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Effect of n-Alkanols on G-Protein α Subunits

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Abstract

Guanine nucleotide binding (G)-proteins are GTP-driven allosteric proteins consisting of a single α subunit and a β and γ heterodimer. Gα subunits function as on/off switches based on the occupancy of the nucleotide-binding site, GTP or GDP, such that any alteration in nucleotide exchange modulates signal output. Our previous work has shown that haloalkanes and ethers inhibit GDP/GTP exchange on αi1, αi2 and αi3 subunits, but not the closely related αo. To test whether individual G-protein sensitivity correlates with n-alkanols potency and hydrophobicity, we studied the effects of n-alkanols of varied chain lengths on GDP/GTP exchange by Gαsubunits. n-alkanols (ethanol, butanol, pentanol, hexanol, heptanol, octanol and nonanol) showed differential effects on guanine nucleotide exchange by Gαi1, Gαi2 and Gαo. Based on our observations, we conclude that n-alkanols interact and modulate the activity of the G-α subunits to different extent, thereby uncoupling pathways known to modulate neuronal excitation.

Introduction

Research into the mechanism of anesthesia has focused on a few ligand-gated ion channels, which appear to be targets for general anesthetic agents [1]. General anesthesia has become so safe since its introduction just over 150 years ago, that the risk associated with it has become almost immeasurably small. Yet it is interesting to note that this progress has been achieved in the absence of a generally accepted hypothesis for the mechanisms of general anesthesia [2].

Previous results support the view that general anesthetics bind directly to specific proteins [3-5]. Most significantly, some important anesthetic targets are identified as a group of transmembrane proteins, such as the GABA-gated chloride channels [6]. Additionally, volatile anesthetics could disrupt central pathways that modulate consciousness, memory, and nociception by blocking receptor signals, which control the responsiveness of both ligand and voltage-gated ion channels [7]. Most of these receptors that are involved with sedation (serotonergic), orientation (adrenergic) and mobility (muscarinic) are coupled to their downstream effectors by heterotrimeric GTP-binding (G) proteins. Receptor activation can lead to G-protein activation through a series of conformational changes. G-proteins are heterotrimeric complexes, composed of three different protein subunits: α, β and γ [8]. The α and β subunits aid the whole protein in binding to the inner surface of the plasma membrane. Attached lipid molecules on α and γ subunits subunits serve as hydrophobic areas that cause the complex to sink into and become localized in the plasma membrane. The α subunit of a G-protein binds GDP when it is inactive, but exchanges GDP for GTP when activated. The GTP-charged α subunit either completely or partially dissociates from the β and γ subunits when in its active state, free to diffuse some distance away on the membrane surface. Depending on the particular G-protein, either the α subunit bound to GTP, or the free βγ heterodimer, or both, may act as the messenger between the receptor site and the downstream effector molecule [9]. When activated, the free α subunit of the G-protein binds to a downstream effector molecule [10-12]. These effectors may be enzymes, such as adenylyl cyclase and phospholipase C Rebecchi and Pentyala [1], or modulated channels [13].

A strong correlation between anesthetic potency and hydrophobicity is well established for most compounds (Meyer-Overton correlation) [14]. Some long chain alcohols have been classified as non-immobilizers that fail to induce anesthesia, as defined by lack of purposeful response to a noxious stimulus, yet they are predicted to be potent anesthetics based on their hydrophobicity. Such exceptions to the Meyer-Overton rule suggest some specificity in the interactions of general anesthetics with their molecular targets. Importantly, they provide a test for relevance to the immobilizing effects of anesthetics when studying possible targets *in vitro*. Previous work has shown that the critical chain length to induce CNS depression for the n-alkanol series, 1- 14 carbons, is 8 carbons, above which the longer chain compounds show a leveling off of potency [15]. Above 12 carbons, the alkanols no longer behave like anesthetics, suggesting that the important molecular sites of action exclude the larger n-alkanols. This kind of study has been extended to potential molecular targets, including ligand-gated ion channels and can be used to narrow the spectrum of targets. Our work attempts to identify the interaction of a homologous series of n-alkanols with Gα subunits.

Materials and Methods

Materials

Short chain n-alkanols (ethanol, propanol, butanol) and long chain n-alkanols (pentanol, hexanol, heptanol, octanol, nonanol) (Sigma Chemical Co.) are used in these experiments. Concentrations of the test compounds in the experiments are measured as described earlier [16]. [^{35}S]GTPγS and Scintiverse were purchased from New England Nuclear, MA. Nitrocellulose filters were obtained from Millipore. All other chemicals used in the study were from Sigma Chemical Co.

G-Protein subunits

Recombinant Gα subunits (Gαi1, Gαi2, and Gαo) were purified as previously described [17]. Gαi1 and Gαo subunits were expressed

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as a His tagged protein with a TEV protease cleavable site and Gαi2, was purchased from Calbiochem. Protein purification is performed by column chromatography with a Duo-Flow FPLC (BioRad). His Tag was cleaved with TEV protease according to manufacturer's protocol (Roche).

Guanine nucleotide binding experiments

Gα subunits are incubated with unlabeled GDP (100 nM) and 5 mM MgCl2 for 2 h at 4°C, prior to treatment with n-alkanols. Nucleotide exchange is initiated by diluting the GDP-charged α subunits (50 ng) into 50 µl of assay buffer (20 mM Hepes, pH 8.0, 1 mM EDTA, 1 mM DTT, 100 mM NaCl, 0.2% cholate) containing 5 mM MgCl2, 2 µCi 35S-GTPγS (10 Ci/mMol) and various concentrations of test chemical, in a 1 ml silanized glass vial sealed with a teflon/rubber septum. The exchange reactions are performed at 30°C for 15 minutes and are terminated with ice-cold stop buffer (10 mM Hepes, 100 mM NaCl, 20 mM MgCl2, pH 7.4), followed by rapid filtration of the samples through HA 0.45 µM nitrocellulose filters. The filters are then washed with 10 ml of the same buffer, dried, suspended in 5 ml of scintiverse, and counted in a liquid scintillation spectrometer.

Statistical analysis

Data are expressed as the mean + S.E. of at least two independent experiments assayed in duplicate. The significance of the difference between means was determined by Student t-test. Analysis of variance was used to determine significance when multiple comparisons were performed. A value of $p < 0.05$ was accepted as statistically significant.

Results and Discussion

The test compounds used in these experiments were ethanol, propanol, butanol, pentanol, hexanol, heptanol, octanol, and nonanol. Each of these increases sequentially in size by one additional carbon atom. Ethanol, a shorter chained n-alkanol, is a two carbon chained alcohol, whereas nonanol, a longer chained n-alkanol, contains nine carbons. As each additional methylene group is added, the chain length, area, and volume is varied. Consistencies in binding can help us make molecular inferences about the G-protein binding site. This site can be affected by size, length, and area, and the homologous series of alkanols enables to determine which molecule has an effect on G-proteins. To test the idea that alkanols affect G-α subunits, the exchange of bound GDP for GTP, a reaction normally stimulated by an activated receptor, was examined.

We tested the effects of n-alkanols on GDP-GTP exchange on Gαi1, Gαi2 and Gαo. We have previously reported that volatile agents destabilize binding of GTP to Gαi Pentyala et al. [16] and promote the association of G-βγ heterodimers with GTP-charged αi2, but not Gαo (Rebecchi & Pentyala 2002) [18]. We have earlier reported that having an amino terminal hexa-His sequence diminished the affinity for GDP and halothane decreased the rate of nucleotide exchange. In this study, we found that the n-alkanol series at physiologically relevant concentrations (less than 100 uM) has no effect on GDP/GTP exchange on His tagged Gαi1 protein (Figure 1: Panel A). Then we extended our studies to different chain length alcohols using His tag clipped Gαi1 protein (Figure 1: Panel B).

We found remarkable difference in terms of the interaction of butanol with different confirmations of G-αi1 (His-tagged and His-clipped). Butanol significantly decreased the nucleotide exchange by Gαi1 subunit. An amino terminal hexa-His sequence was used to help purify the requisite amounts of Gαi1 subunit. Only butanol showed a significant selectivity to this His-clipped protein. This surprising result suggests that the amino terminal region, which provides the main contact surface of heptahelical receptors, is closely coupled to the binding of GDP and the actions of butanol. This implies that the actions of hydrophobic compounds depend upon the state of the protein: either in the free-state or in the coupled-state to either βγ subunits or with the receptor. Further characterization should provide new insights into the mechanism whereby butanol and other volatile agents perturb Gαi1 function.

The effects of n-alkanols, particularly of ethanol, are of interest. We used the His tag clipped Gαo and Gαi2 proteins for the studies as we have earlier observed that clipping the His tag on Gαi1 showed significant effect on the protein by butanol (Figure 1). The closely related α subunit, Gαo, is sensitive to ethanol in a concentration range of 75 – 750 mM (Figure 2). Ethanol also showed significant suppression of GDP/GTP exchange by Gαi2 in a concentration range of 100 – 750 mM (Figure 3) at low concentrations. G-protein signaling pathways have long been considered targets for n-alkanols - especially ethanol. Since the late 80's, numerous studies have reinforced the idea that ethanol perturbs Gαs and Gαi-linked signaling pathways in the brain [19]. Recent work demonstrates that acute ethanol treatment disrupts agonist binding to the delta opioid receptor, decreases the rate of receptor internalization and inhibits agonist stimulated GTPγS binding to Gi/o in the neuronal cell line N18TG2 [20]. Our results are consistent with this report. The interaction site of n-alkanols within the Gαi1 protein's probable binding site is most likely less than four carbons for the short chain (C1- C4) binding site. In essence, the site of ethanol

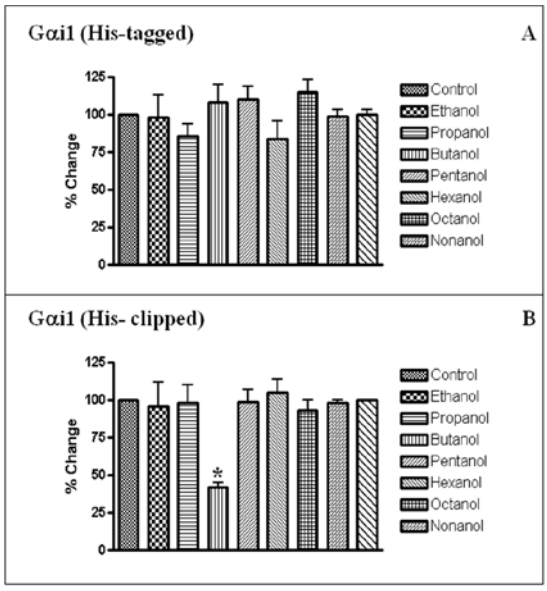

Figure 1: Effects of n-alkanols on GDP/GTPγS exchange from Gαi1 subunit: Gαi1 subunit (His tagged and His tagged clipped) was preloaded with unlabeled GDP as described in Materials and Methods. Samples were diluted into buffer containing [^{35}S]GTPγS and incubated for 15 min at 30°C, a time at which the exchange of nucleotides in the absence of alcohol was approximately 50% complete. N-alkanols at a concentration of 0.05 mM are added to the reaction mixture and the rate reaction was terminated at 30 min with ice-cold stop buffer. The exchange of GTPγS for GDP was determined by trapping the protein on nitrocellulose filters and counting the radioactivity bound to protein. Values are % of control (Gαi1 subunit incubated without alcohol). Each value is mean + SE of at least three experiments assayed in duplicate. * Significant at $p<0.05$.

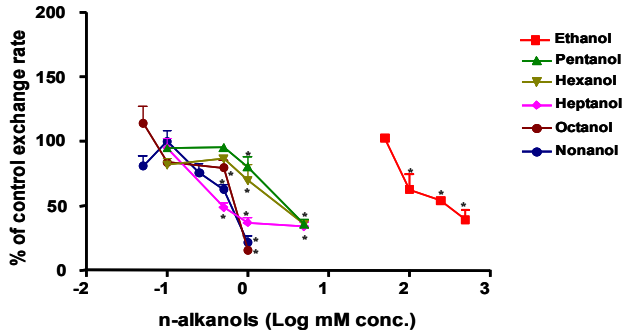

Figure 2: Effects of n-alkanols on GDP/GTPγS exchange from Gαo subunit: Gαo subunit was preloaded with unlabeled GDP as described in Materials and Methods. The exchange of GTPγS for GDP was determined by trapping the protein on nitrocellulose filters and counting the radioactivity bound to protein. Values are % of control (Gαo subunit incubated without alcohol). Each value is mean + SE of at least three experiments assayed in duplicate. * Significant at p<0.05.

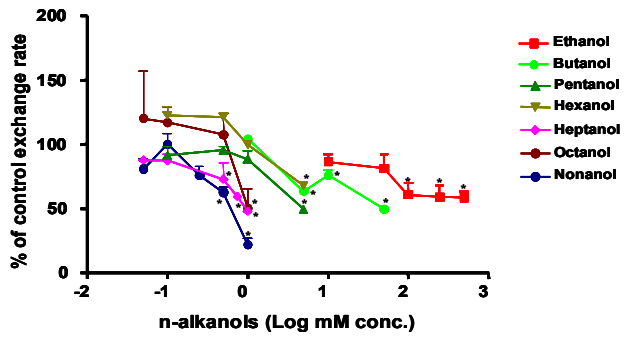

Figure 3: Effects of n-alkanols on GDP/GTPγS exchange from Gαi-2subunit: Gαi2 subunit was preloaded with unlabeled GDP as described in Materials and Methods. The exchange of GTPγS for GDP was determined by trapping the protein on nitrocellulose filters and counting the radioactivity bound to protein. Values are % of control (Gαi2 subunit incubated without alcohol). Each value is mean + SE of at least three experiments assayed in duplicate. * Significant at p<0.05.

binding within the cavity of the Gαo and Gαi2 might be smaller than the volume occupied by long chain alkanols. It is interesting to note that this is on the order of the size of halothane and isofluorane, which are commonly used anesthetics. Long chained alkanols (C5 and beyond), which occupy a much larger volume do not attach to these binding sites. However, these volatile anesthetics still contribute to changes in the rate of GTP-GDP exchange. Therefore, these long chained alkanols must have an affinity for a different binding site. The site of long chain alkanol binding is most likely on the outer surface of the protein, and not within a cavity of the three-dimensional protein – based on crystallographic studies [21]. Clearly more systematic variations in protein, GDP and GTPγS concentrations will be required to completely understand this equilibrium exchange reaction and the effects of alkanols.

Differences in hydrophobicity of individual anesthetic agents alone cannot account for the resistance of Gαo protein subunits to haloalkanes and ethers [16]. The relative resistance to intermediate (C3 - C5) and long chain n-alkanols (C5 and beyond) by Gαi1, Gαi2 and Gαo at concentrations less than 100 uM in this study implies that hydrophobicity is not the sole feature of the agents that disrupt GDP/GTP exchange, in keeping with the overall correlation between anesthetic potency and Gα function. At higher concentrations of more than 100 uM, n-alkanols were found to inhibit the rate of GDP/GTP exchange by G-protein subunits. The results suggest the presence of one or more functional binding sites for the short chain n-alkanols. Whether they share such sites remains to be determined. Our results suggest that various anesthetic compounds interact differentially with different Gα alpha subunits and this interaction might depend upon their conformational state in signaling. Therefore, G-proteins remain a viable part of a more complex and, perhaps, more realistic theory of being the targets for n-alkanols.

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