The second extracellular (E2) loop of G protein-coupled receptors (GPCRs) plays an essential but poorly understood role in the binding of non-peptidic small molecules. We have utilized both orthosteric ligands and allosteric modulators of the M₃ muscarinic acetylcholine receptor (mAChR), a prototypical Family A GPCR, to probe possible E2 loop binding dynamics. We developed a homology model based on the crystal structure of bovine rhodopsin and predicted novel cysteine substitutions that should dramatically reduce E2 loop flexibility via disulfide bond formation, and significantly inhibit the binding of both types of ligands. This prediction was validated experimentally using radioligand binding, dissociation kinetic and cell-based functional assays. The results argue for a flexible “gatekeeper” role of the E2 loop in the binding of both allosteric and orthosteric GPCR ligands.

GPCRs are seven-transmembrane (TM)-spanning proteins representing the largest and most ubiquitously expressed cell surface receptors. GPCRs recognize a plethora of endogenous stimuli, are the target for nearly 50% of all marketed drugs, and are subdivided into three major Families (A, B and C) (1). The largest of these, the Family A GPCRs, include the prototypical examples rhodopsin and the biogenic amine GPCRs.

Despite the relevance of GPCRs to drug discovery, a number of structural and functional questions remain unresolved. To date, the only high-resolution crystal structure of a GPCR that has been published is that of bovine rhodopsin (2). Although proving a useful template for homology modeling of, and predicting ligand-receptor interactions with, GPCR TM regions (3), far less is known about the correlation between loop structures of different GPCRs and those of rhodopsin. The loop regions are clearly vital to the binding of large molecular weight ligands, in particular peptides (4), but their role in molecular mechanisms of GPCRs that utilize small molecules as their endogenous ligands remains largely unresolved. This is because the orthosteric site, i.e., the binding domain for the endogenous agonist (5), for these GPCRs has been mapped within their TM regions (6). However, a number of recent developments in the field point to hitherto unappreciated properties of the extracellular regions of GPCRs, in particular the E2 loop. For example, this region was found to be a negative regulator of C5a receptor activation (7). More recently, random mutagenesis of the M₃ mAChR identified multiple residues in the E2 loop critical for receptor activation (8). In this latter study, however, the bulk of the mutations that exerted significant effects on receptor activation had minimal effects on orthosteric ligand binding, and the role of the E2 loop in contributing to the mode of mAChR ligand binding thus remains largely unexplored.

Intriguingly, for certain small molecule agonist Family A GPCRs, Shi and Javitch (6,9) have proposed that the E2 loop may contribute directly to the orthosteric binding site, by adopting a conformation similar to that found in the crystal structure of inactive state rhodopsin whereby the β4 strand of the loop folds inwards towards the
TM core regions that are crucial for the binding of orthosteric ligands. A key determinant of the correct orientation of the E2 loop is the constraint imposed by a disulfide bond between the loop and the top of TM domain 3; this disulfide is highly conserved amongst other Family A GPCRs, indicating that it plays a widespread role in maintaining an appropriate receptor conformation. However, the orthosteric ligand in rhodopsin, cis-retinal, is covalently attached to its receptor within the orthosteric pocket; for most other GPCRs that utilize diffusible, often hydrophilic, orthosteric ligands, it is difficult to envisage how such ligands actually gain entrance from the extracellular space into the corresponding TM cavity without postulating some form of flexible movement of the E2 loop to accommodate initial ligand access, despite the constraint imposed by the conserved disulfide bond.

The extracellular regions of GPCRs can also be targeted by allosteric modulators (10-12), which co-bind with orthosteric ligands to regulate their function. The best-studied Family A GPCRs in this regard are the mAChRs. Previous studies have proposed that prototypical modulators of mAChRs recognize at least one allosteric site that involves the E2 loop and its environs (13-15), thus highlighting that this region plays an important role in determining not only the actions of orthosteric ligands, but those of novel allosteric ligands as well. In the current study, we have exploited the opportunities afforded by such small molecule modulators to probe their interactions with orthosteric ligands at the M2 mAChR, and to gain novel insight into the role of the E2 loop in this process. Using molecular modeling and mutagenesis, we provide novel evidence of a fundamental role for a requisite flexibility of the E2 loop in the binding of both allosteric and orthosteric GPCR ligands.

Experimental Procedures

Model building and ligand docking- The 7TM helical bundle and extracellular loops of the M2 mAChR were modeled using DeepView (16) with the structure of bovine rhodopsin (1F88 (2)) as a template. The sequence alignment used was based on ref. (17). A disulfide bond between residues C<sup>96</sup> and C<sup>175</sup> was manually added and the structure minimized with the MMFF94s forcefield in Sybyl7.0 (Tripos St. Louis, MO, USA) to correct steric clashes introduced in the modeling process. The completed model was inspected in Sybyl6. For orthosteric ligand docking, N-methylscopolamine (NMS) was built using Sketcher in Sybyl6.92 and the structure was minimized with the Tripos forcefield. The NMS model was saved as a Sybyl MOL2 file then converted to AutoDock PDBQS format using the AutoDock mol2env script in AutoDock 3.0.5 (18). The receptor model structure was converted from Sybyl MOL2 format to AutoDock PDBQS format using the AutoDock mol2topdbqs script. The docking site was selected to centre on the cluster of residues known to bind orthosteric ligands, including D<sup>103</sup>, W<sup>155</sup>, T<sup>190</sup>, T<sup>187</sup>, W<sup>400</sup>, Y<sup>403</sup> and N<sup>404</sup> (3); see also Fig. 1A. A total of 100 docking runs were made using the LGA algorithm, clustered post-docking with an RMSD cut-off of 1.5Å and the docked complexes inspected with Sybyl17.0.

Receptor mutagenesis- The coding sequence of the human M<sub>2</sub> mAChR, obtained from the UMR cDNA Resource Centre (www.cdna.org), was cloned into the Gateway recombination Entry vector, pENTR/D-TOPO, using the pENTR directional TOPO<sup>®</sup> cloning kit (Invitrogen, Mt. Waverley, Australia), following amplification of the gene using the following primers 5'-CACCATGAATAACT CAACAAACTCC-3' (N-terminal forward primer with CACC sequence) and 5'-TTACCTTGTAGCGCCTATGTTC-3' (C-terminal reverse primer). The native stop codon was subsequently mutated to Lys using the QuickChange mutagenesis kit (Stratagene, La Jolla, CA), prior to subcloning of the receptor into pEFS/FRT/V5-DEST Gateway destination vector. Transfer of the M<sub>2</sub> mAChR from pENTR/D-TOPO into pEFS/FRT/V5-DEST was achieved using the LR Clonase enzyme mix kit (Invitrogen) and resulted in in-frame insertion of the V5 epitope tag at the C-terminus of the receptor. This receptor sequence is referred to as “wild-type” throughout this study, and was found to have equivalent pharmacological properties to human M<sub>2</sub> mAChRs studied previously (19) where the native stop codon was intact. Mutations were introduced into the wild-type receptor in pENTR/D-TOPO by site-directed mutagenesis (QuickChange kit). Mutant receptors were subcloned into the pEFS/FRT/V5-DEST vector as described above. Oligonucleotides for site-directed
mutagenesis and DNA sequencing were purchased from GeneWorks (Hindmarsh, Australia). The primers used for all site-directed mutagenesis reactions are detailed in Supplementary Table 1. The integrity of all receptor clones was confirmed by cycle-sequencing with the ABI Prism BigDye Terminator v3.1 ready reaction cycle sequencing kit with reactions analysed on an ABI Prism 373xI 96 capillary automated DNA sequencer (Australian Genome Research Facility, Parkville).

Wild-type and mutant receptors were isogenically integrated into CHO-FlpIn cells (Invitrogen) as follows: 75 cm² flasks with CHO FlpIn cells at 70-75 % confluency were transfected in serum and antibiotic-free DMEM with 1 µg of pEFS/FRT/V5-DEST vector containing the wild type or mutant M₂mAChR gene and 9 µg POG44 vector (containing Flp recombinase) using lipofectamine (75 µl/75 cm² flask) according to the manufacturers recommendations. Selection of cells expressing the receptors was achieved by treatment with 400 µg/ml hygromycin-B every two days until resistant flocules were obtained, prior to passaging (five times). Transfected cells were subsequently maintained in complete DMEM media containing 200 µg/ml hygromycin-B.

[^3]H]NMS binding assays- CHO-FlpIn cells were grown, maintained and harvested for membrane preparation as described previously (19), with the addition of hygromycin B, 200 µg/ml, in the culture medium. For[^3]H]NMS saturation binding, membranes (20-40 µg/ml) were incubated in 500 µl total volume of buffer containing 0.02 to 5 nM[^3]H]NMS (0.1 nM to 10 nM for the V171C+ N419C mutant) for 60 min (120 min for V171C+ N419C) at 37°C. For inhibition binding assays, M₂ CHO-FlpIn cell membranes were incubated as above with 0.2 nM[^3]H]NMS and orthosteric or allosteric ligands at 37°C for 120 min (180 min for V171C+ N419C).[^3]H]NMS dissociation kinetic assays were performed as described previously (19). For some experiments, membranes were pretreated with 80 mM dithiothreitol (DTT) at 37°C for 30 mins followed by washout; subsequent binding assays were then performed as above, but in the presence of 10 mM DTT. Nonspecific binding was defined using 10 µM atropine (100 µM for the V171C+ N419C mutant). Incubations were terminated by rapid filtration and radioactivity was determined by scintillation counting.

ERK1/2 phosphorylation assays- CHO-FlpIn cells were seeded at a density of 40,000 cells/well in 96-well plates and allowed to adhere for a minimum of 4 hours prior to the replacement of growth medium with serum-free DMEM. Following washing and serum starvation overnight, cells were pre-treated for 30 minutes with C3/3-phth at 37°C, then stimulated for 5 minutes with ACh, before lysis using the proprietary lysis buffer supplied with the SureFire™ kit. After processing these samples according to the manufacturer’s instructions, the resulting fluorescence signal of each well was determined with a Fusion-α™ plate reader (Packard), using standard Alphascreen™ settings. Data were normalized to the maximal response elicited by 10% FBS.

Data analysis- Radioligand binding curves were analysed using nonlinear regression (GraphPad Prism 4.03, San Diego, CA) as described previously (19) to derive estimates of radioligand equilibrium dissociation constant (Kₐ) and maximal density of binding sites (Bₘₐₓ). For the dissociation kinetic studies, the decay in radioligand specific binding with time was fitted to a monoexponential function to derive the apparent off rate constant (Kₐ) of[^3]H]NMS dissociation in the absence or presence of each concentration of modulator (19). These Kₐ values were then plotted as a function of modulator concentration, and the resulting concentration-kinetic effect curves were fitted simultaneously with the data derived from the modulator inhibition binding assays, using global nonlinear regression, to an allosteric ternary complex model (20); see Results. Statistical comparisons were by Student’s t-test or one way ANOVA, as appropriate.

RESULTS

Development of a homology model of the M₂ mAChR

The x-ray crystal structure of bovine rhodopsin revealed that the β4 strand of the E2 loop folds into the TM core of the receptor, forming a lid-like structure over the entrance to the orthosteric site. The orientation of the “lid” is
maintained by a highly conserved disulfide bond between a cysteine in the E2 loop and another at the top of TM3. The M2 mAChR (Fig. 1A) is predicted to share these structural features. Using the inactive state bovine rhodopsin structure as a template, we developed a homology model of the M2 mAChR (Fig. 2). As with rhodopsin, the E2 loop of the M2 mAChR is restricted by the disulfide bond between C176 in E2 and C96 at the top of TM3 (yellow residues, Fig. 2A). Loop searching with DeepView (16) identified an appropriate template structure containing a β-turn motif, in keeping with the results of secondary structure prediction for the protein sequence (data not shown). Of particular note is the orientation of the E2 loop in the non-bound receptor, which points in over the orthosteric binding-site crevice (Fig. 2A). This is a feature evident in most models based on rhodopsin (3,6,17,21), and has led to the speculation that the E2 loop may directly contribute to the orthosteric binding pocket (6). We docked the prototypical orthosteric antagonist, NMS, into this region (Fig. 2B). The positively charged ammonium headgroup of NMS interacts with the acidic D103 side chain, which is conserved across all biogenic amine GPCRs; other key interactions between the ligand and protein, consistent with data derived from published studies (3), are also indicated in Fig. 2B.

Constraining E2 loop movement impairs orthosteric ligand access and binding affinity

We next focused on the role of the E2 loop in orthosteric ligand binding. Despite the constraint imposed by the conserved disulfide, the remaining residues in the E2 loop may allow a large degree of freedom. We thus hypothesized that the loop may dynamically adopt a more open conformation, whereby the cleft to the orthosteric pocket becomes more readily exposed, as part of the process for accommodating optimal entry of orthosteric ligands into the TM core.

To directly explore this important possibility, we began with our minimized closed-loop model, where V171 in the E2 loop is predicted to lie within 3.7 Å of N419 near the top of TM7 (red residues, Fig. 2A). We reasoned that mutation of each of these residues to cysteines (V171C+ N419C double mutant) would allow for the formation of a disulfide bond that, together with the highly conserved C96-C176 pair, would greatly restrict the movement of the E2 loop, effectively “locking it down” towards the orthosteric binding site crevice. If a hinge-like opening of the E2 loop is required for orthosteric ligand binding, then the mutation should have a significant inhibitory effect on this property. For additional comparison, we also generated a V171A+ N419A double mutant, which would provide information about the local/direct contribution of these amino acids to ligand binding, as well as individual point mutations (C or A) of each of the V171 and N419 pairs.

As predicted, mutation of V171 and N419 to cysteine led to a significant reduction (approx. 5-fold) in the affinity of the orthosteric antagonist, [3H]NMS, compared to the wild type (Table 1). An even greater inhibitory effect was noted on the binding of the orthosteric agonist, ACh (Figs. 3A-B; Table 1). The competitive interaction between ACh and [3H]NMS was characterized by a biphasic inhibition curve, as has been noted for many agonists of GPCRs, and likely reflects the interaction of the agonist with G protein coupled and uncoupled receptor states (22); nonetheless, it is clear that the ability of ACh to compete with [3H]NMS is markedly impaired at the V171C+ N419C mutant, irrespective of the binding state of the receptor (Table 1). Perhaps more interestingly, the inhibitory effect on radioligand affinity was also accompanied by a profound slowing in the [3H]NMS dissociation rate constant (approx. 24-fold Table 2). Given that a reduction in dissociation rate would be expected to yield an increase in radioligand affinity unless an additional effect was exerted on association rate, our findings suggest that constraining the E2 loop must reduce the [3H]NMS association rate dramatically by a factor of approx. 120-fold in order to yield a 5-fold reduction in radioligand equilibrium binding affinity.

Overall, these results are thus consistent with a mechanism whereby closure of the E2 loop markedly reduces the association of the radioligand with its binding site in the TM regions, but any radioligand that does manage to bind is then not readily able to exit from the pocket due to the loop closure.

The native conformation of residues V171 and N419 also plays a modest role in ligand binding

In contrast to the data obtained at the V171C+ N419C mutant, no significant effect was noted on the affinity of [3H]NMS (Table 1) or its dissociation rate (Table 2) at the V171A+ N419A
mutant, although a modest trend towards a reduction was observed for each parameter. In experiments monitoring the competition between $[^3\text{H}]\text{NMS}$ and ACh, a modest but significant reduction was noted in the value of the low affinity agonist binding state (Table 1; Fig. 3C). Similar results were obtained when we investigated single point mutations (to either A or C) of each of these residues (data not shown). These results suggest that the residues $V^{171}$ and $N^{419}$ on their own may provide some degree of direct contribution to the mode of binding of orthosteric ligands, but cannot account for the dramatic effects observed at the $V^{171}C$ and $N^{419}C$ double mutant.

**Pretreatment with the disulfide bond reducing agent, dithiothreitol (DTT), restores ligand pharmacology towards that of the wild type receptor**

To further confirm the role of disulfide bond formation on the properties of the $V^{171}C$ and $N^{419}C$ mutant, experiments were repeated in the absence or presence of membrane treatment with the disulfide reducing agent, DTT. As shown in Fig. 3A and Table 3, treatment of the wild type receptor did not have a significant effect on the binding affinity of either $[^3\text{H}]\text{NMS}$ or ACh, even though the DTT was likely to have disrupted the conserved disulfide bond between E2 and TM3, the latter experimentally validated by a significant increase in the dissociation rate constant of $[^3\text{H}]\text{NMS}$ at the DTT-treated wild type receptor (Table 2). This finding suggests that, although necessary for receptor folding and delivery to the cell surface, the conserved disulfide bond in the $M_2$ mAChR can subsequently be disrupted with minimal perturbation of orthosteric ligand binding affinity. However, a strikingly different result was obtained at the $V^{171}C$ and $N^{419}C$ mutant. As shown in Fig 3B and Table 3, DTT treatment of this mutant led to a dramatic increase in both affinity states of ACh, approaching those determined at the $V^{171}A$ and $N^{419}A$ mutant (Table 1). For $[^3\text{H}]\text{NMS}$, the resulting dissociation constant was not significantly different from that of the wild type receptor (Table 3). These results validate our novel model prediction of the spatial proximity between the $V^{171}$ and $N^{419}$ residues and, to our knowledge, demonstrate for the first time a requisite role for E2 loop flexibility in the binding of orthosteric mAChR ligands.

**Constraining E2 loop movement impairs allosteric modulator binding affinity**

We next investigated the effects of the mutations on two structurally diverse allosteric modulators (Fig. 1B) known to interact at a common site on the $M_2$ mAChR (23). As outlined in the Introduction, prior studies have suggested that these allosteric modulators bind at a site that is more extracellular relative to the orthosteric site. In terms of the actual mode of modulator binding, two possibilities can be envisaged. The first is that, as with orthosteric ligand binding, the E2 loop adopts an open conformation, moving outwards away from the TM core in order to accommodate modulator binding; in contrast to orthosteric ligands, however, the allosteric modulator would not then move down into the TM binding-site crevice, but rather uses the open E2 loop and its environs to dock, in essence “replacing” the E2 loop. The second possibility is that the E2 loop remains pointing down towards the entrance to the orthosteric site, and the allosteric modulator binds above the loop, utilizing epitopes from this region and perhaps E3 as the allosteric pocket (15). Both possibilities are consistent with the commonly observed experimental finding that prototypical allosteric modulators of the $M_2$ mAChR can profoundly slow the dissociation of orthosteric ligands from the receptor (24).

To quantify the experimental results, we used an allosteric ternary complex model (Fig. 1B), which describes the properties of the modulators in terms of the affinity for the allosteric site (equilibrium dissociation constant, $K_B$) and the cooperativity ($\alpha$) between the orthosteric and allosteric sites when each is occupied by its respective ligand. The latter is a direct measure of the strength and direction of the allosteric effect (10). To further ensure experimental rigor, two paradigms were utilized. The first investigated the effects of the modulator on the dissociation rate of a preformed $[^3\text{H}]\text{NMS}$-$M_2$ mAChR complex; theoretically, this can provide a composite estimate of $K_B/\alpha$ (20). The second paradigm investigated the effect of the modulator on the apparent binding affinity of the radioligand when both were added simultaneously and allowed to approach equilibrium on the
receptor; this can yield individual estimates of $K_B$ and $\alpha$ (19,20).

As shown in Table 1 and Fig. 4A, mutation of V$^{171}$ and N$^{419}$ to cysteine led to a pronounced reduction in the affinity of both allosteric modulators, but did not have a significant effect on the cooperativity with $[^{3}H]NMS$. Treatment of the receptors with DTT led to a marked enhancement of the potency of the modulators at the V$^{171}C+ N^{419}C$ mutant, as would be expected upon breaking of the bonds and return of E2 loop flexibility; the resulting potency estimates of the modulators are not the same as at the wild type receptor, however, and likely reflect the local contribution of the V$^{171}C$ and N$^{419}C$ mutations in the absence of disulfide bond formation (see below). Thus, it can be concluded that, in addition to the effects orthosteric agonists and antagonists, restriction of E2 loop movement impedes the binding of prototypical allosteric modulators of the mAChRs.

Perhaps not surprisingly, mutation of V$^{171}$ and N$^{419}$ to alanine also had a modest but significant effect on the binding affinity of the modulators (Table 1; Fig. 4B), although nowhere near as striking as the cysteine substitutions. As with orthosteric ligand binding, this suggests that substitution of these residues may cause a local conformational perturbation in E2 such that modulator binding affinity is influenced. However, this finding is unlikely to compromise the interpretation of the effects of the V$^{171}C+ N^{419}C$ double mutant, as individual point mutations of V$^{171}C$, V$^{171}A$, N$^{419}C$ or N$^{419}A$ resulted in either no effect or only a modest (approx. 0.5 log unit) reduction in modulator affinity (not shown), comparable to that of the V$^{171}A+ N^{419}A$ double mutant but clearly unable to account for the dramatic effect observed at the V$^{171}C+ N^{419}C$ double mutant. In addition, and to confirm that the effects of DTT on allosteric modulator binding reflect its ability to specifically disrupt disulfide bonds, we repeated the experiments with C$_{7}$/3-phth at the V$^{171}A+ N^{419}A$ double mutant (Fig. 4B). As expected, the resulting estimates of modulator affinity ($pK_B = 5.99 \pm 0.06; n = 4$) and cooperativity ($\log \alpha = -0.97 \pm 0.04$) were similar to those obtained at the untreated V$^{171}A+ N^{419}A$ receptor (Table 1).

The effect of allosteric site mutations on receptor function is consistent with effects on receptor binding

Finally, although the effects of our mutations have been interpreted in terms of changes in binding site affinity and cooperativity, it is possible that the mutants have additional effects on receptor function. To explore this possibility, we determined the potency of C$_{7}$/3-phth as an allosteric inhibitor of ACh-mediated ERK1/2 phosphorylation. Fig. 5 and Table 4 summarize the results of these experiments, where a number of key findings are evident. First, none of the mutations had any effect on basal ERK1/2 phosphorylation, suggesting that we had not affected the propensity of the receptor to shuttle between active and inactive states (8). Second, there was no significant effect of the V$^{171}A+ N^{419}A$ mutant on the maximal ACh-mediated response, in contrast to the key V$^{171}C+ N^{419}C$ mutant; consistent with our hypothesis, constraining the E2 loop markedly reduced ACh efficacy and potency by a likely combination of impeding its interaction with the orthosteric site and reducing receptor expression. Third, application of a functional variant of the allosteric ternary complex model (20), to the data yielded estimates of C$_{7}$/3-phth affinity that are consistent with the expectation that the mutations affect binding interactions within the M$_2$ mAChR, but not receptor function. Differences in cooperativity factors are, of course, expected because the functional assays used ACh as the orthosteric ligand, whereas the binding assays used $[^{3}H]NMS$. In general, the absolute modulator $pK_B$ values shown in Table 4 are somewhat lower than those determined in the binding assays, but this likely reflects differences in the assay conditions. More important is the fact that the relative difference in $pK_B$ values between each mutant and the wild type receptor is similar in the functional assays compared to the binding assays, clearly indicating that the mutations did not introduce additional conformational effects that would lead to the modulator having differential effects on function above and beyond those predicted by their effects on binding.

DISCUSSION
The majority of studies on Family A biogenic amine GPCRs, of which the M₂ mAChR is a prototypical example, have focused on ligand binding in the TM regions. Far less is known about the role of the extracellular regions of these receptors in this process. Recently, however, a number of studies have begun to shed new light on the role of the E2 loop in orthosteric ligand action (6-8). Moreover, the discovery of allosteric ligands that utilize the extracellular regions of the mAChRs to mediate their effects has also provided impetus for further study of the role of GPCR extracellular loops in drug action (12). Despite the presence of a conserved disulfide bond between the E2 loop and the top of TM3, we now provide novel evidence for a requisite flexibility in the E2 loop of the M₂ mAChR necessary for the binding of both allosteric and orthosteric ligands. We hypothesize that this role of the E2 loop extends to other GPCRs (see below).

A role for the E2 loop, and in particular the constraint imposed by the conserved disulfide bond, has been well established in the binding of orthosteric ligands for peptide GPCRs, as well as being important for proper folding, expression, and activation (see ref (9)). The role of the E2 loop in the mode of action of small molecule orthosteric ligand GPCRs is less clear, although an elegant study by Scarselli et al. (8) recently implicated this region in the activation of the M₁ mAChR. The authors of that study also suggested a possible mechanism whereby conformational flexibility in the E2 loop is required for efficient receptor activation. The results from our functional experiments are in accord with this mechanism, as constraint of E2 loop flexibility in the M₂ mAChR has a profound inhibitory effect on the ability of ACh to mediate ERK1/2 phosphorylation (Fig. 5C). However, it is also worth noting that many of the key mutations identified in the study of Scarselli et al. (8) did not actually perturb the binding of orthosteric ligands, at least as assessed in equilibrium binding assays.

Interestingly, other studies on ligand selectivity at α₁a and α₁b adrenoceptors, SHT₁b and SHT₁b receptors, adenosine A₁ and A₂ receptors and dopamine D₂ receptors led to the recent hypothesis that the E2 loop can directly contribute specificity to orthosteric ligand binding by directly forming part of the binding site crevice (6,9), just as it does for rhodopsin. In contrast to rhodopsin, however, the remaining Family A GPCRs almost exclusively utilize diffusible ligands as their endogenous agonists, and it is difficult to envisage how the “closed” E2 loop would accommodate small molecule ligands that utilize the inner TM core of the receptor. We now provide direct evidence that a forced constraint of the E2 loop in this “closed” position, via introduction of a disulfide bond through mutation of V⁺¹⁷¹ and N⁺⁴¹⁹ to cysteine, actually impedes the binding of orthosteric ligands to the M₂ mAChR. It may be argued that this result mainly reflects a localized conformational disruption of multiple amino acids that would otherwise optimally participate in ligand binding, rather than implying a requisite role for a large dynamic movement of the entire loop. However, the latter interpretation is strongly supported by the striking effects we noted of the double cysteine mutation on the binding kinetics of [³H]NMS; a ca. 120-fold reduction in association and ca. 24-fold reduction in dissociation kinetics of the orthosteric ligand are suggestive of a steric “trapping” mechanism promoted by a loop that has profoundly reduced flexibility. In contrast, mutation of V⁺¹⁷¹ and N⁺⁴¹⁹ to alanine, which may also be expected to cause local conformational disruptions, did not significantly affect the kinetics of ligand binding, presumably because loop flexibility was retained.

Importantly, the results of pretreatment with the disulfide reducing agent, DTT, are also consistent with the “trapping” hypothesis for orthosteric ligands, as a subsequent return in loop mobility would be expected to improve ligand affinity, as was observed. It should be noted, however, that one would not necessarily expect the resulting affinity estimates to be identical to the wild type receptor in the DTT experiments, due to the local contribution of mutations in V⁺¹⁷¹ and N⁺⁴¹⁹ to ligand binding (as inferred from the V⁺¹⁷¹ and N⁺⁴¹⁹ to alanine substitutions).

Although our engineered constraint is unlikely to precisely mimic the closed E2 loop as it exists in the native receptor, our findings argue for a requisite flexibility of the E2 loop to allow for access to and egress from the orthosteric binding pocket. This flexibility can also allow the E2 loop to fold over the binding site crevice upon orthosteric ligand binding; indeed, the ability of the receptor to form the novel disulfide bond argues that this conformation is one that can
readily exist for the receptor. Thus, the length and composition of the loop can still contribute to the subtype selectivity that has been suggested by previous studies, as well as modulating the ability of the GPCR to adopt active/inactive states.

Similar considerations apply to the interaction of allosteric modulators with the M<sub>2</sub>mAChR. Previous studies have identified the residues<sup>172</sup> (EDGE<sup>175</sup>) and Y<sup>177</sup> in the E2 loop, as well as N<sup>419</sup>, T<sup>423</sup> and a conserved tryptophan, W<sup>422</sup>, in TM7 as contributing to the allosteric binding of prototypical mAChR modulators such as gallamine, alcuronium and C<sub>r</sub>-/3-phth-like molecules (13-15,25). The classic effects of the prototypical modulators, especially their ability to significantly retard orthosteric ligand dissociation have been interpreted in terms of a “capping” mechanism of the modulator over the orthosteric binding site crevice (9). However, the interaction that leads to modulator binding may be mediated either via attachment from the extracellular face of the receptor over a closed E2 loop, or via a partial “substitution” by the modulator for the E2 loop when the latter adopts an open conformation. Although we cannot discriminate between these two possibilities, our experimental results reveal that loop flexibility is important for either mechanism.

Conceptually, therefore, we propose the following model to accommodate the fact that the same mutations in the E2 loop affect the binding of both orthosteric and allosteric small molecule ligands: We view the E2 loop as a “gatekeeper” with respect to entrance into the orthosteric binding site crevice. Flexibility in the loop is necessary for it to adopt an open conformation such that orthosteric ligands can then proceed into the TM-bound crevice, which represents the major region contributing to direct orthosteric ligand-receptor interactions; the loop can then close over the orthosteric ligands and engage in additional interactions, as inferred from previous studies (12). This mechanism is consistent with the profound effects we observe on orthosteric radioligand binding kinetics. For allosteric modulators, however, the E2 loop is likely to constitute a major (local) contact point for direct modulator-receptor interaction; mutation of V<sup>171</sup> and N<sup>419</sup> to C or A can thus be expected to influence the binding affinity of the modulators, although the stronger inhibitory effects of the cysteine substitutions suggest that E2 loop flexibility is still important for optimal modulator-receptor interactions in this extracellular region.

The findings reported herein are of general relevance beyond the M<sub>2</sub>mAChR. For example, the E2 loop of various Family A GPCRs is a possible target for activating allosteric antibodies (12). In the Family C calcium-sensing receptor, the E2 loop has been implicated in the effects of allosteric modulators, even though their main site of binding is within the TM bundle at this receptor type (26). Similarly, small molecule chemokine CCR5 receptor modulators also utilize extracellular receptor regions (27). Taken together, our findings suggest a fundamental role for a dynamic E2 loop in the binding of not only allosteric modulators, but also for orthosteric ligands at a prototypical Family A GPCR. In addition to contributing to our understanding of the biology of allosteric modulation, it is anticipated that further refinements of this model may allow for a cohesive picture of the allosteric binding pocket.

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Figure 1  The M₃ mAChR possesses topographically distinct binding sites. A) Snake diagram of the M₃ mAChR, indicating amino acid residues previously implicated in the binding of orthosteric ligands (grey) and allosteric modulators (black). The conserved disulfide bond is also shown. White arrows indicate the amino acids mutated in the current study. B) Structures of the allosteric modulators used in this study and the allosteric ternary complex model, which describes allosteric modulator effects in terms of their binding to the free receptor (Kᵢ) and the cooperativity (α) between the orthosteric and allosteric sites when both are occupied. In this model, α > 1 (Log α > 0) denotes positive cooperativity.
(allosteric enhancement of affinity), whereas $\alpha < 0$ (Log $\alpha < 0$) denotes negative cooperativity (allosteric reduction of affinity).

**Figure 2  Homology model of the M$_2$ mAChR.** A) Extracellular view of a minimized M$_2$ mAChR homology model indicating “closed” E2 loop across the entrance to the TM-bound orthosteric binding-site crevice. The highly conserved disulfide bond is indicated in yellow. The residues mutated in the current study, V$^{171}$ and N$^{419}$, are shown in red. Nt, N-terminal domain; E2, second extracellular loop; roman numerals indicated the transmembrane domains. B) Close-up of docked NMS in the orthosteric site. Residues implicated in orthosteric ligand binding are indicated; NMS is shown as rods colored by atom type (carbon, green).

**Figure 3  Novel cysteine mutations in the M$_2$ mAChR impede E2 loop flexibility and orthosteric ligand binding.** A) Effect of DTT (see Experimental Procedures) on the competition between $[^3]$H]NMS and acetylcholine at wild type M$_2$ mAChRs. B) Effect of DTT on the competition between $[^3]$H]NMS and acetylcholine at V$^{171}C+N^{419}C$ mutant M$_2$ mAChRs. C) Competition between $[^3]$H]NMS and acetylcholine at V$^{171}A+N^{419}A$ mutant M$_2$ mAChRs; the dashed line indicates the binding curve at the wild type receptor for comparison. Points represent the mean ± s.e. mean of 3-4 experiments performed in triplicate.

**Figure 4  A flexible E2 loop is vital for the binding of allosteric modulators.** A) Mutation of V$^{171}$ and N$^{419}$ to C leads to a marked reduction in the potency of C$_7$/3-phth to modulate $[^3]$H]NMS binding in both pseudoequilibrium (left panel) and dissociation kinetic (right panel) binding. Exposure to DTT results in a return of modulator potency close to that of the wild type. B) DTT has no effect on the potency of C$_7$/3-phth when investigated at the V$^{171}A+N^{419}A$ M$_2$ mAChR mutant; the dashed line indicates the binding curves determined at the wild type receptor for comparison. For both panels, the curves superimposed on the data represent the best global nonlinear regression curve fit of the allosteric ternary complex model simultaneously to both the pseudoequilibrium and dissociation kinetic experiments. Points represent the mean ± s.e. mean of 4 experiments performed in triplicate.

**Figure 5  The effects of M$_2$ mAChR mutations on receptor function are consistent with their effects on receptor binding properties.** Acetylcholine-mediated ERK1/2 phosphorylation was assessed in intact CHO cells stably expressing each of the indicated constructs using a plate-based signaling assay (see Experimental Procedures). Data were normalized to the maximum response ERK1/2 elicited by 10% FBS and globally fitted to the allosteric ternary complex model. Points represent the mean ± s.e. mean of 6-8 experiments performed in triplicate.
Table 1  Ligand binding and allosteric model cooperativity parameters for mAChR orthosteric and allosteric ligands. Data represent the mean ± s.e. mean of 3-4 experiments performed in triplicate and analysed by nonlinear regression (see Methods).

| Ligand  | Parameter | Wild Type | V^{171}C+N^{419}C | V^{171}A+N^{419}A |
|---------|-----------|-----------|-------------------|-------------------|
| NMS     | pKₐ       | 9.17 ± 0.18 | 8.48 ± 0.14*       | 8.90 ± 0.18       |
|         | Bₘₐₓ (pmol/mg) | 2.28 ± 0.31 | 0.37 ± 0.06*       | 1.85 ± 1.00       |
| Acetylcholine | pKₜₑ | 7.61 ± 0.16 | 6.07 ± 0.23*       | 7.15 ± 0.38       |
|         | (%Rₜₑ)   | 47 ± 6    | 35 ± 4             | 32 ± 10           |
|         | pKₗₑ     | 6.06 ± 0.11 | 3.49 ± 0.16*       | 5.33 ± 0.18*      |
| C₇/3-phth | pKₗₑ     | 6.61 ± 0.03 | 4.45 ± 0.09*       | 5.67 ± 0.05*      |
|         | Log αₑ    | -0.83 ± 0.03 | -0.91 ± 0.10       | -0.63 ± 0.04*     |
| Gallamine | pKₗₑ     | 5.95 ± 0.05 | 4.32 ± 0.09*       | 5.25 ± 0.05*      |
|         | Log αₑ    | -1.55 ± 0.06 | -1.64 ± 0.14       | -1.06 ± 0.06*     |

a Negative logarithm of the radioligand equilibrium dissociation constant.

b Maximum density of binding sites.

c Negative logarithm of the orthosteric ligand apparent equilibrium dissociation constant for the high affinity state. % Rₜₑ denotes the percentage of high affinity sites, shown in parentheses.

d Negative logarithm of the orthosteric ligand apparent equilibrium dissociation constant for the low affinity state.

e Negative logarithm of the allosteric ligand equilibrium dissociation constant for the unoccupied receptor.

f Logarithm of the cooperativity factor for the allosteric interaction.

* Significantly different (p < 0.05) from the untreated wild type receptor, as determined by one way ANOVA.
Table 2  
[^3]H]NMS dissociation rate (K_{off}) constants determined at M_2 mAChR constructs in the absence or presence of DTT treatment (see Methods). Data represent the mean ± s.e. mean from 3 experiments performed in duplicate.

| M_2 Receptor Construct | K_{off} Untreated (min^{-1}) | K_{off} DTT treated (min^{-1}) |
|------------------------|-----------------------------|-------------------------------|
| Wild Type              | 0.72 ± 0.02                 | 0.98 ± 0.05*                  |
| V^{171}C+N^{419}C      | 0.03 ± 0.01*                | 1.03 ± 0.05*                  |
| V^{171}A+N^{419}A      | 0.60 ± 0.05                 | 1.02 ± 0.09*                  |

* Significantly different (p < 0.05) from the untreated wild receptor, as determined by one way ANOVA.
Table 3  Ligand binding and allosteric model cooperativity parameters for mAChR orthosteric and allosteric ligands determined at DTT-treated M_{2} mAChRs. Data represent the mean ± s.e. mean from 4 experiments performed in duplicate.

| Ligand     | Parameter | Wild Type + DTT | V^{131}C+N^{419}C + DTT |
|------------|-----------|-----------------|-------------------------|
| NMS        | pK_{A}^{a} | 9.23 ± 0.01     | 8.97 ± 0.23             |
| ACh        | pK_{H}^{b} | 7.66 ± 0.34     | 6.85 ± 0.17^{*}         |
|            | (%R_{H})  | (41 ± 13)       | (72 ± 7)                |
|            | pK_{L}^{c} | 6.14 ± 0.22     | 4.59 ± 0.47^{*}         |
| C/3-phth   | pK_{B}^{d} | 6.71 ± 0.05     | 6.31 ± 0.05^{*}         |
|            | Log \( \alpha \) | -0.83 ± 0.03 | -0.63 ± 0.03^{*}         |
| Gallamine  | pK_{B}     | 5.71 ± 0.06     | 5.28 ± 0.05^{*}         |
|            | Log \( \alpha \) | -1.19 ± 0.06 | -1.26 ± 0.06             |

\(^{a}\) Negative logarithm of the radioligand equilibrium dissociation constant.

\(^{b}\) Negative logarithm of the orthosteric ligand apparent equilibrium dissociation constant for the high affinity state. \% R_{H} denotes the percentage of high affinity sites, shown in parentheses.

\(^{c}\) Negative logarithm of the orthosteric ligand apparent equilibrium dissociation constant for the low affinity state.

\(^{d}\) Negative logarithm of the allosteric ligand equilibrium dissociation constant for the unoccupied receptor.

\(^{e}\) Negative logarithm of the cooperativity factor for the allosteric interaction between the modulator and the radioligand when both occupy the receptor.

\(^{*}\) Significantly different (p < 0.05) from the DTT-treated wild type receptor (p < 0.05), as determined by Student’s t-test.
Table 4  Allosteric model parameters for the functional interaction between C7/3-phth and acetylcholine at M2 mAChR mutants. Data represents the mean ± s.e. mean from 6-8 experiments performed in triplicate and analysed by nonlinear regression (see Methods).

| M2 Receptor Construct | pK_B^a | Δ Wild Type (vs. Binding)^b | Log α^c |
|-----------------------|--------|-----------------------------|---------|
| Wild Type             | 5.95±0.21 | 0                           | -1.94±0.25 |
| V<sup>171</sup>C+N<sup>419</sup>C | 4.16±0.17* | 1.79 (2.09) | -1.22±0.51 |
| V<sup>171</sup>A+N<sup>419</sup>A | 5.40±0.25 | 0.55 (0.89) | -3.29±0.30* |

^a Negative logarithm of the allosteric modulator equilibrium dissociation constant.

^b Difference between the modulator pK_B value for the mutant relative to that estimated for the wild type receptor; values in parenthesis denote the same calculation using the values obtained from the radioligand binding assays (Table 1).

^c Logarithm of the cooperativity factor governing the allosteric interaction between the modulator and ACh.

* Significantly different (p < 0.05) from the wild type receptor as determined by one way ANOVA.
A

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B

Gallamine

C₇/3-phth

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Critical role for the second extracellular loop in the binding of both orthosteric and allosteric G protein-coupled receptor ligands
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