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REVIEW

Modulation of GPCRs by monovalent cations and anions

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Abstract The recent resolution of G-protein-coupled receptor (GPCR) structures in complex with Na^+ bound to an allosteric modulatory site has renewed interest of the regulation of GPCRs by ions. Here, we summarise key data on ion modulation of GPCRs, obtained in pharmacological, crystallographic, mutagenesis and molecular modelling studies. We show that ion modulation is a highly complex process, involving not only cations but also, rather neglected until now, anions. Pharmacotherapeutic and toxicological aspects are discussed. We provide a mathematical framework for the analysis of ion effects. Finally, we discuss open questions in the field and future research directions. Most importantly, the in vivo relevance of the modulation of GPCR function by monovalent ions must be clarified.

Keywords GPCR · Monovalent cations · Monovalent anions · Modelling · Pharmacology

Introduction

Many G-protein-coupled receptors (GPCRs) exhibit sensitivity to modulation by monovalent cations, e.g. sodium ions (Neve 1991; Martin et al. 1999; Schetz 2005; Ericksen et al. 2009; Schnell and Seifert 2010). Table 1 summarises representative studies for G_s-, G_i- and G_q-coupled receptors. Most studies were performed with cell or organ membranes. When working with cell membranes, the experimentally given ion concentration is the same for the intra- and extracellular side of the analysed receptor, while this is not the case when working with intact cells. In the latter case, Na^+ is the predominant cation extracellularly, while K^+ dominates intracellularly. Initially, the analysis of the effects of monovalent on GPCRs in cell membranes was conducted without an a priori mechanistic hypothesis but rather with the intention to "optimise" experimental conditions for radioligand binding studies and G-protein assays, i.e. high-affinity GTPase assays and [^{35}S]GTPγS-binding assays. With regard to GTPase and [^{35}S]GTPγS-binding assays, the major goal was to enlarge the signal-to-noise window between "basal" G-protein activity and agonist-stimulated G-protein activity.

The functional studies on ion effects are very heterogeneous in terms of the systems studied, parameters analysed and results obtained. Particularly, the rank order of efficacy/potency of cations is highly system-dependent, and in some cases, anions have more influence than cations, pointing to a highly complex modulation of GPCRs by ions (Table 1). Because of the complexity of the effects of monovalent cations, for a long time, it was difficult to frame them into an overarching pharmacological concept, although the differential effects of various salts clearly indicated that ion effects exhibit some degree of specificity (see examples in Table 1).

The differential effects of various salts on receptor/G-protein coupling suggested some sort of "binding site" for ions on signalling proteins, a hypothesis that developed post hoc based on experimental data. In the late 1980s and early 1990s, the concept of constitutive GPCR activity was developed. This concept assumes that receptors exist in an inactive (R) and an active (R^*) state and that they isomerize between

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Table 1 Effects of cations and anions on selected GPCRs

GPCR	Test system	Parameters	Cations (relative potency or efficacy)	Anions (relative potency or efficacy)	Ion concentrations	Further important findings or additional comments	Ref.
Histamine (HA) receptors							
gpH_1R ($G\alpha_q$)	Guinea pig brain membranes	Effect of Na^+ on HA- and antagonist potency in [^{3}H]MEP binding	$Na^+ > Li^+ > K^+ \sim Rb^+$ (chlorides)	n.d.	100 mM	Negligible effect of 100 mM Na^+ on potency of *d*-CP, PRO and MEP (in 50 mM Tris).	Chang and Synder (1980)
	Guinea pig cerebellum homogenates	[^{3}H]QMDP binding (inhibition)	$Li^+ \sim Na^+ > K^+ > Cs^+ \sim Rb^+$ (chlorides)	$Cl^- \sim SO_4^{2-} \sim NO_3^-$ (all Na^+ salts)	5–200 mM	Na^+—but not Li^+—inihibits [^{3}H]QMDP binding more effectively than [^{3}H]MEP binding.	Treherne et al. (1991)
		[^{3}H]MEP binding (inhibition)	$Li^+ >> Na^+$ (chlorides)	n.d.	Li^+, 40 mM; Na^+, 100 mM		
		Effect of Na^+ and Tris on antagonist potency in [^{3}H]-MEP binding	Na^+ Tris-HCl also inhibits [^{3}H]MEP binding	n.d.	Na^+, 100 and <2.5 mM; Tris, 10, 50, 200 and <2.5 mM	Na^+ effect on potency of (+)-QMDP>MEP (in 50 mM Tris); Na^+ effect declines at higher Tris-concentrations; Na^+ effect on antagonist potency, BC~(+)-CP~Tri>(+)-QMDP>>PRO~MEP>TEM~TER (10 mM Tris).	Gibson et al. (1994)
hH_3R ($G\alpha_i$)	hH_3R , co-expressed with $G\alpha_{i1-3}$, $G\alpha_o$ and $G\beta_1\gamma_2$ in Sf9 cell membranes	[^{3}H]NAMH binding steady-state GTPase assay	GTPase assay: Na^+: potency of HA↓, of THIO↑; inhibition of signalling by $Li^+ \sim Na^+ \sim K^+$	GTPase assay: inhibition of signalling by $Cl^- < Br^- < I^-$	0–150 mM	No effect of Na^+ (100 mM) on [^{3}H]-NAMH affinity but increase of B_{max}; strongest effect of Na^+ on basal hH_3R-signalling, when $G\alpha_{i3}$ was co-expressed.	Schnell and Seifert (2010)
hH_4R ($G\alpha_i$)	$hH_4R+G\alpha_{i2}+G\beta_1\gamma_2$ in Sf9 cell membranes	Steady-state GTPase assay	Na^+ (chloride)	n.d.	0–120 mM	hH_4R-signalling in GTPase assays is Na^+-insensitive.	Schneider et al. (2009)
Dopamine (DA) receptors							
pD_2R	Porcine anterior pituitary membranes	binding with [^{3}H]NPA (agonist) and [^{3}H]spiperone (antagonist)	Na^+ (chloride)	n.d.	100 mM	Na^+: ↓D_2R-agonist affinity and ↑[^{3}H]-spiperone affinity; ↓K_D and ↓number of binding sites of [^{3}H]-NPA; effects partially augmented by Mg^{2+}	Watanabe et al. (1985)
rD_2R	Transfected HEK-293 cell membranes	[^{3}H]methylspiperone binding	Na^+ (chloride)	n.d.	140 mM	Na^+: ↑affinity for agonists and antagonists of a class of D_4R-selective 1,4-DAP compounds.	Ericksen et al. (2009)
Adrenergic receptors							
$h\alpha_2AR$	"Untreated" platelet membranes	[^{3}H]EPI (agonist) and [^{3}H]YOH (antagonist) binding; *N*-methyl-D-glucamine used as Na^+-"substitute" in Na^+-free controls	$Na^+ > Li^+ > K^+$ (chlorides); EC_{50} (Na^+): 5–15 mM; K^+ more potent in modifying antagonist than agonist binding	n.d.	0–120 mM	Effects of Na^+: ↓agonist ([^{3}H]-EPI) affinity, ↑antagonist ([^{3}H]-YOH) association, without influencing dissociation.	Limbird et al. (1982)
	Digitonin-solubilised α_2AR from platelets					Effects of Na^+: ↑[^{3}H]YOH association and ↑dissociation. ↑[^{3}H]EPI dissociation; Na^+ may bind to receptor protein.	
$h\alpha_2AR$	Platelets, purified by gel-filtration in the presence and absence of Na^+ (*N*-methyl-D-glucamine as Na^+-substitute	EPI-induced platelet aggregation release of [^{3}H]5-HT containing granules	Na^+ (chloride) removal of extra-platelet Na^+	n.d.	137 mM	Absence of Na^+: ↓EPI-induced platelet aggregation and ↓[^{3}H]5-HT release; Na^+ ineffective on thrombin response and on EPI-mediated inhibition of PGE_1-induced cAMP accumulation.	Connolly and Limbird (1983)

Table 1 (continued)

GPCR	Test system	Parameters	Cations (relative potency or efficacy)	Anions (relative potency or efficacy)	Ion concentrations	Further important findings or additional comments	Ref.
hα_2AR	Platelets purified similar as in Connolly and Limbird (1983)	EPI- and thrombin-induced platelet aggregation; displacement of [^{3}H]YOH by EPI; determination of membrane potential	Na^+ (chloride) manipulation of intra-platelet Na^+ by monensin and ouabain (↑) or incubation in Na^+-free buffer (↓)	n.d.	13–138 mM (intra-platelet); 0 or 136 mM (extra-platelet)	High $[Na^+]_i$: ↑EPI- (but not thrombin-) induced platelet aggregation, ↓EPI and [^{3}H]YOH affinity; low $[Na^+]_i$: ↑EPI affinity; EPI affinity independent of membrane potential; Na^+ acts on intracellular site.	Motulsky and Insel (1983)
hβ_2AR, fused to $G_{S\alpha S}$ or $G_{S\alpha L}$	Membrane preparations from baculovirus-infected Sf9 cells	[^{3}H]DHA binding: competition with ISO; competition with SAL in the presence of GDP	Elimination of high-affinity agonist binding by Li^+, Na^+ and K^+ ineffective GTPγS effect on high-affinity agonist binding incomplete in presence of Na^+ and K^+	n.d.	150 mM	Na^+ converts a part of $G_{S\alpha L}$ into the high-affinity conformation for GDP.	Seifert (2001)
		AC activity assay	Increase of AC activation by ISO: Li^+~Na^+~K^+~Rb^+~Cs^+ (biphasic effect of K^+, Rb^+ and Cs^+) reduction of AC inhibition by ICI: Li^+>Na^+>K^+>Rb^+>Cs^+ (chlorides)	Increase of AC activation by ISO, reduction of AC inhibition by ICI: I^->Br^->Cl^-; reduction of GTPγS-induced AC activation: I^->Br^->Cl^-	0–150 mM	Na^+ effect on β_2AR-$G_{S\alpha L}$: ↑agonist stimulation and ↓inverse agonist inhibition of AC activity; only weak Na^+ effects on β_2AR- $G_{S\alpha S}$; in general stronger influence of anion than of cation radius	
Opioid receptors							
δOR-Gα_{i1} (Cys^{351}-Ile^{351}) fusion protein (PTX resistant)	PTX-treated and PTX-untreated membranes from transfected HEK-293 cells	binding of [^{3}H]DADLE (agonist) or [^{3}H]naltrindol (antagonist)	↓[^{3}H]DADLE binding: Na^+ >>Li^+>K^+>NMDG; two Na^+ sites (K_d=7.9 mM and 463 mM) but only low-affinity sites for Li^+ and K^+ ↑[^{3}H]naltrindol binding: Na^+>K^+~Li^+	↓[^{3}H]DADLE bdg.: I^->Br^->Cl^-	Monovalent cations, 0–800 and 145 mM (anions)	Similar results for PTX-treated and PTX-untreated membranes→effect not restricted to δOR-Gα_{i1}(Cys^{351}-Ile^{351}), but also true for endogenous G$\alpha_{i/o}$ proteins in HEK cells; absence of monovalent ions: high basal [^{35}S]GTPγS binding and inability of DADLE to induce a signal. Characterisation of hydrophobic membrane interior by DPH: low-affinity effects of monovalent cations probably caused by interaction with the polar head region of lipid bilayers	Vosahlikova et al. (2014)
		DADLE-induced [^{35}S]GTPγS binding	↑B_{max}: Na^+>K^+~Li^+; ↑EC_{50} : Li^+>K^+>Na^+; ↓basal [^{35}S]GTPγS binding: Na^+>Li^+~K^+; effect of Li^+ significant at 1-2 mM	↓Basal and DADLE-induced [^{35}S]GTPγS bdg.: I^->Br^->Cl^-	Na^+, K^+, Li^+, 100 and 200 mM; Li^+, 0.5–2 mM; 145 mM (anions)		
		Laurdan generalised polarisation (Laurdan GP_{ex})	Laurdan GP_{ex} unchanged by 150-200 mM Na^+, Li^+, K^+; ↓increase of Laurdan GP_{ex} by 800 mM of Li^+>Na^+>K^+>Cs^+~ ion-free	n.d.	150 and 200 mM		
		By steady-state fluorescence anisotropy of DPH	No effect: Na^+, K^+, Li^+, Cs^+	n.d.	100, 200 and 800 mM		

Table 1 (continued)

GPCR	Test system	Parameters	Cations (relative potency or efficacy)	Anions (relative potency or efficacy)	Ion concentrations	Further important findings or additional comments	Ref.
δOR	NG108-15 cells (rat glioma/mouse neuroblastoma hybrid); PTX treated and PTX untreated	Basal, agonist (DADLE)-stimulated and antagonist (ICI-174864)-inhibited high-affinity GTPase activity	↓Basal GTPase activity: Na^+>Li^+>Rb^+>K^+ (150 mM, chlorides). K^+ slightly stimulates at <50 mM; ↑total receptor-dependent activity Δ(DADLE-ICI) Na^+~Li^+~Rb^+~K^+>>NMDG (all 150 mM)	n.d.	0–200 and 150 mM	Na^+ and K^+ slightly increase DADLE-induced activity at concentrations <100 mM but reduce activity in the presence of ICI-174864 (inv. agonist). Receptor/Gα ratio of ligand-occupied receptors unchanged by Na^+, but modulation of "empty" GPCR-Gα interaction.	Costa et al. (1990)
			Gradual ↑ of Na^+/K^+ ratio: decline of basal activity stronger than of activity in the presence of ligand; Δ(DADLE-ICI) unaltered, % DADLE effect increases with higher Na^+/K^+ ratio; Na^+ effect declines with increasing intrinsic efficacy of ligands (positive or negative)	n.d.	150 mM (sum of Na^+ and K^+)	Na^+ mainly modulates basal activation of Gα by "empty" receptors in intact membranes. In the presence of ligands, Na^+ effect is highest with neutral antagonists.	
			Mg^{2+} <10 μM: increase of GTPase activity, no Na^+- and ligand effects; Mg^{2+} >10 μM: effects of Na^+ and ligands	n.d.	Combination of Na^+ or K^+ (both 150 mM) with increasing Mg^{2+} concentrations (1 nM–10 mM)	Reduction of GTPase activity by PTX: weak at Mg^{2+} <1 μM, but stronger at Mg^{2+} >1 μM. Na^+ effect occurring at Mg^{2+} >1 μM is PTX sensitive	
	$Gα_{i/o}$ subunits, purified from bovine brain		No effect of Na^+ on basal GTPase activity of purified bovine $Gα_{i/o}$.	n.d.	150 mM	Na^+ effect is partially restored after addition of $Gα_{i/o}$ to PTX-treated membranes.	
m/r μOR	Membranes from transfected CHO cells	High-affinity [^{35}S]GTPγS binding	↓Basal [^{35}S]GTPγS binding Na^+>K^+, $IC_{50,\ Na+}$=30 mM. Na^+ effect reduced in presence of partial agonists and absent with full agonists net and % stimulation of [^{35}S]GTPγS binding: full agonists: increasing from 0–150 mM of Na^+ partial agonists: max. at 50–75 mM, inhibition at higher Na^+ (120 mM: Na^+>K^+)	n.d.	0–150 mM	Absence of Na^+ increases basal [^{35}S]GTPγS binding (PTX-sensitive effect), reduces GDP affinity of Gα to the same extent as DAMGO (full agonist) and increases affinity and B_{max} of high-affinity [^{35}S]GTPγS binding only in the presence of GDP (similar to agonists). B_{max} of [^{35}S]GTPγS binding w/o Na^+ is reduced after agonist pretreatment of cells (DAMGO). Agonist-induced stimulation of [^{35}S]GTPγS binding w/o Na^+: absent in CHO-membranes; 40 % in rat thalamus. CHO and rat thalamus: Na^+ reduces potency of full agonists more effectively than of partial agonists. Na^+ increases efficacy of full agonists much more than of partial agonists.	Selley et al. (2000)
	Rat thalamic membranes		↓Basal [^{35}S]GTPγS binding Na^+>K^+, $IC_{50,\ Na+}$=40 mM net stimulation of [^{35}S]GTPγS binding: Na+effect on partial agonist>full agonists. % [^{35}S]GTPγS binding: increased by Na^+ for full agonists, but decreased for partial agonists (120 mM: Na^+>K^+)				

Table 1 (continued)

GPCR	Test system	Parameters	Cations (relative potency or efficacy)	Anions (relative potency or efficacy)	Ion concentrations	Further important findings or additional comments	Ref.
Neuropeptide Y receptors							
$hY_2R+G\alpha_{i2}+G\beta_1\gamma_2$	Membranes from Sf9 cells	Steady-state GTPase assay	Reduction of hPP-stimulated GTPase activity: Na^+~K^+~Li^+. Increase of basal GTPase activity by Li^+ (no effect of Na^+ and K^+)	Reduction of hPP-stimulated GTPase activity: I^->Br^->Cl^- increase of basal GTPase activity by Li^+ salts: I^->Br^->Cl^-	0–150–50 mM	Effect of 50 mM Na^+ on hY_4R depends on ligand and co-expressed Gα subunit: basal GTPase activity more sensitive to Na^+ with $G\alpha_{i2}$ than with $G\alpha_o$, $G\alpha_{i2}$: Na^+ increases EC_{50} value of hPP and GW-1229, $G\alpha_o$: Na^+ increases EC_{50} value of GW-1229 but not of hPP, $G\alpha_{i2}$ or $G\alpha_o$: Na^+ increases E_{max} of both GW-1229 and hPP	Pop et al. (2011)
$hY_4R+G\alpha_{i2}$ (or $G\alpha_o$)+ $G\beta_1\gamma_2$+RGS4			hPP-stimulated GTPase activity: high cation sensitivity; Na^+>K^+~Li^+	Reduction of hPP-stimu-lated GTPase activity: I^->Br^->Cl^-			
Chemoattractant and chemokine receptors							
hFPR1+$G\alpha_{i2}$+-$G\beta_1\gamma_2$	Membranes from Sf9 cells	[^{35}S]GTPγS binding assay	Na^+>>K^+ (chlorides); IC_{50} (Na^+)=5 mM	n.d.	0–50 mM (Na^+); 0–150 mM (K^+)	Na^+ reduces basal G-protein activity and efficacy of the inverse agonist CsH. Na^+ increases stimulation by the agonist fMLF	Wenzel-Seifert et al. (1998)
	FPR1-transfected HEK293 cells		Same Na^+ effects as in Sf9 cell system	n.d.	100 mM	Na^+ effects confirmed in FPR1-transfected HEK293 cells	
hFPR1	HL-60 cell membranes	[^{35}S]GTPγS-binding assay	Na^+>Li^+>K^+>choline~NMDG fivefold reduction of fMLF potency by Na^+	n.d.	50 mM	Reduction of basal [^{35}S]GTPγS binding and increase of relative fMLF signal	Gierschik et al. (1991)
hCXCR4+$G\alpha_{i2}$+-$G\beta_1\gamma_2$	Membranes from Sf9 cells	GTPase activity (stimulation with SDF-1α and inhibition by AMD3100)	Basal GTPase activity: Na^+, K^+, Li^+ ineffective reduction of SDF-1α efficacy: chlorides, bromides: Na^+~K^+~Li^+ iodides: K^+>Na^+>Li^+ reduction of SDF-1α potency: chlorides: K^+>Na^+~Li^+	Basal GTPase activity: no effect of Cl^-, Br^-, I^- reduction of SDF-1α signal: I^->Cl^->Br^-	0–150 mM	Biphasic effect on SDF-1α-induced GTPase activity by NaCl, NaBr, KCl and KBr (stimulation at <50 mM, inhibition at higher concentrations). No biphasic effect of Li^+-salts and iodides. Weak stimulation of basal activity by LiI (cf. Pop et al. 2011)	Kleemann et al. (2008)
hFPR1, hC5aR, $hLTB_4R$ (BLTR), hPAFR+$G\alpha_{i2}$+-$G\beta_1\gamma_2$	Membranes from Sf9 cells	[^{35}S]GTPγS-binding assay	Stimulation w/o Na^+: LTB_4>PAF>fMLF~C5a. Inhibition by Na^+: Na^+ efficacy: FPR1~C5aR>BLTR~PAFR. Na^+ potency: FPR1> C5aR~BLTR>PAFR	n.d.	0–300 mM	Na^+-mediated stabilisation of the inactive state of hFPR1, hC5AR, $hLTB_4R$ and hPAFR unmasks distinct levels of constitutive activity	Seifert and Wenzel-Seifert (2001)
hFPR1 and purino-ceptors	Bt_2cAMP-differentiated HL-60 cells	fMLF-induced inward currents	Na^+ or Ca^{2+} required for fMLF signal; inhibited by SK&F 96365	n.d.	~140 mM	Na^+ and Ca^{2+} pass non-selective cation channels permeability of non-selective cation channels higher for Na^+ than for Ca^{2+}	Krautwurst et al. (1992)
		fMLF-induced β-glucuronidase release	Increased by Na^+; Ca^{2+} acts synergistically				
		fMLF-induced O_2^- formation	Increased by Na^+; Ca^{2+} acts synergistically				

Table 1 (continued)

GPCR	Test system	Parameters	Cations (relative potency or efficacy)	Anions (relative potency or efficacy)	Ion concentrations	Further important findings or additional comments	Ref.
		High-affinity GTPase assay	Stimulation of basal GTP hydrolysis (chlorides): Li^+~K^+>Na^+>choline. Na^+-induced increase of absolute fMLF signal (chlorides): Li^+~K^+>Na^+>choline		25 and 50 mM	No change of relative fMLF-induced GTPase signal by Li^+, Na^+, K^+ or choline; results differ from Na^+ effect shown in refs. (Wenzel-Seifert et al. 1998; Gierschik et al. 1991)	
FPR1	HL-60 cell membranes	[^{3}H]fMLF high-affinity binding	Inhibition of [^{3}H]fMLF binding: Na^+>Li^+>K^+>choline	n.d.	0–200–100 mM	Na^+ reduces high-affinity binding sites of [^{3}H]fMLF and increases low-affinity sites. Na^+ effect is PTX resistant and independent of Mg^{2+}. Na^+ and GTPγS effects on [^{3}H]fMLF binding are additive and have distinct mechanisms.	Gierschik et al. (1989)
		High-affinity GTPase assay	Na^+ reduces basal activity and increases EC_{50} of fMLF; increase of relative fMLF signal: Na^+>Li^+>K^+>choline			PTX pretreatment of HL-60 cells prior to membrane preparation causes stronger reduction of basal GTPase activity than addition of 100 mM of Na^+ alone	
Cannabinoid receptors							
Rat CB_x receptors	Membranes from rat cerebellum, striatum and hippocampus	AC assay CB_xR agonists used: CP-55940 and WIN 55212-2	Na^+ ineffective in cerebellar membranes, but increase of CB_xR-mediated AC inhibition in striatal membranes. Na^+-induced increase of IC_{50} of WIN 55212-2 in striatum and cerebellum; no effect of Na^+ on IC_{50} of CP-55940	n.d.	0–150 mM	Differential effects of Na^+ in cerebellum and striatum or hippocampus. Differences not confined to CB_xR but also observed with $GABA_BR$ agonist Baclofen and with OR agonist D-Ala-Enk. Differences in Na^+ effect on CB_xR-mediated inhibition of AC activity are most likely caused on the level of $G\alpha_i$ stimulation, but not by direct Na^+ effects on AC or on CB_xR ligand binding.	Pacheco et al. (1994)
		High-affinity GTPase assay. CB_xR agonists used: CP-55940 and WIN 55212-2	Na^+ reduces GTPase activity in cerebellar membranes but increases activity in striatal and hippocampal membranes (maximum at 50 mM). Na^+-induced reduction of IC_{50} of WIN 55212-2 in cerebellum, but increase of WIN IC_{50} in striatum; no effect of Na^+ on IC_{50} of CP-55940				
		[^{3}H]WIN55212-2 radioligand binding	Na^+ causes decrease of [^{3}H]WIN55212-2 binding to both cerebellar and striatal membranes				

Table 1 (continued)

GPCR	Test system	Parameters	Cations (relative potency or efficacy)	Anions (relative potency or efficacy)	Ion concentrations	Further important findings or additional comments	Ref.
hCB_1R and hCB_2R+ $G\alpha_{i2}$+$G\beta_1\gamma_2$	Sf9 cell membranes	High-affinity steady-state GTPase assay with CP-55940 (agonist) or AM-251 (inverse agonist)	Na^+ effect on CB_1R: reduction of basal GTPase activity and inverse agonistic effect of AM-251; increase of agonism of CP-55940. CB_2R is Na^+ resistant.	n.d.	150 mM	Differential effects of Na^+ on CB_1R and CB_2R	Nickl et al. (2008)

Most studies regarding the effects of monovalent ions were performed with G_i/G_o-coupled receptors. It should be emphasised that the majority of relevant studies were performed with membrane preparations, i.e. in the absence of physiologically occurring ion gradients between the extra- and intracellular side of the membrane. Far less studies were conducted with G_s- and G_q-coupled GPCRs. This table is not meant to be comprehensive. Rather, we tried to compile data from key publications for GPCRs where large ion effects were observed. The effects of ions were studied on GPCRs of various species, using membranes from organs, cell culture lines and cell membranes expressing recombinant receptor. Most relevant studies on ion effects were performed with membranes. With few exceptions, only one given salt was studied in any given assay format. However, in native cells, at least KCl and NaCl are present simultaneously. The effects of salts were examined in radioligand binding studies, guanine nucleotide exchange assay (GTPase activity or GTPγS binding) or effector system assays (adenylyl cyclase assay). The table shows that the systems analysed were very heterogeneous so that direct side-by-side comparison of several GPCRs, if not studied in one and the same study, is impossible. It should also be kept in mind that charged buffer substance used in assays can have an impact on GPCRs, but this aspect has only been rarely studied. Most pronounced effects of ions were observed in the ≥50 mM range, but in some systems, even ion concentrations ≤10 mM exhibited effects. It should also be mentioned that there is no universal potency/efficacy pattern for cations and anions. The ion effects are highly system and GPCR dependent. Most studies focused on cation effects, but in several cases where anions were specifically studied, anion effects were much more pronounced than cation effects

n.d. not determined, *MEP* mepyramine, *BC* benzilylcholine, *Bt_2cAMP* dibutyryl cyclic AMP, *CsH* cyclosporine H, *CP* chlorpheniramine, *DA* dopamine, *DADLE* Tyr-D-Ala-Göy-Phe-D-Leu (enkephaline derivative), *1,4-DAP* 1,4-disubstituted aromatic piperazine/piperidine, *DHA* dihydroalprenolol, *DPH* 1,6-diphenyl-1,3,5-hexatriene, *EPI* epinephrine, *fMLF* formyl-methionyl-leucyl-phenylalanine, *hPP* human pancreatic polypeptide, *5-HT* 5-hydroxytryptamin, serotonin, *NMDG N*-methy-D-glucamine (sodium substitute), *OR* opioid receptor, *PGE_1* prostaglandine E_1, *[^{3} H]-QMDP* [^{3} H]-(+)-*N*-methyl-4-methyldiphenhydramine, *SK&F 96365* non-selective cation channel inhibitor, *TRI* triprolidine, *PRO* promethazine, *PTX* pertussis toxin, *SAL* salbutamol, *TEM* temelastine, *TER* terfenadine, *YOH* yohimbine, *$[Na^+]_i$* intra-platelet sodium

these two states (Lefkowitz et al. 1993). The equilibrium between these two states is different for any given receptor, and when agonist-independent (constitutive) *R* to *R** isomerization is sufficiently high, measurable basal G-protein- and effector activation emerges (Seifert and Wenzel-Seifert 2002). This basal G-protein and effector activity can be reduced by inverse agonists that stabilise the *R* state. Furthermore, it was noted for several receptors including β_2-adrenoceptor and chemoattractant receptors that in addition to inverse agonists, Na^+ can effectively reduce constitutive GPCR activity (see examples in Table 1) giving rise to the concept that Na^+ acts as universal allosteric inverse agonist (Seifert and Wenzel-Seifert 2001). This pharmacological concept was confirmed by the recent elegant studies showing crystal structures of GPCRs bound to Na^+ (Liu et al. 2012; Fenalti et al. 2014; Miller-Gallacher et al. 2014; Katritch et al. 2014). The crystal structures show that the Na^+ binds in an allosteric binding site near the highly conserved $Asp^{2.50}$ (Liu et al. 2012; Fenalti et al. 2014; Miller-Gallacher et al. 2014; Katritch et al. 2014). Based on the simulation data available in literature, so far it can be assumed that the monovalent cations bind to the allosteric binding site by coming from the extracellular side (Selent et al. 2010; Yuan et al. 2013; Wittmann et al. 2014b). In this binding trajectory, the cation has to pass the orthosteric ligand binding site (Selent et al. 2010; Yuan et al. 2013; Wittmann et al. 2014b). An entry of the monovalent cation coming from the intracellular side has not been described until now and is unlikely because the intracellular side of the receptor is, compared with the extracellular side, more positively charged. For example, according to the amino acid sequence, the extracellular domains of the hH_4R exhibit an elementary net charge of −5, whereas the intracellular domains exhibit an elementary net charge of +22 (Brunskole et al. 2011). Furthermore, it can be assumed that the binding trajectory for monovalent anions to GPCRs starts from the intracellular side of the receptor, anions ultimately binding between the transmembrane domains at the intracellular side of the hH_4R (Wittmann et al. 2014b).

Molecular modelling approach

Based on crystal structures, e.g., the crystal structures of the human H_1 receptor (Shimamura et al. 2011), human D_3 receptor (Chien et al. 2010), human adenosine A_{2A} receptor ($A_{2A}R$) (Liu et al. 2012) or human δ-opioid receptor (DOPR) (Fenalti et al. 2014), homology models of the GPCR of interest can be modelled (Wittmann et al. 2014b). After docking of monovalent ions to the allosteric binding site, their interactions with the GPCR can be studied (Wittmann et al. 2014b). However, GPCRs, embedded in a lipid bilayer are not rigid structures. Rather they show a distinct flexibility and undergo dynamic processes. Thus, it is state of the art to perform molecular dynamic (MD) simulations with the GPCR embedded in a lipid bilayer and surrounded by water molecules and ions in order to observe its dynamics on a molecular level (Selent et al. 2010; Yuan et al. 2013; Wittmann et al. 2014b). These simulations are of special interest, because they allow observing the binding pathways from ions or the binding and unbinding of water from outside of the GPCR into an allosteric binding site. Another important technique is to illustrate an energetical term, e.g. an interaction energy, by a hypersurface (Wittmann et al. 2014b). Although such hypersurfaces represent intersections, often only considering two coordinates or two courses of interest, valuable information can be obtained, e.g. preferred binding areas with similar energy can be identified.

In the following, the methods used within this study are described briefly: The simulations were performed according to protocols, described previously (Wittmann et al. 2014b). Briefly, the homology model of the inactive hH_3R was constructed using the crystal of the hH_1R (Shimamura et al. 2011) as template, using establishes protocols (Wittmann et al. 2014b). Internal water molecules were included and the monovalent cation (Li^+, Na^+, K^+, Rb^+, Cs^+) was docked manually into its allosteric binding site according to Fenalti et al. (2014), as described (Wittmann et al. 2014b). The resulting receptor models were placed manually in a POPC lipid bilayer, intra- and extracellular water molecules and an appropriate number of cations and Cl^-, solved in the extra- and intracellular water were added, as described (Wittmann et al. 2014b). For the monovalent cations, appropriate force field parameters (Joung and Cheatham 2008) were used. Subsequent to a 5-ns equilibration phase for each system (force constants 250 kJ/(mol nm^2) for the first 2.5 ns and 100 kJ/(mol nm^2) for the second 2.5 ns were put onto the backbone atoms of the TM domains of hH_3R), the 50 ns productive phases were performed. Each MD simulation was performed at least three times using different seed values (Wittmann et al. 2014b). During the 50-ns productive simulation, the overall conformation of the hH_3R and the cation in its allosteric binding site remained stable. It should be noted that the aim of the present simulations was to predict the preferred area of the cation in its allosteric binding site of hH_3R. For this aim, the simulation time of 50 ns is appropriate. It was not the aim to study the whole binding pathway of the cation from the extracellular water into the allosteric binding site of hH_3R. The standard Gibbs energy of solvation of the cation for the transfer from the orthosteric to the allosteric binding site ($\Delta\Delta G^o$) was calculated for each cation using a protocol, described previously (Wittmann et al. 2014b). Each simulation was performed for 9 ns and was repeated two times, using a slightly different starting position of the cation in the orthosteric or allosteric binding site.

Impact of crystal structures and molecular modelling onto understanding of ion sensitivity on a molecular level

For many years, an allosteric binding site near the highly conserved $Asp^{2.50}$ was suggested as binding site for the monovalent cations (Neve et al. 1991; Ceresa and Limbird 1994). This hypothesis was proven in mutagenesis studies by exchanging the aspartate for asparagines (Neve et al. 1991; Ceresa and Limbird 1994; Schetz and Sibley 2001; Schnell and Seifert 2010). The next milestone was the solution of the crystal structures of the $A_{2A}R$ (Liu et al. 2012) and of the DOPR (Fenalti et al. 2014), with a sodium ion bound in this allosteric binding site. The crystal structures revealed insights into the interaction of the Na^+ with the amino side chains in the allosteric site, especially $Asp^{2.50}$ and $Ser^{3.39}$ (Liu et al. 2012; Fenalti et al. 2014). However, also the coordination of the ion by water molecules was shown to be of relevance (Liu et al. 2012). Although mutagenesis studies and crystal structures provided important insights into ion-sensitivity of GPCRs, the picture on a molecular level has to be completed by molecular modelling studies, especially MD simulations, because with this technique, the dynamics of different processes can be studied quite well (Selent et al. 2010; Yuan et al. 2013; Shang et al. 2014; Wittmann et al. 2014b). For the dopamine D_2 receptor (D_2R) and the μ-opioid receptor (MOPR), the complete binding of a sodium ion from the extracellular side of the receptor via the orthosteric into the allosteric binding site was shown, giving insights into the entry of a monovalent cation and the time scale of the entire binding process (Selent et al. 2010; Yuan et al. 2013). However, crystallographic studies have not yet addressed the question how anions affect GPCR function. Last but not least, structure-activity relationships for cations have not yet been examined in the crystallographic studies.

Movement of monovalent cations and anions to their binding sites

It was shown by MD simulations at the D_2R (Selent et al. 2010) and opioid receptors (Yuan et al. 2013; Shang et al. 2014) and hH_4R (Wittmann et al. 2014b) that a Na^+ can bind from the extracellular side into the allosteric binding site of the receptor. The overlay of snapshots at different time steps of a MD simulation of hH_4R shows the flexibility of the most important amino side chains as well as the preferred areas for Na^+ and Cl^- (Fig. 1a). A comparison of the simulation results suggests that the cation is caught by negatively charged glutamate or aspartate at the extracellular surface of the receptor (Fig. 1a (I)). However, a comparison of the amino acid sequences, forming the extracellular surface of GPCRs, suggests that negatively charged amino acids are located in different areas on the extracellular surface of various receptors. Thus, individual entry pathways for different GPCRs are likely. In case of hH_4R, Glu^{160} catches the Na^+ whereas Glu^{163} undergoes a conformational change and guides the Na^+ into the orthosteric binding site (Wittmann et al. 2014b). As illustrated, this part of the process is very fast, while the cation remains for a longer period in the orthosteric binding site (Fig. 1a (IIa, IIb); Fig. 1b). Because of the additional negatively charged $Glu^{5.46}$, the Na^+ also binds sporadically there (Fig. 1a (IIa)). However, the preferred binding area in the orthosteric binding site is near $Asp^{3.32}$ (Fig. 1a (IIb)). Afterwards, the Na^+ again binds very fast into the allosteric binding site (Fig. 1a (III)), where it remains stable again for a longer

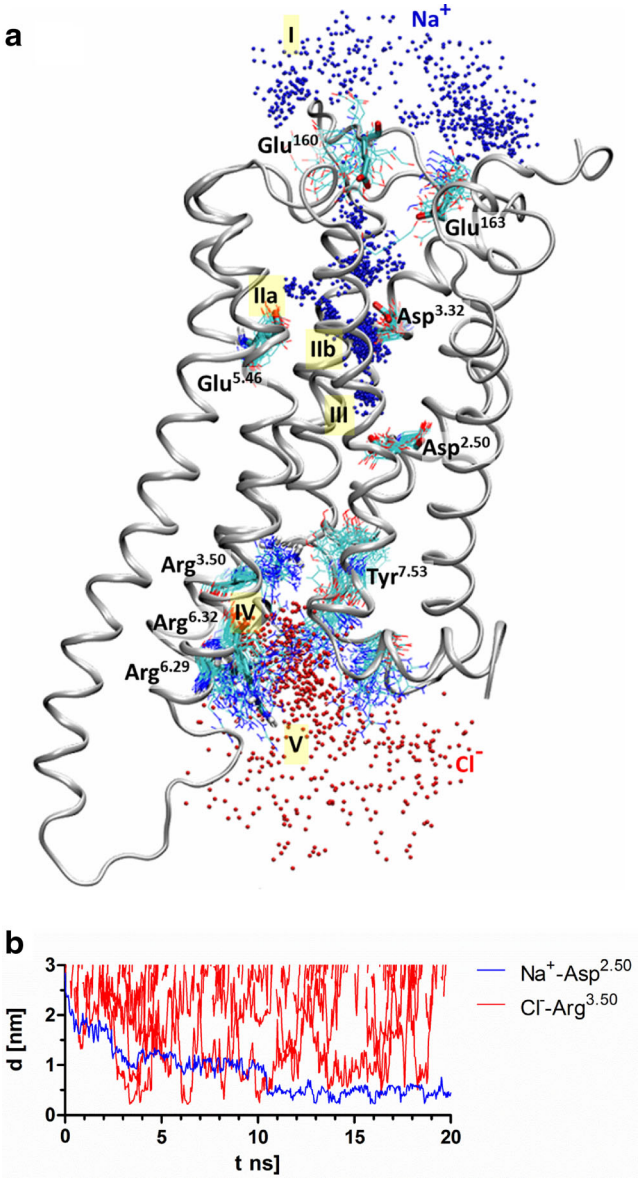


Fig. 1 Movement of monovalent cations and anions to their binding site. **a** Binding pathway of Na^+ and Cl^- to hH_4R as determined by MD simulations (Wittmann et al. 2014b). **b** Time course of the distance of Na^+ to $Asp^{2.50}$ and Cl^- to $Arg^{3.50}$ at hH_4R, observed during MD simulations

Table 2 Physicochemical properties of monovalent cations and anions

	Monovalent cations					Monovalent anions			Refs.
	Li^+	Na^+	K^+	Rb^+	Cs^+	Cl^-	Br^-	I^-	
r_{ion} (Å)	0.60	0.98	1.33	1.49	1.65	1.81	1.96	2.20	
r_{hyd} (Å)	1.98	2.38	2.74	2.88	2.96	3.13	3.28	3.50	Joung and Cheatham (2008)
ΔG^o_{hyd} (kJ/mol)	−471.4	−369.9	−297.1	−274.5	−253.2	−373.7	−346.0	−311.3	Joung and Cheatham (2008)
n_{hyd}	4	5	6	8	8	6	6	7	Soper and Weckström (2006), Varma and Rempe (2006), Mähler and Persson (2012)

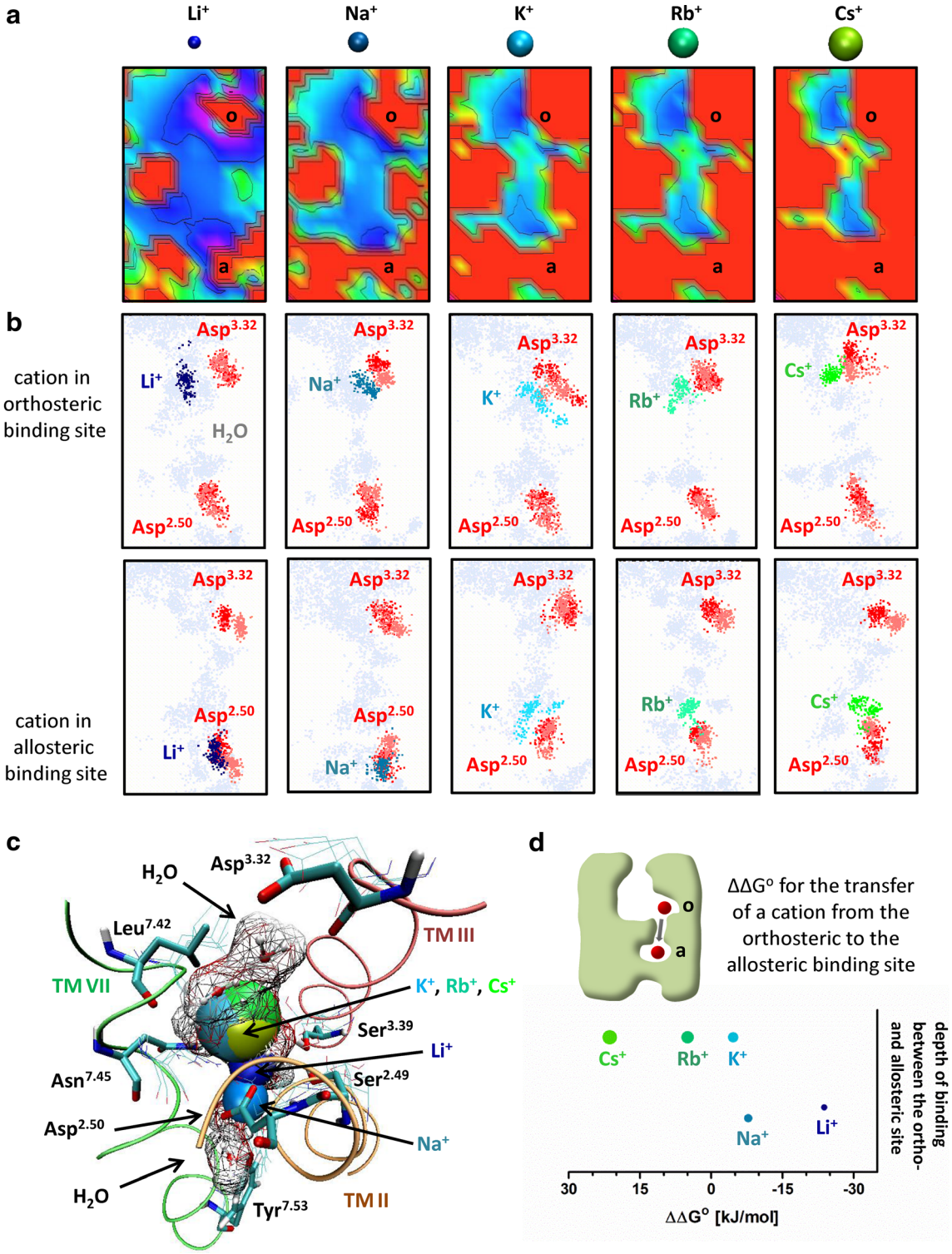
period of time. In hH_4R, a monovalent cation (Na^+) and monovalent anion (Cl^-) were observed to bind simultaneously to the allosteric binding site or the intracellular part between the TM domains, respectively. Whereas one and the same Na^+ ion is stable in the allosteric binding site, a strong fluctuation of the Cl^- regarding the distance to $Arg^{3.50}$ was observed (Fig. 1a (IV)). These distinct pair configurations remain stable for about 0.5 ns. However, due to the strong fluctuation of the Cl^-, a Cl^- was observed to be in close contact to $Arg^{3.50}$ during the complete simulation time. Similar to the Na^+, the Cl^- is caught by positively charged amino acids at the intracellular surface of the receptor. At hH_4R, these are predominantly $Arg^{6.29}$ and $Arg^{6.32}$ (Fig. 1a (V)).

Signature of the allosteric cation binding site on a molecular level

In biophysical and physiological processes, ion-specific effects play an important role because biological systems are affected when salt concentrations are varied or distinct ions are replaced (Lo Nostro and Ninham 2012). Because of the differences in size, interaction properties (e.g. Coulomb- or Lennard-Jones interaction energies) and hydration shell of monovalent cations (Table 2), those cations exhibit different physicochemical properties and were ordered in the so-called Hofmeister (1888) series. Additionally, anion and cation specificity was described for several biological systems, e.g. proteins or even enzymatic activity by experimental techniques and molecular simulations (Collins et al. 2007; Yang et al. 2010; Friedman 2011; Rembert et al. 2012; Lo Nostro and Ninham 2012; Stepankova et al. 2013). Thus, differences in interaction of monovalent cations with GPCRs in the allosteric binding site are expected. To elucidate these differences on a molecular level, different molecular modelling techniques can be used. The interaction energy at an intersecting plane through the channel between the ortho- and allosteric binding site of a representative GPCR, the human histamine H_3-receptor (hH_3R) shows that there are two preferred areas for cations, first, around the highly conserved $Asp^{3.32}$ of the orthosteric and second, around the highly conserved $Asp^{2.50}$ around the allosteric binding site (Figs. 2 and 3).

We considered Na^+, Ka^+, Li^+, Cs^+ and Rb^+. While only Na^+ and K^+ physiologically modulate GPCR function, the latter three cations provide extremely valuable tools for the analysis of ion effects on GPCRs because they differ from each other in physicochemical properties (Table 2). Moreover, Li^+ is unique in this series because this cation is highly effective in the treatment of manic-depressive (bipolar) disorders (Beaulieu et al. 2009; Marlinge et al. 2014; Tselnicker et al. 2014) and there is recent interest in Li^+ as drug for the treatment of neurodegenerative diseases (Forlenza et al. 2014). Although the concentrations at which the Li^+ affects GPCR function in vitro are much higher than the therapeutic plasma concentrations in patients (~1 mM) (Grandjean and Aubry 2009), it cannot be excluded that Li^+ interacts with GPCRs in vivo. One limitation of the studies conducted with Li^+ on GPCRs in vitro is the fact that Li^+ was only studied alone but not together with Na^+ or K^+ (Gierschik et al. 1991; Seifert 2001). With increasing size of the cation in the series $Li^+ \rightarrow Na^+ \rightarrow K^+ \rightarrow Rb^+ \rightarrow Cs^+$, the energetically favoured area decreases (Fig. 2a). Additionally, in the same series, the

Fig. 2 Signature of the allosteric cation binding site on a molecular level. ▶ **a** Interaction energy (Coulomb- and Lennard-Jones) surface of a cation (Li^+, Na^+, K^+, Rb^+ and Cs^+) within the area of the orthosteric site, allosteric site and the binding channel of hH_3R; *o* position of the $Asp^{3.32}$ in the orthosteric binding site, *a* position of the $Asp^{2.50}$ in the allosteric binding site; *purple and dark blue*, energetically preferred areas for a monovalent cation; *yellow and orange*, energetically disfavoured areas for a monovalent cation. **b** Preferred areas for the cation (Li^+, Na^+, K^+, Rb^+ and Cs^+) around the orthosteric and allosteric binding site based on an overlay of snapshots of a molecular dynamic simulation of the corresponding cation in the hH_3R. *Red*, position of the carboxy oxygens of $Asp^{3.32}$ and $Asp^{2.50}$; *grey*, position of the oxygens of water molecules (H_2O). **c** Overlay of Li^+, Na^+, K^+, Rb^+ or Cs^+ in the allosteric binding site of hH_3R; shown are representative snapshots of MD simulations; most important amino acids are shown as *sticks*; representative water molecules between the orthosteric and allosteric binding site are shown as *wireframe surface*. **d** estimation of the change in standard Gibbs energy of solvation of the cation for the transfer from the orthosteric to the allosteric binding site ($\Delta\Delta G^o$). All data shown within this figure were obtained by calculations as described previously (Wittmann et al. 2014a, b).



transition of the cation through the connecting channel becomes energetically more and more disfavoured (Fig. 2a). It is well known that also water molecules are an important key player in interaction of a cation with the allosteric binding site (Liu et al. 2012; Katritch et al. 2014). Furthermore, previous modelling studies showed that water molecules connect the orthosteric and the allosteric binding site (Fig. 2b, c) (Wittmann et al. 2014b). The interaction of a cation with a GPCR is a dynamic process. The preferred areas of water molecules and cations based on snapshots of MD simulations

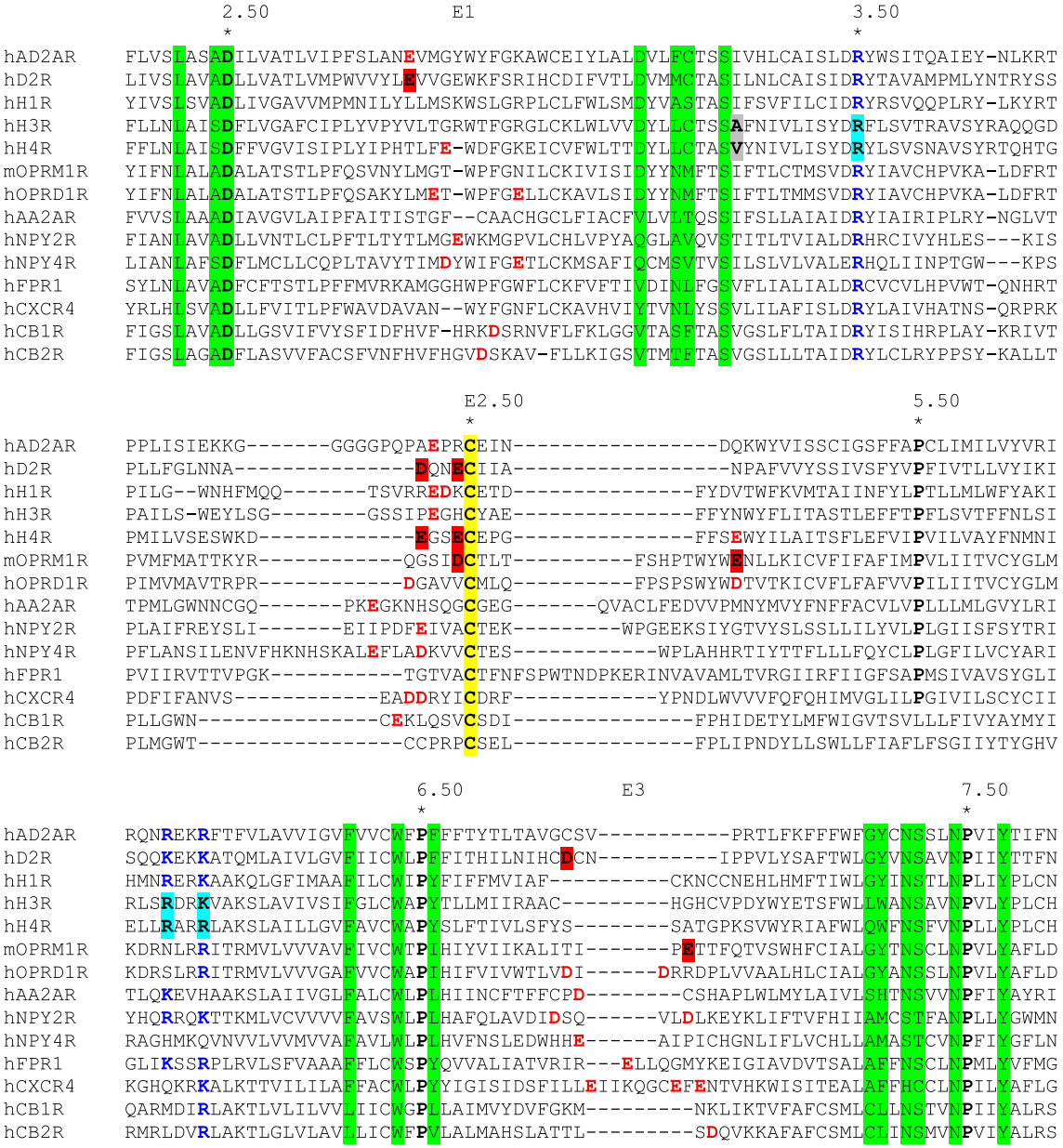


Fig. 3 Sequence alignment of different GPCRs, regarding amino acids being important for sensitivity to monovalent cations and anions. *Asterisk*, highly conserved amino acids according to Ballesteros-Weinstein nomenclature; *yellow boxes*, highly conserved cysteine residues, forming a disulphide bridge between the E2-loop and the transmembrane domain III; *green boxes*, most important amino acids forming the sodium binding channel between the orthosteric and the allosteric binding site; *red boxes*, negatively charged amino acids, observed in MD simulations to catch at Na^+ at the extracellular surface and to guide the cation into the orthosteric binding site (Wittmann et al. 2014b; Selent et al. 2010; Yuan et al. 2013); *red bold letters*, negatively charged amino acids at the extracellular surface which could be involved in catching monovalent cations; *cyan boxes*, positively charged amino acids, observed in MD simulations to interact with monovalent anions at the intracellar part between the transmembrane domains of a GPCR (Wittmann et al. 2014b); *blue bold letters*, positively charged amino acids at the intracellular surface between the transmembrane domains of a GPCR which could be involved in interaction with monovalent anions; *grey boxes*, amino acids which may be responsible for differences in Na^+ sensitivity between hH_3R and hH_4R (Wittmann et al. 2014b). (UniProtKB accession codes: human α_{2A} adrenergic receptor (hADA2AR), P08913; human dopamine D_2 receptor (hD_2R), P14416; human histamine H_1 receptor (hH_1R), P35367; human histamine H_3 receptor (hH_3R), Q9Y5N1; human histamine H_4 receptor (hH_4R), Q9H3N8; mouse μ-type opioid receptor (mOPRM1R), P42866; human δ-type opioid receptor (hOPRD1), P41143; human adenosine A_{2A} receptor (hAA2AR), P29274; human neuropeptide Y receptor type 2 (hNPY2R), P49146; human neuropeptide Y receptor type 4 (hNPY4R), P50391; human *N*-formyl peptide receptor (hFPR1), P21462; human chemokine receptor type 4 (hCXCR4), P61073; human cannabinoid receptor 1 (hCB_1R), P21554; human cannabinoid receptor 2 (hCB_2R), P34972)

around the orthosteric and allosteric as well as the connecting channel of the hH_3R are shown for the series $Li^+ \rightarrow Na^+ \rightarrow K^+ \rightarrow Rb^+ \rightarrow Cs^+$ (Fig. 2b). In both cases, the cation bound in the orthosteric or allosteric site and water molecules are found in the cation binding channel (Fig. 2b). While the preferred binding area of the analysed cations is very similar for the orthosteric binding site, there are obvious differences in the preferred binding for Li^+ and Na^+ on the one hand and K^+, Rb^+ and Cs^+ on the other hand: While Li^+ and Na^+ bind deeper into the allosteric pocket, K^+, Rb^+ and Cs^+ bind preferably at the area between the binding channel and the upper part of the allosteric binding site (Fig. 2b, c). Here, the hydration by water molecules or coordination by amino acid side chains and the preferred hydration number (Table 2) play a role. These differences lead to distinct signatures of cation binding in the allosteric site. Consequently, binding of different monovalent cations to the allosteric site may result in distinct interaction networks within the receptor, and these different interaction patterns may result in different receptor conformations. This hypothesis is supported by the highly heterogeneous cation effects in various systems (Table 1). Thus, like for conventional GPCR ligands, GPCR-specific structure-activity relationships for various cations are emerging.

The cation signatures shown (Fig. 2a, b), may be similar between aminergic GPCRs, but different to GPCRs with no aspartate at position 3.32 (Fig. 3), e.g. the $A_{2A}R$, because for the latter receptor, the preferred region for cation binding in the orthosteric binding site is missing. Furthermore, within the family of aminergic GPCRs more subtle differences in amino acids forming the binding channel are present (Fig. 3): for example, the hH_3R is highly Na^+ sensitive (Schnell and Seifert 2010), whereas the closely related human histamine H_4-receptor (hH_4R) shows no sensitivity regarding sodium ions (Schneider et al. 2009), although Na^+ probably binds to the allosteric binding site (Schneider et al. 2009). Molecular modelling studies suggest that a reason for this may be the differences in amino acid sequence between hH_3R and hH_4R at positions 3.40 and 7.42 (Wittmann et al. 2014b). Additionally, differences in the change in standard Gibbs energy for the transfer of a monovalent cation from the orthosteric to the allosteric binding site ($\Delta\Delta G^o$) have to be considered, dependent of the nature of the monovalent cation. In the series $Li^+ \rightarrow Na^+ \rightarrow K^+ \rightarrow Rb^+ \rightarrow Cs^+$, the standard Gibbs energy increases (Fig. 2d). Based on this data, it can be suggested that there is a rising preference for binding of a monovalent cation in the series $Cs^+ \rightarrow Rb^+ \rightarrow K^+ \rightarrow Na^+ \rightarrow Li^+$. This trend regarding the basal GTP hydrolysis was also shown previously by experimental studies at hH_3R for $K^+ \rightarrow Na^+ \rightarrow Li^+$ (Schnell and Seifert 2010). The standard Gibbs energy for the transfer from the orthosteric to the allosteric site strongly depends on the nature of the monovalent cation. Furthermore, taking into account the high similarity of the allosteric binding site between different GPCRs (Fig. 3), this trend may be detected for other GPCRs, considering the heterogeneous cation effects in various systems. The calculations suggest that the binding of Li^+ to the allosteric binding site is energetically strongly favoured compared with Na^+ (Fig. 2d). This observation may be of relevance for the therapeutic effect of Li^+, e.g. in therapy of bipolar disorders (Beaulieu et al. 2009; Marlinge et al. 2014; Tselnicker et al. 2014). In this context, it may be interesting to study the binding of Li^+ in the allosteric binding site at different GPCRs by MD simulations or even to obtain crystal structures.

Impact of monovalent ions onto GPCRs—a structural perspective

As shown exemplarily for the hH_3R by molecular modelling studies, monovalent cations can bind stably into the orthosteric and allosteric binding site of a GPCR (Wittmann et al. 2014b) (Fig. 2c). Furthermore, MD simulations revealed that monovalent anions preferably bind between the intracellular parts of the transmembrane (TM) helices (Wittmann et al. 2014a). However, in contrast to cations in the allosteric site, a specific anion binding site was not yet identified in crystallographic studies. In case of hH_3R, monovalent anions bind closely to $Arg^{3.50}$, $Arg^{6.29}$ and $Arg^{6.32}$ (Wittmann et al. 2014a) (Fig. 1a). The analysis of the percentage of conservation of positively charged amino acids located at the intracellular surface between the TM domains suggests that $Arg^{3.50}$, $Lys/Arg^{6.29}$ and $Lys/Arg^{6.32}$ (Fig. 3) are promising candidates for an anion binding site. Moreover, due to the complete conservation of $Arg^{3.50}$ in GPCRs, this amino acid may represent the anion binding site, whereas the other positively charged amino acids in this region, which are not fully conserved, e.g. $Lys/Arg^{6.29}$ and $Lys/Arg^{6.32}$ may guide the anion to the proposed allosteric anion binding site in a GPCR-specific manner (Fig. 3).

The binding of anions in the intracellular part of the GPCR may have two different consequences: First, the anion may keep TM VI and TM II closely together by electrostatic interaction so that opening of the intracellular part of the receptor—equivalent to receptor activation—is suppressed. Thus, anions bound in the intracellular part of the GPCR may stabilise its inactive state. Second, the binding of the anion may block binding of the G protein to the GPCR in the active state. Thus, it may be worthwhile to analyse crystal structures of GPCRs with regard to anion binding. As illustrated by the scheme (Figs. 1a and 4) and as shown by MD simulations, a monovalent cation and monovalent anion can bind simultaneously to the allosteric binding site and the intracellular part between the TM domains, respectively. The minimum distance between both monovalent ions is in the ~16–25-Å range (Wittmann et al. 2014a). Clearly, this

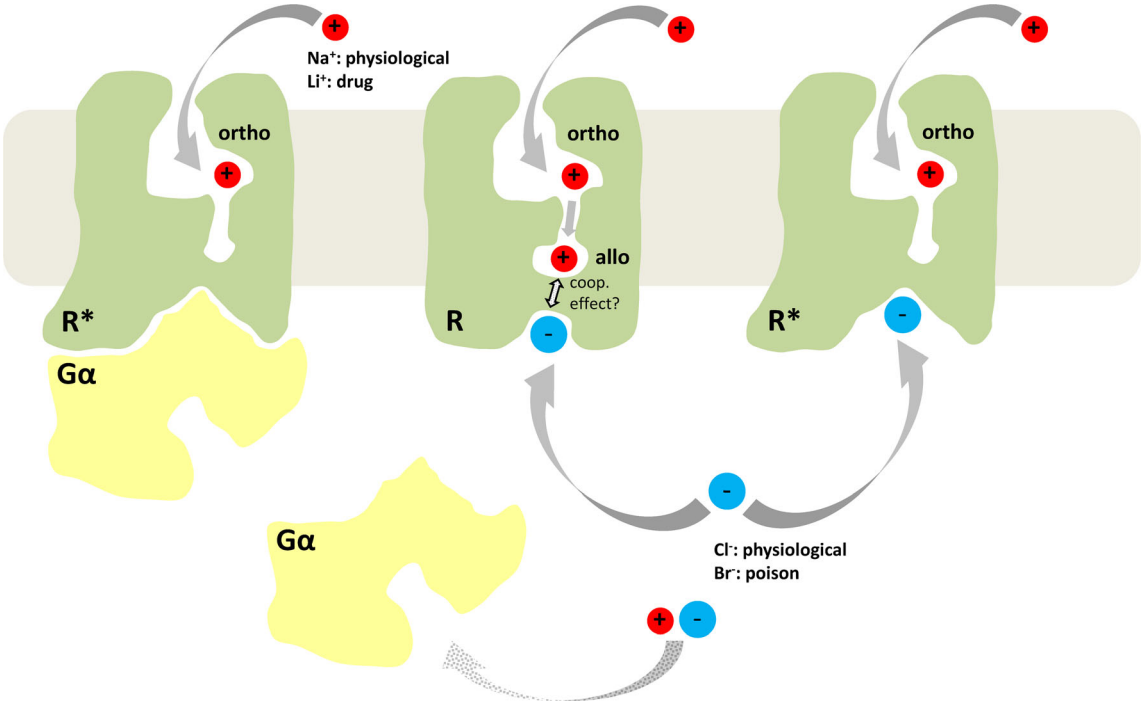


Fig. 4 Impact of monovalent cations and anions on GPCR function: possible pharmacological interventions. This scheme illustrates the influence of monovalent cations and anions onto a GPCR in the inactive (*R*) and active (*R**) state and onto the Gα subunit of a G-protein. Monovalent cations, e.g. Na^+ were shown to bind into the orthosteric and allosteric site of several GPCRs (Selent et al. 2010; Liu et al. 2012; Yuan et al. 2013; Fenalti et al. 2014; Wittmann et al. 2014b), while monovalent anions were detected in MD simulations to bind in the intracellular part of the *R* and active *R** state of GPCRs, e.g. the hH_3R (Wittmann et al. 2014a). Additionally, the scheme demonstrates that also an interaction of monovalent cations or anions with the Gα subunit of a G-protein has to be considered. The 7TM-topology of GPCRs and the globular shape of Gα as evident from X-ray crystallography is intentionally not depicted here to focus on the monovalent ion binding sites in these proteins

distance is too large for a direct electrostatic interaction. However, binding of the cation and anion may result in changes of the interaction network and consequently influence each other, e.g. result in a more favoured stabilization of the inactive receptor. In support for an anion binding site in GPCRs are data showing that in several cases, anions exhibit a much more pronounced effect on receptor/G-protein coupling than cations (Table 1). However, to this end anions have not yet been systematically analysed at GPCRs. By analogy to cations, anions with different physiochemical properties (Cl^-, Br^-, I^-) (Table 2) can be used as pharmacological tools to analyse anion binding sites. It is possible that some of the CNS effects observed in bromide intoxication (James et al. 1997; Baird-Heinz et al. 2012) are the result of altered GPCR function. The assumed interactions of anions with GPCRs at the intracellular side are intriguing in the light of the fact that anions can also modulate the function of G-proteins (Higashijima et al. 1987). Thus, it is conceivable that anions act as allosteric modulators of receptor/G-protein coupling. However, there were only very few follow-up studies on the seminal work of Higashijima and colleagues (Seifert 2001).

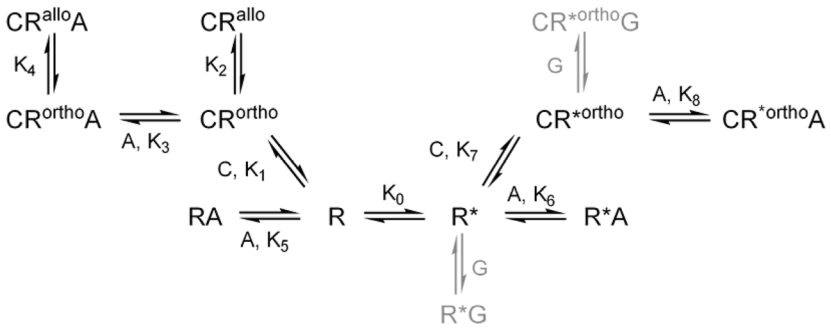
While it will be very difficult to achieve exquisite specificity in the effects of ions for a given receptor, the development of allosteric modulators, either positively or negatively modulating ion-receptor interaction, is feasible. With regard to cation entry to the orthosteric site, it should actually be quite easy to obtain such modulators because cation entry sites at the extracellular domains of GPCRs are very heterogeneous (Fig. 3), offering an unique allosteric opportunity for achieving receptor selectivity. Likewise, the intracellular domains, where the tentative anion binding site is located (Fig. 1a), is highly heterogeneous. However, since this domain is located at the intracellular site of the membrane, allosteric modulators must be more hydrophobic to reach this target. The same considerations apply to modulators of anion interactions with G-proteins. In principle, G-proteins are less suitable targets than GPCRs because they couple to many GPCRs, increasing the probability of side effects.

Monovalent ion mediated impact on binding and signalling—a mathematical description

In order to understand the influence of ligand binding onto signal transduction by GPCRs, the concept of an equilibrium between inactive *R* and active receptors *R** was developed (Seifert and Wenzel-Seifert 2002). Assuming, efficacy is proportional to the concentration of the active receptor–G-protein

complex, an analytical description of the receptor function becomes possible. A first extension of this quantitative model dealing with the impact of sodium ions on GTP hydrolysis induced by the hH_3R was established (Wittmann et al. 2014a). Experimental evidence for an impact of anions on the GTP hydrolysis was shown for some GPCRs including the hH_3R (Schnell and Seifert 2010). In order to establish a quantitative description of the influence of monovalent cations and anions on the efficacy an extension of the classical model regarding the GPCR-ion interaction is presented in Fig. 5a. The resulting equation for the efficacy *E*, shown in Fig. 5b, is a complex equation, including all equilibrium constants. Because the concentration of cations C is present in the enumerator and denominator, an increase in concentration of cations can lead

a



b

mass action laws

$$K_0 = \frac{R*}{R} \qquad K_5 = \frac{RA}{R \cdot A_0}$$

$$K_1 = \frac{CR^{ortho}}{R \cdot C_0} \qquad K_6 = \frac{R*A}{A_0 \cdot R*}$$

$$K_2 = \frac{CR^{allo}}{CR^{ortho}} \qquad K_7 = \frac{CR*^{ortho}}{R* \cdot C_0}$$

$$K_3 = \frac{CR^{ortho}A}{A_0 \cdot CR^{ortho}} \qquad K_8 = \frac{CR*^{ortho}A}{A_0 \cdot CR*^{ortho}}$$

$$K_4 = \frac{CR^{allo}A}{CR^{ortho}A}$$

conservation of matter

$$R_0 = R + R* + CR^{ortho} + CR^{allo} + CR^{ortho}A + CR^{allo}A + RA + R*A + CR*^{ortho} + CR*^{ortho}A$$

$$A_0 = A + RA + R*A + CR^{ortho}A + CR^{allo}A + CR*^{ortho}A$$

$$C_0 = C + CR^{ortho} + CR^{allo} + CR^{ortho}A + CR^{allo}A + CR*^{ortho} + CR*^{ortho}A$$

final equation for the efficacy

$$E = f \cdot R_0 \cdot \frac{K_0 \cdot (1 + C_0 \cdot K_7)}{1 + K_0 + C_0 \cdot (K_1 + K_0 \cdot K_7 + K_1 \cdot K_2) + A_0 \cdot (K_5 + K_0 \cdot K_6) + C_0 \cdot A_0 \cdot (K_1 \cdot K_3 + K_0 \cdot K_7 \cdot K_8 + K_1 \cdot K_3 \cdot K_4)}$$

Fig. 5 Mathematical model to describe the sensitivity of a GPCR to monovalent cations or anions. **a** Schematic presentation of the different binding processes; *R* inactive receptor without any ligand or ion, *R** active receptor without any ligand or ion, *C* monovalent cation, CR^{ortho} inactive receptor with a monovalent cation in the orthosteric binding site, CR^{allo} inactive receptor with a monovalent cation in the allosteric binding site, *A* monovalent anion, $CR^{ortho}A$ inactive receptor with the monovalent cation in the orthosteric binding site and the monovalent anion between the intracellular transmembrane domains, $CR^{allo}A$ inactive receptor with the monovalent cation in the allosteric binding site and the monovalent anion between the intracellular transmembrane domains, *RA* inactive receptor with the monovalent anion between the intracellular transmembrane domains, *R*A* active receptor with the monovalent anion between the intracellular transmembrane domains, $CR*^{ortho}$ active receptor with the monovalent cation in the orthosteric binding site, $CR*^{ortho}A$ active receptor with the monovalent cation in the orthosteric binding site and the monovalent anion between the intracellular transmembrane domains; *grey shaded*, these equilibriums are not considered in the calculations under (**b**). **b** For the equilibriums, described under (**a**), the corresponding mass action laws and conservation of matter are given; considering the approximation that each active receptor-ion complex without an anion between the intracellular part of the TM domains (*R**, $CR*^{ortho}$) interacts with a G-protein, the efficacy can be calculated as described (Wittmann et al. 2014a); the given efficacy *E* represents the absolute efficacy of G-protein activation and is not calculated relative to a full agonist; *f* represents a proportional factor (Wittmann et al. 2014a); the binding of agonists to the GPCR, as already described (Wittmann et al. 2014a) is not included within the mathematical model shown here; the mentioned terms for all species represent the molar concentrations

to an increase as well as a decrease in efficacy. In contrast, an increase of the anion concentration always leads to a decrease in efficacy (Fig. 5b).

In general, the quantitative analysis of the impact of monovalent cations and anions onto GPCRs, as already performed for the hH_3R (Wittmann et al. 2014a), and consequently the calculation of the corresponding equilibrium constants will provide a more detailed insight into the interaction between ions and GPCRs on a molecular level. However, the quantitative treatment of the experimental data in order to evaluate binding constants regarding the different cation and anion complexes requires large experimental data sets with high precision.

Remaining questions and future directions

The combination of pharmacological, mutagenesis, molecular modelling and crystallography techniques has provided significant insights into the high complexity of the interactions of monovalent cations and anions with GPCRs. However, there are still many open questions: MD simulations indicate that monovalent ions, like Li^+ and Na^+ compared with K^+, Rb^+ and Cs^+, bind into different parts of the allosteric binding sites of GPCRs, resulting in structural differences in the allosteric site. Thus, more crystal structures of different GPCRs showing sensitivity to monovalent cations in experimental studies should be solved, paying special attention to binding of Li^+ because of its pharmacological relevance. As exemplarily shown for the hH_3R, MD simulations provide hints that anions bind preferably between the intracellular parts of the TM domains in the inactive as well as in the active state of a GPCR. Whereas MD studies showed that the cation binding area in the allosteric site of a GPCR is more focused, the anion binding site is much wider. Although no single amino acid could be identified to be important for anion binding until know, there are some strong candidates, e.g. $Arg^{3.50}$ and positively charged amino acids at the positions 6.29 or 6.32 that can be probed by mutagenesis. Additionally, crystallographic studies should examine possible anion binding sites in the intracellular GPCR portions between the TM domains. Furthermore, synergistic cooperative effects of cation and anion binding sites in GPCRs need to be studied. As an important next step towards understanding the effects of anions on GPCRs, it will be necessary to systematically analyse various anions in the presence of a fixed cation. Based on the highly heterogeneous effects of cations and anions on various GPCRs, it is reasonable to assume that any given GPCR interacts with cations and anions differently. Future studies should also examine various anions and cations in combination, an aspect that has not yet been fully recognised. In vitro studies will remain the backbone of future research in monovalent ion research because precise manipulation of cation and anion concentrations in intact cells is hardly possible. One should be aware of the fact that the variations of ion concentrations under pathophysiological in vivo conditions are not as large as under experimental in vitro conditions. Thus, one should be careful by transferring experimental results to pathophysiological conditions. However, despite this discrepancy, such experimental studies substantially increase our understanding of the impact of monovalent cations and anions on GPCRs on a molecular level.

It should be emphasised that both extra- and intracellular monovalent ion concentrations are not static. Specifically, both hyperosmolarity and hypoosmolarity syndromes are known (May and Jordan 2011; Lombardo et al. 2013). Overall, very little research has been performed so far on the impact of changes of extracellular monovalent ion concentrations on GPCR function in intact cells. In one notable study, it was observed that the constitutive activity of the luteinizing hormone receptor in transfected COS-7 cells as assessed by cAMP accumulation was markedly enhanced by removal of NaCl from the extracellular medium, sucrose serving as isotonic substitute (Cetani et al. 1996). Similar studies can be readily performed for any given GPCR, and we anticipate that such studies will reveal substantial differences in the monovalent ion modulation of GPCRs. During the past 20 years, such studies were probably not performed because they were perceived as "descriptive", but in light of the recently resolved GPCR crystal structures in complex with Na^+, such studies will gain mechanistic momentum. Moreover, intracellular Na^+ and Cl^- concentrations are dynamically regulated by multiple mechanisms and change in numerous diseases including cardiovascular diseases and pulmonary diseases (Coppini et al. 2013; Simon Bulley and Jaggar 2014). Furthermore, GPCRs can activate Na^+ entry into cells (Krautwurst et al. 1992). Hence, it is conceivable that changes in extra- and intracellular monovalent ion concentrations modulate GPCR functions in a subtle manner in health and disease.

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