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NEURONAL EXPRESSION OF cAMP-SPECIFIC PHOSPHODIESTERASE 7B mRNA IN THE RAT BRAIN

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Abstract—cAMP plays an important role as second messenger molecule controlling multiple cellular processes in the brain. cAMP levels depend critically on the phosphodiesterases (PDE) activity, enzymes responsible for the clearance of intracellular cAMP. We have examined the regional distribution and cellular localization of mRNA coding for the cAMP-specific phosphodiesterase 7B (PDE7B) in rat brain by *in situ* hybridization histochemistry. PDE7B mRNA is specifically distributed in rat brain, preferentially in neuronal cell populations. The highest levels of hybridization are observed in olfactory tubercle, islands of Calleja, dentate gyrus, caudate-putamen and some thalamic nuclei. Positive hybridization signals are also detected in other areas, such as cerebral cortex, Purkinje cells of the cerebellum and area postrema. By double *in situ* hybridization histochemistry, we found that 74% and 79% of the cells expressing PDE7B mRNA in striatum and olfactory tubercle, respectively, were GABAergic cells (expressing glutamic acid decarboxylase mRNA), in contrast with the lack of expression in the few cholinergic cells (expressing choline acetyltransferase mRNA) present in those two areas (around 0.4% in olfactory tubercle). In the thalamic nuclei, a majority of cells containing PDE7B mRNA also expresses a glutamatergic marker (76.7% express vesicular glutamate transporter vGluT1 and 76% express vGluT2 mRNAs). Almost all PDE7B expressing cells in dentate gyrus (93%) were glutamatergic.

These results offer a neuroanatomical and neurochemical base that will support the search for specific functions for cAMP dependent PDEs and for the development of specific PDE7 inhibitors.

Key words: *in situ* hybridization, PDE, glutamatergic cells, GABAergic cells, cholinergic cells.

cAMP and cGMP play a key role in signal transduction in a wide variety of cellular responses. In brain, cAMP has been implicated in sensory functions, synaptic plasticity, learning and memory. Thus, information about how the intracellular cAMP levels are regulated will help to understand the mechanisms underlying these functions. Intracellular levels of cAMP are controlled not only by its synthesis by the enzyme adenylyl cyclase (Houslay and Milligan, 1997), but also by its degradation through the action of cyclic nucleotide phosphodiesterases (PDE), which catalyze the hydrolysis of 3′,5′-cyclic nucleotides into 5′-nucleoside monophosphates (Beavo, 1995; Conti and Jin, 1999; Francis et al., 2002; Houslay, 1998).

PDEs have been so far classified into 11 families (PDE1–PDE11). They have different regulatory properties and intracellular location, with particular isoforms being expressed in a cell-specific manner (Conti and Jin, 1999). Families 4, 7 and 8 specifically hydrolyze cAMP, with PDE7 and PDE8 having a higher affinity for this substrate. Two members of the PDE7 family have been cloned, PDE7A (Michaeli et al., 1993), and PDE7B (Hetman et al., 2000; Sasaki et al., 2000). The distribution of PDE7A transcripts in the CNS, was first determined in mouse brain by RNase protection assays (Bloom and Beavo, 1996). Later, PDE7A mRNA was visualized by *in situ* hybridization in a preliminary study in a few adult and embryonic rat brain regions (Hoffmann et al., 1998), in rat brain and peripheral organs (Miró et al., 2001), and in some areas of the human brain (Pérez-Torres et al., 2003). Recently, alterations on the mRNA levels of PDE7A in postmortem human brains with Alzheimer's disease has been reported (Pérez-Torres et al., 2003).

Different *in vivo* models have been developed suggesting an implication of cAMP in learning, memory processes and other brain functions. Treatment of aged mice with rolipram, a PDE4 inhibitor, ameliorated the physiological and the memory defects of the age-related spatial memory loss (Bach et al., 1999). Administration of rolipram, a selective PDE4 inhibitor, induced long-term potentiation in the CA1 field of mouse hippocampus (Barad et al., 1998). These findings suggest the involvement of cAMP in synaptic plasticity during learning and memory processes. Since PDE7 has high affinity for cAMP, this enzyme could play an important role in maintaining cAMP levels at the basal intracellular levels. It has been shown that PDE 7A plays a role in the activation and/or proliferation of T cells (Li et al., 1999) and is upregulated in human B-lymphocytes (Lee et al., 2002). A few PDE7 inhibitors have been described (Barnes et al., 2001; Castro et al., 2001; Martinez et al., 2000; Pitts et al., 2004).

The cellular localization of the PDEs in brain is an important step forward for the understanding of their function. Here we describe experiments aimed at the analysis of both regional and cellular expression of the mRNA coding for PDE7B in neuronal populations expressing specific neurotransmitters of the adult rat brain. Our study

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Abbreviations: AC, adenylyl cyclase; ChAT, choline acetyltransferase GAD, glutamic acid decarboxylase; GFAP, glial fibrillary acidic protein; MBP, myelin binding protein; PBS, phosphate-buffered saline; PDE, phosphodiesterase; vGluT1, vesicular glutamate transporter 1; vGluT2, vesicular glutamate transporter 2.

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reveals that PDE7B mRNA has a restricted distribution in the brain being especially abundant in forebrain structures, where it is expressed in different neuronal populations with excitatory and inhibitory neurotransmitters.

EXPERIMENTAL PROCEDURES

Tissue preparation

Adult male Wistar rats ($n=5$; 200–300 g) were purchased from Iffa Credo (Lyon, France). Animal care followed the Spanish legislation on "Protection of animals used in experimental and other scientific purposes" in agreement with the European (E.E.C) regulations (O.J. of E.C. L358/1 18/12/1986). The animals were killed by decapitation. All efforts were made to minimize the number of animals used and their suffering. The brains were frozen on dry ice and kept at −20 °C. Tissue sections, 14 μm thick, were cut on a microtome-cryostat (Microm HM500 OM, Walldorf, Germany), thaw-mounted onto APTS (3-aminopropyltriethoxysilane; Sigma, St. Louis, MO, USA) -coated slides, and kept at −20 °C until used.

Hybridization probes

For the detection of PDE7B mRNA two 45 base-oligodeoxyribonucleotides that were complementary to bases 556–600 and 1390–1434 (GenBank acc. no. NM_080894) were used. These regions were chosen because they share no similarity with other members of the different PDE families. Most of the results shown in the present work were done using the first PDE7B oligonucleotide.

Glutamatergic cells were recognized by the presence of the mRNA coding for both vesicular glutamate transporters (vGluT1 and vGluT2): vGluT1 with two oligonucleotides complementary to bases 127–172 and 1756–1800 (GenBank acc. no U07609) and vGluT2 with two oligonucleotides complementary to the bases 466–510 and 2156–2200 (GenBank acc. no AF271235). GABAergic cells were identified by the presence of the enzyme synthesizing GABA, glutamic acid decarboxylase (GAD) that in adult brain exists as two major isoforms, GAD65 and GAD67. Two oligonucleotides for each isoform mRNA were made: bp 159–213 and 514–558 (GenBank acc. no NM_012563) and bp 191–235 and 1600–1653 (GenBank acc. no NM_017007). Cholinergic cells were distinguished by the presence of the enzyme choline acetyltransferase (ChAT) mRNA with two oligonucleotides complementary to bases 571–618 and 1321–1368 of the rat ChAT cDNA sequence (Ishii et al., 1990).

We also identified the non-neuronal population by the presence of the glial fibrillary acidic protein (GFAP) and myelin binding protein (MBP) mRNAs, markers for astrocytes and oligodendrocytes, respectively. Two oligonucleotides complementary to bases 225–274 and 1194–1242 were made for the GFAP mRNA (Brenner et al., 1990) and one oligonucleotide complementary to bases 179–223 (GenBank acc. no M25889) for MBP mRNA. The oligonucleotides were all synthesized and HPLC purified by Isogen Bioscience BV (Maarsden, The Netherlands). Evaluation of the oligonucleotide sequences with basic local alignment search tool of EMBL and GenBank databases indicated that the probes do not show any significant similarity with mRNAs other than their corresponding targets in the rat.

Oligonucleotides for PDE7B mRNA were labeled at their 3′-end using [α-^{33}P]dATP (3000 Ci/mmol; New England Nuclear, Boston, MA, USA) for the *in situ* hybridization histochemistry experiments and terminal deoxynucleotidyltransferase (Oncogene Research Products, San Diego, CA, USA), purified using QIAquick Nucleotide Removal Kit (Qiagen GmbH, Hilden, Germany; Tomiyama et al., 1997). ChAT, GAD and vGluT oligonucleotides (100 pmol) were non-radioactively labeled with the same enzyme and Dig-11-dUTP (Roche Diagnostics GmbH, Mannheim, Germany) according to a previously described procedure (Schmitz et al., 1991).

In situ hybridization histochemistry procedure

The protocols for single- and double-label *in situ* hybridization histochemistry were based on previously described procedures (Landry et al., 2000; Tomiyama et al., 1997) and have been already published (Serrats et al., 2003). Frozen tissue sections were brought to room temperature, fixed for 20 min at 4 °C in 4% paraformaldehyde in phosphate-buffered saline (PBS; 1× PBS: 8 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 136 mM NaCl, 2.6 mM KCl), washed for 5 min in 3× PBS at room temperature, twice for 5 min each in 1× PBS, and incubated for 2 min at 21 °C in a solution of predigested pronase (Calbiochem, San Diego, CA, USA) at a final concentration of 24 U/ml in 50 mM Tris–HCl pH 7.5, 5 mM EDTA. The enzymatic activity was stopped by immersion for 30 s in 2 mg/ml glycine in 1× PBS. Tissues were finally rinsed in 1× PBS and dehydrated through a graded series of ethanol. For hybridization, radioactively labeled and non-radioactively labeled probes were diluted in a solution containing 50% formamide, 4× SSC (1× SSC: 150 mM NaCl, 15 mM sodium citrate), 1× Denhardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), 10% dextran sulfate, 1% sarkosyl, 20 mM phosphate buffer pH 7.0, 250 μg/ml yeast tRNA and 500 μg/ml salmon sperm DNA. The final concentrations of radioactive and Dig-labeled probes in the hybridization buffer were in the same range (approximately 1.5 nM). Tissue sections were covered with hybridization solution containing the labeled probe/s, overlaid with Nescofilm coverslips (Bando Chemical Inc., Kobe, Japan) and incubated overnight at 42 °C in humid boxes. Sections were washed four times (15 min each) in 0.6 M NaCl, 10 mM Tris–HCl pH 7.5 at 60 °C, and once in the same buffer at room temperature for 30 min.

Development of radioactive and non-radioactive hybridization signal

Hybridized sections were treated as described by Landry et al., 2000. Briefly, after washing, the slides were immersed for 30 min in a buffer containing 0.1 M Tris–HCl pH 7.5, 1 M NaCl, 2 mM $MgCl_2$ and 0.5% bovine serum albumin (Sigma, Steinheim, Germany) and incubated overnight at 4 °C in the same solution with alkaline-phosphatase-conjugated anti-digoxigenin-F(ab) fragments (1:5000; Roche Diagnostics GmbH). Afterward, they were washed three times (10 min each) in the same buffer (without antibody), and twice in an alkaline buffer containing 0.1 M Tris–HCl pH 9.5, 0.1 M NaCl, and 5 mM $MgCl_2$. Alkaline phosphatase activity was developed by incubating the sections with 3.3 mg nitroblue tetrazolium and 1.65 mg bromochloroindolyl phosphate (Roche Diagnostics GmbH) diluted in 10 ml of alkaline buffer. The enzymatic reaction was blocked by extensive rinsing in the alkaline buffer containing 1 mM EDTA. The sections were then briefly dipped in 70% and 100% ethanol, air-dried and dipped into Ilford K5 nuclear emulsion (Ilford, Mobberly, Chesire, UK) diluted 1:1 with distilled water. They were exposed in the dark at 4 °C for 6 weeks, and finally developed in Kodak D19 (Kodak, Rochester, NY, USA) for 5 min, and fixed in Ilford Hypam fixer (Ilford).

For film autoradiography, some hybridized sections were exposed to Biomax-MR (Kodak) films for 2–4 weeks at −70 °C with intensifying screens. Consecutive sections were stained with Cresyl Violet for anatomical reference.

Analysis of the results

The average densities of PDE7B mRNA in different brain regions were evaluated semi quantitatively on film autoradiograms with the aid of an image analysis system (MCID M4; Imaging Research, St. Catherines, Ontario, Canada).

Tissue sections were examined in bright- and dark-field in a Wild 420 macroscope (Leica, Heerbrugg, Germany), in a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany) and in a