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Allosteric sodium in class A GPCR signaling

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Abstract

Despite their functional and structural diversity, G protein-coupled receptors (GPCRs) share a common mechanism of signal transduction via conformational changes in the seven-transmembrane (7TM) helical domain. New major insights into this mechanism come from the recent crystallographic discoveries of a partially hydrated sodium ion that is specifically bound in the middle of the 7TM bundle of multiple class A GPCRs. This review discusses the remarkable structural conservation and distinct features of the Na^+ pocket in this most populous GPCR class, as well as the conformational collapse of the pocket on receptor activation. New insights help to explain allosteric effects of sodium on GPCR agonist binding and activation, and sodium's role as a potential co-factor in class A GPCR function.

Keywords

GPCR activation; allosteric modulation; biased signaling; conserved pocket; sodium ion; water binding

Early phenomena attributed to a specific sodium-dependent modulation of GPCR function

G protein-coupled receptors (GPCRs) are the largest superfamily of membrane proteins in the human genome and have key roles in human physiology and in the action of more than 30% of therapeutic drugs [1]. The binding of a ligand stabilizes conformational changes in the receptor, which trigger the activation of intracellular (IC) effectors such as G proteins

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and arrestins [2], leading to a cascade of cellular responses. Although all GPCRs share a common seven-transmembrane (7TM) architecture, they can be divided into four major classes in humans, A, B, C, and Frizzled (F) (see Glossary), that have very little sequence homology between them [3,4]. Out of 826 human GPCRs, more than 700 belong to Class A [3,4] and have several highly conserved functional motifs in their 7TM domains, including *D(E)RY* in helix II, *FxxCWxP* in helix VI, *NPxxY* in helix VII, and the 'hydrogen bond network' between helices I, II, III, VI, and VII [5]. Although the importance of these motifs in GPCR signaling was known for years, their precise roles in signal transduction are only now being revealed, principally due to technological breakthroughs leading to a flurry of GPCR structures being captured in different activation states and complexes [6,7]. One of the most exciting major findings concerns the crystallographic discovery in class A GPCRs of a conserved allosteric binding site for a sodium ion [8], an essential ion that is implicated in many physiological functions.

The first hints of a specific allosteric effect of Na^+ on class A GPCR function can be traced to a study performed 40 years ago [9]. This seminal work found that Na^+ negatively modulates agonist binding to the opioid receptors, without significantly affecting the binding affinity of antagonists. In the absence of other biochemical assays, this 'sodium effect' was employed to differentiate opioid agonist from opioid antagonist candidate drugs [10]. Later, similar biochemical phenomena were observed for more than 20 diverse GPCRs, including adrenergic, dopaminergic, serotonergic, neurotensin, and other receptors (Table 1). These allosteric effects were usually described at physiologically relevant Na^+ concentrations (~140 mM), supporting its biological role. Follow-up mutagenesis studies implicated a conserved acidic $D^{2.50}$ (Ballesteros–Weinstein numbering [11]) residue in helix II as being critical for the sodium-dependent effects, suggesting that Na^+ acts via binding at a specific site within the helical bundle. Moreover, substitution of $D^{2.50}$ with uncharged residues (Table 1) dramatically reduced agonist-dependent signaling of some GPCRs, while maintaining ligand binding and often basal signaling, thereby indicating a specific role for this putative 'sodium site' in agonist-mediated GPCR signal transduction. Only now, however, has high-resolution crystallography of GPCRs provided the critical structural insights that are essential for unraveling the molecular underpinnings of these biochemical phenomena, as well as for illuminating the functional and physiological consequences of sodium binding to GPCRs.

Identification of Na^+ in the crystal structures of class A GPCRs

The recently solved high-resolution (1.8 Å) structure of the A_{2A} adenosine receptor (A_{2A}AR) [8] was the first to reveal a Na^+/water cluster in the middle of the 7TM helical bundle, thereby providing a detailed description of a GPCR allosteric site (Figure 1A,B). The Na^+ in the A_{2A}AR is coordinated by two highly conserved residues, $D^{2.50}$ and $S^{3.39}$, and three water molecules. These water molecules belong to a nearly continuous water-filled passage connecting the A_{2A}AR extracellular (EC) and IC sides. Importantly, the reliable detection of Na^+ in this allosteric site was enabled by high-resolution crystal structures and the unambiguous location of at least five oxygen atoms, a hallmark of a Na^+ coordination shell. Sodium ions can be identified then by their characteristic coordination geometry and short distances (2.2–2.6 Å) to the oxygen atoms [12]. Although a retrospective analysis of

the earlier medium resolution structures (2.4–2.9 Å) suggests that some spherical electron densities in close proximity to $D^{2.50}$ (previously modeled as water) are compatible with Na^+ (Figure 1D), these structures could not provide unequivocal evidence for sodium at this site.

Within 18 months of the discovery of the Na^+ in the $A_{2A}AR$ structure, direct crystallographic evidence for sodium in an identical position was found in inactive-state GPCR structures of an adrenergic receptor (β_1AR) [13], a protease-activated receptor (PAR1) [14], and an opioid receptor (δ-OR) [15] (Figure 1C–F). These crystal structures together represent three of the four major branches (α, δ, γ) in the class A GPCR tree [4]. In each of these high-resolution structures, the Na^+ is coordinated by a salt bridge to $D^{2.50}$ together with four additional polar interactions with receptor side chains and water molecules. The most striking similarities can be found between the $A_{2A}AR$ (PDB identifier: 4EIY) and β_1AR (PDB identifier: 4BVN) [13] structures. Not only are all residues of the pocket conserved chemically and conformationally with $RMSD_{ALL\_ATOM}$ = 0.8 Å (except $Y^{7.53}L$, which was introduced as a thermostabilizing mutation in β_1AR [13]), but also the positions of Na^+ and eight water molecules of the cluster are preserved to within 0.3 Å RMSD. Considering that $A_{2A}AR$ and β_1AR share only ~32% sequence identity in their 7TM domains and have different ligands, this high level of structural conservation of the Na^+ and water cluster is truly remarkable and suggests its key functional role. Interestingly, pharmacological analysis in the same study [13] shows that high Na^+ concentrations do not impact agonist binding in b1AR, suggesting that Na^+ specific binding can be easily overlooked by classical allosteric effect assessment.

Distinct types of sodium pockets in inactive GPCRs

The common binding of the Na^+ in 7TM bundles, however, does not require an identical pocket structure to that found in the $A_{2A}AR$ and β_1AR. Interestingly, opioid receptors (in the γ-branch) present a structural variation on the Na^+/water coordination motif, whereas conformations of the 15 conserved residues of the pocket remain similar to $A_{2A}AR$ and β_1AR. Indeed, the 1.8 Å resolution structure of the δ-OR [15] (PDB identifier: 4N6H) reveals the pivotal importance of another position, 3.35, for sodium binding in opioid receptors (Figures 1E and 2C). Although in many GPCR structures the 3.35 position has a hydrophobic residue that points towards the lipidic membrane interface, the δ-OR structure has an $N^{3.35}$ side chain pointed to the interior of the sodium pocket. The oxygen of the $N^{3.35}$ side chain in the δ-OR structure thereby directly coordinates the Na^+ (at 2.45 Å distance) by occupying the same spatial positions as a water molecule found in the $A_{2A}AR$ structure. At the same time, the nitrogen of the $N^{3.35}$ side chain replaces another water molecule seen in the $A_{2A}AR$ structure, and participates in a water-mediated hydrogen bonding network. Notably, $N^{3.35}$ has the same conformation in the other inactive-state opioid receptor structures [16–18], and is conserved in 78 human GPCRs (mostly of the γ- and δ-branches), where it can also be involved in Na^+ coordination.

The crystal structure of inactive PAR1 (a member of the δ-branch of GPCRs) [14] represents a dramatic deviation in the pocket configuration (Figure 1F), with seven residues of the pocket (1.53, 3.43, 3.35, 6.48, 7.45, 7.46, and 7.49) being different from those in the $A_{2A}AR$. Moreover, in the PAR1 structure the Na^+ is shifted by about 1.5 Å towards the IC

side and is coordinated by acidic side chains two residues, D2.50 and D7.49. Although the 7.49 position of the NPxxY motif is conserved as N7.49 in 86% of all class A GPCRs, one can notice that other 7% of class A (PAR1 and other 51 receptors) have Asp side chains in both the 2.50 and 7.49 positions. Interestingly, many other δ-branch GPCRs and most of the 380 human olfactory receptors have a second acidic residue in the pocket located in a different position, 3.39. Those GPCRs with two negatively charged acidic side chains within the ion coordination shell are likely to have distinctive ion-binding properties and might potentially accommodate not only monovalent but also divalent cations.

The β-branch of class A GPCRs is currently represented by only one crystal structure of the NT1 neurotensin receptor (NTSR1), which was solved in an ‘active-like’ state. As discussed below, the sodium site is collapsed in the known active-like state GPCR structures, and therefore sodium binding in this NTSR1 structure is not expected [19]. At the same time, biochemical experiments [20] provide convincing evidence for $D^{2.50}$-dependent Na^+ binding and allosteric effects at NTSR1 [19,20] and other β-branch receptors, such as the gonadotropin releasing hormone (GnRHR) and urotensin receptors (Table 1). The key polar residues coordinating the Na^+ and water cluster (positions 1.50, 2.50, 3.39, 7.46, 7.49, and 7.53) are also conserved in NTSR1 (Figure 2C), supporting a putative Na^+ binding site. Variations in some non-polar residues corresponding to the pocket, however, suggest distinct features of the Na^+ pocket of β-branch receptors that remain to be crystallographically characterized.

It is likely that other structural variations can be found in the sodium pocket across all class A GPCR families, which could significantly affect both the sodium-binding profile and the sodium-dependent allosteric effects. For example, access to the sodium pocket can vary dramatically, from a rather open passage in the opioid [15,18] and muscarinic [21,22] receptor structures to a more restricted passage in $A_{2A}AR$ and β_1AR. The presented examples, however, suggest that despite differences in detail, a specific sodium-binding site may be a common feature that is preserved in many receptors from all four major branches of class A GPCRs, in which $D^{2.50}$ and the key polar side chains of the pocket are conserved.

Conservation of Na^+ binding across class A GPCR families and branches

Sequence analysis of all class A GPCRs confirms an exceptionally high conservation of the pocket (Figure 2 and Figure S1 in the supplementary material online). In fact, the pocket (as defined in $A_{2A}AR$) combines 15 of the 34 residue positions that are conserved in the majority of the non-olfactory class A GPCRs (Figure 2C). The pocket encompasses the three previously identified conserved motifs: (i) $F^{6.44}$ and $W^{6.48}$ of the *FxxCWxP* motif in helix VI; (ii) $N^{7.49}$ and $Y^{7.53}$ of the *NPxxY* motif in helix VII; and (iii) a 3D cluster of conserved polar residues in helices I, II, III, VI, and VII that is sometimes referred to as a hydrogen bonding network [5]. The only conserved cluster that is not a part of the sodium pocket is the ‘DRY’ motif in helix III located closer to the IC side of GPCRs. Remarkably, all 15 residues of the $A_{2A}AR$ sodium pocket are exactly conserved in 45 GPCRs, mostly in the aminergic and adenosine subfamilies, but also in sphingosine-1 phosphate and some orphan receptors, as well as in the γ-branch opioid and somatostatin receptors (Figure S1 in the supplementary material online). A simple estimation shows that the concomitant

conservation of this group of residues is about ~2,500 fold higher than expected if the residues of the pocket mutated independently (Table S2 in the supplementary material online), suggesting strong evolutionary pressure for conservation of the pocket configuration as a whole. Most other receptors in α– and γ-branches have only minor variations in the pocket, with at least 12 residues of the pocket preserved as in A_{2A}AR (Figure S1 in the supplementary material online).

Across all four branches of the non-olfactory class A GPCRs, the residues of the sodium pocket vary more significantly, although seven key acidic and polar side chains remain conserved. These highly conserved positions include $D^{2.50}$ (90% conserved as Asp), $N^{1.50}$ (97% Asn), $S^{3.39}$ (75% Ser), $N^{7.45}$ (70% Asn, or 90% as any polar side chain), $S^{7.46}$ (66% Ser and 75% as any polar side chain), $N^{7.49}$ (75% Asn, and 20% as Asp), and finally $Y^{7.53}$ (89% Tyr). The variations between known Na^+ binding GPCRs are mainly observed in non-polar residues of the pocket, suggesting that a GPCR's compatibility with sodium binding is largely defined by these key polar positions.

The olfactory receptors also apparently have highly conserved pockets that are compatible with Na^+, although binding and allosteric effects of Na^+ in this large family of GPCRs remains to be experimentally established. All olfactory receptors have an acidic residue 2.50 (83% as Asp and 17% as Glu) and a conserved pattern of polar residues. This pattern is, however, quite distinct from non-olfactory receptors: for example, the olfactory GPCRs have an acidic residue 3.39 (80% as Glu and 14% as Asp), and a histidine at 6.44 (98% as His).

It is also important to note, that in the other human GPCR classes (B, C, and F), none of the residues corresponding to the sodium pocket is conserved. Apparently, these functionally and evolutionary distinct GPCR classes do not have a common Na^+ binding site in their 7TM domains, although this does not exclude a possibility that some of these GPCRs are modulated by Na^+ binding in the other parts of the receptor.

Class A GPCRs lacking a putative Na^+ pocket have distinctive properties

Our analysis also suggests that those 36 of the class A receptors (~5%) that lack acidic residues $D(E)^{2.50}$ (Table S1 in the supplementary material online) may have functional properties that are distinct from other class A GPCRs and, in most cases, do not possess ligand-modulated signal transduction. Thus, of these 36 proteins, 26 are described as 'pseudogene', 'non-signaling', 'decoy', 'constitutively active orphan', or 'putative/probable' GPCRs, as annotated by the IUPHAR or UniProt databases. Among these 26 proteins, there are also three orphan LGR receptors (LGR4–6) that act via binding to Frizzled receptors [23], as well as the NT2 neurotensin receptor (NTSR2). Unlike its NTSR1 homolog, which is fully functional, NTSR2 lacks the $D^{2.50}$ side chain, and although it still binds neurotensin, NTSR2 is not modulated by allosteric sodium and does not signal in response to neurotensin [19,20].

The next group of nine receptors in Table S1 that lack $D(E)^{2.50}$ may still have a normal signal transduction capability in response to diffusible ligands due to an alternative acidic residue supporting sodium binding. This group comprises six olfactory receptors that have

another acidic residue in the pocket, $E^{3.39}$, as well as three receptors with either $D^{3.39}$, $D^{7.49}$ or $D^{7.50}$ residues, all in the sodium pocket. Among these receptors is GnRHR, which has a neutral $N^{2.50}$ side chain, but an acidic $D^{7.49}$ side chain. Indeed a recent study shows that sodium has a modest (but significant) allosteric effect on GnRHR [24], suggesting that an alternative acidic residue in the pocket can still support sodium binding.

This remarkable correlation between the absence of the allosteric Na^+ binding site on the one hand and the lack of an established ability of the receptor to signal in response to small molecule ligands on the other hand further corroborates importance of the allosteric Na^+ for class A GPCRs. Another interesting exception that only confirms this rule is discussed in the next section.

Lack of Na^+ binding in visual opsins

Rhodopsin (OPSD) and other visual opsins (OPSB, OPSG, and OPSR) represent an interesting exception that deserves a closer look. The last line in Table S1 shows the absence of $D^{2.50}$ or any other acidic side chain in the allosteric pocket of blue opsin (OPSB). Moreover, all four opsins lack polar side chains in the other two crucial sodium-coordinating positions, 3.39 and 7.45 (Figure S1 in the supplementary material online), which is likely to abolish specific Na^+ binding. Analysis of the high-resolution (2.2 Å) crystal structure of bovine rhodopsin [25,26] also suggests an absence of sodium binding in the 7TM pocket. Indeed, among four water molecules found in proximity of the $D^{2.50}$ side chain in this structure, none has distances and coordination that are compatible with a sodium ion. Moreover, access from the aqueous EC space into the 7TM bundle core in the rhodopsin structure [25] (and probably in homologous opsins) is completely blocked by the EC loops and N terminus, which makes Na^+ binding in the allosteric pocket even less likely.

The visual opsins therefore present a rare case of signaling class A GPCRs that apparently lack specific sodium binding. However, the function of these receptors also presents a major exception among all human class A GPCRs in that opsins are activated by photochemical *11-cis* to *all-trans* isomerization of a covalently bound retinal, rather than by a diffusible ligand. Such photochemical isomerization provides abundant energy for the receptor activation ($\Delta G \sim 35$ kcal/mol [27]), which is several fold higher than the energy contributed by small diffusible ligands. This observation suggests that the presence of the allosteric sodium may not be required for receptors activated by large chemical energy such as opsins, while being critical for effective signaling by small diffusible ligands in most class A GPCRs.

Activation involves structural rearrangements in the Na^+ pocket

Further evidence for a key functional role of the Na^+ cluster in the modulation of conformational transitions comes from an analysis of active state structures of class A GPCRs (Figure 3). Our comparison of inactive- and active-state crystal structures of $A_{2A}AR$ and β_2AR [28,29] reveals that the Na^+ and water pocket collapses in size from ~200 to <70 $Å^3$ due to the activation-related movements of the TM helices. In particular, an inward movement of helix VII at the NPxxY motif and an outward movement of helix VI (both associated with receptor activation [28,29]) are the key rearrangements that are responsible

for the pocket collapse. Conformational analysis and molecular dynamics studies for $A_{2A}AR$ suggest that such a collapsed pocket in the activated states of GPCRs is incompatible with Na^+ binding [30]. Similar observations regarding the collapse of the Na^+ pocket can be made by examining the other recently solved crystal structures of either fully or partially activated GPCRs (Figure 3). In all of these structures, the sodium pocket is reduced in size and often split into smaller cavities, which do not provide adequate coordination for a Na^+ and its hydration shell waters. The shapes and locations of the residual pockets vary between these activated structures, probably reflecting differences between receptor types and activation stages. Note, however, that the most pronounced helical shift, as well as the most dramatic split and relocation of the pocket, has been observed in the fully activated complex of β_2AR with G protein [28,29], suggesting that Na^+ relocation is a critical part of the GPCR activation process.

Functional studies and challenges

Although biochemical evidence for the allosteric effects of sodium on agonist binding exists for a number of diverse GPCRs (Table 1), a detailed understanding of the functional sodium effect on GPCR signaling in living cells is complicated by several factors. Some evidence for sodium's impact on GPCR function was obtained by direct measurements of G protein binding to isolated cell membranes [31–33] or G protein-mediated signaling in whole cells [34,35] as a function of Na^+ concentration. However, the presence of sodium-conducting ion channels and transporters, and an overall dependence of cell signaling on physiological concentrations of Na^+, can profoundly affect a reliable delineation of the direct functional effects of Na^+ on GPCRs.

Mutagenesis of the conserved sodium-coordinating residues provides an important alternative for evaluation of the functional effects of sodium. The majority of such mutagenesis studies have been focused on the 2.50 position, showing that in various class A GPCRs, $D^{2.50}$ replacement by an uncharged residue can abolish or drastically reduce agonist-induced G protein binding and activation [36–43] (see also [44]). Some studies have also demonstrated that $D^{2.50}$ substitutions can abrogate the allosteric effects of G protein on agonist binding [37,45]. In many of these cases, it was shown that $D^{2.50}$ mutants can retain surface expression, and maintain (or even improve) agonist binding properties [45], suggesting that $D^{2.50}A$ and $D^{2.50}N$ mutants can maintain a correct fold for binding while affecting specific aspects of signal transduction. Mutagenesis studies have also been performed on the other sodium coordinating residues, 7.49, 7.45, and 3.39, by changing them to non-polar side chain residues. Similar to $D^{2.50}$ substitutions, these substitutions resulted in the disruption of normal ligand dependent signaling [43,46,47], suggesting that Na^+ itself is a major component of the signaling mechanism.

The importance of allosteric Na^+ binding is further supported by gain-of-function experiments, in which the introduction of acidic residues in the sodium pocket partially restores signaling function in $D^{2.50}N$ mutants. Such studies were performed for the $5\text{-}HT_{2A}$ serotonin receptor, showing that whereas the $D^{2.50}N$ mutant abrogated coupling to G protein, a $D^{2.50}N/N^{7.49}D$ double mutant capable of binding Na^+ regained most of the functional activity [46]. Similarly, a $D^{2.50}N/N^{7.49}D$ double mutant partially restored

function for the μ-opioid receptor [48], whereas in sodium-dependent GnRHR, these residues are already reversed as $N^{2.50}$ and $D^{7.49}$ in the wild type protein [49].

The constitutive activity of GPCRs can be dramatically affected by Na^+ concentration as well as by mutations in $D^{2.50}$ and other sodium-coordinating residues. Most of the published results are consistent with the notion that Na^+ stabilizes the inactive state and thereby reduces basal G protein activity [35,50–52], although the effects may vary somewhat between GPCRs [13,53] and between functional readouts of signaling [20]. An interesting example is provided by viral GPCRs (vGPCRs), which are close homologs to human receptors but lack characteristic residues of the sodium pocket [54]. Thus, a vGPCR from Kaposi's sarcoma-associated herpes virus (KSHV) is closely related to chemokine receptors but has mutations in the key conserved residues of the sodium pocket (2.50, 3.39, 7.45, and 7.49) that render it constitutively active.

Allosteric sodium site residues modulate signaling bias

Apart from G protein mediated signaling, GPCR function involves other independent pathways, including those mediated by β-arrestin [55]. Selective up- or down-regulation of these pathways by ligands or mutations often leads to so-called biased signaling or functional selectivity, which is of key importance for GPCR biology and pharmacology [56–59]. Several studies suggested that changes in the allosteric sodium pocket can also lead to pronounced signaling bias: for example, in the angiotensin 1 (AT1R) receptor [60]. More recently, dramatic signaling bias effects of the sodium site were observed for δ-OR signaling [15]. Site-directed mutagenesis and functional assays in this study show that $D^{2.50}A$ substitution, as well as some other substitution of the sodium-coordinating residues, such as $N^{7.45}A$ and $N^{7.49}A$, can still retain G protein-mediated signaling for at least some agonists, as observed previously [61]. This study found, however, that these mutations transform classical δ-opioid antagonists, such as naltrindole, into potent β-arrestin-biased agonists [15]. A special role was also described for the $N^{3.35}$ side chain, which participates in Na^+ coordination in δ-OR (Figure 1E). Substitution of this residue either reduces ($N^{3.35}V$) or abrogates ($N^{3.35}A$) the allosteric effects of sodium on ligand binding, while at the same time dramatically augmenting constitutive arrestin-mediated signaling.

These data support the notion that the allosteric sodium in the δ-OR has a more profound impact on regulating β-arrestin efficacy, whereas its effects on canonical G-protein signaling may depend on specific ligands and specific residue mutations. Therefore, sodium-coordinating residues and sodium itself can act as selective 'efficacy switches' for distinct GPCR functional pathways.

Possible mechanisms of sodium as a co-factor in GPCR signaling

An essential role for the Na^+ and water cluster in GPCR function is supported by: (i) the structural features of Na^+ binding in the center of the 7TM bundle in the inactive state receptors; (ii) an exceptionally high conservation of the pocket in ligand activated class A GPCRs; (iii) dramatic activation-related changes in the Na^+ pocket; and (iv) strong allosteric effects of the Na^+ on constitutive and ligand-dependent GPCR signaling. One key aspect of the Na^+ interactions with GPCRs is that, unlike most allosteric modulators, Na^+ is present at

high physiological concentrations (~140 mM) in most EC environments, which ensures near-saturation of the specific Na^+ binding sites in GPCRs with $EC_{50}(Na^+) < 50$ mM [30]. This omnipresence in class A GPCRs and involvement in major aspects of signaling suggest a role of sodium as a co-factor that is essential for proper receptor function. Although the exact nature of Na^+ involvement in signal transduction has only just begun to be understood, we suggest several hypothetical mechanisms (Figure 4) that can be further tested experimentally and computationally:

i. Na^+ bound at D2.50 along with the water cluster stabilize the inactive state [8,30] (Figure 4a,b,c), creating a potential barrier that diminishes basal activity [52] and reduces agonist affinity (see refs in Table 1). In some GPCRs, e.g. b1AR, Na^+ appears to stabilize ligand-free receptor without affecting the equilibrium between inactive and active states [13].

ii. Agonist binding disrupts the Na^+/water cluster (Figure 4d) and relocates Na^+ towards the intracellular side (Figure 4e), thus reducing the energy barrier for receptor activation, and, thus, facilitating signaling. Disruption of the cluster also allows formation of a new pattern of hydrogen bonds in the pocket, which may stabilize active states of the receptor.

iii. The Na^+ and water cluster facilitates coupling between the inward movement of helix VII (which compresses the pocket) and the outward movement of helix VI (which allows the Na^+ to escape towards the cytoplasm). Note that helix VI has been associated with G protein signaling, whereas helix VII has been associated with β-arrestin signaling in previous studies of GPCRs [63]. Disruption of the sodium cluster (Figure 4E,F) may facilitate the inward movement of helix VII and uncouple it from the outward movement of helix VI, thereby differentially affecting activation of G protein and arrestin pathways, as observed in δ-OR mutants [15].

iv. The Na^+ enters the binding pocket from the EC solvent [64], thereby following a strong concentration gradient and electrostatic potential (Figure 4A). By contrast, entrance of the Na^+ from the cytoplasm is very unlikely because it goes against the electrochemical potential and the Na^+ would have to overcome repulsion of the highly positively charged protein interface on the IC side of GPCRs. On receptor activation and collapse of the sodium pocket, the ion is dislocated towards the IC side (Figure 4E), where it can interact with $D^{3.49}$ of the D(E)RY motif in helix III, freeing $R^{3.50}$ for interactions with G protein [65].

v. On receptor activation and pocket relocation, the Na^+ can be further released into cytoplasm (Figure 4F). The entrance for the new ion from the EC solvent would be blocked by the bound agonists, thereby locking the activated receptor into a sodium-free state until agonist dissociation. Such a ‘locking’ mechanism can help to explain the surprising stability of specific ligand-induced activated states, which have been described crystallographically in several GPCRs [19,28,66,67]. It may also be implicated in persistent signaling of some receptors, including the emerging evidence for signaling of internalized GPCRs in early endosomes [68–70].

vi. The transmembrane transfer of Na^+ is promoted by both membrane electrostatic potentials and a Na^+ concentration gradient (with a combined $\Delta G \sim 3$ kcal/mol [71]). Because it is coupled to conformational changes in the receptor's 7TM bundle, Na^+ transfer can provide an energy source, assisting GPCR signaling by small molecules (Figure 4F).

vii. The Na^+ translocation may also help to explain an emerging evidence for the voltage sensing and the gating currents in GPCRs coupled to activation of the receptors [72–74]. Mechanisms of such voltage-dependence in GPCRs are likely to be different from those observed in ion channels, and remain largely unknown.

The above working hypotheses are not mutually exclusive and may work concomitantly. Thus, for example, the sodium cluster can serve as a soft barrier up to a certain stage in activation, its disruption and potentially dislocation of Na^+ into the cytoplasm would leave the receptor in a quasi-stable activated state. Though the mechanistic illustrations in Figure 4 are oversimplified, theoretical predictions of potential measurable effects of Na^+ may aid the planning of experiments to solve outstanding questions on sodium's role in GPCR function (Box 1).

Box 1

Outstanding questions

- Which of the remaining untested, human, class A G protein-coupled receptors (GPCRs) specifically bind Na^+ at physiological concentrations, and how do the variations in the pocket affect sodium affinity and allosteric effects?
- What exactly is the functional role (or roles) of the Na^+ in GPCR signal transduction, basal signaling, and coupling/decoupling between G protein and β-arrestin signaling pathways?
- When the sodium site collapses on receptor activation, where does the Na^+ go?
- If the Na^+ is transported across the membrane following the membrane electrochemical potential, how could such energy coupling benefit small molecule signaling via GPCRs?
- Can other monovalent ions (e.g., K^+) bind to the $D^{2.50}$ pocket in a physiologically/therapeutically relevant way considering the lower concentrations of these ions in the extracellular solvent?
- How does the presence of two acidic residues in the pocket (e.g., in protease-activated receptor (PAR1), olfactory receptors, and other receptors) change ion affinity and selectivity, and could it support the binding of divalent ions (e.g., Ca^{2+}) at physiological concentrations?
- How does changing concentrations of Na^+ (e.g., in neuron synapses or endosomes that carry internalized GPCRs [68,70]) affect the spatio-temporal profile of GPCR signaling?

- Could the Na^+ site confer a key evolutionary advantage to class A GPCRs, the youngest but most sprawling of all human GPCR classes [88]?

Practical implications for GPCR studies

The knowledge of sodium's impact on GPCR function may have immediate practical implications for GPCR structural and functional biology, beyond their early use as a screen for ligand agonism activity [9]. Thus, the allosteric effects of sodium can inform the choice of optimal salt conditions for structural studies. For example, antagonist-bound inactive state receptors have been crystallized in high NaCl concentrations [8,75], whereas crystallization of agonist-bound receptors in activated states favors low Na^+ conditions [19]. Rationally designed mutations in the sodium pocket can also modulate the receptor's ability to adopt an inactive, active, or biased signaling state [15], enhance surface expression in cells [76], or stabilize a specific state for structure-based drug discovery and biochemical screening assays. It is also clear that structural knowledge of Na^+ binding and its importance in GPCR conformational changes should be taken into account in the efforts to build predictive atomistic models of GPCR activation mechanisms [77], and the first steps in this direction are already yielding interesting insights [30,64,78].

The sodium pocket may also serve as a target for allosteric and bitopic ligands with unique functional features. The size and properties of the sodium-binding pocket in $A_{2A}AR$/β_1AR and other GPCR structures suggest that it can accommodate small (MW~200–300 Da) molecules carrying a positively charged group. Indeed, this might be the case for the diuretic drug amiloride and its derivatives, for which several studies (Table 1) demonstrated binding to several GPCRs from class A α- and β-branches. The binding of amilorides to GPCRs was shown to (i) compete with Na^+, (ii) disappear on $D^{2.50}$ mutations, and (iii) allosterically reduce agonist binding [24,79–87]. Flexible docking of amilorides into the $A_{2A}AR$ sodium pocket [8,30] suggests their snug fit into the pocket, with the positively charged guanidine group forming a salt bridge to $D^{2.50}$ (Figure 5). The predicted position of the amiloride scaffold is further compatible with the binding of the derivatives with bulky N5 substitutions, which protrude towards the orthosteric ligand binding pocket and may impact not only agonist but also some antagonist binding [24,30].

Although the affinities of known amilorides to GPCRs are in the micromolar range, more effective allosteric or bitopic molecules targeting the allosteric sodium pocket could have novel functional properties desirable for tool compounds or potential therapeutic applications.

Concluding remarks

The 'sodium effect' on GPCR agonist binding has captivated researchers for more than 40 years. Only now, however, have high-resolution crystallographic studies revealed a common structural basis for Na^+-specific binding in the center of the 7TM helical bundle, which explains this and other sodium effects on GPCRs. The observed activation-related collapse of the sodium pocket implicates a specific role for the Na^+ in the signal transduction mechanism, where the ion translocates towards or into the cytoplasm. The exceptionally

high evolutionary conservation of the sodium binding site in most class A GPCRs (and its absence in light-activated visual opsins, non-signaling GPCRs, or GPCRs of other classes) also points to a critical role of the Na^+ in the function of those class A GPCRs that are activated by small diffusible ligand binding in the 7TM domain. These crystallographic and evolutionary insights, combined with biochemical evidence, support the physiological omnipresence of sodium as a key co-factor in GPCR functional mechanisms. The robust 3D structural platform in combination with biochemical, biophysical, and computational approaches opens a path towards deciphering further specific details and variations in the Na^+-dependent mechanisms in about 680 receptors of class A GPCRs. Some of the remaining key questions regarding the functional role of sodium are challenging, but the answers could help to clarify GPCR signaling mechanisms and enable the discovery of new allosteric and bitopic ligands with distinct functional properties.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Glossary

Allosteric modulation	modification of the orthosteric ligand binding and/or receptor signaling by another ligand or ion that binds to a distinct (allosteric) site
Ballesteros–Weinstein residue numbering	uses the X.YY format to denote the transmembrane helix number (X) and residue position (YY) relative to the most conserved residue in this helix (X.50). The numbering is used to refer to structurally equivalent residue positions in different G protein-coupled receptors (GPCRs)
Biased signaling (or functional selectivity)	preferential signaling via certain signaling pathways by specific ligands (or in a mutant receptor), as compared to endogenous ligands (or wild-type receptors). In many cases, signaling bias was observed between G protein and β-arrestin-dependent pathways
Bitopic ligand	ligand binding to both allosteric and orthosteric sites
Co-factor	a non-protein chemical compound that is required for the protein's biological activity
Constitutive (or basal) activity	receptor signaling in the absence of a bound ligand

GPCR classes and branches	about 826 human GPCRs are classified into four classes (A, B, C, and F) on the basis of sequence similarity. Class A comprises about 715 GPCRs, subdivided into α, β, γ and δ branches (or groups), as well as a separate group of ~380 olfactory receptors
Orthosteric ligand	ligand that binds to the same site of the receptor as the endogenous agonist

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Highlights

- A Na binding site is found in high-resolution structures of several diverse GPCRs
- This allosteric site in the middle of 7TM domain is highly conserved in class A GPCRs
- Specific binding of Na^+ explains its biochemical and functional effects on GPCRs
- Collapse of the pocket and Na^+ relocation are involved in GPCR signaling

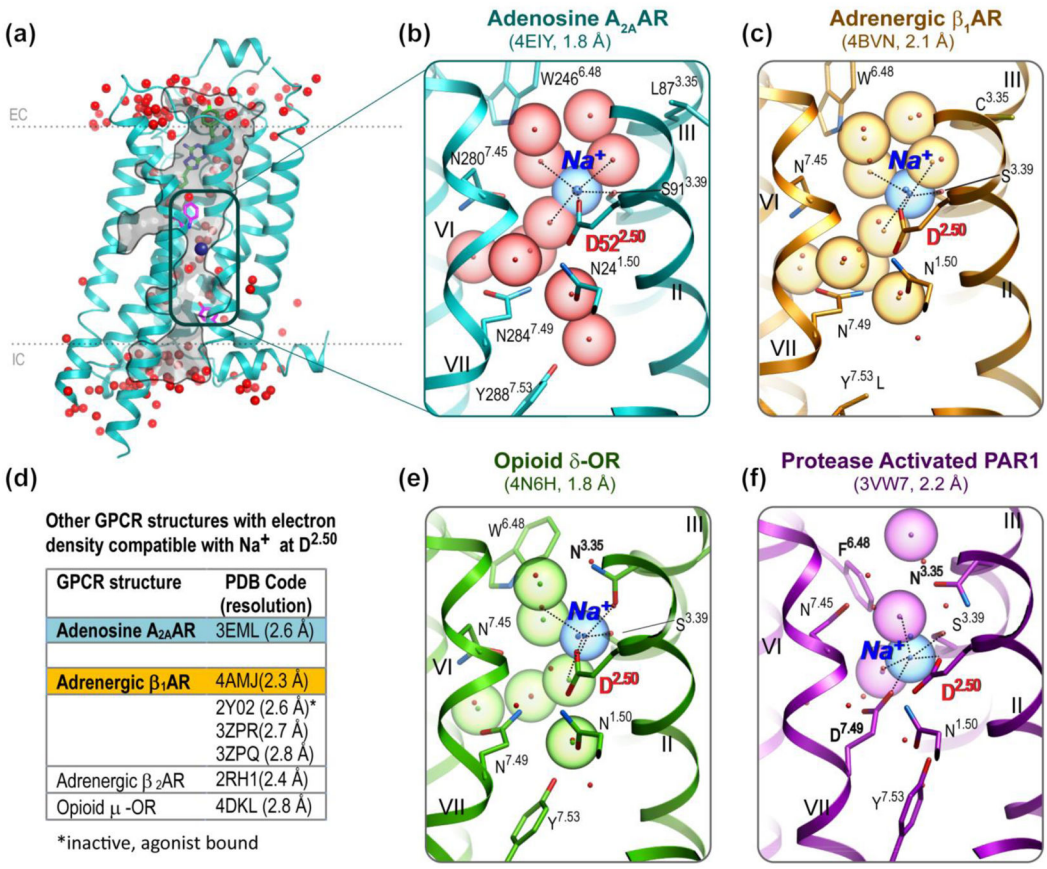

Figure 1.

Na^+ and water cluster detected in G protein-coupled receptor (GPCR) structures. **(A)** The high-resolution A_{2A} adenosine receptor (A_{2A}AR) structure [8] shown with waters (red spheres) and Na^+ (blue sphere) in the narrow passage connecting the extracellular (EC) and intracellular (IC) sides of the receptor. **(B)** Close-up of the A_{2A}AR allosteric pocket comprising Na^+ and a cluster of 10 water molecules. Acidic residue $D^{2.50}$ and all residue positions of the pocket involved in polar interaction with the cluster are shown as sticks. Roman numerals show numbering of the transmembrane helices. **(C)** Close-up of the β_1AR allosteric pocket [13], colored orange. Ten water molecules and the Na^+ position from A_{2A}AR are shown for comparison as red dots and a blue dot, respectively. Note the $Y343^{7.53}L$ mutation. **(D)** A list of medium-resolution structures (2.3–2.8 Å) [18,75,104–107] with electron densities in the proximity of $D^{2.50}$ that are potentially compatible with sodium binding. **(E)** Close-up of the δ-opioid receptor (δ-OR) allosteric pocket [15], colored green. **(F)** Close-up of the protease-activated receptor (PAR1) allosteric pocket [14], colored magenta.

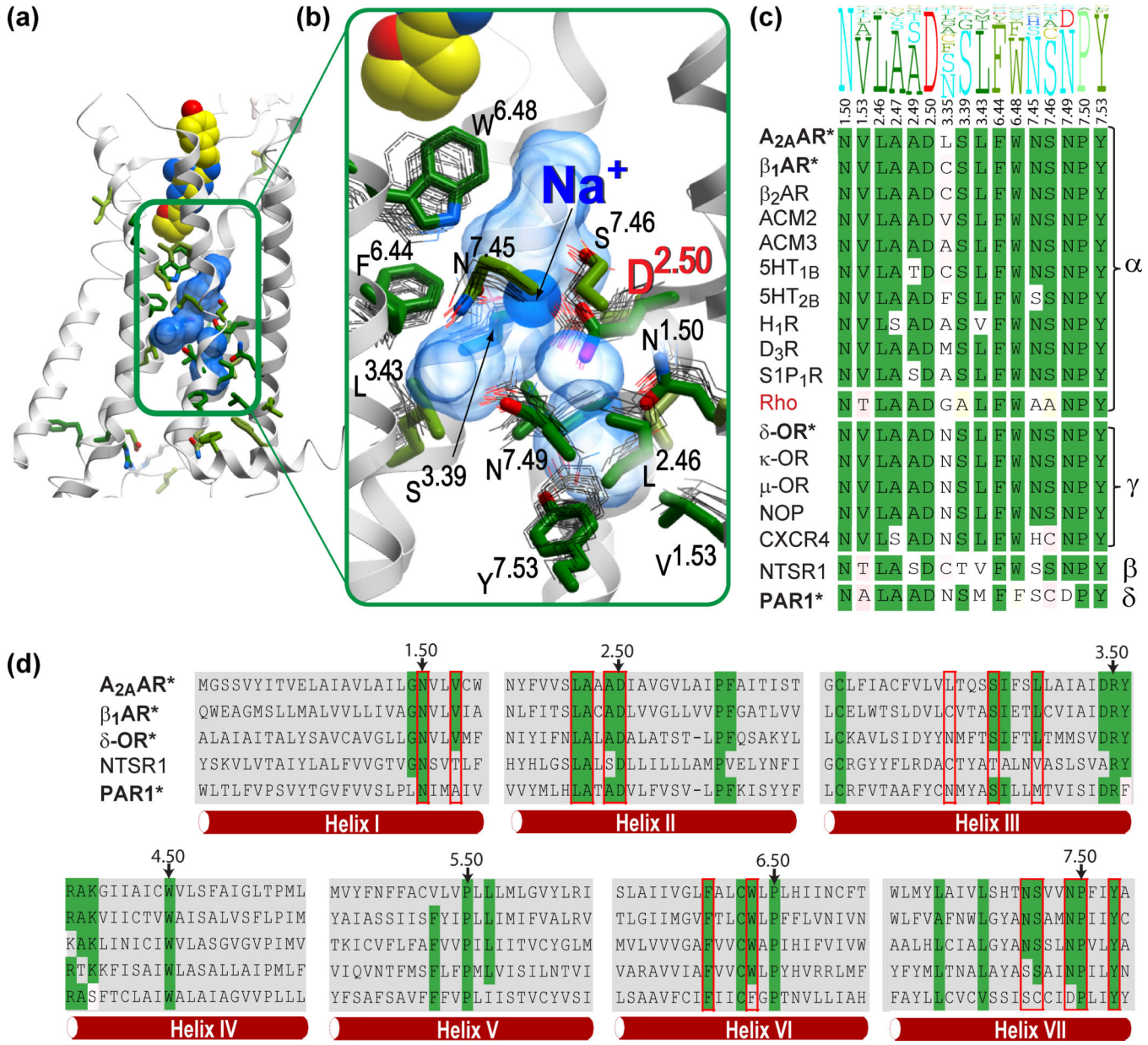

Figure 2.

Structural and sequence conservation of the Na^+ and water pocket in G protein-coupled receptors (GPCRs). **(A)** Overview of the A_{2A} adenosine receptor ($A_{2A}AR$) crystal structure, showing residues with higher than 50% conservation in all non-olfactory class A GPCRs as sticks with green carbons. **(B)** A close-up of the central allosteric pocket (transparent blue surface), showing the side chains located within 5 Å from the 10 waters of the sodium ion/water cluster (green sticks: $A_{2A}AR$; gray thin lines: the corresponding side chains of the overlaid GPCR crystal structures in inactive state). The helix VII backbone in the foreground has been removed for clarity. **(C)** Sequence conservation of the 16 pocket residues. The top part shows the residue conservation profile in all non-olfactory class A GPCRs, where the height of the residue letter represents the share of the residue in this

position. The bottom part shows individual residues in all available class A crystal structures, with conserved residues highlighted in green. Greek letters on the right denote the four major branches of class A GPCRs. Receptors with sodium binding determined by a high-resolution crystal structure are in bold and marked with '*'. Rhodopsin, which lacks a Na^+ binding pocket, is shown in red. **(D)** Sequence alignment in seven-transmembrane helices of representative class A GPCRs from all four major branches. Residues of the Na^+ pocket are highlighted by red boxes, and conserved residues are highlighted in green. The most conserved residues in each helix (X.50) are marked by arrows.

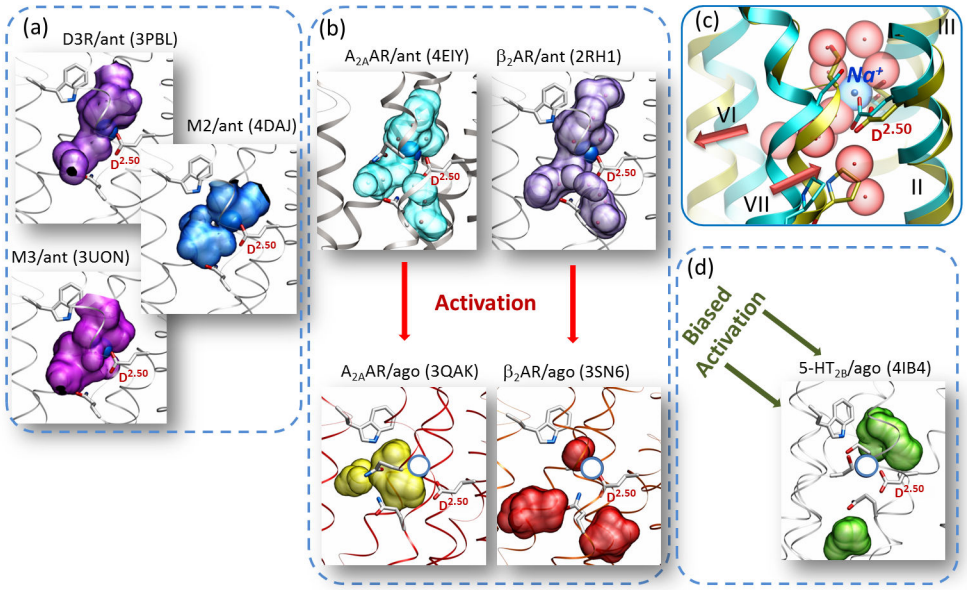

Figure 3.

Activation involves collapse of the sodium pocket in class A G protein-coupled receptors (GPCRs). **(A)** Activation-related conformational changes, in particular inward movement of helix VII and outward movement of helix VI, cause collapse and relocation of the allosteric pocket in A_{2A} adenosine receptor (A_{2A}AR), which makes it incompatible with binding of the Na^+ and water cluster. **(B)** Collapse of the pocket between inactive and activated states found in A_{2A}AR structures (PDB identifiers 4EIY and 3QAK, respectively) is even more pronounced in β_2-adrenergic receptors ((PDB identifiers 2RH1 and 3SN6). **(C)** Most other inactive class A GPCR structures have pockets that are compatible with sodium binding. **(D)** The pocket is also collapsed in the arrestin-biased activated structure of $5HT_{2B}$ (right bottom panel). Allosteric pockets are shown by semitransparent surfaces, and the $D^{2.50}$ side chain is labeled in all panels. The position of Na^+ in the A_{2A}AR-antagonist complex (PDB identifier 4EIY) superimposed on the other inactive structures is shown as dark blue spheres, whereas Na^+ superimposed on the active-state structures is indicated as empty circles in light blue.

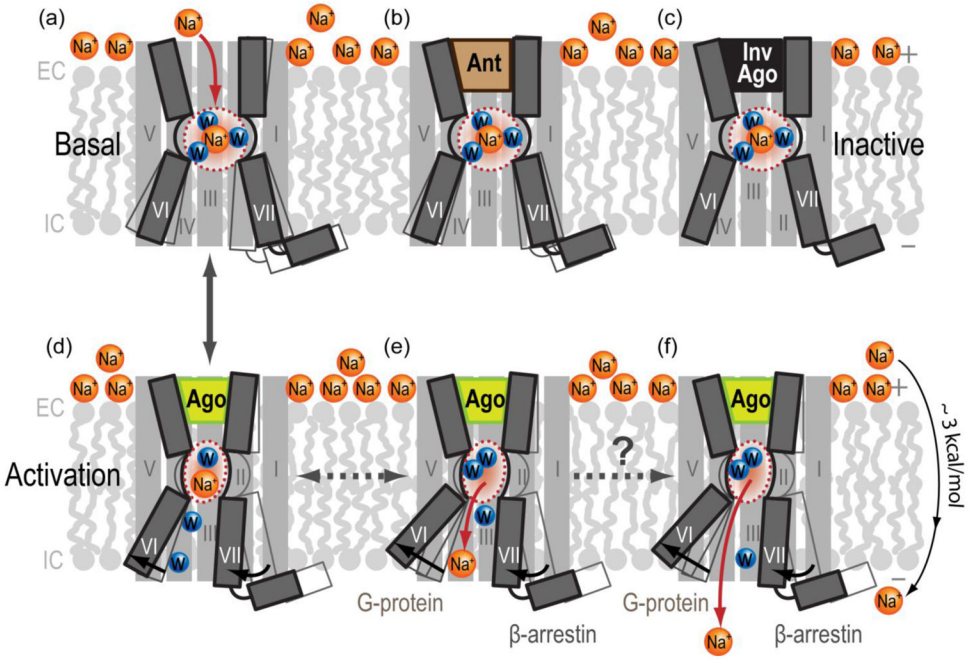

Figure 4.

Hypothetical mechanisms of Na^+ involvement in GPCR activation. **(A)** In the inactive apostate receptors, sodium gains access from the extracellular side into the conserved allosteric pocket, where it forms a network of ionic and polar interactions as a part of Na^+ and water cluster. **(B)** Antagonist (Ant) or **(C)** inverse agonist (InvAgo) binding in the orthosteric pocket is compatible with allosteric Na^+ binding and can further stabilize the inactive state. **(D)** GPCR activation by agonists (Ago), as seen in crystal structures of activated receptors, involves an inward movement of helix VII and an outward movement of helix VI, leading to a partial collapse/reshaping of the allosteric pocket. The resulting collapse of an optimal Na^+/water cluster potentiates displacement of Na^+ along the internal passage towards the intracellular side of membrane, where it can either **(E)** engage in transient interactions with other conserved acidic residues [e.g., $D(E)^{3.49}$ of the D(E)RY motif] and G proteins or **(F)** exit the protein altogether, traversing the membrane along the Na^+ electrochemical gradient, estimated at about 3 kcal/mol.

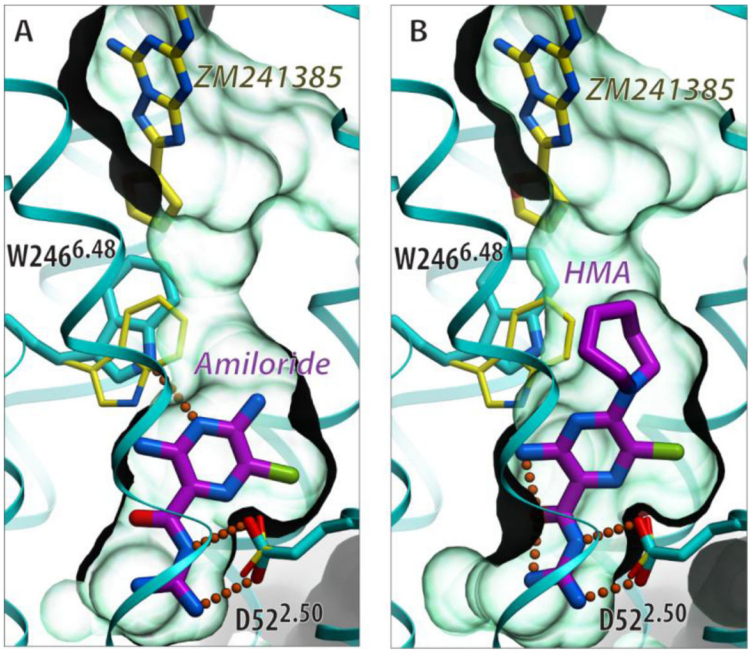

Figure 5.

Predicted binding of amilorides in the conserved allosteric Na^+ pocket. The flexible docking poses of amiloride **(A)** and 5-(N,N-Hexamethylene)amiloride (HMA) **(B)** in the sodium pocket of inactive $A_{2A}AR$ suggest that both ligands optimally fit the sodium cavity with only slight conformational changes from the crystal structure (PDB identifier: 4EIY). Neither amiloride ligand (shown with carbon atoms colored magenta) makes direct contact with the orthosteric antagonist ZM241385 (with yellow carbons, positioned as in 4EIY). Their impact on ZM241385 binding can be mediated by the shift in the $W246^{6.48}$ side chain, which is predicted to be especially pronounced for HMA, in agreement with the stronger negative modulation of antagonist binding by HMA. The receptor is shown by a cyan cartoon with the carbon atoms of the side chains of $D52^{2.50}$ and $W246^{6.48}$ colored cyan when shown in the crystal structure conformation and colored in yellow for the flexible model conformation. The binding cavity is shown as a semitransparent surface in light green.

Table 1

Published evidence for allosteric effects of sodium and amilorides, and their dependence on $D^{2.50}$ mutations

GPCR families	Na^+ allosteric effect on ligand binding	$D^{2.50}$ controls allosteric effects of Na^+	$D^{2.50}$ mutation effects on G protein coupling and activation	Allosteric effect of amiloride and analogs
Adenosine[a] [89][b]	A1 [79,90], A2A[a] [8,30,79], A3 [79,81]	A1 [90], A2A[a] [80], A3 [81]	–	A1 [79], A2A[a] [8,30,79,80], A3 [79,81]
Adrenergic[a]	α[91], $\alpha 2_A$ [92– 94]	$\alpha 2_A$ [92–94]	$\alpha 2_A$ [93,94]	α2 [82], α1 [83]
Dopamine [64,95]*	D2 [84,96]	D2 [84,96]	D2 [84,96]	[84], D1, D2, D3, D4 [85]
Muscarinic	–		M3 [45], M1 [97], M1, M2, M3, M4 [98]	[86]
5HT	–		2A [46], 1B [87]	1B [87]
Opioid[a]	[9,10,31], μ-OR [34], NOP [99,100], δ-OR [15]	δ-OR[a] [15,61]	All subtypes [48]	–
Somatostatin	SSTR2 [101]	SSTR2 [101]	–	–
Neurotensin	NTSR1 [19,20]	NTSR1 [19,20]	NTSR1 [19,20]	–
Gonadotropin- releasing hormone	GnRHR [24]	–	GnRHR [49]	GnRHR [24]
Urotensin	UTR [102]	UTR [102]	UTR [102]	–
Cannabinoid	CB1 [41]	CB1 [41]	CB1,CB2 [41,53]	–
Angiotensin	–		AT_1R [36]	–
Endothelin	–		ET_AR, ET_BR [39]	–
Cholecystokinin	–		CCK_B [103]	–
Bradykinin	B2 [50]	B2 [50]	B2 [50]	–

[a] G protein-coupled receptor (GPCR) subfamilies and subtypes for which crystallographic evidence of Na^+ binding in the conserved pocket have been obtained.

[b] Review and/or modeling studies.