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Deuteration as a tool in investigating the role of protons in cell signaling

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ABSTRACT

Background: The mechanisms underlying the inhibitory effects of deuterium oxide (D_2O; heavy water) are likely to provide insight into the fundamental significance of hydrogen bonds in biological functions. Previously, to begin elucidating the effect of D_2O on physiological functions in living cells, we studied the effects of D_2O on voltage-sensitive Ca^{2+} channels in AtT 20 cells and showed that actin distribution, Ca^{2+} currents, and β-endorphin release were affected. However, the molecular mechanisms underlying the inhibitory effects of D_2O in whole animals and living cells remain obscure, especially in the effects of D_2O on the cell signaling.

Methods: We investigated the molecular mechanisms underlying the inhibitory effects of D_2O on the IP_3-mediated Ca^{2+} signaling pathway using Ca^{2+} imaging and micro-calorimetric measurements in mGluR1-expressing CHO cells.

Results: DHPG-induced Ca^{2+} elevations were markedly reduced in D_2O. Moreover, the Ca^{2+} elevations were completely suppressed in H_2O after receptor activation with DHPG in D_2O, recovering gradually in H_2O medium. Without prior stimulation in D_2O, however, DHPG-induced Ca^{2+} elevations in H_2O were not affected. Micro-calorimetric measurements showed reduced total DHPG-evoked heat generation in D_2O, while initial heat production and absorption associated with receptor activation were found to be larger. The reduction of DHPG-induced Ca^{2+} elevation and heat generation in D_2O medium may be due to decreased amount of IP_3 by the reduced hydrolysis of PIP_2.

General significance: Protein structure changes due to the replacement of hydrogen with deuterium will induce the inhibitory effects of D_2O by reduction of the frequency of –OH bonds.

1. Introduction

For all types of living systems, evidence indicates that water is a ubiquitous and critical element. Both hydrophilic and hydrophobic interactions between cellular components have important roles in the establishment of micro- and macro-molecular structures and physiological function [1].

In general, water is a mixture of nine stable isotope forms; natural water is 99.7% H_2O and contains about 145 ppm of D_2O. Water in mammalian tissue contains 5–6 ppm or higher D_2O. Commercially available heavy water can be 99.8% or more D_2O [2].

Deuteration (substitution of H_2O with D_2O) has been used successfully as a nondestructive method to study the involvement of water in both the cytoskeletal structure and the molecular mechanisms of various functions in living cells [3–5]. Deuteration induces a number of observable effects that can be useful in understanding the contribution of –OH groups to biological function. Over the past four decades, the biological effects of D_2O have been reported in whole animals, animal cells, microorganisms, and macromolecules [1]. It is widely believed that D_2O has deleterious effects on whole organisms leading to death or potentially suppresses tumor genesis at specific concentrations [6–8], even though the chemical characteristics of D_2O are quite similar to H_2O [3–5]. Studies of the toxic effects of D_2O, however, are rarely conducted currently and the proposed mechanism so far is scattered largely, such as hydrophobic bond formation [9], stabilizing hydrophobic bonds [10], tubulin polymerization [11], persistence in phosphatidylinositol (PI) turnover and calcium movement [12], and accelerate actin assembly [13].

In our previous work examining the mechanism underlying the inhibitory effects of D_2O, we found that 90% (v/v) D_2O buffer increased the amount of actin filament in the cell soma and decreased it in cell processes, whereas β-tubulin was not affected in AtT 20 cells [14]. Associated with this, Ca^{2+} imaging demonstrated that high-K^+-induced Ca^{2+} entry was completely blocked when D_2O medium was replaced

Abbreviations: D_2O, deuterium oxide; DHPG, S-3,5-dihydrophenylglycine; IP_3, inositol trisphosphate; mGluR1, metabotropic glutamate receptor type 1; PLC, phospholipase C; PIP_2, phosphatidylinositol 4,5-bisphosphate

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again with H_2O medium. Furthermore, use of deuterated internal solution in whole-cell patch electrodes reduced Ca^{2+} currents that recovered when the extracellular solution was also deuterated, suggesting that the D_2O gradient across the plasma membrane is critical to Ca^{2+} channel kinetics. Radioimmunoassay revealed that high-K^+-induced β-endorphin release increased during D_2O treatment and decreased upon D_2O wash out. We concluded that fast H to D exchange (H/D) and slow D to H exchange (D/H) in the protein may contribute to these effects described here, including the hysteresis effect on Ca^{2+} entry [14].

Generally, it is well known that there are two independent physiological effects of D_2O. In the solvent isotope effect, the motility of monovalent cations is reduced by about 20%, whereas the dielectric constant is unchanged [15].

As a solvent, D_2O increases the stability of soluble proteins, likely by increasing hydrophobic bond formation, thereby influencing their functionality [10,16]. D_2O is also proposed to bind hydrogen in proteins [17], although the enthalpic and entropic effects of D_2O appear to have little overall effect in some cases [18]. The effects of D_2O on nerve conduction and muscle contraction have been extensively studied, and recently research has focused on functional proteins in the plasma membrane: Na^+–K^+ ATPase, Na^+–H^+ antiporter, and voltage-dependent Ca^{2+} channels [19–22]. These effects have been attributed to the solvent isotope effect.

Another effect is the isotope exchange effect on functional proteins, also known as the H/D exchange effect [23]. When cells or molecules are exposed to D_2O, the H/D exchange effect, deuteration of N–H, S–H, and O–H bonds occurs rapidly [24]. In short, an effect in which switching from H to D is reversible is likely due to the solvent isotope effect. In contrast, an effect that is fast with H–D substitution, but slow with D–H substitution is likely to be due to the H/D exchange effect [24].

In 1960, Gross and Spindel demonstrated that the percentage of fertilized sea urchin eggs decreases linearly with increased D_2O content (10% to 70%) in the external medium [25]. Despite increasing data, the mechanism underlying D_2O-induced sterility remains unknown. Recent evidence established that fertilization involved inositol-trisphosphate (IP_3)-mediated Ca^{2+} elevation [26,27], raising the possibility that D_2O has an inhibitory effect on this intracellular signaling pathway. If this is the case, D_2O may affect many cellular functions via disruption of this ubiquitous signaling pathway.

In the present study, we compared the effect of D_2O with H_2O on the IP_3-mediated Ca^{2+} signaling pathway using Ca^{2+} imaging and micro-calorimetric measurements in mGluR1-expressing CHO cells.

2. Materials and methods

2.1. Chemicals

In the present report, the term “ordinary water” refers to H_2O, while “heavy water” refers to water with a content of at least 99.8% D_2O molecules. DHPG was purchased from Tocris Cookson (Bristol, UK). U73122 was purchased from Calbiochem (San Diego, CA). Fura-2 free acid and thapsigargin were purchased from Molecular Probes (Eugene, OR). Fura-2-AM was supplied by Dojindo (Kumamoto, Japan). D_2O was purchased from CDN isotopes (Quebec, Canada). All other reagents were purchased from Wako Pure Chemicals (Osaka, Japan), were of analytical grade, and were used without further purification.

2.2. Fura-2 fluorescence spectra

Excitation spectra for fura-2 were measured in Ca^{2+}/EGTA buffers prepared with H_2O and 90% D_2O. Fura-2 free acid (2 μl) was diluted in 2 ml of the buffers to a final concentration of 1 μM and excited at 300 to 400 nm (band width 2.5 nm), and the emission was monitored at 510 nm (band width 2.5 nm) using a fluorescence spectrometer (Hitachi F-2500, Tokyo, Japan). Free [Ca^{2+}] in the buffers was attained using the titration method described previously [28]. Briefly, the titration was performed starting with 2 ml of 100 mM KCl, 10 mM K_2H_2EGTA, 1 μM fura-2, and 10 mM K-MOPS (pH = 7.3). Discarding 0.2 ml of this solution and replacing it with 0.2 ml of 100 mM KCl, 10 mM K_2Ca^{2+} EGTA, 1 μM fura-2, and 10 mM K-MOPS (pH = 7.3) resulted in 9 mM K_2H_2EGTA and 1 mM K_2Ca^{2+}EGTA in the solution. Subsequent iterations to reach n mM Ca^{2+}EGTA and (10–n) mM EGTA (n = 2 to 10) were performed by discarding $2/(11-n)$ ml and replacing with an equal volume of the Ca^{2+}-containing stock solution (10 mM Ca^{2+}). For the calculation of $[Ca^{2+}]_i$, 151 nM was used as the apparent dissociation constant between Ca^{2+} and EGTA in 100 mM KCl (pH = 7.2) at 20 °C, leading to the equation; $c = 151n/(10-n)$, where c and n denote free Ca^{2+} concentration (nM) and total Ca^{2+} concentration (mM), respectively.

2.3. Cell culture

Wild-type CHO cells and mGluR1-expressing CHO cells were a generous gift from Prof. S. Nakanishi at Kyoto University. They were cultivated in a high glucose minimal essential medium supplemented with 10% fetal bovine serum (Gibco BRI, Grand Island, NY). Cells were grown to confluence in a monolayer culture in tissue culture flasks in a humid air, 5% CO_2 atmosphere incubator at 37 °C. For Ca^{2+} imaging experiments, the cells were plated on ϕ35-mm glass-bottomed culture dishes and further incubated for 2 days in the CO_2 incubator before use.

2.4. Ca^{2+} imaging

CHO cells were rinsed twice with BSS (130 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM D-glucose, and 10 mM HEPES, pH = 7.4; Sigma Chemical Co., St Louis, MO). The cells were loaded with 5 mM fura-2-AM in BSS and incubated for 30 min at room temperature (24–26 °C) before washing three times with BSS. The cells were allowed to stabilize for 30 min in BSS prior to the experiment. The cells were placed on a fluorescence microscope (Axioplan2; Zeiss, Germany), continuously perfused with BSS (2 ml/min), and observed using a 60× objective lens (0.9 NA). Fura-2 was excited by alternately applying 0.3-s UV light flashes at 340 and 380 nm (100 W Xenon lamp) using a wheel-type filter changer (Hamamatsu Photonics C4312; Hamamatsu, Japan) and fluorescence images (512 × 512 pixels) at 510 nm were collected every 3.6 s using a high-speed cooled-CCD camera (Hamamatsu Photonics C4880) equipped with an intensifier (Hamamatsu Photonics C2400-80) and stored on a personal computer using Argus-50 software (Hamamatsu Photonics). $[Ca^{2+}]_i$ was estimated by normalization with calibration analysis using the Argus-50 software using a calibration curve for H_2O or 90% D_2O (Fig. 1A).

2.5. Micro-calorimetry

Micro-calorimetric measurements were performed at 30 °C using a VP-ITC micro-calorimeter (Microcal Inc., Northampton, MA) using the isothermal titration method [29,30]. CHO cells were washed twice with BSS, suspended in oxygenated H_2O- or 90% D_2O-BSS (0.5×10^5 to 2×10^5 cells/ml), and placed in the measurement chamber (1.35 ml). They were allowed to equilibrate for 10 to 20 min with mild stirring before starting experiments. After collecting initial heat flux measurements for 1 min, DHPG was injected into the measurement chamber within 10 s (15 μl stock solution, 2.7 mM in BSS) and the heat flux was measured every second. The heat flux measured using wild-type CHO cells was considered to be baseline and was subtracted from the heat flux obtained using mGluR1-expressing CHO cells to yield the mGluR1-mediated heat flux. This controlled for the dilution effect of