Immunohistochemical Localization of the InsP$_4$ Receptor GTPase-Activating Protein GAP1$^{IP4BP}$ in the Rat Brain

Armando P. Signore,$^1$ Flavia O’Rourke,$^2$ Xinghua Lu,$^2$ Maurice B. Feinstein,$^2$ and Hermes H. Yeh$^{1,2,3,*}$

$^1$Program in Neuroscience, University of Connecticut Health Center, Farmington
$^2$Department of Pharmacology, University of Connecticut Health Center, Farmington
$^3$Department of Neurology, University of Connecticut Health Center, Farmington

The distribution of GAP1$^{IP4BP}$, a GTPase-activating protein showing high affinity and stereospecificity for inositol 1,3,4,5-tetrakisphosphate (InsP$_4$), was investigated by Western blot and immunohistochemistry of rodent brain with polyclonal antibodies generated against the carboxy-terminus of the cloned protein. GAP1$^{IP4BP}$-like immunoreactivity was found throughout the brain, most notably in the pyriform cortex, neocortex, hippocampus, striatum, and cerebellar cortex. However, the most striking immunolabeling was consistently localized to area CA1 of the hippocampus and the central, medial, and intercalated nuclei of the amygdala. Western blot analysis of the corresponding brain regions corroborated these immunohistochemical observations. The regionally specific expression of GAP1$^{IP4BP}$ provides the prerequisite neuroanatomical substrate toward elucidating the functional role of InsP$_4$ and GAP1$^{IP4BP}$ in the central nervous system. J. Neurosci. Res. 55:321–328, 1999.

Key words: hippocampus; inositol-phosphate; metabotropic receptor

INTRODUCTION

The activation of receptors in neural tissues that are linked to phospholipase C increases intracellular levels of inositol 1,4,5-trisphosphate (InsP$_3$), resulting in release of intracellular stores of Ca$^{2+}$ (Fisher and Agranoff, 1987). Inositol 1,3,4,5-tetrakisphosphate (InsP$_4$), formed by the ATP-dependent phosphorylation of InsP$_3$ catalyzed by InsP$_3$ 3-kinase, has also been implicated in cellular calcium signaling (Irvine and Moor, 1986; Berridge and Irvine, 1989; Cullen et al., 1990; Loomis-Husselbee et al., 1996; O’Rourke et al., 1996; Tsubokawa et al., 1996). InsP$_4$ may have a synergistic effect with InsP$_3$ (Cullen et al., 1990) or may exert a direct effect via its own receptor (Morris et al., 1987; Bird et al., 1991; Gawler et al., 1991; Kachintorn et al., 1993; Wilcox et al., 1993; Bird and Putney, 1996; Loomis-Husselbee et al., 1996; Tsubokawa et al., 1996). Evidence for a direct effect of InsP$_4$ includes the demonstration that InsP$_4$ opens calcium channels in neurons (De Waard et al., 1992; Tsubokawa et al., 1994; Tsubokawa et al., 1996), enhances calcium influx in endothelial cells (Lüchhoff and Clapham, 1992), and induces calcium efflux from inside-out platelet plasma membrane vesicles (O’Rourke et al., 1996). InsP$_4$ also appears to regulate influx of calcium subsequent to unloading of intracellular stores by InsP$_3$ (Putney, 1986; Cullen et al., 1990; Gawler et al., 1991; Bird and Putney, 1996).

High-affinity binding sites for radiolabeled InsP$_4$ have been reported in whole brain (Theibert et al., 1991, 1992; Challiss et al., 1991; Cullen and Irvine, 1992; Parent et al., 1994; Stricker et al., 1995; Smith and Nahorski, 1996), specifically in the hippocampus (Parent et al., 1994) and cerebellum (Cullen and Irvine, 1992; Theibert et al., 1992). Several putative InsP$_4$ binding proteins of different properties have been isolated (Donié and Reiser, 1991; Cullen et al., 1995a; Theibert et al., 1991, 1992), one of which is an Mr$_r = 104$ kD protein that was purified from pig (Cullen et al., 1995a) and human (O’Rourke et al., 1996) platelet membranes. The human cDNA was cloned from a cDNA library from bone marrow, sequenced, and identified as a member of the GAP1 family of GTPase-activating proteins (GAPs) and designated as GAP1$^{IP4BP}$ (Cullen et al., 1995b; Lockyer et al., 1997). The GAP1$^{IP4BP}$ was shown to activate Ras and Rap GTPase and to bind InsP$_4$ with high affinity and...
exquisite sensitivity. It contains a highly conserved GTPase-activating domain, domains for Ca\(^{2+}\)-dependent and Ca\(^{2+}\)-independent binding to phospholipids, and a single pleckstrin homology (PH) domain with a Bruton tyrosine kinase motif that binds both InsP\(_4\) and phospholipids (Fukuda et al., 1996; Lockyer et al., 1997). GAP1\(^{IP4BP}\) enhances the GTPase activity of Rap and Ras in vitro, thereby terminating their activity (Cullen et al., 1995b; Yamamoto et al., 1995). The Ras GAP (GTPase activating protein) activity is inhibited by phospholipids and stimulated by InsP\(_4\) (Cullen et al., 1995b), suggesting that competition between InsP\(_4\) and phospholipid may affect membrane binding of this protein (Cullen et al., 1995b; Lockyer et al., 1997).

To understand the functions of GAP1\(^{IP4BP}\) further, we transfected human erythroleukemia (HEL) cells with a vector to express antisense cDNA and obtained cloned cells with greatly diminished GAP1\(^{IP4BP}\) protein (Lu et al., 1999). The GAP1\(^{IP4BP}\)-deficient cells had substantially augmented Ca\(^{2+}\) influx when stimulated by thrombin, a Ca\(^{2+}\) mobilizing mitogen, or when the intracellular pool of Ca\(^{2+}\) was released by thapsigargin. The effect of antisense knockout of GAP1\(^{IP4BP}\) was shown to be due to the enhanced activity or expression of a hyperpolarizing Ca\(^{2+}\)-activated K\(^{+}\) channel that increases Ca\(^{2+}\) influx. A possible link to Ras is suggested because a Ras/Raf kinase-mediated pathway has been shown to increase Ca\(^{2+}\)-activated potassium current in fibroblasts (Huang and Rane, 1994). Thus, apart from any effects that may be mediated by InsP\(_4\), GAP1\(^{IP4BP}\) itself appears to control an intracellular signal transduction pathway that can regulate the synthesis and/or activity of a potassium channel that modulates Ca\(^{2+}\) influx.

As a prerequisite for understanding the functions of GAP1\(^{IP4BP}\) in the nervous system, we describe the regionally selective distribution of GAP1\(^{IP4BP}\)-like immunoreactivity (GAP1\(^{IP4BP}\)-LI) in the rat brain by using polyclonal antibodies generated against the carboxy-terminal sequence of human GAP1\(^{IP4BP}\) protein (Cullen et al., 1995b; Loomis-Husselbee et al., 1996). Intense immunolabeling was found in the limbic regions, in particular the CA1 subfield of the hippocampus, amygdala, and olfactory-associated cortical structures. Prominent GAP1\(^{IP4BP}\)-LI was also detected in neocortex, striatum, and cerebellar cortex.

MATERIALS AND METHODS

Western Blot Analysis

Adult female Long-Evans rats were asphyxiated with carbon dioxide, and their brains were removed. The cerebellum was detached, and the hippocampal CA1 and CA3 subfields and neocortex were microdissected from brain slices. The dissected brain tissue was individually homogenized in Dounce homogenizers on ice in a solution containing a cocktail of protease inhibitors and detergent to solubilize intact protein: 20 mM sodium bicarbonate, 1 mM EDTA, 10% CHAPS, 20 μM leupeptin, 20 μM pepstatin, and 0.5 mM phenyl methyl sulfonyl fluoride, pH 7.2. These steps must be carried out rapidly to avoid generation of proteolytic fragments that react with the antisera. The detergent-solubilized samples were assayed by using Coomassie blue reagent and were adjusted to a protein concentration of 5.0 mg/ml with additional buffer plus SDS at a final concentration of 2%, followed by immediate boiling for 10 min. Aliquots of 50 μg total protein were loaded in individual wells for electrophoresis on 7.5% SDS-PAGE gels followed by blotting onto an Immobilon membrane with a semi-dry blotting apparatus (Pharmacia, Piscataway, NJ), as previously described (O’Rourke et al., 1996). All further incubations and washes were performed at room temperature. Blots were incubated for 1 hr with blocking solution containing 5% powdered non-fat milk in phosphate buffered saline (PBS) with 0.1% Tween 20 (PBS-TWEEN). After the blocking step the blots were probed with three anti-GAP1\(^{IP4BP}\) antisera, one provided by Dr. PJ. Cullen (used at a dilution of 1:1,000) and two commercially raised anti-GAP1\(^{IP4BP}\) antisera (designated 51–3 and 52–3) or preimmune sera (diluted to 1:500) in blocking buffer for an additional 2 hr. All antisera were raised against the last 20 amino acid residues of the carboxy-terminus sequence (GDKSFQSYIRQQSETSTHSI) of the human GAP1\(^{IP4BP}\). The blots were then washed twice with PBS-TWEEN for 15 min and three times for 5 min each and then incubated for an additional 45 min with goat anti-rabbit peroxidase conjugated secondary antibody (Calbiochem, San Diego, CA) diluted 1:5,000 in PBS-TWEEN, followed by washing as described for the primary antibody. The washed immunoblot was incubated with chemiluminescence reagents as described in the kit (Kirkegard and Perry, Gaithersburg, MD) and exposed to X-ray film for 1 min. Bands on the films were quantified using a Molecular Dynamics (Sunnyvale, CA) laser densitometer and ImageQuant software.

Cultures enriched in astrocytes were prepared according to modification of previously described methods (McCarthy and DeVellis, 1980). Cells derived from neonatal rat cortical tissue were plated onto plastic culture plates and maintained in modified Eagle’s medium supplemented with 5% heat-inactivated fetal calf serum. After 5–7 days in culture, the plates were shaken at 200 rpm overnight to remove oligodendrocytes and microglia. The remaining cells, enriched in astrocytes, were suspended, plated onto new culture dishes, and allowed to grow to confluence. Cells in culture plates
were solubilized and analyzed by Western blotting, as described above.

Immunohistochemistry

Adult rats received an intraperitoneal injection of a lethal dose of sodium pentobarbital prior to intra-aortic perfusion by using PBS with 1.0 U/ml heparin (pH 7.4) and then PBS containing 4% paraformaldehyde (Fisher Scientific, Springfield, NJ) to fix the tissue. The brains were removed, immersed in PBS containing 30% (w/v) sucrose, and 25-µm sagittal and coronal sections were collected by using a cryostat (Model HM 505; Microm, Walldorf, Germany). The sections were stored in a cryoprotective solution containing 30% (w/v) sucrose, 30% (v/v) ethylene glycol, and 1% (w/v) polyvinylpyrrolidone in PBS. Sections were first incubated for 1 hr in a blocking solution consisting of 10% normal goat serum and 0.5% Triton X-100 in PBS. The GAP1IP4BP antiserum from Dr. P.J. Cullen was used at a dilution of 1:500, and polyclonal antiserum lots 51–3 and 52–3 (Advanced Chemtech, Louisville, KY) were used at dilutions of 1:100 to 1:500. Sections were incubated in the primary antibodies at 4°C for 18–24 hr and then with biotinylated goat anti-rabbit secondary antibody (1:200 in PBS and 1% goat serum) for 4–12 hr. An avidin-biotin–conjugated horseradish peroxidase kit (Vector, Burlingame, CA) was used for detection, and after brief incubation in ammonia-PBS (PBS, pH 7.4, with ammonium hydroxide) the reaction product was visualized by using 0.14 mM 3,3'-diaminobenzidine (DAB), 0.003% H2O2, and 0.03% nickel ammonium sulfate in ammonia-PBS. Control sections were processed in parallel but without the primary antisera. Monoclonal anti-GFAP was used at a dilution of 1:1,000 to identify astrocytes in brain sections. Bound anti-GFAP was detected with goat anti-mouse secondary antibody at 1:200 and visualized after peroxidase–anti-peroxidase and DAB reactions. A rodent atlas (Paxinos and Watson, 1997) was used to confirm anatomical locations. Images were digitized in Photoshop 4.0 (Adobe Systems, Mountain View, CA). Unless otherwise stated, all chemicals were obtained from Sigma (St. Louis, MO).

RESULTS

We first established the specificity and potency of the GAP1IP4BP antiserum by Western blotting of detergent-solubilized brain tissue and cellular homogenates (Fig. 1). Human blood platelets, which contain high amounts of GAP1IP4BP (O’Rourke et al., 1996), served as positive control. We used a rabbit GAP1IP4BP antiserum obtained from Dr. P.J. Cullen and two additional antisera (anti-GAP1IP4BP lots 51–3 and 52–3) that were commercially generated against the identical 20–amino acid carboxy-terminal region of the GAP1IP4BP protein. The specificity of the first GAP1IP4BP antibody was demonstrated by Cullen et al. (1995b) and Lockyer et al. (1997). On Western blots, each of the antisera detected a single band at ~Mr = 100 kDa protein band in human blood platelets (Fig. 1, lane f), and in the striatum, hippocampus, basal forebrain, and cerebellum that were microdissected from the adult rat brain (Fig. 1, lanes a–c, e).

All three antisera were employed in parallel immunohistochemical experiments. Of the three antisera, the one obtained from Dr. P.J. Cullen consistently produced the most intense immunostaining. Although lots 51–3 and 52–3 of the commercially prepared GAP1IP4BP antisera were suitable for Western blot analysis, they were not as effective for immunohistochemical demonstration of GAP1IP4BP-LI in brain sections. Even though all antisera were raised against the identical carboxy-terminal portion of the GAP1IP4BP peptide, they may have preferentially recognized different epitopes with different accessibility to the protein in fixed tissue compared with the SDS/heat-denatured proteins on Western blots.

Figure 2 illustrates the localization of GAP1IP4BP-LI in cryosections from the rat brain by using the GAP1IP4BP antiserum obtained from Dr. P.J. Cullen. A sagittal section (Fig. 2A) and a series of coronal sections are shown arranged in a rostral-to-caudal sequence (Fig. 2B–H). GAP1IP4BP-LI is present in gray matter throughout the brain, especially in the hippocampal CA1 subfield (Figs. 2A,E–G, 3A) and cerebellum (Figs. 2A, 5B).
addition, distinct cellular immunostaining was found in the amygdala (Figs. 2E, 3C), olfactory bulb (Fig. 2B), olfactory (Fig. 2C–G), and neocortices (Fig. 2, all sections, and 5A). Control cryosections processed without the primary antibody (Fig. 2I) or with preimmune sera substituting for primary antibody displayed no immunoreactivity (data not shown).

Pyramidal cells in the CA1 subfield of the hippocampus displayed the most intense GAP1IP4BP-LI (Fig. 3A), showing the soma as well as the apical dendrites extending into the stratum radiatum (Fig. 3B, arrowhead). Immunostaining of pyramidal cells in the CA2–4 subfields and granule cells in the dentate gyrus was considerably weaker (Fig. 3A). By using the same antisera, the presence of GAP1IP4BP-LI was further verified by Western blotting of tissue obtained by microdissecting CA1 and CA3 hippocampal subfields, cerebellum, and cerebral cortex (data not shown). Within the amygdaloid nuclear complex, the central, medial, and intercalated nuclei showed intense GAP1IP4BP-LI (Fig. 3C,D). At the cellular level, GAP1IP4BP-LI appeared to be concentrated along the cell membrane, outlining the somata of individual neurons in CA1 (Fig. 3B) and amygdala (Fig. 4).

Fiber tracts displayed only background levels of immunostaining (Figs. 2, 3A) and, at higher magnification, profiles displaying GAP1IP4BP-LI were sparse and limited to small, punctate oligodendrocyte-like cell bodies. In sections processed immunohistochemically for glial fibrillary acidic protein, an astrocyte-specific marker, immunopositive cells were distinct from cells displaying GAP1IP4BP-LI, indicating that the cells containing GAP1IP4BP-LI were not astrocytes. Furthermore, GAP1IP4BP-LI was undetectable in Western blots of cultured astrocytes (Fig. 1d).
GAP1 IP4BP-LI was also found in other brain regions, notably the neocortex and cerebellum. In the neocortex (Fig. 5A) comparable immunostaining was found among all the layers, except layer I, which consistently displayed the weakest immunoreactivity. In the cerebellar cortex, GAP1 IP4BP-LI was evenly distributed throughout the Purkinje and granule cell layers (Fig. 5B). The staining was diffuse, but the contours of individual Purkinje cells, along with their proximal dendritic arbor, were discernible. Cell bodies situated along the entire thickness of the molecular layer, presumably those of basket/stellate cells, were also evident.

DISCUSSION

This is the first immunohistochemical demonstration of the specific localization of the InsP₄ receptor protein GAP1 IP4BP in the brain. The present study includes a display of not only its occurrence but, more importantly, the regional selectivity of the pattern of distribution of GAP1 IP4BP-LI. Most impressive is the differential expression in CA1 vis-à-vis other subfields of the hippocampus. Staining was also strong in several other nuclei, in particular the amygdala, pyriform cortex, and cerebellum. In all areas GAP1 IP4BP-LI was concentrated along the membrane around the soma as well as proximal dendrites, but not in the nucleus or axon of individual neurons. This membrane location is consistent with the characterization of radiolabeled InsP₄ binding sites in isolated plasma membrane fractions of brain, platelets, and HEL cells (Theibert et al., 1991; Cullen and Irvine, 1992; Cullen et al., 1994; O’Rourke et al., 1996; Tsubokawa et al., 1996). It is also consistent with findings that InsP₄ affects calcium flux across the plasma mem-

Fig. 3. Regionally specific expression of GAP1 IP4BP-like immunoreactivity (LI) in the hippocampus and amygdaloid nuclei. A: In the hippocampus, GAP1 IP4BP-LI is strong in the CA1 subfield and weaker in CA2, CA3, and dentate gyrus (DG). White tracts (corpus callosum, CC) show virtually no staining. B: CA1 cells in the stratum pyramidale (SP) show staining of somata, with dendrites extending into the stratum radiatum (SR) but not into the stratum oriens (SO). C: Cells in the amygdala (arrow; central, medial, and intercalated nuclei) are also immunoreactive. D: GAP1 IP4BP-LI is also primarily in somata and proximal processes of amygdaloid cells. Bars: (A,C) 1.0 mm; (B,D) 25 µm.
branes of CA1 hippocampal pyramidal neurons (Tsubokawa et al., 1996), endothelial cells (Lückhoff and Clapham, 1992), and platelets (O’Rourke et al., 1996). Furthermore, it is in agreement with the membrane-delimited localization of expressed GAP1IP4BP protein in COS cells and HeLa cells (Lockyer et al., 1997) and HEL cells (O’Rourke and Feinstein, unpublished). We also observed some weaker GAP1IP4BP-LI within the cells, probably representing protein in the cytosol and/or in internal membranes. In platelets, GAP1IP4BP was found in internal membrane fractions, and about 10% of the total was in the soluble fraction (O’Rourke et al., 1996).

The location of GAP1IP4BP-LI in the plasma membrane, which is probably attributable to its phospholipid and InsP4 binding PH domain (Lockyer et al., 1997), is in sharp contrast to that of other brain-enriched GAPs, such as GAP1m (Lockyer et al., 1997), neurofibromin (Gregory et al., 1993), and p120GAP (Gibbs et al., 1988). This suggests unique regulatory roles for the different GAPs and a specific role for GAP1IP4BP related to some function of the plasma membrane. Our review of the GenBank

Fig. 4. GAP1IP4BP-like immunoreactivity (LI) is localized, but not limited to the somatic and proximal dendritic membrane. Photomicrograph of cells from the amygdala show GAP1IP4BP-LI outlining pyramidal cells (arrows), suggesting that immunoreactivity is present in the plasma membrane. The cytoplasmic region extending into the proximal portions of dendrites (arrowheads) is also immunoreactive. Bar, 25 µm.

Fig. 5. GAP1IP4BP-like immunoreactivity in the neocortex and cerebellum. A: Immunoreactive cell bodies can be seen throughout the neocortical layers. B: In the cerebellum, diffuse staining is seen in the granule cell layer (GC), and somatic profiles of individual Purkinje cells are clearly evident (arrow). Bars, 200 µm.
databases showed that GAP1IP4BP is the human counter-
part of oligodendrocyte GAPIII (Baba et al., 1995) and
bovine brain R-Ras GAP (Yamamoto et al., 1995). Each
of these GAP cDNAs codes for a very highly conserved
834–amino acid protein with predicted molecular weights
of 95–96 kDa. The human, rat/mouse, and bovine forms
are 95% identical and 97–98% conserved. Furthermore,
the 20–amino acid carboxy-terminal peptide of human
GAP1IP4BP used to raise the antisera differs from rodent
GAPIII by only one amino acid (T829/I), making it highly
likely that the latter is the GAP1 IP4BP -LI observed in the
rat brain sections. Messenger RNA for GAPIII protein
was most highly expressed in rat brain, including cortex,
hippocampus, cerebellum, and oligodendrocytes, but not
astrocytes (Baba et al., 1995), in agreement with our
measurements of expressed protein.

Receptors linked to phosphoinositide metabolism,
the generation of InsP3 and InsP4, and the mobilization of
Ca2+ have been implicated in regulating neuronal plastic-
ity (Henzi and MacDermott, 1992; Berридge, 1993;
Putney and Bird, 1993; Tsubokawa et al., 1996). Calcium
is involved in learning and memory (Molinari et al.,
1996), and altered calcium homeostasis has been impli-
cated in aging (Disterhoft et al., 1996; Hartmann et al.,
1996), ischemia-induced cell death, a model of epilepto-
genesis in the rat hippocampus (Faas et al., 1996), and
neurodegeneration. In addition, proteins of the Ras super-
family are involved in gene expression, cell proliferation,
and apoptosis. Therefore, it is noteworthy that the pres-
ence of GAP1IP4BP-LI is most evident in brain regions that
harbor a high degree of plasticity, i.e., structures of the
limbic system, neocortex, striatum, and cerebellum. Be-
because the neuroanatomical localization of a protein is a
key step in ultimately establishing its functions in the
brain, the expression and pattern of distribution of
GAP1IP4BP-LI demonstrated in the present study provide
the anatomical basis for guiding further functional stud-
ies.

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