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Distribution of PDE8A in the nervous system of the Sprague-Dawley rat

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ABSTRACT

Phosphodiesterases (PDEs) are essential regulators of cyclic nucleotide signaling. Little is known of the distribution and function of the cyclic adenosine monophosphate (cAMP) hydrolyzing PDE8A family.

Employing immunohistochemistry and Western blots this study maps the distribution of PDE8A in the brain of adult male Sprague-Dawley rats and in the trigeminal ganglion.

PDE8A was confined to neuronal perikaryal cytoplasm and to processes extending from those perikarya. The neurons exhibiting PDE8A-immunoreactivity were widely distributed in the forebrain, brain stem, and cerebellum. Strongly immunoreactive neurons were located in the olfactory bulb, the septal area, zona incerta, and reticular nucleus of the thalamus. Less immunoreactivity was seen in the hippocampus and cerebral cortex. Intense staining was detected in both the substantia nigra and the sensory trigeminal nucleus. In cerebellum PDE8A immunoreactivity was located not only in the Purkinje cells, but also in the granular cells as well as the parallel fibres in the molecular layer. PDE8A immunoreactivity was represented in the epithelial lining of the choroids plexus, the dura mater, and the neurons of the trigeminal ganglion.

The localization of the cAMP degrading PDE8A may indicate a role for PDE8A in cAMP signaling related to pain transmission, motor function, cognition and olfaction.

1. Introduction

The cyclic nucleotide phosphodiesterases (PDEs) comprise a group of enzymes which hydrolyze the second messenger molecules, cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP); such PDEs being important modulators of cyclic nucleotide mediated signal transduction (Beavo, 1995). The enzymes were isolated from rat brain in the early 1970s (Strada et al., 1974; Uzunov and Weiss, 1972). PDEs regulate the localization, duration and amplitude of cyclic nucleotide signaling within subcellular domains (Baillie et al., 2005; Cooper, 2005; Wilson et al., 2008). The PDEs show different modes of regulation, cellular distribution, and intracellular location. Such diversities, in conjunction with recent advances in the development of selective inhibitors of specific PDEs, have led to the introduction of clinically important new treatment principles of inhibiting PDEs important for specific diseases (Boolell et al., 1996; Menniti et al., 2007; Michelakis et al., 2002).

Eleven PDE families have been identified (Beavo, 1995; Bender and Beavo, 2006; Lugnier, 2006) varying in substrate specificity and divided into three major groups: the cAMP selective PDE4, PDE7 and PDE8; the cGMP selective PDE5, PDE6, PDE9; and the dual selective, PDE1, PDE2, PDE3, PDE10 and PDE11, hydrolysing both cAMP and cGMP.

Though much is known of the initial PDE families described (PDE1–PDE6), there has been less focus on the more recently discovered PDE7–PDE11. We have previously described the distribution of PDE9–11 in parts of the rat brain (Kruse et al., 2009); however, partly because selective inhibitors have not been available, not much is known of the cAMP degrading PDE8A.

Abbreviations: AM, amygdala; AOP, anterior olfactory nucleus; pars, posterior; Ap, area postrema; CA1, CA1 region of hippocampal gyrus; Gr, nucleus gracilis; Gi, gigantocellular reticular nucleus; Cu, nucleus cuneatus; DC, dorsal cochlear nucleus; DLG, dorsal nucleus of the lateral geniculate nucleus; EPl, external plexiforme layer of olfactory bulb; Fr, frontal neocortex; fr, fasciculus retroflexus; GP, globus pallidus; HDB, diagonal band of Broca, horizontal part; ic, internal capsule; icp, inferior cerebellar peduncle; Ion, inferior olivary nucleus; Igsc, intermediate gray layer of superior colliculus; IMM, intermediomedial cell column of lamina VII; Ipn, interpeduncular nucleus; Lar, lateral reticular nucleus; lo, lateral olfactory tract; Mo, motor cortex; Mrn, medullary reticular nucleus; MS, medial septal nucleus; Mt, mamillothalamic tract; NX, nervus vagus; Och, optic chiasm; Opt, optic tract; PAG, periaqueductal gray; PBG, parabigeminal nucleus; Pc, posterior commissure; Pir, piriform cortex; Pn, pontine reticular nucleus; Rn, red nucleus; Sc, superior colliculus; Sn, substantia nigra; Soln, solitary nucleus; sp5, spinal trigeminal tract; Sp5n, spinal trigeminal nucleus; Rt, reticular nucleus; STR, striatum; VC, ventral cochlear nucleus; VDB, diagonal band of Broca, vertical part.

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Primarily, previous studies have located PDE8 mRNA but not protein, which may the more functionally relevant (Kobayashi et al., 2003; Perez-Torres et al., 2003). Two isoforms of PDE8 exist; PDE8A and PDE8B, both hydrolysing cAMP and each with at least 5 different transcripts (1–5) (Omori and Kotera, 2007; Wang et al., 2001). PDE8B has previously been located in the brain by Northern blot and *in situ* hybridisation (Kobayashi et al., 2003; Perez-Torres et al., 2003). Knowing the distribution of PDE in specific tissues is a prerequisite in determining as to whether or not single PDE enzymes could be new drug targets. We have focused on describing the hitherto undetermined distribution of PDE8A protein in the rat brain, and trigeminal ganglion.

2. Materials and methods

2.1. Antibody characterisation

Affinity purified rabbit polyclonal antibody against PDE8A was from Fabgennix, USA (#PD8A-121AP). Biotinylated swine anti-rabbit IgG from Dako, Denmark (#0353). HRP-conjugated donkey-anti-rabbit IgG from GE Healthcare, Denmark. The specificity of the PDE8A antibody for Western was confirmed by pre-incubation of antibody with blocking peptide (~40× molar excess) for 1 h at 4 °C prior to Western blotting against human recombinant protein and positive control lysate (from PDE8A-transfected COS cells) as well as rat whole brain, rat hippocampus, and rat testes. Without pre-incubation, a band of 94–96 kDa is observed on 4–12% gradient SDS-gels with a few weak additional bands present below and one above (~120 kDa). None of those additional bands disappear after pre-incubation. The predicted size of rat PDE8A is 93.3 kDa. For IHC the specificity was reconfirmed by pre-absorption (Fig. 4F) of the antibody for 48 h at 4 °C on a rotor with 50 μg antigenic peptide pr ml prior to staining.

The antibody is raised against a peptide containing amino acids 693–713 (ENGETDKNQEVINTMLRTPEN) of human PDE8A which shows 47% overall identity with rat PDE8A. The peptide was post-synthetically modified and coupled to KLH (Keyhole Limpet Hemocyanin) to increase antigenicity.

2.2. Chemicals and kits

ECL Advance Western blotting Detection Kit was supplied by GE Healthcare, Denmark, and RC DC Protein Assay Kit was supplied by Bio-Rad Laboratories, Denmark. ABC–streptavidin horseradish peroxidase complex was supplied from Vector Labs, Burlingame, CA, USA (#PK-6100). Tissue-Tek® O.C.T™ Compound was supplied by Sakura Finetek, Denmark. Biotinylated tyramide was manufactured as described by Hannibal et al. (1995). Pertex was supplied from HistoLab, Sweden (#00840).

2.3. Animals

Male Sprague-Dawley (200–300 g) rats were supplied by Charles River (Sulzfeld, Germany; Taconic Farms, Germantown, NY). The animals were kept under a 12/12 light–dark schedule with free access to food and water. The animals were acclimatized for at least 1 week prior to decapitation. All experiments with animals were performed in accordance with the guidelines of EU *Directive 86/609/EEC* (approved by the Danish Council for Animal Experiments).

2.4. Tissue sample isolation for Western blotting

Male Sprague-Dawley rats were sedated, decapitated and the brains including the upper part of the spinal cord kept cool and isolated over a period of 4–6 h following Krebs saline perfusion. Tissues were homogenised immediately in lysis buffer (150 mM NaCl, 50 mM Tris–HCl (pH 7.4), 0.5% Triton-X100, 5 mM EDTA, 10 μM leupeptin, 1 mM PMSF, 10 nM calyculin A, 4000 KIU (Kallikrein Inhibitor Units) aprotinin (Trazylol©), 5 mM pepstatin, 50 mM NaF, 10 mM β-glycerolphosphate and 1 mM ortho-vanadate), centrifugated at 14,000 rpm and supernatant stored at −80 °C prior to Western blotting.

2.5. Western blotting

The cleared lysates were adjusted to 30 μg total protein pr. sample and run using 9% SDS-PAGE. The proteins were transferred to nitrocellulose membranes (Hybond ECL, Amersham Biosciences) using wet electro blotting. The membranes were blocked for 1 h using 2% ECL Advance Blocking Agent (GE Healthcare, Denmark), incubated overnight at 4 °C in primary antibody solutions, washed briefly TBS-T (Tris buffered saline + 0.01% Tween-20) and then incubated with secondary antibody for 1 h at room temperature. Antibody dilutions were 1:500 for the primary antibody and 1:40,000 for the secondary HRP-conjugated antibody. Membranes were developed using ECL Advance Western Blotting Detection Kit. Image capture was done using a LAS-1000 digital camera unit (Fujifilm Denmark).

2.6. Immunohistochemistry

Perfusion fixation and cutting of sections: The rats were anesthetized with tribromethanol (500 mg/kg) and perfused through the left ventricle of the heart with ice cold heparinized (15000 IU/l) phosphate-buffered saline (pH 7.4, PBS) for 2 min followed by 4% formaldehyde in 0.1 M phosphate buffer (pH 7.4) for 15 min. The brains were removed and post-fixed in the same fixative overnight. Brains were then transferred to 25% sucrose in PBS for at least 48 h, frozen in crushed dry ice, and stored at −80 °C until sectioning. Series of coronal cryostat sections of the brains were cut in; (a) 40 μm in thickness or (b) 16 μm in thickness. The 40 μm sections were transferred to phosphate buffered saline (PBS) and immunoreacted as free floating sections. The 16 μm sections were mounted on Menzel SuperFrost slides (Thermo Fischer Scientific, Denmark).

Further, 6 μm thick coronal brain sections were cut from brains and trigeminal ganglia, which had been fixed and embedded in paraffin. Those sections were deparaffinized in xylol, rehydrated in series of ethanols and washed in PBS.

Immunohistochemical procedures: Both the 40 μm thick free floating cryostat sections and the slide mounted 16- and 6 μm thick sections were washed 2 × 5 min in PBS and incubated for 15 min in 5% swine serum in PBS to decrease unspecific binding. That was followed by incubation with the rabbit polyclonal anti-PDE8A IgG diluted 1:500 in 0.3% Triton X-100 and 1% BSA in PBS overnight at 4 °C. The sections were then washed 3 × 2 min in washing buffer: 0.25% BSA, 0.1% Triton X-100 in PBS (PBS-BT), and then incubated for 1 h at room temperature with secondary biotinylated swine anti-rabbit antibody diluted 1:500 in washing buffer. The sections were washed again 3 × 2 min in PBS-BT and incubated in ABC–streptavidin horseradish peroxidase complex diluted 1/200 in PBS-BT for 45 min. After washing, the sections were incubated with biotinylated tyramide diluted 1:100 in PBS + 0.005% H_2O_2 for 5–7 min at room temperature and washed 3 × 2 min in the washing buffer. Amplification of signal by biotinylated thyramide was necessary for obtaining the staining of PDE8 in the sections. Such was followed by the second incubation of 30 min with ABC-VectaStain. The sections were washed in 0.05 M Tris–HCl buffer, pH 7.6, and incubated for 5–15 min at room temperature with a solution of 0.05% DAB (3,3′-diaminobenzidine) and 0.01% H_2O_2 in Tris–HCl buffer, pH 7.6. After washing in distilled water, the free floating sections were mounted on glass slides in 0.5% gelatine in water and all sections were dried in the fume hood. That was followed by cover slipping with Pertex®.

Sections incubated with the diluted antiserum, to which 50 μg of the epitope was added, were used as immunohistochemical controls.

The sections were photographed with a Zeiss AxioCam HR CCD camera. The digitalized images were assembled by use of Adobe Photoshop.

Drawing of serial sections: Drawing of coronal immunoreactions was effected using Leica microfilm reader on plain white paper. Results were scanned as JPEG files and corrected in Adobe Illustrator 10.

3. Results

3.1. Forebrain

Olfactory bulb: In coronal sections through the rostral part of the olfactory bulb, a collection of strongly PDE8A-immunoreactive (IR) neurons are located in the ventral part of the anterior olfactory nucleus (Figs. 1A and 3A). In the dorsal part of the olfactory bulb, a collection of strongly IR neurons is present exhibiting a thin crescent-shaped area close to the lateral border of the nucleus (Fig. 1A).

IR neurons are located ventrally in the caudal part of the olfactory bulb, corresponding to the posterior olfactory nucleus (Fig. 1A); however large neurons endowed with several processes are seen scattered in the more medial part of the bulb. A weak immunoreaction is also observed in the majority of neurons within the granular layer of the piriform cortex (Fig. 1C and D).

Septal area: Strong IR neurons are located in the vertical and horizontal band of Broca (Figs. 1B, C, and 3B) and in the medial septal nucleus (Fig. 1C). Moderate IR neurons are present in the deep part of the piriform cortex in the area of the endopiriform nucleus (Fig. 1B and D). Some IR neurons are present, scattered in the lateral part of striatum (Fig. 1B and C).

Anterior thalamic and striatal area: In a coronal section through the rostral part of the median eminence, large IR neurons were observed in globus pallidus (Fig. 1E). IR neurons were also present in the striatum (Figs. 1E and 3C). Further, moderately stained neurons were observed in the thalamic nuclei, especially in the anteriodorsal thalamic nucleus (Fig. 1E). In the coronal section, hippocampus is present and in both the pyramidal layer