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Neurons and microvessels express the brain glucose transporter protein GLUT3

(Northern blot/Western blot/immunocytochemistry/transport)

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ABSTRACT **To elucidate glucose transport mechanisms in brain and to demonstrate the cellular expression of the brain-type glucose transporter (GLUT3), antisera to a synthetic peptide corresponding to the C terminus were prepared and used as probes for this isoform of the facilitative glucose transporter family. Immunocytochemistry of frozen sections of dog and rat brain demonstrated GLUT3 antigen in pyramidal cell bodies and processes, in microvessels, and in intima pia or glia limitans. Immunoanalysis of Western blots identified a protein (M_r, 45,000) that was present in both neuron/neuropil and microvessel fractions. The presence of the GLUT3 message in brain was confirmed by Northern blot analysis and by amplifying and partially sequencing GLUT3 cDNA by PCR. These findings demonstrate a neuron glucose transporter in tissue and suggest that GLUT3 may play an important role in brain metabolism under physiological and pathophysiological conditions.**

The functional activity of the brain is critically dependent on glucose as an energy source. For glucose to be utilized in brain metabolism, it must be transported initially through the walls of cerebral blood vessels and subsequently through the plasma membranes of glial cells and neurons. Although glucose transport through the microvascular wall has been studied extensively (1–3), little is known about the process by which glucose enters the neuron. The erythroid glucose transporter (GLUT1) (492 amino acids) is abundant in brain microvessels (4) and specifically in luminal and abluminal endothelial cell membranes (5), but immunocytochemical studies have failed to identify this transporter in neurons (6). Glucose thus appears to enter the neuron by an alternative transport mechanism.

Recent evidence indicates that a family of glucose transporter-like proteins is present in mammalian tissues, with each transporter expressing a unique pattern of tissue distribution (7). The nucleotide sequence of a 496-amino acid isoform (GLUT3, the brain-type glucose transporter) having 64.4% identity with GLUT1 has been reported, and the mRNA encoding this protein was found to be most abundant in adult human cerebrum relative to other tested tissues (8). The existence of one or more additional glucose transporters in brain is supported by a report that antibodies to GLUT1 are capable of immunoprecipitating only 30% of the [^{3}H]cytochalasin B-labeled glucose transporters in rat brain membranes (9). These findings have led us to search immunocytochemically for the specific location of GLUT3 in brain. Related goals of the study were to demonstrate that the GLUT3 gene is expressed in brain tissue from nonhuman species and that the C-terminal amino acid sequence of GLUT3 is sufficiently similar in other species to permit cross-reactivity of antisera raised to this sequence. Expression of GLUT3 in dog brain was therefore studied by using Northern blots and PCR.

MATERIALS AND METHODS

Antisera. A 13-amino acid peptide corresponding to the C-terminal end of human GLUT3 (Asn-Ser-Ile-Glu-Pro-Ala-Lys-Glu-Thr-Thr-Thr-Asn-Val) (8) was synthesized at the Microchemical Facility of the Institute of Human Genetics (University of Minnesota). A cysteine residue was added at the N-terminal end to facilitate conjugation of the peptide to carrier proteins. The peptide was purified by reversed-phase high-performance liquid chromatography and linked to keyhole limpet hemocyanin or ovalbumin before immunizing rabbits. Rabbit antiserum to the C terminus of GLUT3 conjugated to keyhole limpet hemocyanin was designated G3A1. Rabbit antiserum to the C terminus of GLUT3 conjugated to ovalbumin was designated G3A3. Antiserum to the C terminus of GLUT1 has been characterized (5).

Northern Blots. Probes for GLUT3 and GLUT1 mRNA were prepared from full-length cDNA clones. The cDNAs were labeled with ^{32}P by nick-translation and used to probe Northern blots of mRNA extracted from canine cerebral cortex. The pBS/hMGT3 plasmid containing a 2.6-kilobase (kb) GLUT3 cDNA was provided by Graeme I. Bell (Howard Hughes Medical Institute, University of Chicago). The pGEM-3 plasmid containing a 2.47-kb GLUT1 cDNA was obtained from the American Type Culture Collection (no. 59630/59631). Northern hybridization conditions have been reported (8).

PCR. Oligonucleotide primers for GLUT3 were used in the PCR to amplify cDNA derived from dog cerebral cortex poly(A)$^+$ mRNA. Similar results were obtained with mRNA preparations from three dog brains. The primers were an upper strand 5′ consensus primer (primer A) corresponding to a region of high homology (bases 660–683) within GLUT1, -2, -3, and -4 and a lower strand 3′ primer (primer Q) specific for the C terminus of GLUT3 (bases 1711–1730). Reverse transcription was performed on 1.0 μg of poly(A)$^+$ mRNA from canine cerebral cortex using oligo(dT) priming and avian myeloblastosis virus reverse transcriptase (Amersham). Approximately 6 ng of first-strand cDNA synthesis product was used as template in 100 μl of a mixture containing 10 μl of 10× PCR buffer (Cetus), 40 pmol of primer A, 20 pmol of primer Q, 2.5 units of *Taq* polymerase, and each dNTP at 200 μM (final concentration). A Perkin–Elmer/Cetus thermocycler was programmed as follows: 1 min at 94°C, 2 min at 58°C, 2 min at 72°C for 30 cycles, and a final extension of 6 min at 72°C. This pair of primers yielded a predicted 1014-base-pair (bp) product.

To increase the specificity of a GLUT3 product, an upper-strand internal 5′ primer (primer B; bases 765–788) was

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Abbreviations: GLUT1, erythroid glucose transporter; GLUT3, brain-type glucose transporter.

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selected and used in conjunction with primer Q. Reactions were performed as described above with 20 pmol of each primer and 1 μl of the 1014-bp product as template. A Savant thermocycler was programmed as follows: 1 min at 94°C, 1 min at 42°C, 2 min at 72°C for 30 cycles, and a final extension of 6 min at 72°C. These primers yielded a predicted 966-bp amplification product.

Samples of the 966-bp band were separated by electrophoresis on a 1% agarose gel, removed from the agarose (Gene Clean, Bio 101, La Jolla, CA), ethanol precipitated, and reconstituted in Tris/EDTA buffer, pH 8.0. The product was sequenced by a modified Sequenase protocol (United States Biochemical) adapted for the direct sequencing of PCR products by the dideoxynucleotide chain-termination method. The sequence obtained with a fourth primer (primer C; upper-strand bases 1620–1640) was used in conjunction with the sequence obtained with primer Q to yield 176 continuous bases of sequence at the C terminus of canine GLUT3. Because primer Q anneals at a stringent temperature of 58°C–62°C, we assume that the final 20 bases at the 3′ end of canine GLUT3 cDNA are similar or identical to the human GLUT3 sequence.

Western Blots. Samples of canine and rat cerebral cortex were separated into microvessel and nonvascular (neuron/neuropil) fractions before electrophoresis and blotting. To prepare these fractions, the tissue was gently disrupted with a Potter–Elvehjem tissue grinder, passed through 350- and 110-μm mesh nets, and centrifuged (1000 × *g*; 10 min) as described (10). This pellet was resuspended in 20% dextran and centrifuged (2500 × *g*; 15 min) to separate the microvessels (pellet) from the nonvascular fraction (buoyant layer). The microvessels were washed, collected using a 20-μm mesh net, and lysed in distilled water. Microvessel cell membranes were collected by centrifugation (1000 × *g*). The nonvascular fraction was washed three times in phosphate-buffered saline and collected by centrifugation (1000 × *g*). This fraction was not subjected to distilled water lysis. Both fractions were solubilized and subjected to polyacrylamide gel electrophoresis followed by transfer blotting onto nitrocellulose membranes. Human erythrocyte ghosts were also prepared for electrophoresis and blotting. GLUT1 and GLUT3 were visualized by using a gold-labeled secondary antibody and silver enhancement (Amersham) or a biotinylated secondary antibody and streptavidin/alkaline phosphatase (GIBCO). Negative controls consisted of nonimmune rabbit serum and the transporter-specific antisera preabsorbed with their respective synthetic peptides (10–500 μM).

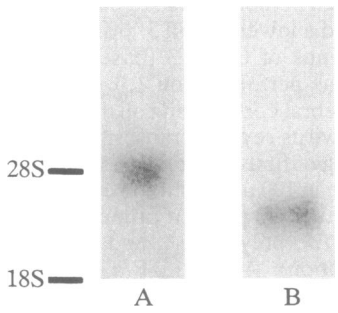

FIG. 1. Northern blots of canine cerebral cortex mRNA (30 μg per lane) probed with ^{32}P-labeled GLUT3 (lane A) and GLUT1 (lane B) cDNAs. The 4.1-kb GLUT3 mRNA transcript is present in canine cerebral cortex and migrates at a higher apparent molecular weight than the GLUT1 transcript. Hybridization conditions for the two probes were identical, but because of differences in autoradiogram exposure times (5 days in lane A; 1 day in lane B) the autoradiographic densities do not indicate the relative abundances of the two mRNAs. Positions of 28S and 18S ribosomal RNA are indicated.

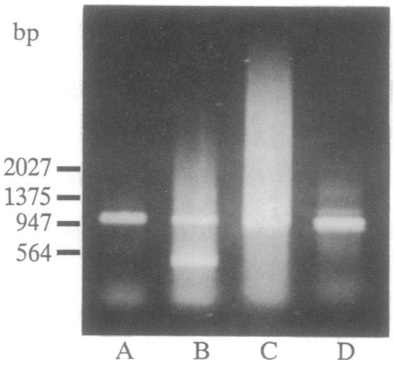

FIG. 2. Amplification of GLUT3 cDNA fragments derived from canine brain by PCR. Standards were λ DNA/*Hin*dIII + *Eco*RI and are indicated on the left-hand scale as bp. Lane A, control product. An upper-strand 5′ consensus primer for GLUT1, -2, -3, and -4 (bases 660–683) and a lower-strand 3′ primer (GLUT3; bases 1711–1730) were used with a 2.6-kb GLUT3 insert in plasmid pBS/hMGT3 as template. A band predicted to be 1014 bp was observed. Lane B, the same primers produced an identical band (1014 bp) when used with cDNA derived from canine cerebral cortex as template. A second, smaller band (650 bp) was also observed and, based on sequence analysis, is apparently unrelated to glucose transporters. Lane C, an upper-strand internal 5′ primer (GLUT3; bases 765–788) was used with the same lower-strand 3′ primer and the 1014-bp amplification product of lane B as template. These primers yielded a predicted 966-bp amplification product. Lane D, control product. The same primers as in lane C were used with a 2.6-kb GLUT3 insert in plasmid pBS/hMGT3 as template. A similar 966-bp product was detected. These bands were not visualized in negative control lanes (not shown) in which no template was used.

Tissue fractions from four brains (two dog and two rat) were analyzed separately, and all yielded similar results.

Immunocytochemistry. Brain tissue was collected from four adult rats that were decapitated and from four mongrel dogs after rapid exsanguination while all animals were under deep halothane anesthesia. The tissue was snap frozen in isopentane chilled to −70°C with liquid nitrogen. Frozen sections of brain were cut in a cryostat, thaw-mounted on silane-coated slides (11), and transferred to formal acetic fixative (6) for 5 min without drying. The transporter-specific antisera were used at dilutions of 1:300 (12-min incubation at 37°C) or 1:600 (overnight incubation at 4°C). Bound antibody was detected by using a biotinylated secondary antibody and peroxidase-conjugated streptavidin (Biogenex Laboratories,

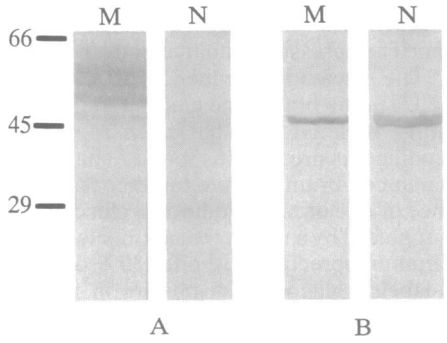

FIG. 3. Immunoblots of microvessel (lanes M) and neuron/neuropil (lanes N) membrane proteins (75 μg per lane) from canine cerebral cortex. Molecular weights (× 10^{-3}) of standards are indicated on the left. Bound antibody was detected by using a biotinylated secondary antibody and streptavidin/alkaline phosphatase. (*A*) GLUT1-specific antiserum (1:800). GLUT1 was observed only in microvessel membrane proteins as a broad band at M_r 46,000–60,000. (*B*) GLUT3-specific antiserum (G3A1; 1:400). GLUT3 was detected as a narrow band (M_r, 45,000) in both microvessel and neuron/neuropil membrane fractions.

San Ramon, CA). Negative controls consisted of nonimmune rabbit serum and the transporter-specific antisera preabsorbed with the GLUT1 and GLUT3 synthetic peptides (10–500 μM).

RESULTS AND DISCUSSION

The GLUT3 cDNA probe identified a 4.1-kb GLUT3 transcript in mRNA extracts of canine cerebral cortex (Fig. 1). Similar results were obtained when Northern blots were prepared with separate mRNA extracts from three dog brains. This finding is consistent with Northern blot analyses of human tissues (8, 12) performed with GLUT3 cDNAs in which hybridizations to 4.1- and 2.7-kb transcripts were observed. Hybridizations to 3.6- and 3.2-kb GLUT3 transcripts have been reported for other species (13, 14). The GLUT3 mRNA migrated at a different location than the canine GLUT1 mRNA, indicating the absence of cross-hybridization under the stringent hybridization conditions used.

Amplification of cDNA derived from canine cerebral cortex mRNA using the PCR resulted in a 966-bp product (Fig. 2). The nucleic acid sequence was obtained for 176 bases of the 3′ end of this product. This sequence shows 98.7% homology to the human sequence and indicates that the 13 amino acids of the human C-terminal peptide used for im-

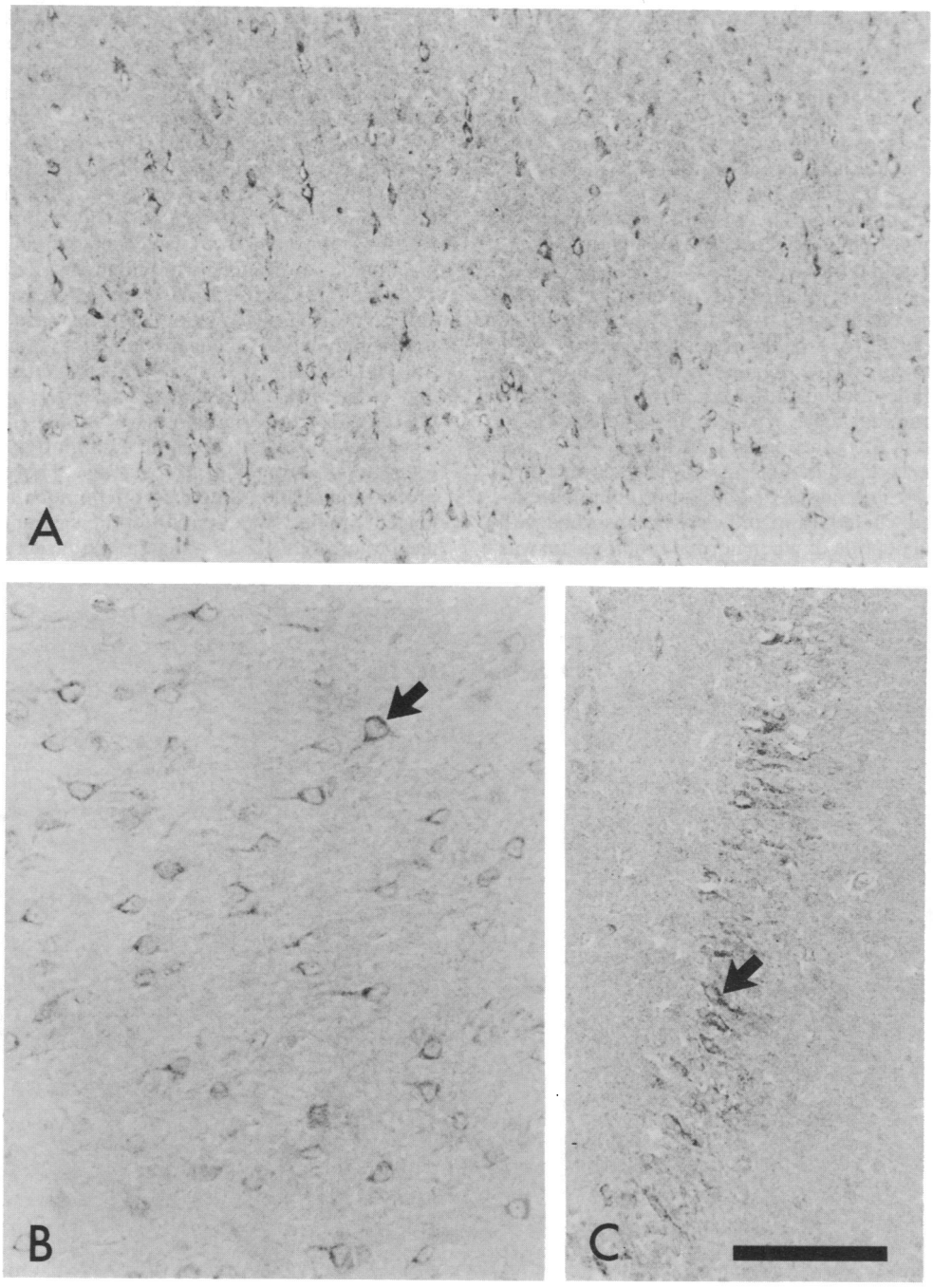

FIG. 4. Immunocytochemistry of rat brain coronal frozen sections using G3A1 antiserum to GLUT3 (1:300). Bound antibody was detected by using a biotinylated secondary antibody and peroxidase-labeled streptavidin. The chromogen was aminoethylcarbazole. (*A*) Cerebral cortex. Immunoreactivity was observed in pyramidal cells located throughout cerebral cortex. (*B*) Cerebral cortex, high power. Reaction product (arrow) was abundant in perikaryon, extending into the proximal region of neuronal cell processes. (*C*) CA1 region of hippocampus. Pyramidal cells contained various amounts of reaction product (arrow). (*A* and *C*, bar = 160 μm; *B*, bar = 80 μm.)

munization are similar or identical to the C terminus of canine GLUT3. Thus, the G3A1 and G3A3 antisera should be effective as probes for canine GLUT3 in Western blots of brain membrane proteins, and they may also be useful for detection of GLUT3 in tissues of other species.

The results of Western blots using the immunogold/silver enhancement technique and the biotin/streptavidin technique were identical. GLUT1 was detected as a broad band (M_r, 47,000–64,000) in blots of membrane proteins derived from brain microvessels (Fig. 3). However, this isoform was not detected in the nonvascular (neuron/neuropil) fraction. These results corroborate our earlier findings (5) and those of others (4, 15). In contrast, GLUT3 was detected with G3A1 as a distinct, relatively narrow band (M_r, 45,000) both in the neuron/neuropil fraction and in cerebral microvessel membranes. GLUT3 was not detected in human erythrocyte ghosts, and control blots were negative in all cases. These results indicate the following: (*i*) Unlike GLUT1, which appears to be located exclusively in vessels, GLUT3 is present in the nonvascular cell fraction as well as in isolated microvessels. (*ii*) When compared with the broad GLUT1 band, the narrow GLUT3 band suggests that GLUT3 is a more homogeneous molecular species and may be glycosylated more uniformly than GLUT1. (*iii*) Although it consists of a slightly longer amino acid sequence than GLUT1 (7), the GLUT3 isoform migrates with an apparent smaller molecular weight. This suggests that, relative to GLUT1, GLUT3 is glycosylated with fewer or smaller oligosaccharides.

Differences between the G3A1 and G3A3 antisera were apparent in immunocytochemical stains of frozen sections. G3A1 stained pyramidal cells of rat brain, whereas G3A3 stained microvessels of both dog and rat brain. G3A3 also stained a cellular layer surrounding cerebral cortex that corresponds to the glia limitans or intima pia. Immunoreactivity was abolished when the antisera were preabsorbed with GLUT3 C-terminal peptide or when normal rabbit serum was substituted for the primary antibody. Preabsorbing with GLUT1 C-terminal peptide had no effect on the staining characteristics of either antiserum.

When G3A1 was used as the primary antiserum, immunoreactivity was found in pyramidal cells throughout rat cerebral cortex and also in some hippocampal neurons (Fig. 4). Reaction product appeared to be located in neuronal cytoplasm (perikaryon) and in the bases of processes extending from neuronal cell bodies. Hippocampal pyramidal cells exhibited various amounts of GLUT3 antigen. Some of these neurons were moderately stained, but many contained only traces of antigen. Little positive staining was observed in other areas of brain.

With G3A3 as the primary antiserum, both dog and rat microvessels were stained. The strongest responses were observed in cerebral cortex (Fig. 5). Capillaries were stained uniformly, suggesting that capillary endothelial cells may contain the GLUT3 transporter. Staining of arterioles and venules was largely confined to a distinct layer, probably composed of astrocytic processes or leptomeningeal cells, surrounding the vessel wall. This conclusion is based partly on the location of the stained layer (16) and the fact that the layer remained associated with neuropil in instances in which the arteriole had contracted away from neuropil during fixation or freezing. It is also based on the intense staining of glia limitans or intima pia of both dog and rat as noted above. The potential glucose transport function for this surface layer has been discussed previously in the case of GLUT1 (5).

The implication of the immunocytochemistry results is that the two antisera G3A1 and G3A3 contain differing abundances of antibodies recognizing different epitopes on the GLUT3 C-terminal amino acid sequence. G3A1 and G3A3 were raised against the same 13-amino acid peptide conjugated to different carrier proteins. Therefore, the carrier protein may have influenced the immune responses of challenged rabbits. Whether different carrier proteins may be used to create GLUT3 antisera that target specific brain cell types requires additional study. The results also suggest that

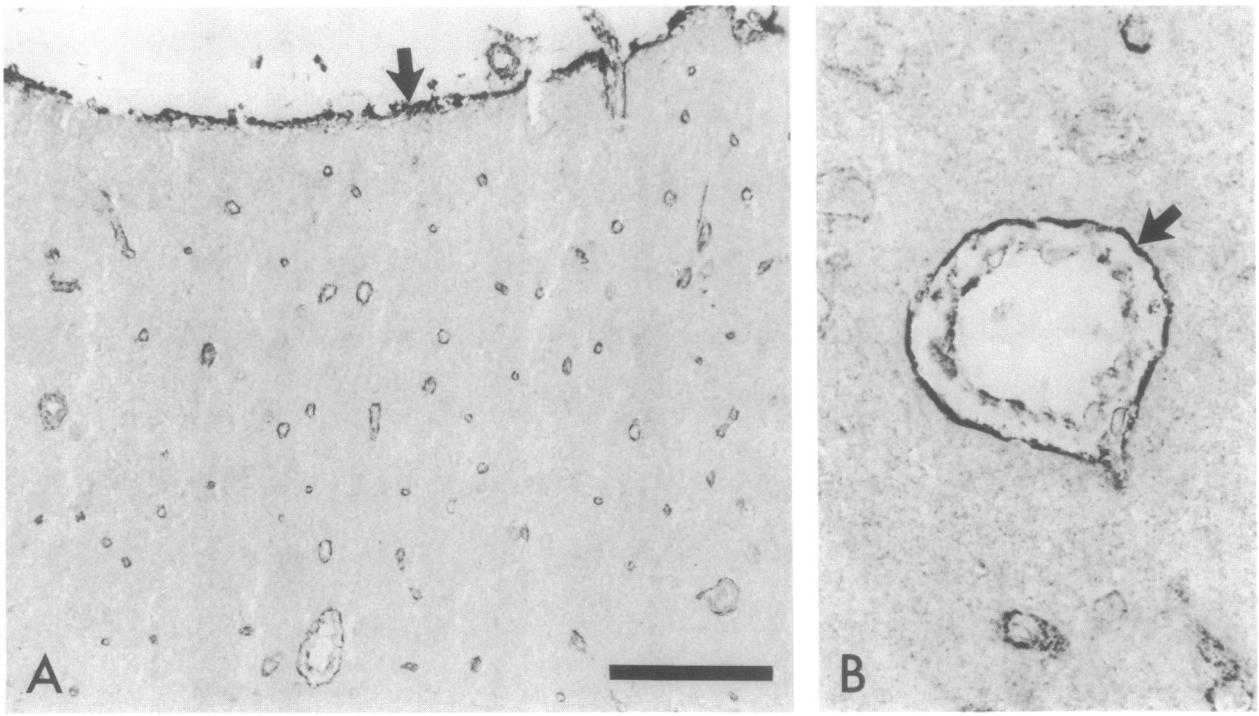

FIG. 5. Immunocytochemistry of canine brain frozen sections using G3A3 antiserum to GLUT3 (1:300). Bound antibody was detected by using a biotinylated secondary antibody and peroxidase-labeled streptavidin. The chromogen was aminoethylcarbazole. (*A*) Cerebral cortex. Reaction product was associated with microvessels and glia limitans or intima pia (arrow). (*B*) Cerebral arteriole. Immunoreactivity was present in a layer (arrow), probably composed of astrocytic processes or leptomeningeal cells, surrounding the smooth muscle cells of the arteriolar wall. (*A*, bar = 160 μm; *B*, bar = 40 μm.)

the GLUT3 C terminus in pyramidal cells and microvessels may not be equally available to the two antisera or that it may exist in two structural (secondary or tertiary) forms specific to neuronal or vascular cells. We have observed that the G3A1, but not the G3A3, epitope survives perfusion with buffered formaldehyde, and this observation lends support to the hypothesis of conformation-dependent epitopes. Finally, these results do not exclude the possibility that other brain cell types (fusiform and stellate neurons, astrocytes, oligodendrocytes) may contain GLUT3 transporters that were undetectable under the conditions used in this study.

In a recent report (15), the concentration of immunoreactive GLUT1 in bovine microvessel membranes (10.8 ± 0.9 pmol/mg) and the concentration of total glucose transporters as defined by D-glucose-displaceable [^{3}H]cytochalasin B binding (11.7 ± 3.5 pmol/mg) were determined. Although no statistical difference was detected, the errors in these measurements allow for the presence of significant amounts of additional transporter isoforms. On the basis of these results and our current observations, we conclude that GLUT3 constitutes a significant, but quantitatively minor, glucose transporter at the blood–brain barrier.

Our results indicate that the GLUT3 isoform is found in the brains of at least two mammalian species and that it may be a major transporter for glucose entry into pyramidal cells. In rat brain, the immunocytochemical finding that GLUT3 C-terminal antigen is present in pyramidal cells and microvessels is consistent with the finding that antibodies to GLUT1 peptides were incapable of precipitating most cytochalasin B-labeled rat brain transporter (9). Individual neurons varied widely in staining intensity and thus, apparently, in their GLUT3 content. Additional study may determine whether specific populations of neurons exist with respect to expression of GLUT3.

The presence of GLUT3 in neuronal cytoplasm contrasts sharply with the distribution of GLUT1 in cerebral microvessel endothelial cells and may be related to differences in function and regulatory mechanisms of these two transporters. An ultrastructural immunogold study of canine brain (5) indicated that most endothelial GLUT1 was associated with luminal and abluminal membranes and little was associated with cytoplasmic structures. Thus, the brain endothelium, which facilitates transcellular transport of glucose, does not have a significant quantity of intracellular GLUT1-type transporters available for recruitment to the plasma membrane in response to regulatory stimuli. GLUT3, on the other hand, is clearly associated with cytoplasmic structures, and its cellular distribution may be similar to that of the insulin-sensitive transporters of basal adipocytes. An immunogold study of cultured adipocytes (17) indicated that treatment with insulin induced rapid translocation of transporters from cytoplasm to plasma membrane, increasing the transporter labeling of the plasma membrane 3-fold. Glucose utilization by neurons is believed to vary greatly in response to physiological stimulation and changes in cellular energy demands. Therefore, the cytoplasmic localization of major quantities of GLUT3 in neurons offers the possibility that this transporter may be under dynamic regulatory control. Alternatively, the observations may represent the trafficking of glucose transporters in pathways of synthesis and degradation.

The involvement and regulation of the GLUT3 isoform in brain under pathophysiological conditions remains to be defined. The GLUT1 transporter in cerebral microvessels of human brain tumors exhibits reduced expression (18) but is not altered in hippocampus after ischemia (19). The finding that the GLUT3 isoform of the glucose transporter family is present in brain microvessels and pyramidal cells suggests that this transporter may play an important role in brain metabolism. Abnormalities affecting glucose transport or transport regulation in brain could have important consequences for neuronal function.

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