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Research report

Mechanism of noradrenaline-induced α1-adrenoceptor mediated regulation of Na-K ATPase subunit expression in Neuro-2a cells

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ABSTRACT

Rapid eye movement sleep (REMS) plays an important role in maintaining brain excitability by regulating noradrenaline (NA) level and Na-K ATPase activity. We showed earlier that REMS deprivation (REMSD) associated elevated NA increased neuronal, while decreased glial Na-K ATPase activity. However, our knowledge was insufficient on how the REMSD-associated effect is sustained particularly under chronic condition. Using Neuro-2a cells as a model, we investigated the molecular mechanism of NA-induced increase in mRNA expression of Na-K ATPase subunit and the enzyme activity. The cells were treated with NA in the presence or absence of either α1- or β-adrenoceptor (AR) antagonists, Ca^{++}-channel blocker or SERCA-inhibitor, and PKA or PKC inhibitor. We observed that NA acting on α1-AR increased Na-K ATPase activity and mRNA expression of the catalytic α1- and α3-Na-K ATPase subunits in the Neuro-2a cells. Further, PLC and PKC mediated modulation of intracellular Ca^{++} played a critical role in inducing the mRNA expression. On the other hand NA, acting on β-AR up-regulated expression of the regulatory β1-subunit of Na-K ATPase. The involvement of SP1 as well as phospho-CREB transcription factors in the NA-mediated increased expression of various subunit isoforms was established. The results of this study along with that of earlier reports support our proposed working model of NA-induced increase in mRNA expression of specific Na-K ATPase subunit leading to increased Na-K ATPase activity. The findings help us understand the molecular mechanism of NA-induced increased brain excitability, for example, upon REMSD including under chronic condition.

1. Introduction

The brain integrates divergent inputs from far and near to control most functions of the body directly or indirectly. The excitable property of the neurons is critically responsible for instantaneous as well as optimum functioning of the brain. The enzyme Na-K ATPase plays a pivotal role in maintaining neuronal transmembrane potential gradient and excitability (Larsen et al., 2016). Several behaviours and factors affect the Na-K ATPase activity and thus, modulate the brain excitability (Kaphzan et al., 2013; Lingrel et al., 2007; McCarren and Alger, 1987). Under normal condition rapid eye movement sleep (REMS), an essential cognitive state, maintains brain excitability by modulating the Na-K ATPase activity (Gulyani and Mallick, 1993; Mallick et al., 1999). Consistent studies showed that the REMS maintains brain level of noradrenaline (NA), which modulates the brain Na-K ATPase activity and brain excitability (Khanday et al., 2016; Mallick et al., 2000; Mallick and Adya, 1999). These findings led us to propose that normally REMS maintains brain excitability and thus, serves house-keeping function of the brain (Mallick and Singh, 2011).

The Na-K ATPase actively exchanges Na^+/K^+ across the plasma membrane in all mammalian cells, including the neurons and is responsible for maintenance of transmembrane electrical potential gradient. It plays a critical role in executing normal neuronal activity and function (Mobasheri et al., 2000). The Na-K ATPase is a transmembrane tetrameric protein consisting two each of α- and β-subunits. The α-subunit is responsible for the catalytic and transport properties of the enzyme, while the β- is regulatory subunit. Both, the α- and β-subunits of Na-K ATPase exist in multiple isoforms, which are encoded by different genes and follow developmental as well as tissue specific expression patterns (Lingrel et al., 2003; Orlowski and Lingrel, 1988; Sweadner, 1989). Although α1-, α3- and β1-subunits are usually expressed in all neuronal cells (Juhaszova and Blaustein, 1997), α2-isoform has been reported to be expressed by some neuronal cells only (Moseley et al., 2007). The existence of isoforms with varying kinetic properties and expression patterns suggests their specific role.

The activity of an enzyme may be regulated allosterically as well as transcriptionally. We have consistently studied REMS deprivation (REMSD) associated NA mediated allosteric regulation of Na-K ATPase

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activity in neurons and glia in vivo and in vitro (Amar and Mallick, 2015; Amar et al., 2016; Baskey et al., 2009; Mallick and Singh, 2011). However, for sustained effects, including upon REMSD, the synthesis of the enzyme is likely to be modulated. As the brain contains neurons and glia, we needed to study the effect of NA on both those cells. We have recently reported the effect of NA on glial Na-K ATPase activity and mRNA expressions (Amar et al., 2017). For a better understanding of REMSD associated NA-mediated altered functioning of the brain and behaviour, in this study we explored the molecular mechanism of NA-induced regulation of neuronal Na-K ATPase subunit expression. We have analysed the involvement of adrenergic receptors (AR), second messenger(s) and specific transcription factors in NA-induced regulation of Na-K ATPase subunits expression. We used Neuro-2a cells as a model system as a practical approach for convenience to exclude the influence of obvious, unavoidable confounding factors encountered while conducting in-vivo studies. The use of Neuro-2a in this study may be supported by the facts that these are rodent (mouse) c1300-neuroblastoma derived cells (Klebe and Ruddle 1969), possess Na-K ATPase and AR (Baskey et al., 2009; Kimelberg, 1974; Manthey et al., 2010). Also, due to the presence of several neuronal properties, Neuro-2a cells have been extensively used for studying neuronal biology including differentiation, axonal growth, signalling and functions (Dasgupta and Milbrandt, 2007; Evangelopoulos et al., 2005; Furmanski et al., 1971).

2. Material and methods

2.1. Chemicals

Tris-Base, HEPES, noradrenaline (NA); α1-AR antagonist, prazosin (PRZ); β-AR antagonist, propranolol (PRN); adenosine tri-phosphate (ATP); Na-K ATPase inhibitor, ouabain; Dulbecco's Modified Eagle's Media (DMEM); N, *N*-dimethyl acetamide (N,N-DA) and dimethyl sulfoxide (DMSO); protease inhibitor cocktail; Penicilline-Streptomycin solution; L-type Ca^{++}-channel blocker, nifedipine (Nif) and sarcoendoplasmic reticulum calcium transport ATPase (SERCA)-inhibitor, cyclopiazonic acid (CyA) were procured from Sigma-Aldrich, U.S.A. Cell permeable cyclic adenosine monophosphate (cAMP) analogue, dbcAMP; cell permeable protein kinase-A (PKA) inhibitor, H89 and protein kinase-C (PKC) inhibitor, chelerythrine chloride (CC), phospholipase C (PLC) antagonist, U73122 were obtained from Tocris, U.S.A. Trizol® reagent; RNA-zap; TURBO DNA-free kit; power SYBER-PCR master mix; Superscript-III first strand cDNA synthesis kit; Fetal Bovine Serum (FBS) were procured from Life Technologies, U.S.A. Tricholoroacetic acid (TCA) and other chemicals used were of analytical grade.

2.2. Cell culture studies

Neuro-2a cell line, procured from National Centre for Cell Sciences, Pune, India, were cultured in DMEM, supplemented with 10% heat inactivated FBS and 1% antibiotic mix (penicillin/streptomycin). The cells were maintained in a humidified atmosphere containing 5% CO_2 at 37 °C in an incubator (Thermo Forma, USA). Cells at 70–80% confluency were used in all experiments and kept in serum free condition for 6 h before treatments.

2.3. Cell viability assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)-assay was conducted to test the cytotoxicity of NA on Neuro-2a cells. The cells were seeded in 96-well culture plates at a density of 2×10^4cells/well and treated with 1, 10, 50, 100, 250, 500 and l000 μM of NA for 12 h. After treatment, the culture medium was replaced with a medium containing 20 μl MTT (5 mg/ml dissolved in phosphate buffered saline (PBS), pH 7.2) for 4 h at 37 °C and 5% CO_2. After 4 h, the formed crystals were dissolved in 200 μl DMSO. Absorbance in each well was read at 570 nm using a microplate reader (BioTek Power Wave XS) and corrected by subtracting the absorbance of blank readings. The values were expressed as percentage of absorbance with reference to untreated cells (mean ± SEM).

2.4. Treatments with NA and agonists/antagonists of downstream signalling molecules

For studying the involvement of specific-AR, the cultured Neuro-2a cells were treated with NA (100 μM) for 12 h in the presence and absence of PRZ (50 μM) or PRN (50 μM). To study the role of the second messenger, cAMP, and PLC the cultured cells were treated with dbcAMP (750 μM) and antagonist of PLC, U73122 (10 μM) for 12 h and to check the involvement of downstream target PKC, Neuro-2a cells pre-treated with CC (10 μM) were treated with NA for 12 h. Suitable vehicle controls were carried out whenever DMSO (0.10%) was used as a solvent to dissolve various antagonists.

2.5. Membrane preparation from cell-culture

The membrane fractions from the Neuro-2a cells were prepared essentially following the method described earlier (Amar et al., 2017; Lai et al., 2013). Briefly, 1×10^7 Neuro-2a cells were used for membrane fraction isolation. Confluent monolayers from control and treated cells grown in 100 mm petri dishes (Corning, U.S.A) were washed twice with ice cold phosphate buffered saline (PBS) and harvested by scraping. The cells were pelleted at 1000*g* for 5 min. The cell pellet was then re-suspended in 500 μl ice cold homogenizing buffer A (containing 50 mM HEPES, 10 mM KCl, protease inhibitor cocktail) and passed 10 times through 26G needle attached to 1 ml syringe. The suspension was maintained on ice for 10 min and after centrifugation (1000*g* for 10 min) the post-nuclear fraction was further centrifuged (45,000*g* for 45 min) to get crude membrane. The crude membrane pellet was re-suspended in buffer B (0.32 M sucrose, 12 mM Tris, 1 mM EDTA, 0.1% Triton-X 100 and protease inhibitor cocktail, pH 7.4) and protein concentration was determined by Bradford method (Bradford, 1976).

2.6. Estimation of Na-K ATPase activity

Ouabain sensitive Na-K ATPase activity in the membrane prepared from cultured Neuro-2a cells was estimated as reported earlier (Amar and Mallick, 2015; Mallick et al., 2000). The effect of NA on Na-K ATPase activity in the presence and absence of PRZ or PRN was estimated. The reaction mixture contained 100 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 3 mM ATP and 50 mM Tris (pH7.4). An aliquot (30–40 μg protein) of the membrane fraction was incubated with the reaction mixture at 37 °C for 20 min and ATP was used as the substrate, while ouabain (1 mM) was used as a specific blocker of Na-K ATPase. The reaction was stopped by adding 1 ml of 10% ice-cold TCA and the mixture was centrifuged at 800*g* for 5 min. The supernatant was collected for estimation of released inorganic phosphate following the method of Fiske and Subbarow (Fiske and Subbarow, 1925) using UV–vis spectrophotometer (Perkin-Elmer, U.S.A). The released phosphate was an estimate of ouabain sensitive Na-K ATPase activity and has been expressed as micromoles of Pi released/mg protein/h.

2.7. RNA isolation and qPCR

Total RNA from the control and treated Neuro-2a cells was extracted using Trizol. The contaminating DNA was removed from the isolated RNA by DNase treatment using TURBO DNA-free Kit. Equal (1 μg) amount of total RNA was reverse transcribed by Superscript-III first strand cDNA synthesis kit. qPCR for different subunit isoforms of Na-K ATPase was performed with power SYBR-PCR master mix. Equal amount (1 μg) of cDNA, gene specific primers (Table 1) and master mix were subjected to qPCR using the ABI Prism 7500 FAST Real-Time PCR