± 0.06</td>
</tr>
<tr>
<td>Pirenzepine</td>
<td>8.03 ± 0.08</td>
<td>7.73 ± 0.01</td>
<td>8.24 ± 0.04</td>
<td>7.65 ± 0.10</td>
<td>7.89 ± 0.23</td>
</tr>
<tr>
<td>Carbachol</td>
<td>4.89 ± 0.14</td>
<td>5.58 ± 0.19</td>
<td>5.18 ± 0.01</td>
<td>4.68 ± 0.12</td>
<td>4.53 ± 0.12</td>
</tr>
<tr>
<td>Gallamine</td>
<td>5.04 ± 0.06</td>
<td>3.94 ± 0.07 (100)</td>
<td>4.00 ± 0.04 (100)</td>
<td>2.35 ± 0.03 (100)</td>
<td>7.02 ± 0.02 (100)</td>
</tr>
<tr>
<td>Brucine</td>
<td>4.58 ± 0.02°</td>
<td>4.49 ± 0.02 (100)</td>
<td>5.02 ± 0.03 (100)</td>
<td>N.A.*</td>
<td>4.79 ± 0.03 (100)</td>
</tr>
</tbody>
</table>

N.A., not applicable; wt, wild type.

* Maximal densities in [3H-NMS] binding sites (Bmax, fmol/mg protein) are from saturation experiments.

° EC50,dis is from curve fitting to eq. 8 with Bmax,dis constrained to 100%.

° Cooperativity factor (negative logarithm).

° EC50, dis was used for curve fitting according to eq. 5 because of nearly neutral cooperativity (Data Analyses).

° Not applicable. Over a 1–300 μM concentration range, brucine did not significantly modify equilibrium [3H]NMS binding at the mutants.
residue in Bo(15,22)Pz binding. Bo(15)Pz was actually severely impacted by all mutations (with a 10-fold decrease in affinity at the W405F and W400A mutants). Bo(22)Pz displayed an intermediate profile, with a weaker contribution of the W405 residue (especially when replaced by Ala). Altogether, these findings provide additional support to the delineation of two subgroups of BoPz ligands with differential sensitivity to W405 (10–12 series) or W400 (15–22 series) mutations. They also reinforce the idea that their Bodipy moiety, depending on linker length, may explore distinct (or partially overlapping) allosteric epitopes, including (15–22 series) or not (10–12 series) the key W400 gallamine residue.

To get deeper insight into the role of the two tryptophan residues, kinetic studies were carried out to compare [3H]NMS and BoPz dissociation rates at the four mutants (Table 5).

[3H]NMS dissociation was slightly retarded in all mutants, with a more significant impact of the W405 substitution. These findings corroborate previous reports on [3H]NMS dissociation from M1 W400A/F (Matsui et al., 1995) and M2 homologous W400A and W405A (Prilla et al., 2006) mutants. As none of these mutations have relevant effects on [3H]NMS affinity, association and dissociation steps are probably retarded to the same extent. Thus, W405, and to a minor degree W400, may have a structural role in maintaining proper homologous W400A and W405A (Prilla et al., 2006) mutants.

These findings corroborate previous reports on [3H]NMS and BoPz dissociation rates at the four mutants (Table 5).

Comparison of BoPz ligand binding properties at the EGFP(17)hM1 receptor and its four mutants as assessed from equilibrium [3H]NMS and FRET binding measurements.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>EGFP-wt hM1</th>
<th>EGFP-W405A</th>
<th>EGFP-W405F</th>
<th>EGFP-W400A</th>
<th>EGFP-W400F</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H]NMS (pKᵢ, − log M)</td>
<td>8.02 ± 0.04</td>
<td>7.77 ± 0.05</td>
<td>7.42 ± 0.05</td>
<td>8.25 ± 0.02</td>
<td>8.00 ± 0.01</td>
</tr>
<tr>
<td>FRET</td>
<td>7.91 ± 0.04</td>
<td>7.60 ± 0.10</td>
<td>7.42 ± 0.06</td>
<td>8.12 ± 0.08</td>
<td>8.01 ± 0.01</td>
</tr>
<tr>
<td>[3H]NMS (pKᵢ, − log M)</td>
<td>7.51 ± 0.01</td>
<td>7.20 ± 0.01</td>
<td>6.99 ± 0.09</td>
<td>7.49 ± 0.06</td>
<td>7.19 ± 0.05</td>
</tr>
<tr>
<td>FRET</td>
<td>7.67 ± 0.01</td>
<td>7.35 ± 0.06</td>
<td>7.08 ± 0.04</td>
<td>7.66 ± 0.09</td>
<td>7.51 ± 0.03</td>
</tr>
<tr>
<td>[3H]NMS (pKᵢ, − log M)</td>
<td>7.77 ± 0.05</td>
<td>7.06 ± 0.04</td>
<td>6.80 ± 0.06</td>
<td>6.60 ± 0.05</td>
<td>6.94 ± 0.04</td>
</tr>
<tr>
<td>FRET</td>
<td>7.97 ± 0.01</td>
<td>7.19 ± 0.07</td>
<td>6.94 ± 0.08</td>
<td>6.88 ± 0.02</td>
<td>7.41 ± 0.01</td>
</tr>
<tr>
<td>[3H]NMS (pKᵢ, − log M)</td>
<td>7.74 ± 0.07</td>
<td>7.60 ± 0.06</td>
<td>7.18 ± 0.04</td>
<td>7.17 ± 0.07</td>
<td>7.14 ± 0.09</td>
</tr>
<tr>
<td>FRET</td>
<td>7.92 ± 0.09</td>
<td>7.77 ± 0.07</td>
<td>7.15 ± 0.12</td>
<td>7.19 ± 0.01</td>
<td>7.27 ± 0.04</td>
</tr>
</tbody>
</table>
sequence identity between M1 and M3; 45.0% between M1 and M2), the M3 receptor was selected as the template to model M1 structure.

3-Quinuclidinyl benzilate (QNB) and tiotropium bind in a remarkably similar pose to M2 and M3 receptors (Kruse et al., 2012) that is likely to represent a conserved binding mode for structurally close anticholinergics. We were therefore interested in examining whether this could also apply to pirenzepine binding in our M1 model (Supplemental Fig. 2). This was indeed the case, as all three antagonists are enclosed in a highly conserved aromatic cage, establish an ionic bond with Asp3.33, and fit a common three-dimensional pharmacophore defined by a positively charged nitrogen atom in an aliphatic 6-membered ring, a carbonyl group, and two aromatic rings. This allowed us to build the M1-pirenzepine complex, to show (Fig. 7B) that pirenzepine binding pose well mimicks that of the two other antagonists in M2 and M3 crystal structures, and to validate our M1 model, at least from the orthosteric site point of view.

A more global image of the M1 receptor model is provided in Fig. 7B, which shows that the cavity in the 7TM M1 receptor core displays a highly hydrophobic region at the junction between the orthosteric site and the allosteric vestibule, in the vicinity of the side chain of Trp101 in TM3. The two residues which were mutated in this study, W400 and W405, sit, respectively, in the vestibule and at the TM7/lipid bilayer interface. They form the ends of a network of aromatic residues bridging TM helices (including the Tyr106-Tyr381-Tyr404 triad but also Tyr82, Tyr85, Trp91, Trp101, Trp157, and Tyr408) and may lock its conformation. This M1 model provides the structural foundations for further discussion of our experimental data on fluorescent pirenzepine derivatives.

**Discussion**

We investigated in much detail the binding properties of a series of fluorescent Bodipy-pirenzepine derivatives at EGFP-fused M1 receptors. A combination of radioligand and FRET binding experiments and of mutagenesis and molecular modeling studies was necessary to demonstrate their bitopic nature: they bind, through their pirenzepine pharmacophore, to the orthosteric site and simultaneously occupy, through their Bodipy moiety, the M1 allosteric vestibule (Fig. 7). Whereas long (15–22 atoms) linkers allow the fluorophore to access the gallamine/brucine site, shorter (10–12 atoms) linkers confine it within a neighboring but separate allosteric subdomain.

Pirenzepine and BoPz compounds display similar affinities (10 nM range), suggesting a weak contribution of the fluorophore to overall binding energy of the derivatives. Such a view is supported by the observation of monophasic and unchanged dissociation kinetics for all BoPz derivatives, whatever the protocol which is applied. Thus, the fluorescent ligands display a homogenous binding mode, and probably firmly anchor to the receptor through binding of their pirenzepine moiety within the orthosteric site. One should mention, however, that the Bo(5) fragment has allosteric affinity in the low micromolar range, and that Bo(15,22)Pz binding is clearly impacted by W400A/F mutations, whereas that of pirenzepine is much less affected. These latter findings suggest that both the linker and the pirenzepine pharmacophore help the Bodipy moiety reach a local concentration high enough to interact with allosteric residues.

When examined from the orthosteric point of view, all BoPz derivatives exhibit either competitive-like (when tested against [3H]NMS and other orthosteric compounds under equilibrium conditions) or allosteric (as afforded from their ability to alter [3H]NMS dissociation kinetics) properties. Such a mixed orthosteric/allosteric behavior is often observed for bitopic ligands (Mohr et al., 2010; Daval et al., 2012; Valant et al., 2012). Other explanations include a strong negative binding cooperativity with [3H]NMS (if one considers BoPz affinity ratios at free and [3H]NMS-occupied receptors) or a differential positioning of these derivatives, depending on orthosteric site occupancy. Although retardation of [3H]NMS dissociation occurs at high BoPz concentrations, fluorophore-driven exclusion and occupancy of the allosteric vestibule by the bulky BoPz derivatives are unlikely. An alternate possibility is to consider an interaction of their pirenzepine moiety with a peripheral site that sterically hinders [3H]NMS egress from the orthosteric pocket. Indeed, real-time FRET monitoring of Bo(10)Pz binding kinetics at EGFP-fused M1 receptors indicated that an initial fast and low affinity binding event, occurring at the receptor surface close to EGFP, precedes ligand translocation within the transmembrane core (Ilién et al., 2009). Molecular dynamic simulations of tiotropium binding to M2 and M3 receptors also revealed the possibility for an orthosteric ligand to pause within the allosteric vestibule while entering or dissociating from the orthosteric pocket (Kruse et al., 2012).

FRET experiments provided further information on BoPz properties, now regarded as individual tracers. Indeed, equilibrium and kinetic FRET data indicated that 1) all BoPz

---

**TABLE 5**

Effect of point mutations in the EGFP(Δ17)hM1 receptor on dissociation kinetics of [3H]NMS and BoPz tracers

<table>
<thead>
<tr>
<th>Tracer</th>
<th>EGFP- wt hM1</th>
<th>EGFP- W405A</th>
<th>EGFP- W405F</th>
<th>EGFP- W400A</th>
<th>EGFP- W400F</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H]NMS</td>
<td>6.9 ± 0.3</td>
<td>2.3 ± 0.1</td>
<td>2.5 ± 0.4</td>
<td>3.6 ± 0.3</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td>Bo(10)Pz</td>
<td>5.9 ± 0.5</td>
<td>Biphase</td>
<td>24 ± 1</td>
<td>9.0 ± 0.6</td>
<td>9.4 ± 0.5</td>
</tr>
<tr>
<td>Bo(12)Pz</td>
<td>15.4 ± 0.4</td>
<td>47 ± 4</td>
<td>39 ± 6</td>
<td>21 ± 2</td>
<td>21 ± 2</td>
</tr>
<tr>
<td>Bo(15)Pz</td>
<td>13.7 ± 1.6</td>
<td>97 ± 7</td>
<td>75 ± 6</td>
<td>368 ± 15</td>
<td>60 ± 5</td>
</tr>
<tr>
<td>Bo(22)Pz</td>
<td>15.5 ± 0.5</td>
<td>28 ± 2</td>
<td>48 ± 2</td>
<td>152 ± 8</td>
<td>62 ± 1</td>
</tr>
</tbody>
</table>
derivatives involve an orthosteric binding component; 2) Bo(10,12)Pz affinity is allosterically modulated by brucine (potentiation) and gallamine (inhibition), and thus, their capacity to alter [3H]NMS dissociation rate probably originates from the recruitment of allosteric epitopes that do not overlap with the classic allosteric domain; 3) Bo(15,22)Pz, in marked contrast with the former ligands, inherits allosteric properties from occupancy of the gallamine/brucine site; and 4) Bo(15,22)Pz dissociation proceeds similarly, whatever the agent (atropine, brucine, or gallamine) selected to prevent tracer reassociation to the receptor, as expected for concomitant binding of the fluorescent pirenzepine derivative and either allosteric modulator. (D) Predicted binding pose for Bo(22)Pz in the M1 receptor. All images reflect the same view and scale, with the exception of (A), which is magnified 1.75 times. For the sake of clarity, muscarinic antagonists are presented as CPK-colored sticks, and hydrogen atoms are omitted. In (B–D), pirenzepine (white) is shown behind TM3, which is transparent. In (C and D), the 7TM cavity is positioned as a dashed area, and the site of fusion (receptor N terminus) of eGFP is indicated.

Mutagenesis experiments, focused on the impact of W400A/F and W405A/F point mutations on orthosteric, allosteric, and BoPz ligand binding properties, provided additional support of the idea that the Bodipy moiety in Bo(10,12)Pz and Bo(15,22)Pz derivatives reach distinct allosteric binding epitopes. Indeed, Bo(10,12)Pz binding is slightly, but significantly, altered by mutations of the W405 residue, whereas Bo(15,22)Pz, gallamine, and brucine display marked losses in affinity at the W400A/F mutants. However, Bo(15)Pz and, to a lesser extent, Bo(22)Pz also show a significant reduction in affinity upon Ala/Phe substitution of the W405 residue. Thus, a preferential location of their fluorophore within the gallamine site does not exclude the possibility for Bodipy, provided the linker is long enough, to explore neighboring domains within the allosteric vestibule (Fig. 7). Keeping in mind that mutagenesis data do not prove a direct implication of these Trp residues, one cannot exclude the destabilization of the aromatic network to which they belong to be responsible for alterations in global positioning of BoPz derivatives within the receptor (Hulme et al., 2007; our modeling studies). Indeed, variations in off rates for [3H]NMS (slower) and fluorescent tracers (faster) from the four M1 mutants may result from a partial collapse of the allosteric lid, hindering [3H]NMS egress from the orthosteric domain or the fluorophore of BoPz ligands to properly anchor to an allosteric site.

These data highlight the prominent role of linker length in the definition of the bitopic nature and the selection of different allosteric binding poses for BoPz compounds. Previous reports already indicated that the fusion of bulky groups (including fluorophores) to pirenzepine was well tolerated in terms of affinity, provided the linker was long enough (at least 6–10 methylenes) to relax the constraints imposed by pirenzepine binding to the M1 orthosteric site (Karton et al., 2007; our modeling studies).
1991; Tahtaoui et al., 2004). Although the nature (polyethylene glycol or isopeptidic) of the linker seems here of marginal importance, the examples of Bo(5) and of similar derivatives (Daval et al., 2012) clearly indicate that the linker conveys affinity and negative binding cooperativity to these molecules. Valuable insights into linker-guided positioning of the fluorophore of Bo(10,12)Pz and Bo(15,22)Pz compounds were gained from M1 modeling and ligand docking studies. The structures of all ligand-receptor complexes were built assuming a fixed pose for pirenzepine (substituted or not) we found to perfectly fit a three-dimensional pharmacophore (Fig. 7A) common to QNB and tiotropium in M2 and M3 (Kruse et al., 2012) receptors, respectively. Interestingly, the distal N-methyl group of pirenzepine (the anchor point for the linker) is not oriented toward the larger opening between the orthosteric and allosteric modules (Fig. 7B). Instead, it faces a narrow channel (between Asp105, Tyr404, and Tyr408 residues), filled with a water molecule in the M2 structure (Haga et al., 2012). In BoPz-M1 complexes (Fig. 7, C and D), the linker passes through this channel, hence replacing the water molecule, to reach the most hydrophobic region of the cavity (Fig. 7B; brown shaded area), near Trp101. This configuration fixes the position of the first seven atoms of the linker (close to pirenzepine), thereby restricting possibilities for positioning the fluorophore, especially if the linker is short. Experimental support of this view is provided by the measurement of a 10-fold-faster off rate of the Bo(10)Pz compound from the W101 AM1 mutant (unpublished data). In Bo(10)Pz-M1 complexes (Fig. 7C), the Bodipy group locates at the entrance of the vestibule, in an opening between TM2 and TM7, yet in a position compatible with concomitant binding of brucine or gallamine. When presented as volumes, these allosteric modulators best evidence plugs over the orthosteric pocket (Supplemental Fig. 3). Long linkers, such as in Bo(22)Pz (Fig. 7D), fold into the allosteric cavity near W400 and allow the fluorophore to locate between extracellular loops 2 and 3, so that the binding of brucine and gallamine is prevented.

These models nicely account for most of our experimental observations, including the estimation of a distance (10 Å) between the Bodipy boron atoms in Bo(10)Pz- and Bo(22)Pz-receptor complexes, which rather well coincides with the average difference in EGFP-Bodipy separation (5 Å) determined between the Bodipy boron atoms in Bo(10)Pz- and Bo(22)Pz-brucine and gallamine is prevented. Long linkers, such as in Bo(22)Pz (Fig. 7D), fold into the allosteric cavity near W400 and allow the fluorophore to locate between extracellular loops 2 and 3, so that the binding of brucine and gallamine is prevented.

As the fluorophore, according to linker

also demonstrate that a fluorophore fused to an orthosteric compound is not an innocent bystander, as it may confer an allosteric texture to the resultant tracer. Unless carefully controlled, this may lead to data misinterpretation, especially when working with bioamine receptors which contain vicinal orthosteric and allosteric modules. Thus, the rational design and in-depth study of bitopic molecules may provide, in addition to advantages expected from the orthosteric and allosteric moieties within a single molecule, valuable guidelines to decipher “hidden” dual drug binding properties.

Acknowledgments

The authors thank Stéphanie Riché for technical assistance and Patrick Wehrung for high-resolution mass spectrometry and high-performance liquid chromatography analyses.

Authorship Contributions

**Participated in research design:** Daval, Galzi, Ilien.

**Conducted experiments:** Daval, Kellenberger, Bonnet, Utard, Ilien.

**Contributed new reagents or analytic tools:** Bonnet.

**Performed data analysis:** Daval, Kellenberger, Ilien.

**Wrote or contributed to writing of the manuscript:** Kellenberger, Galzi, Ilien.

References


Address correspondence to: Dr. Brigitte Ilien, Unité Biotechnologie et Signalisation cellulaire, UMR 7242 CNRS, Université de Strasbourg, Ecole Supérieure de Biotechnologie de Strasbourg, 300 Bvd S. Brant - BP 10413, 67412 Illkirch, France. E-mail: brigitte.ilien@unistra.fr