

The Influence of Monovalent Cations on Trimeric G Protein G_i1 α Activity in HEK293 Cells Stably Expressing DOR-G_i1 α (Cys³⁵¹-Ile³⁵¹) Fusion Protein

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Summary

The effect of monovalent cations on trimeric G protein G_i1 α was measured at equimolar concentration of chloride anion in pertussis-toxin (PTX)-treated HEK293 cells stably expressing PTX-insensitive DOR-G_i1 α (Cys³⁵¹-Ile³⁵¹) fusion protein by high-affinity [³⁵S]GTP γ S binding assay. The high basal level of binding was detected in absence of DOR agonist and monovalent ions and this high level was inhibited with the order of: Na⁺ > K⁺ > Li⁺. The first significant inhibition was detected at 1 mM NaCl. The inhibition by monovalent ions was reversed by increasing concentrations of DOR agonist DADLE. The maximum DADLE response was also highest for sodium and decreased in the order of: Na⁺ > K⁺ \approx Li⁺. Our data indicate i) an inherently high activity of trimeric G protein G_i1 α when expressed within DOR-G_i1 α fusion protein and determined in the absence of monovalent cations, ii) preferential sensitivity of DOR-G_i1 α to sodium as far as maximum of agonist response is involved.

Key words

δ -opioid receptor (DOR) • Monovalent ions • G_i1 α protein

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Introduction

The opioid receptors (μ -, δ - and κ -OR) have long been known to use inhibitory G proteins of G_i/G_o family for signal transduction because most of opioid-induced signals can be effectively blocked by

pertussistoxin (PTX). Each opioid receptor may interact with five different forms of G_i/G_o proteins (G_i1 α , G_i2 α , G_i3 α , G_o1 α , G_o2 α). Therefore, consequences of OR stimulation by various OR ligands are difficult to predict and the mechanism of action does not follow the straightforward and clearly defined sequence of molecular events (Law *et al.* 2000, Tso and Wong 2003).

HEK293 cells stably expressing fusion proteins between OR and PTX-resistant forms of G α subunits represent an advantageous model for comparison of coupling efficiency of a given type of G protein with the same OR (Moon *et al.* 2001b, Massotte *et al.* 2002). In these cells, endogenously expressed G proteins of G_i/G_o family may be effectively blocked by PTX-treatment prior to membrane isolation and 1:1 ratio between receptor and the cognate G protein provides the most simple and clearly defined stoichiometry. The fusion proteins were constructed between DOR and forms of α subunit of G_i1 protein in which cysteine³⁵¹ was mutated to range of amino acids (Moon *et al.* 2001a, Bahia *et al.* 1998). All these mutated forms of G_i1 α protein were pertussis-toxin insensitive. The fusion protein containing isoleucine in position 351, DOR-G_i1 α (Cys³⁵¹-Ile³⁵¹), when stably expressed in HEK293T cells, exhibited the high DOR agonist-stimulated activity.

We have taken advantage of using this cell line with the aim to improve methodological conditions for detection of opioid-stimulated, PTX-sensitive trimeric G protein activity in the fore brain cortex. This activity was too low to be quantitatively compared among different membrane preparations isolated from control and morphine-treated rats as the increase of high-affinity

[³²P]GTPase or [³⁵S]GTPγS binding was no more than 110 % of the basal level when measured in 100 mM NaCl. Simultaneously, we also wanted to measure the sensitivity of the basal activity of G proteins to different monovalent ions as sodium ions (Na⁺) stabilize the non-active R state of GPCRs and decrease the basal GDP/GTP exchange of G_i/G_o proteins (Mullaney *et al.* 1996, Burford *et al.* 2000, Moon *et al.* 2001a, b, Massotte *et al.* 2002, Seifert and Wenzel-Seifert, 2002). Constitutive activity of GPCR and inverse agonism represent a pharmacologically important phenomena and monovalent ions modulate GPCR-initiated signaling in not yet fully understood mechanism (Giershick *et al.* 1989, 1991, Lefkowitz *et al.* 1993, Samama *et al.* 1994, 1997, Bond *et al.* 1995, Leff 1995, Seifert and Wenzel-Seifert 2002, Milligan *et al.* 1995, Milligan 2003, Schnell and Seifert 2010).

Materials and Methods

Materials

DOR agonist DADLE [(2-D-alanine-5-D-leucine)-enkefalin = Tyr-D-Ala-Gly-Phe-D-Leu] was purchased from Sigma (E7131). Complete protease inhibitor cocktail was from Roche Diagnostic, Mannheim, Germany (cat. no. 1697498).

Cell culture

HEK293 cells stably expressing δ-OR-G_i1α (Cys³⁵¹-Ile³⁵¹) fusion protein were cultivated in DMEM (Sigma) supplemented with 2 mM (0.292 g/l) L-glutamine and 10 % v/v new born calf serum at 37 °C as described by Moon *et al.* (2001a). Geneticin (800 μg/ml) was included in the course of cell cultivation. The cells were grown to 60-80 % confluency, treated for 16 hours with pertussis toxin (25 ng/ml) and harvested by centrifugation for 10 min at 1800 rpm (1500xg). The cell sediment was snap frozen in liquid nitrogen and stored at -80 °C until use.

Preparation of membrane fraction

Plasma membrane-containing P2 particulate fraction was prepared as described before by Moon *et al.* (2001a). Membranes were snap frozen in liquid nitrogen, stored at -80 °C and used only once. Protein was determined by Lowry method.

Agonist-stimulated [³⁵S]GTPγS binding

Membranes (20 μg protein per assay) were

incubated with (total) or without (basal) 1 μM DADLE in final volume of 100 μl of reaction mix containing 20 mM HEPES, pH 7.4, 3 mM MgCl₂, 2 μM GDP, 0.2 mM ascorbate and [³⁵S]GTPγS (1-2 nM) for 30 min at 30 °C. The binding reaction was discontinued by dilution with 3 ml of ice-cold 2 mM HEPES, pH 7.4, 0.15 mM MgCl₂ and immediate filtration through Whatman GF/C filters on Brandel cell harvester. Radioactivity remaining on the filters was determined by liquid scintillation using Rotiszint Eco Plus cocktail. The effect of increasing concentrations of NaCl, KCl and LiCl on [³⁵S]GTPγS binding was determined by "one-point assay" using constant concentration of 1 μM DADLE; when analyzing the dose-response curves, DADLE concentration was increased from 2×10⁻⁹ to 1×10⁻⁶ M DADLE (8 concentrations). Non-specific GTPγS binding was determined in parallel assays containing 10 μM GTPγS. The binding data were analyzed by GraphPadPrism 4; EC₅₀ and B_{max} values were calculated by fitting the data with rectangular hyperbola.

[³H]DADLE binding study

Membranes (10 μg protein per assay) were incubated with increasing concentrations of [³H]DADLE (0.07-38.5 nM) in final volume of 100 μl of binding mix containing 75 mM Tris-HCl, pH 7.4, 12.5 mM MgCl₂, 1 mM EDTA ± 200 mM NaCl (60 min at 30 °C). The bound and free radioactivity was separated by filtration through Whatman GF/B filters in Brandel cell harvester. Filters were washed 3x with 3 ml of ice-cold incubation buffer and placed in 5 ml of scintillation cocktail (Rotiszint Eco Plus). The non-specific binding was defined as that remaining in the presence of 10 μM non-radioactive DADLE.

Results

Monovalent ions affect substantially GPCR-induced response of trimeric G proteins in general (Giershick *et al.* 1989, 1991, Seifert 2001, Schnell and Seifert 2010), but many previous studies dealing with the influence of OR agonists on G_i/G_o proteins were carried out in the presence of 100 mM sodium chloride (Mullaney *et al.* 1996, Burford *et al.* 2000, Moon *et al.* 2001a, b, Massotte *et al.* 2002, Bourova *et al.* 2003). Under such conditions, the basal activity of G proteins in NaCl free medium, its sensitivity to sodium and other monovalent ions and the effect of OR agonists under varying concentrations of these ions could not have been

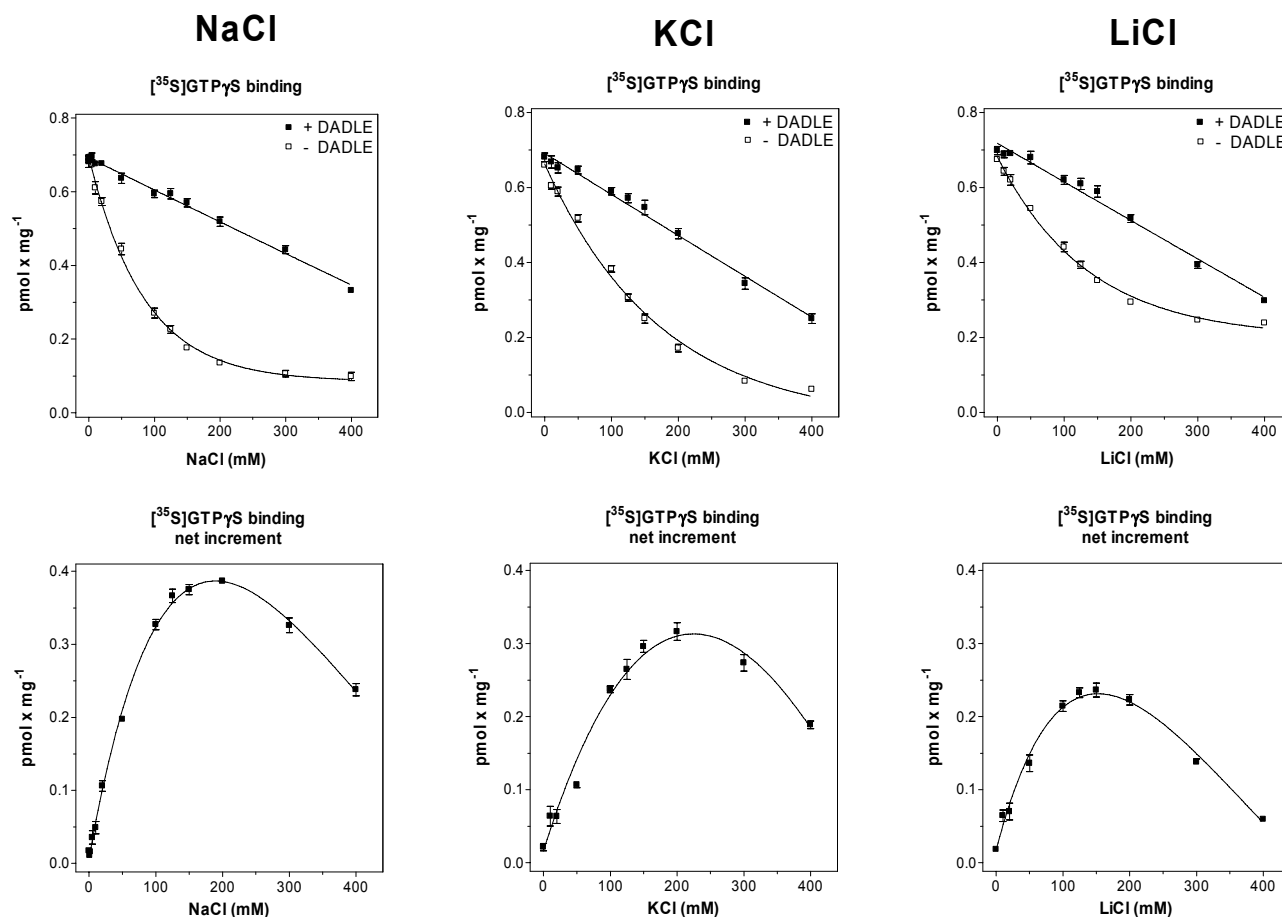


Fig. 1. The effect of increasing concentrations of monovalent cations on the basal and DADLE-stimulated high-affinity [35 S]GTP γ S binding in membranes isolated from PTX-treated cells. *Upper columns.* High-affinity [35 S]GTP γ S binding was measured in the absence ($-$ DADLE) or presence ($+$ DADLE) of 1 μ M DADLE plus increasing concentrations of NaCl (left), KCl (middle) or LiCl (right) in membranes isolated from PTX-treated cells. *Lower columns.* Net-increment of agonist-stimulation Δ_{DADLE} was calculated as the difference between DADLE-stimulated ($+$ DADLE) and basal level of binding ($-$ DADLE). Data were expressed as pmol \times mg $^{-1}$ and represent the means \pm SEM of 3 experiments performed in triplicates.

determined. Therefore, in this work, the effect of increasing concentrations of different monovalent cations Na $^{+}$, K $^{+}$, Li $^{+}$ on the basal level of [35 S]GTP γ S binding was analyzed in the absence or presence of DOR agonist DADLE. These experiments were performed in membranes isolated from PTX-treated HEK293 cells stably expressing PTX-insensitive DOR-G $_i$ 1 α (Cys 351 -Ile 351) fusion protein with the aim to get information about the coupling between the defined type of OR (DOR) and the defined type of G protein (G $_i$ 1 α).

The basal level of high-affinity [35 S]GTP γ S binding was measured in monovalent ion-free medium, subsequently, increasing concentrations of NaCl were added and the effect of these cations was determined in the absence ($-$ DADLE) or presence of 1 μ M DADLE ($+$ DADLE). These measurements were performed in membranes isolated from PTX-treated cells with the aim to characterize specifically the response of G $_i$ 1 α (Cys 351 -

Ile 351) protein. Data shown in Fig. 1 (upper left panel, $-$ DADLE) indicated the high sensitivity of the basal level of binding to inhibition by sodium chloride in the absence of DOR agonist. The first significant decrease of [35 S]GTP γ S binding was detected at 1 mM NaCl ($p < 0.05$), the 50 % inhibition was reached at 62 mM NaCl. The increase of NaCl concentrations above 200 mM (89 % inhibition) had relatively small effect on [35 S]GTP γ S binding indicating that non-specific effect of ionic strength on G $_i$ 1 α protein activity was not significant.

The profile of NaCl effect was dramatically altered by agonist addition (Fig. 1, upper left panel, $+$ DADLE). The exponential decrease was changed for shallow inhibition curve continuously decreasing from zero to 400 mM NaCl, however, the difference between agonist-stimulated and the basal level of binding (Δ) increased steeply when increasing sodium concentration

from zero to 100 mM (Fig. 1, lower left panel); the highest net-increment of DADLE stimulation was detected at 200 mM NaCl ($\Delta_{\text{NaCl}} = 0.39 \text{ pmol} \times \text{mg}^{-1}$).

The effect of potassium and lithium on the basal level [^{35}S]GTP γ S binding was less efficient (Fig. 1, upper middle and right panels, -DADLE): 50 % inhibition was detected at 98 mM KCl and 88 mM LiCl, respectively; at 200 mM KCl (LiCl), the decrease of [^{35}S]GTP γ S binding represented 79 % (82 %) of maximum inhibition at 400 mM KCl (9 %) and LiCl (36 %).

Net-increment of DADLE stimulation decreased in the order of NaCl > KCl > LiCl [$\Delta_{\text{NaCl}} = 0.39 \text{ pmol} \times \text{mg}^{-1} > \Delta_{\text{KCl}} = 0.31 \text{ pmol} \times \text{mg}^{-1} > \Delta_{\text{LiCl}} = 0.23 \text{ pmol} \times \text{mg}^{-1}$, lower panels in Fig. 1]. Thus, sodium was more potent than potassium and lithium when inhibiting the high basal level of binding measured in the absence of ions; sodium was also more efficient when supporting the agonist response of G $_i$ 1 α protein. The specificity and high sensitivity of G $_i$ 1 α to sodium suggest that relatively small change in Na $^+$ concentration, assumingly in the close vicinity of intracellular side of PM, may be effective towards functional coupling sites between DOR and G $_i$ 1 α . Data presented in Fig. 1 support this view as the first significant difference between DADLE-stimulated and the basal level of [^{35}S]GTP γ S binding was detected at 1 mM NaCl ($p < 0.05$).

As expected and due to the inhibitory effect of PTX on the endogenous set of G proteins of G $_i$ /G $_o$ family, the basal level of [^{35}S]GTP γ S binding in membranes isolated from PTX-treated cells ($0.63 \text{ pmol} \times \text{mg}^{-1}$) was lower than that in PTX-untreated cells ($0.83 \text{ pmol} \times \text{mg}^{-1}$) and increasing concentrations of DADLE had no effect on [^{35}S]GTP γ S binding in the absence of ions (data not shown).

The inhibitory effect of sodium on agonist binding parameters of DOR was tested in the last part of our work (Fig. 2). At 200 mM NaCl, the maximum binding capacity (B_{max}) of [^3H]DADLE represented 67 % of B_{max} value determined in the absence of sodium chloride. This results agree well with the high-stability of ternary complex [H-R-G] formed between OR agonist DADLE and DOR-G $_i$ 1 α (Cys 351 -Ile 351) protein (Moon *et al.* 2001a).

Discussion

When trying to measure the OR-stimulated G protein activity in rat brain (Bourova *et al.* 2009, 2010), we had to deal with the persisting problem of the

high basal activity of G proteins detected by both high-affinity [α - ^{32}P]GTPase and [^{35}S]GTP γ S binding assays. Under such conditions, the stimulation by OR agonists was low and not significantly different from the basal level. Our membrane preparations were extensively washed in the course of isolation procedure, so, the presence of endogenous OR ligands was unlikely. Therefore, we have studied the model HEK293 cell line expressing DOR-G $_i$ 1 α (Cys 351 -Ile 351) fusion protein, in which endogenous proteins of G $_i$ /G $_o$ family were rendered inactive by exposure of intact cells to PTX. As previous studies of these and other cell lines expressing OR included high concentrations of NaCl in the assay mix for determination of G protein activity (Mullaney *et al.* 1996, Burford *et al.* 2000, Moon *et al.* 2001a, b, Massotte *et al.* 2002, Bourova *et al.* 2003) and none of these studies analyzed the concentration dependency and specificity of sodium effect, in this work, we have made the detailed analysis of the effect of different monovalent cations on functional coupling between DOR and G $_i$ 1 α protein. Being aware of important role of monovalent anions in this respect (Schnell and Seifert, 2010), equimolar concentration of the same anion (Cl $^-$) was used in all [^{35}S]GTP γ S binding assay media.

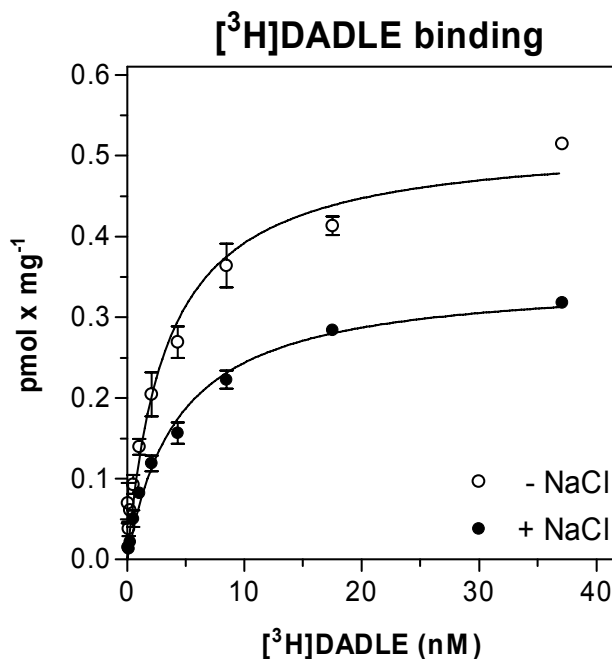


Fig. 2. Inhibition of [^3H]DADLE binding by sodium chloride. Saturation of [^3H]DADLE binding sites was measured as described in Methods in the presence (\circ) or absence (\bullet) of 200 mM NaCl. The data represent average of 3 experiments \pm SEM.

In membranes isolated from PTX-treated DOR- $G_{i1\alpha}$ cells, where the endogenous G proteins of G_i/G_o family were effectively blocked, Na^+ acted like an efficient inhibitor of the high basal level of high-affinity [^{35}S]GTP γ S binding to $G_{i1\alpha}$ which was measured in the absence of monovalent ions and DOR agonist (Fig. 1, upper columns). This basal level of $G_{i1\alpha}$ protein activity was decreased with the order of: NaCl ($EC_{50} = 62$ mM) > LiCl ($EC_{50} = 88$ mM) > KCl ($EC_{50} = 98$ mM) (Fig. 1, upper panels). Please notice that sodium concentrations effecting the basal level of [^{35}S]GTP γ S binding were lower than those used when testing the non-specific effect of ionic strength on GPCR-induced signaling (>200 mM). DADLE had no effect on [^{35}S]GTP γ S binding in the absence of ions; sodium chloride was more efficient than lithium and potassium chlorides when inhibiting the high basal level of [^{35}S]GTP γ S binding.

Maximum DADLE-stimulated response, expressed as the net-increment of agonist stimulation (Fig. 1, lower panels) was also highest for sodium and decreased in the order of $Na^+ > K^+ > Li^+$ [$\Delta_{NaCl} = 0.39$ pmol \times mg $^{-1}$ > $\Delta_{KCl} = 0.31$ pmol \times mg $^{-1}$ > $\Delta_{LiCl} = 0.23$ pmol \times mg $^{-1}$]. Thus, $G_{i1\alpha}$ protein expressed within DOR- $G_{i1\alpha}$ fusion protein exhibited the high basal activity which was fully sensitive to monovalent cations as described before for other G proteins coupled to constitutively active receptors; furthermore and similarly to a non-fused G proteins, it exhibited preferential sensitivity to sodium as far as the maximum of DOR agonist response was involved.

Conclusions

Our results obtained by analysis of the effect of different monovalent cations on coupling between the *defined* type of OR (DOR) and the *defined* type of PTX-sensitive G protein ($G_{i1\alpha}$) both present within DOR- $G_{i1\alpha}$ (Cys 351 -Ile 351) fusion protein in 1:1 stoichiometry indicated preferential sensitivity of this fusion protein to sodium. The inhibition of the very high basal level of [^{35}S]GTP γ S binding ($Na^+ > K^+ > Li^+$) as well as stimulation by δ -opioid agonist DADLE ($Na^+ > K^+ \approx Li^+$) proceeded with almost identical order as far as different monovalent cations were involved. This conclusion is

fully in line with the original data of Koski *et al.* (1983) indicating preferential sensitivity of opioid-stimulated signaling to sodium in neuroblastoma x glioma NG108-15 cells independently expressing endogenous OR and PTX-sensitive G proteins of G_i/G_o family. Obviously, in this cell line, both OR and G proteins were expressed independently.

The study of OR induced change in intracellular, steady-state concentrations of sodium (12 mM Na^+) and considerations about the physiological or pathophysiological significance of our data has to be addressed in the future as the high basal activity of trimeric G proteins may reflect constitutive activity of GPCR; relatively high sensitivity of the basal level of high-affinity [^{35}S]GTP γ S binding or [^{32}P]GTPase activity to the inhibitory effect of sodium in 0-50 mM range might be considered in states of altered permeability of the cell membrane resulting in an increase of intracellular sodium or fluctuations in intracellular concentrations of this ion. *In our case, the first significant decrease of the basal level of [^{35}S]GTP γ S binding was detected at 1 mM NaCl.* Similar data were obtained when analyzing the high-affinity [^{32}P]GTPase in isolated plasma membranes from brain cortex (data not shown). The increase of intracellular sodium in 0-20 mM range was shown to induce dramatic change in structural organization of membrane-water interface at the intracellular side of plasma membrane (Zicha *et al.* 1993). This area of plasma membrane is also crucial for functional interaction between GPCR and $G\alpha$ subunits of trimeric G proteins (Lefkowitz *et al.* 1993, Milligan *et al.* 1995, Milligan 2003).

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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