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$GABA_A$ergic stimulation modulates intracellular protein arginine methylation

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HIGHLIGHTS

- The benzodiazepine subtype-$2\alpha_1 2\beta_2 1\gamma_2$ is expressed in P19 cells.
- Stimulating $GABA_A$ergic pathway produces an intracellular alkalinization.
- Stimulating $GABA_A$ergic pathway modulates downstream PRMT activity.
- The $GABA_A$ergic mediated PRMT activation is impaired in a mouse model of FXS.

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ABSTRACT

Changes in cytoplasmic pH are known to regulate diverse cellular processes and influence neuronal activities. In neurons, the intracellular alkalization is shown to occur after stimulating several channels and receptors. For example, it has previously demonstrated in P19 neurons that a sustained intracellular alkalinization can be mediated by the Na^+/H^+ antiporter. In addition, the benzodiazepine binding subtypes of the $GABA_A$ receptor mediate a transient intracellular alkalinization when they are stimulated. Because the activities of many enzymes are sensitive to pH shift, here we investigate the effects of intracellular pH modulation resulted from stimulating $GABA_A$ receptor on the protein arginine methyltransferases activities. We show that the major benzodiazepine subtype ($2\alpha_1$, $2\beta_2$, $1\gamma_2$) is constitutively expressed in both undifferentiated P19 cells and retinoic acid differentiated P19 neurons. Furthermore stimulation with diazepam and, diazepam plus muscimol produce an intracellular alkalinization that can be detected ex vivo with the fluorescence dye. The alkalinization results in significant perturbation in protein arginine methylation activity as measured in methylation assays with specific protein substrates. Altered protein arginine methylation is also observed when cells are treated with the $GABA_A$ agonist muscimol but not an antagonist, bicuculline. These data suggest that pH-dependent and pH-independent methylation pathways can be activated by $GABA_A$ergic stimulation, which we verified using hippocampal slice preparations from a mouse model of fragile X syndrome.

1. Introduction

The maintenance of intracellular pH is crucial for cell survival [12]. Accordingly; cells have several redundant means of sensing and regulating intracellular pH [3]. Among the most prominent sentinels are several types of ion transporters and receptors.

The Na^+/H^+ antiporter is shown to constitutively expressed in embryonal carcinoma P19 cells, and the retinoic acid induced P19 neurons differentiation is coupled to downstream effectors by PRMT activity [20]. Thus, a variety of PRMT substrates exhibit a unique pH dependence, which can be altered by NH_4Cl-mediated depolarization. Indeed, applying NH_4Cl to P19 cells in vitro results in sustained intracellular alkalinization which can be easily monitored using pH sensitive fluorescent dyes such as SNARF-1AM [20].

Abbreviations: PRMT, protein arginine methyltransferase; GABAγ, -amino butyric acid; SAH, S–adenosyl–homocysteine; KO, knock-out; FXS, fragile X syndrome; WT, wild-type; RA, retinoic acid.

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Signaling through GABA results synaptic inhibition and the receptors that mediate this inhibition are the ionotrophic $GABA_A$ receptors. Under certain conditions these receptors can produce a transient intracellular alkalinization. This occurs when specific subtypes of the receptor that respond to benzodiazepams assemble into functional receptors. For instance the subtype containing two α_1 subunits, two β_2 subunits, and one γ_2 subunit, the major subtype found in brain [14] is one example. But other receptor subtypes containing the γ_2 *subunit*, which is required for forming the benzodiazepine binding pocket, can also mediate this type of signaling. Thus, adding diazepam to hippocampal slices that had been previously stimulated result in a marked increase in the magnitude and extent of intracellular alkalinization [1].

P19 neurons are shown to produce functional $GABA_A$ [5,16] receptors. To test the hypothesis that the PRMT activities are modulated by $GABA_A$ receptor mediated intracellular alkalinization, we investigated whether $GABA_A$ agonist/antagonists were coupled downstream to protein arginine methylation using P19 cells and hippocampus slide preparation. The results of these studies, reported here suggest that $GABA_A$ receptors are linked to protein arginine methylation by pH-dependent and pH-independent pathways.

2. Materials and methods

2.1. Buffers and chemicals

HMTase Buffer is prepared as described [9]. PBS and SNARF-1AM were purchased from Invitrogen. Protease inhibitors were purchased from Roche. Muscimol and bicuculline were obtained from Ascent. Diazepam, and SAH were obtained from Sigma. [^{3}H]-*S*-Adenosyl-L-methionine 15 Ci/mmol was purchased from MP Biomedical, Inc.

2.2. Proteins, peptides and antibodies

Histone H3 and H4 were purchased from Roche Applied Biosciences. Myelin basic protein (mouse) was obtained from Sigma. NOLA1 was purchased from GenWay Biotech, Inc. Biotinylated SmD1 peptide, biotin-KREAVAGRGRGRGRGRGRGRGRGGPRR, was synthesized by CPC Scientific. The antibody to the β subunit of the $GABA_A$ receptor, anti-dimethylarginine antibody SYM10 pAb, and anti-Histone H4(R3)^{SMe2} pAb were purchased from Millipore. The antibody to the α_1 subunit of the $GABA_A$ receptor was purchased from GenWay Biotech, Inc. Anti-Hsp70c mAb was obtained from Stress-Gen. Anti-MAP2 antibody was from Santa Cruz, and anti-PAPB mAB was obtained from Cell Signaling.

2.3. Cell culture

P19 cells were cultured as described [19]. Differentiation of rapidly growing cultures into neuronal and glial cells was accomplished accordingly [13]. All of the results presented here correspond to cells cultured for 6–8 days in the presence or absence of 0.1 μM RA.

2.4. Mice and hippocampal slices

WT and fragile X KO mice were obtained from the fragile X animal colony at the New York State Institute for Basic Research (IBR) [21]. WT control animals were C57X FVB. The animals were treated in accordance with the principles and procedures of the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The mice were euthanized by CO_2 asphyxiation as instructed by the local Institutional Animal Care and Use Committee at IBR.

Hippocampal slices were prepared as described [6]. The slices were treated or not treated with 10 μM diazepam and 100 μM muscimol for 15–30 min and subsequently harvested for protein or RNA.

2.5. RNA isolation and RT-PCR

RNA was extracted from P19 cells or mouse tissues using RNAeasy mRNA mini columns (Qiagen). One microgram of RNA was used to prepare first strand cDNA (Invitrogen). Aliquots of the cDNAs were amplified using the following primers: $GABA_A$–α_1, F-5′AGCTATACCCCTAACTTAGCCAGG and R-5′AGAAAGCAATTCTCGGTGCAGAGGAC; $GABA_A$-β_2, F-5′TGAGATGGCCACATCCGAAGCAGT and R-5′CTCACGGAAGGCTGTAGTTTAGTTCA; $GABA_A$–γ_{2S}, F-5′AAGAAAAACCCTGCCCCTACCATTG and R-5′GTCTCCATAAGATTCAGCGAATAAC. Primers are based on the sequences of mouse $GABA_A$ receptor subunits α_1, β_2 and γ_{2S}; GenBank accession numbers NM_012050, NM_008070, NM_177408, respectively. Reaction products were resolved on agarose gels along with appropriate size markers.

2.6. Western blotting and immunofluorescence microscopy

Proteins were prepared from cultured cells or mouse tissues for Western blotting as described [18]. The blots were detected by primary antibody SYM10 or Hsp70c and corresponding HRP-conjugated secondary antibody (Pierce), and developed using the PicoTag system (Pierce).

Cells were processed for immunohistochemistry as described [19]. The cells were incubated with designated primary antibody and then with secondary antibody (Alexa Fluor 488 or Alexa Fluor 568, Molecular Probes). Fluorescence was detected with an Eclipse 90i dual laser-scanning confocal microscope (NIKON). Images were acquired at 20–40× magnification Equivalent regions of interest (ROI) within each image are presented.

2.7. In vitro methylation assays

In vitro methylation by the endogenous methyltransferases (MTs) of the proteins in cell lysates or various substrate proteins was performed as described [20], so was the *in vitro* methylation of the biotinylated-SmD1 peptide [20].

2.8. pH-Dependent activation of P19 cells

Retinoic differentiated P19 cells were loaded with the pH-sensing fluorescent dye SNARF-1AM, as described [20]. The cells were treated or not treated with diazepam (10 μM), musicmol (100 μM), bicuculline (60 μM) or combination of them. The images were captured and the results were computed as described [20].

2.9. Satirical analysis

One-way ANOVA and student Newman–Keuls post-hoc testing were used.

3. Results

3.1. Mouse P19 cells express functional $GABA_A$ receptors

PRMT in embryonic P19 cells is shown to be modulated by intracellular alkalinization occurring through the Na^+/H^+ antiporter [20]; however there are other receptors whose activation can alter intracellular alkalinization. One of these is the $GABA_A$ receptor [1]. To determine whether $GABA_A$ergic stimulation also results in altered PRMT-dependent methylation we first determined whether

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