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THE DENSE CORE VESICLE PROTEIN IA-2, BUT NOT IA-2β, IS REQUIRED FOR ACTIVE AVOIDANCE LEARNING

G. N. CARMONA,[a] T. NISHIMURA,[a] C. W. SCHINDLER,[b] L. V. PANLILIO[b] AND A. L. NOTKINS[a]*

[a] *Experimental Medicine Section, Laboratory of Sensory Biology Branch, National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, USA*

[b] *Preclinical Pharmacology Section, Behavioral Neuroscience Research Branch, National Institute on Drug Abuse, National Institutes of Health, Baltimore, MD, USA*

Abstract—The islet-antigens IA-2 and IA-2β are major autoantigens in type-1 diabetes and transmembrane proteins in dense core vesicles (DCV). Recently we showed that deletion of both IA-2 and IA-2β alters the secretion of hormones and neurotransmitters and impairs behavior and learning. The present study was designed to evaluate the contribution to learning of each of these genes by using single knockout (SKO) and double knockout (DKO) mice in an active avoidance test. After 5 days of training, wild-type (WT) mice showed 60–70% active avoidance responses, whereas the DKO mice showed only 10–15% active avoidance responses. The degree of active avoidance responses in the IA-2 SKO mice was similar to that of the DKO mice, but in contrast, the IA-2β SKO mice behaved like WT mice showing 60–70% active avoidance responses. Molecular studies revealed a marked decrease in the phosphorylation of the cAMP response element-binding protein (CREB) and Ca^{2+}/calmodulin-dependent protein kinase II (CAMKII) in the striatum and hippocampus of the IA-2 SKO and DKO mice, but not in the IA-2β SKO mice. To evaluate the role of CREB and CAMKII in the SKO and DKO mice, GBR-12909, which selectively blocks the dopamine uptake transporter and increases CREB and CAMKII phosphorylation, was administered. GBR-12909 restored the phosphorylation of CREB and CAMKII and increased active avoidance learning in the DKO and IA-2 SKO to near the normal levels found in the WT and IA-2β SKO mice. We conclude that in the absence of the DCV protein IA-2, active avoidance learning is impaired. © 2014 Published by Elsevier Ltd. on behalf of IBRO.

Key words: autoantigens, type-1 diabetes, dopamine, CREB, CAMKII.

INTRODUCTION

The insulinoma-associated proteins IA-2 and IA-2β, also known, respectively, as ICA512 and phogrin, are transmembrane proteins of dense core vesicles (DCV) and are found in neuroendocrine cells throughout the body (Lan et al., 1996; Lu et al., 1996; Takeyama et al., 2009). Based on sequence analysis both are members of the receptor-like protein tyrosine phosphatase (PTP) family, but are enzymatically inactive on standard PTP substrates because of two critical amino acid substitutions in the PTP domain (Lan et al., 1994; Magistrelli et al., 1996). Recently, however, IA-2β was shown to have low phosphatidylinositol phosphatase activity (Caromile et al., 2010). Structurally IA-2 and IA-2β consist of an intracellular, transmembrane and luminal domain and show 74% identity in their intracellular domain, but only 26% identity in their luminal domain. IA-2 is 979 and IA-2β 986 amino acids in length. In humans, the genes for IA-2 and IA-2β are located, respectively, on chromosomes 2q35 and 7q36 and in mice on chromosomes 1 and 12 (Leiter et al., 1997; Saeki et al., 2000).

IA-2 and IA-2β have been of particular interest to the diabetes community because both are major autoantigens in type 1 diabetes (Notkins, 2007; Achenbach et al., 2008). Autoantibodies to these proteins appear years before the onset of clinical disease and in combination with other diabetes-associated autoantibodies have become predictive markers for this disease (Notkins, 2007). Studies on the biological function of these proteins by knockout experiments in mice and knockdown and overexpression experiments in neuroendocrine-secreting cell lines have shown that they affect the half-life and, in turn, the number of DCV (Harashima et al., 2005; Cai et al., 2011).

Alterations in the secretion of hormones (e.g. insulin) and neurotransmitters (e.g. dopamine, serotonin, glutamate) (Nishimura et al., 2009), secondary to the decreased expression of IA-2 and IA-2β, result in a variety of abnormalities including alterations in glucose tolerance, reproduction, behavior, learning and circadian

*Corresponding author. Address: National Institutes of Health, Building 30, Room 106, MSC4322, 9000 Rockville Pike, Bethesda, MD 20892-4322, USA. Tel: +1-301-496-4535; fax: +1-301-402-4163.
E-mail address: anotkins@mail.nih.gov (A. L. Notkins).

Abbreviations: ANOVA, analysis of variance; CAMKII, Ca^{2+}/calmodulin-dependent protein kinase II; CREB, cAMP response element-binding protein; CS, conditioned stimulus; DCV, dense core vesicles; DKO, double knockout; EDTA, ethylenediaminetetraacetic acid; GBR-12909, a selective dopamine uptake inhibitor; IA-2 and IA-2β, insulinoma associated proteins; NOD, non-obese diabetic; PTP, protein tyrosine phosphatase; SDS, sodium dodecyl sulfate; SKO, single knockout; UCS, unconditioned stimulus; WT, wild-type.

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rhythm (Kubosaki et al., 2004, 2006; Kim et al., 2009; Nishimura et al., 2009).

Our initial learning and behavior experiments focused primarily on double knockout (DKO) mice in which both IA-2 and IA-2β were knocked out (Nishimura et al., 2009). The present experiments employing genetic, molecular, pharmacologic and behavioral approaches were initiated to determine the effect of the knockout of the individual IA-2 and IA-2β genes on learning and behavior as evaluated by an active avoidance test. These experiments showed that IA-2, but not IA-2β, is required for normal learning in the active avoidance test.

EXPERIMENTAL PROCEDURES

Mice

Targeted disruption of the C57BL6 mouse IA-2 and IA-2β genes was described previously (Saeki et al., 2002; Kubosaki et al., 2004, 2005). The targeted alleles were backcrossed to the C57BL/6J genetic background for eight (IA-2) and four (IA-2β) generations, and heterozygotes were crossed to give double heterozygotes. Double heterozygotes then were interbred to generate lines of wild-type (WT) (IA-$2^{+/+}$/IA-$2\beta^{+/+}$) mice, two lines of single knockout (SKO) mice [IA-2-KO (IA-$2^{-/-}$/IA-$2\beta^{+/+}$) and IA-2β-KO (IA-$2^{+/+}$/IA-$2\beta^{-/-}$)], three-allele mutants (IA-$2^{+/-}$/IA-$2\beta^{-/-}$), and DKO mice (IA-$2^{-/-}$/IA-$2\beta^{-/-}$). Mice used in the current study were generated by breeding animals of the same genotype within each line, except that male IA-$2^{-/-}$/IA-$2\beta^{-/-}$ mice were bred to three-allele female (IA-$2^{+/-}$/IA-$2\beta^{-/-}$) mice to generate DKO mice because female DKO mice are infertile (Kubosaki et al., 2006). IA-$2^{-/-}$/IA-$2\beta^{+/+}$ mice on the non-obese diabetic (NOD) background (IA-2 NOD SKO) were generated as described previously (Kubosaki et al., 2004). Male mice were used in the learning experiments to avoid endocrine changes related to the estrous cycle. All mice were genotyped before use and each mouse was bred, aged and analyzed in the same facility according to National Institutes of Health guidelines. Lights were on from 6:00 AM to 6:00 PM and food and water were available *ad libitum*. Behavioral experiments were generally conducted between 5:00 AM and 3:00 PM. All experimental procedures were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committee.

Active avoidance learning

Training was conducted using a fully automated shuttle-box located in a ventilated and sound-protected procedure room. The shuttle box (47 × 18 × 26 (h) cm) (Ugo Basile, Comerio Italy) was divided into two compartments with an opening between compartments. A light and sound (670 kHz and 70 dB) was used as the conditioned stimulus (CS) and preceded by 3-s unconditioned stimulus (UCS) of an electric foot shock of 0.3 mA. Animals could avoid the UCS by moving from one compartment into the other within 3 s after the onset of the CS. The UCS lasted for a total of 3 s. At the end of this period the trial was terminated and after a 30- s inter-trial interval a new trial was initiated. There were two training sessions per day each consisting of 50 trials. Thus, over the 5 days of the experiment each of the animals underwent 500 trials. The daily number of sessions and trials selected for this study was based on published papers using the active avoidance paradigm as an index of learning in mice (Smith et al., 2002; Trigo et al., 2008). The number of trials on which active avoidance responses were made by each animal when avoiding the UCS and the number of times each animal failed to escape the UCS were recorded by a computer attached to the automated shuttle-box. In treatment experiments designed to restore active avoidance learning, the selective dopamine uptake inhibitor GBR-12909 (20.0 mg/kg) or an equal volume of saline was administered intraperitoneally and the animals were returned to their home cages for 30 min before initiation of active avoidance testing.

Western blotting

Mice were decapitated, their brains were quickly removed, and their hippocampus or striatum were rapidly dissected with the dissecting plate on ice. Samples were immediately homogenized in 10 vol. of ice-cold 80 mM Tris–HCl buffer (pH 7.4) containing 150 mM NaCl, 2 mM EDTA, 0.4 mM DTT and 0.1% sodium dodecyl sulfate (SDS). The homogenates were centrifuged at 15,000*g* at 4 °C for 30-min and supernatants were collected. Protein concentration was determined by the Bradford protein assay. Equal amounts of proteins (50υg) from each group were separated on SDS–polyacrylamide gel electrophoresis (PAGE) gel and electrophoretically transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA, USA), blocked, and probed overnight at 4 °C with antibodies. Polyclonal anti-CREB and anti-phospho-CREB antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Monoclonal anti-CamKII and polyclonal anti-phospho-CamKII antibodies were purchased from Abcam (Cambridge, MA, USA).

Statistics

The principal dependent variable for the behavioral experiments was the percentage of trials with an active avoidance response during each daily session. The independent variables were genotype (a between-group variable) and session number (a within-subjects variable). Statistical analysis for consisted of a two-way analysis of variance (ANOVA) with repeated measures followed by Bonferroni post hoc analysis to evaluate behavioral data using GraphPad Prism (version 6; GraphPad Software, San Diego, CA, USA). For the data in Fig. 5, in addition to genotype and session number, drug pretreatment (saline versus GBR-12909) was also included as a between-group independent variable. Data in Fig. 5 were analyzed using restricted maximum-likelihood estimation (Proc Mixed, SAS Institute, Cary, NC, USA), and significant interaction effects from this