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Synaptotagmin IV is an immediate early gene induced by depolarization in PC12 cells and in brain

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ABSTRACT **Subtractive library construction and differential screening were used to identify a cDNA for a cell type-specific immediate early gene induced in rat PC12 pheochromocytoma cells. Sequencing identified the protein product of this gene as rat synaptotagmin IV (SytIV). Synaptotagmins are synaptic vesicle proteins thought to play a role in depolarization-induced, calcium-mediated exocytosis and neurotransmitter release. SytIV mRNA accumulation is transiently induced in PC12 cells by potassium depolarization, calcium ionophore, ATP, and forskolin. In contrast, growth factors and phorbol 12-myristate 13-acetate induce little or no SytIV mRNA accumulation. Kainic acid-induced seizures in rats are followed by accumulation of SytIV message in the hippocampus and piriform cortex. The SytIV gene may provide a direct link between depolarization-induced neuronal gene expression and subsequent modulation of synaptic structure and function.**

We and others have identified "primary response," or immediate early genes (IEGs) whose transcription is elevated by mitogenic stimulation of fibroblasts in the presence of protein synthesis inhibitors (reviewed in ref. 1). Many of the mitogen-induced IEGs are also induced in neurons following experimental seizures and/or more specific modes of neuronal stimulation (2, 3). Previous studies of both IEGs induced in PC12 pheochromocytoma cells (4–7) and genes induced in brain following experimentally induced seizures (8–11) have identified many IEGs that are also inducible in other cell types.

We wish to identify neuron-specific IEGs. We combined subtractive hybridization, to reduce the frequency of "housekeeping genes" and genes induced in all cell types, and differential screening, to identify cDNAs that are induced in PC12 cells by a variety of stimuli. We have identified an inducible form of synaptotagmin that is the rat homologue of synaptotagmin IV (SytIV) (12). The SytIV gene is inducible in PC12 cells by depolarization, calcium ionophore, forskolin, and ATP but is relatively unresponsive to mitogens. Kainic acid seizures induce SytIV expression in the hippocampus and piriform cortex.§

MATERIALS AND METHODS

Cell Culture and RNA Isolation. PC12 cells were cultured on collagen (Collaborative Research)-coated dishes by modifications of conditions described previously (13). FAO hepatoma cells were cultured in α minimal essential medium with 10% fetal bovine serum. Rat1 cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. PC12 groups received cycloheximide (CHX, 10 μg/ml), conditioned medium, and one of the following treatments: nerve growth factor (NGF, 50 ng/ml), basic fibroblast growth factor (bFGF, 10 ng/ml), epidermal growth factor (EGF, 10 ng/ml), phorbol 12-myristate 13-acetate (PMA, 50 ng/ml), KCl (50 mM), forskolin (50 μM), or a mixture of these inducers. RNA was prepared (13) after 3 hr. RNA was also prepared from PMA/CHX-treated FAO cells and EGF/PMA/CHX-treated Rat1 cells. PC12 and FAO poly(A)$^+$ RNAs were prepared with PolyATtract (Promega), and Rat1 poly(A)$^+$ RNA with FastTrack (Invitrogen).

Library Construction and Plasmid Preparation. A directional λ ZAP II (Stratagene) cDNA library was constructed for each poly(A)$^+$ RNA (PC12$^+$, FAO$^+$, and Rat1$^+$). Clones were converted to plasmids with the Exassist/Solr *Escherichia coli* system (Stratagene).

Subtractive Hybridization and Preparation of an Induced, Cell Type-Enriched PC12 Library. Plasmid DNAs were linearized with *Not* I restriction endonuclease, which cuts the vector 5′ of the cDNA insert and the site of amplification (see below). Antisense cRNA was prepared from each library by use of T7 RNA polymerase, followed by treatment with RNase-free DNase I (Megascript; Ambion, Austin, TX). PC12$^+$ single-stranded DNA was synthesized by reverse transcribing the PC12$^+$ cRNA with an upper ZAP primer (5′-CGGGCTGCAGGAATTC-3′; Integrated DNA Technologies, Coralville, IA).

FAO and Rat1 cRNAs were biotinylated with super-XX-PAB (Clontech). PC12$^+$ single-stranded DNA (10 μg) was prehybridized with 25 μg of RNA transcribed from pBluescript plasmid (Stratagene) without insert. Biotinylated FAO$^+$ and Rat1$^+$ cRNAs (200 μg) were added, and hybridization was continued. Streptavidin was added, and the streptavidin–biotin-RNA·single-stranded DNA hybrids and excess streptavidin–biotin-RNA were removed by repeated phenol/chloroform extractions (14, 15). After concentration, the subtraction was repeated. Twice-subtracted PC12$^+$ single-stranded DNA was PCR amplified by using the upper ZAP primer and a lower ZAP primer, 5′-CCCCTCGAGTTTTTTTTTTT-3′, designed to hybridize to poly(A) tails and the *Xho* I cloning-site region. PCR-amplified DNA (650 ng) was cut with *Eco*RI and *Xho* I and ligated to Uni-ZAP XR (*Eco*RI/*Xho* I digested, phosphatase-treated λ ZAP II; Stratagene), packaged with Gigapack Gold II (Stratagene), and amplified in Sure *E. coli* cells (Stratagene).

Screening the Subtracted Library for Induced, PC12-Restricted cDNAs, and Isolation of Near-Full-Length Clones. Twenty 10-cm plates, each containing ≈3000 phage on a lawn of XL1-Blue *E. coli* (Stratagene), were inoculated in a set pattern with phage containing cDNAs for the S2 ribosomal protein (a constitutive clone), TIS8/egr-1 (an inducible clone), and tyrosine hydroxylase (a PC12-specific clone). Three ni-

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Abbreviations: CHX, cycloheximide; IEG, immediate early gene; SytI–SytIV, synaptotagmin I–IV; PMA, phorbol 12-myristate 13-acetate; NGF, nerve growth factor; bFGF, basic fibroblast growth factor; EGF, epidermal growth factor.

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§The sequence reported in this paper has been deposited in the Genbank data base (accession number L38247).

trocellulose filter (Schleicher & Schuell) lifts were made. The first filter was hybridized with ^{32}P-labeled cDNA prepared from uninduced PC12 cells. The second was hybridized to ^{32}P-labeled cDNA prepared from ligand-treated PC12 cells. The third was hybridized to ^{32}P-labeled cDNA prepared from induced FAO cells. Filters were exposed to film so that the S2 spots were of equal intensity. Clones that appeared to show greater intensity with the probe from induced PC12 cells relative to untreated PC12 cells and show little or no hybridization to the FAO$^+$ probe were picked, plaque purified, gridded, subjected to filter lifts, and rescreened with PC12$^+$, PC12, FAO$^+$, and Rat1$^+$ probes. Inserts that appeared induced and restricted to PC12 cells were amplified with T3 and T7 primers (Stratagene). PCR product was labeled with [α-^{32}P]dCTP and used for preliminary Northern analyses of differential expression in PC12 cells and cell-type specificity. Clones to be analyzed further were converted to plasmids. Longer cDNA clones were isolated by screening the original PC12$^+$ library; 5′-ends were cloned with the 5′-AmpliFINDER RACE kit (Clontech).

Preparation of the SytI Probe. PC12 cell total RNA served as template for Moloney murine leukemia virus reverse transcriptase, using SytI primer sequence 5′-GCCGAATCCCACTATGTGGGCAGATGC-3′. PCR was carried out with this primer and 5′-GCGCTCGAGAAGGGAGGAAAGAACGCC-3′, spanning the sequence between aa 95 and 421. PCR product was cloned with the Invitrogen TA cloning kit. Product was verified by sequencing.

Northern Analysis. RNA from cultures was isolated by LiCl precipitation (16). RNA from tissues was isolated according to Chomczynski and Sacchi (17). Denatured RNA samples were subjected to electrophoresis, transferred to Nylon membranes (Biotrans, ICN), fixed by irradiation, and hybridized with radioactive probes prepared from gel-purified inserts. Filters were exposed to film at −80°C with an intensifying screen.

DNA Sequencing. Sequencing was carried out by the University of California, Los Angeles, Sequencing Core Facility on an Applied Biosystems instrument.

Kainate Treatment, Preparation of Brain Sections, and *in Situ* Hybridization. Male Sprague–Dawley rats on a 12-hr light/dark cycle received food and water ad libitum. Kainate was administered by subcutaneous injection (10 mg/kg) in 0.9% NaCl. Animals were sacrificed 4 hr after initiation of seizures. Controls were not handled prior to sacrifice. Rats were anesthetized with methoxyflurane and decapitated. Brains were removed and frozen in −30°C isopentane (methyl butane), and 10-μm sections were cut and stored at −70°C. The SytIV oligonucleotide 5′-TGGGGTTGGTTTTGGGGAAATTGCCATTGAGG-3′ (539–570, corresponding to aa 99–110) and the SytI oligonucleotide 5′-ATCTGACTGCGGATGTTGGTTGCTGAAGCACTTTC-3′ (nt 359–393 of the 5′ untranslated region) were end-labeled with [α-[^{35}S]thio]dATP by terminal deoxynucleotidyltransferase (Promega). Incorporation yields ranged from 75% to 85%. Slides were incubated for 30 min in 4% paraformaldehyde/phosphate-buffered saline (PBS), washed three times in PBS (30 min), and dehydrated through graded ethanol solutions. Probes were diluted to ≈5000 cpm/μl in hybridization buffer [50% formamide/4× standard saline citrate (SSC)/5× Denhardt's solution/1% SDS/10% dextran sulfate/0.1 M dithiothreitol with poly(A) at 25 μg/ml and poly(C) at 25 μg/ml]. Sections were hybridized overnight (43°C) and washed for 20 min in 4× SSC, several hours in 2× SSC, and 30 min in 0.15× SSC (55°C). Slides were dehydrated through graded ethanol solutions with 0.3 M ammonium acetate, dried, and exposed to film (Amersham).

Quantitation of Autoradiographs. Films were analyzed with a computerized image analysis system (DUMAS system from Drexel University, Philadelphia) and BRAIN software (Drexel University). Relative optical densities were measured for CA1, CA3, dentate gyrus, parietal cortex, and piriform cortex. Statistical analyses were performed with STATISTICA software (Statsoft, Tulsa, OK). This system consists of ANOVAs for group and region, followed by the Newman–Keuls post-hoc analysis. P values < 0.01 were considered statistically significant.

RESULTS

Cloning a cDNA for an Inducible SytIV from PC12 Cells. PC12 cultures were treated individually with NGF, EGF, bFGF, PMA, forskolin, or KCl or with a mixture of these inducers, in the presence of CHX. After 3 hr, cells were harvested and pooled, and poly(A)$^+$ RNA was prepared. Poly(A)$^+$ RNA was also prepared from PMA/CHX-treated FAO hepatoma cells and PMA/EGF/CHX-treated Rat1 fibroblasts. cDNA libraries were prepared in λ Uni-ZAP (XR) II for each RNA, plasmid cDNA libraries were created, and cRNA was synthesized from each plasmid library. Single-stranded cDNA was prepared from the PC12-induced cRNA. The FAO-induced and Rat1-induced cRNAs were biotinylated and used to twice subtract the PC12 single-stranded cDNA. The PC12 cDNA remaining after subtraction was subjected to PCR amplification with λ ZAP primers designed to amplify inserts with poly(A) tails. After digestion with *Eco*RI and *Xho* I, a PC12 cell type-restricted phase library was prepared. Triplicate filter lifts were screened with ^{32}P-labeled cDNAs prepared from poly(A)$^+$ RNAs from PC12 cells treated with inducers, untreated PC12 cells, and PMA-treated FAO cells. Clones expressed in stimulated PC12 cells, but not in stimulated FAO cells, were plaque purified. Their inserts were amplified and used for Northern analysis of RNA from PC12 cells and other rat cell lines. One clone that hybridized to PC12 RNA and did not give a significant signal with other cells was used to identify a near-full-length clone from the original PC12 cDNA library. Sequencing identified the encoded protein as the rat homologue of SytIV (ref. 12 and Fig. 1). Although our initial screen of the library identified only the SytIV gene, a subsequent rescreen of the library also identified a cDNA for secretogranin as a depolarization/forskolin inducible gene whose message levels are not altered by growth factors (data not shown). Secretogranin was previously identified as a depolarization-inducible gene by screening of a cDNA library prepared from the brains of animals subjected to depolarization (11).

SytIV Is Encoded by an IEG That Is Induced by Depolarization, Calcium Ionophore, ATP, and Forskolin in PC12 Cells. The PC12 cDNA library from which we cloned rat SytIV was prepared from cells induced with growth factors, depo-

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M. Syt4  ------------------------------------------------------------  50
R. Syt4  MAPITTSRVEFDEIPTVVGIFSAFGLVFTVSLFAWICCQRRSAKSNKTPP            50

M. Syt4  --------------------Q----------V-G-T---------------------  100
R. Syt4  YKFVHVLKGVDIYPENLSSKKKFGGDDKSEAKRKAALPNLSLHLDLEKRD          100

M. Syt4  -------A-----------------T------N----------------------  150
R. Syt4  LNGNFPKTNPKAGSSSDLENVTPKLFPETEKEAVSPESLKSSTSLTSEEK        150

M. Syt4  --K---------------------------------------------------------  200
R. Syt4  QEWLGTLFLSLEYNFEKKAFVVNIKEAQGLPAMDEQSMTSDPYIKMTILP        200

M. Syt4  ----R--------------G-------I----------------------------  250
R. Syt4  EKKHKVKTRVLRKTLDPVFDETFTFYGVPYPHIQELSLHFTVLSFDRFSR        250

M. Syt4  --------I------------------------------------------------  300
R. Syt4  DDVIGEVLVPLSGIELSDGKMLMTREIIKRNAKKSSGRGELLVSLCYQST        300

M. Syt4  ------------------------------------------------------------  350
R. Syt4  TNTLTVVVLKARHLPKSDVSGLSDPYVKVNLYHAKKRISKKKTHVKKCTP        350

M. Syt4  ------------------------------------------------------------  400
R. Syt4  NAVFNELFVFDIPCESLEEISVEFLVLDSERGSRNEVIGRLVLGATAEGS        400

M. Syt4  -------------------------  425
R. Syt4  GGGHWKEICDFPRRQIAKWHMLCDG  425
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FIG. 1. Predicted amino acid sequences of mouse (M.) and rat (R.) SytIV. Transmembrane domains are indicated by the dashed underline. C2 domains are indicated by the solid underlines. Dashes indicate identical amino acids.

larizing agents, and other agents. Both potassium depolarization and the calcium ionophore A23187 cause calcium to enter neurons and stimulate neurotransmitter release. Both agents induced SytIV message accumulation in PC12 cells. Forskolin, a stimulator of adenylate cyclase and neurotransmitter release, also induced an increase in SytIV message. Finally, ATP, a PC12 secretogogue that causes an influx of calcium, induced SytIV mRNA accumulation (Fig. 2*A*). However, growth factors (NGF, EGF, bFGF, and interleukin 6) induced little or no elevation in SytIV mRNA. PMA, a potent inducer of many IEGs in PC12 cells (13), slightly but reproducibly suppressed SytIV mRNA. In contrast to SytIV induction, growth factors and PMA were better inducers of the TIS8/NGFI-A/Egr1 gene than were depolarizing conditions or secretagogues (data not shown).

We utilized an agent that elevates cAMP (forskolin), an agent that increases intracellular calcium (A23187), and one naturally occurring secretogogue (ATP) to determine the time courses of SytIV mRNA accumulation. SytIV mRNA peaked in PC12 cells 2–8 hr after forskolin, ATP, or A23187 addition. For ATP and forskolin, message levels returned to baseline by 24 hr (Fig. 2*B*). Potassium depolarization also caused a peak in SytIV exposure, at 3–5 hr (data not shown). For both A23187 and KCl induction, PC12 cells came off the plate after 8–10 hr. SytIV was also induced by forskolin and KCl in PC12 cells that were differentiated by exposure to NGF for 7–10 days (data not shown).

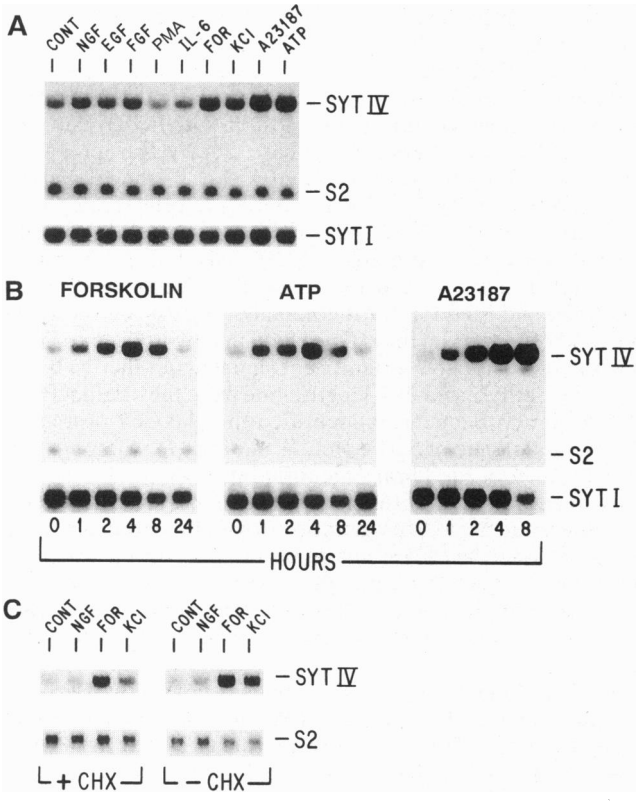

FIG. 2. SytIV is induced in PC12 cells by depolarization, calcium ionophore, ATP, and forskolin. (*A*) Induction of SytIV in PC12 cells by individual inducers. PC12 cells were treated with NGF (50 ng/ml), EGF (20 ng/ml), bFGF (20 ng/ml), PMA (50 ng/ml), interleukin 6 (IL-6, 50 ng/ml), forskolin (FOR, 50 μM), KCl (50 mM), A23187 (10 μM), or ATP (200 μM). CONT (control), no addition. Cells were harvested 3 hr after additions. RNAs were prepared and subjected to electrophoresis and transfer to membranes. Each lane was loaded with 10 μg of RNA. The membrane was hybridized with a mixture of ^{32}P-labeled SytIV and S2 cDNA probes. S2, a cDNA for a ribosomal protein, was used to normalize for RNA loading. Filters were exposed to film, then stripped, hybridized to a SytI probe, and reexposed. (*B*) Time course of SytIV induction. PC12 cells were treated for the times shown with forskolin (50 μM), ATP (200 μM), or A23187 (10 μM). RNAs were isolated and subjected to Northern analysis. After exposure to film, the filters were stripped, rehybridized with a SytI probe, washed, and reexposed. (*C*) SytIV is encoded by an IEG. PC12 cells were exposed to CHX alone (10 μg/ml) or to NGF (50 ng/ml), forskolin (50 μM), or KCl (50 mM), in the presence or absence of CHX. Cells were harvested after 3 hr. RNA was subjected to Northern analysis.

PC12 cells express SytI and -III but not SytII (18). It was of interest to determine whether SytI message is inducible. Filters containing RNAs from the time-course experiments (Fig. 2*B*) and experiments to determine the SytIV response to individual inducers (Fig. 2*A*) were stripped and reprobed with a SytI cDNA. Neither calcium mediators nor forskolin induced SytI mRNA accumulation in PC12 cells. A slight decrease in SytI message was often observed 4–8 hr after exposure to depolarizing agents or secretagogues (Fig. 2*B*). Growth factors and PMA did not modulate SytI mRNA levels (Fig. 2*A* and data not shown).

To determine whether SytIV is an IEG, induced by activation of preexisting transcription factors, we examined SytIV mRNA accumulation in PC12 cells stimulated in the presence of CHX. Both forskolin and elevated potassium, but not NGF, induced SytIV mRNA accumulation in the presence of CHX (Fig. 2*C*); hence, SytIV is encoded by a stimulus-specific IEG.

Fig. 2 *A–C* show three separate experiments in which depolarization or forskolin induced SytIV induction and illustrate the variability observed in induction in PC12 cells. The variability is based primarily on differences in basal levels (high in Fig. 2*A*, intermediate in Fig. 2*B*, and low in Fig. 2*C*). Fig. 2 *A* and *C* demonstrate the lack of neurotrophin induction relative to depolarization or forskolin stimulation. Induction of TIS8/Egr-1 in these same experiments was substantially greater in response to EGF, FGF, NGF, or PMA than in response to KCl or forskolin (data not shown), demonstrating the preferential induction of SytIV by the latter two agents despite a robust signal transduction activation in these cells by growth factors.

SytIV Is Expressed Only in Brain and Neuroendocrine Tissue. We isolated RNA from rat brain regions and other tissues and analyzed these RNAs with probes for SytI and SytIV. Like SytI mRNA, SytIV mRNA was found in all brain regions (Fig. 3). The concentration gradients of SytI and SytIV mRNAs were generally rostral to caudal, with the brainstem having the lowest level of both messages. The pituitary gland, a major neuroendocrine organ, was the only other tissue to exhibit substantial amounts of SytIV mRNA. In contrast, SytI mRNA was low in the pituitary, relative to brain. No SytI or SytIV mRNA was detectable in heart, muscle, liver, intestine, kidney, lung, or thymus (data not shown).

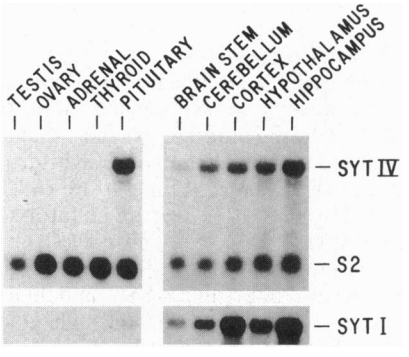

FIG. 3. Comparison of SytI and SytIV expression in brain and neuroendocrine tissues. RNA samples from each of the rat tissues and brain regions indicated in the figure were subjected to Northern analysis with SytI, SytIV, and S2 probes. Each lane was loaded with 10 μg of RNA.

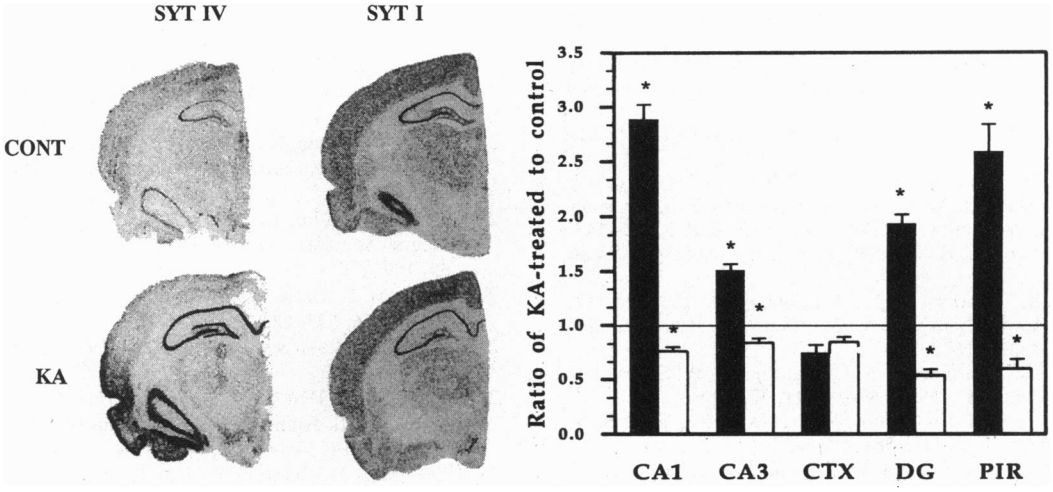

FIG. 4. Kainic acid-induced seizures are followed by increased levels of SytIV mRNA in brain. Five male rats were injected subcutaneously with kainic acid (KA, 10 mg/kg). Four hours after seizures initiated, these rats and five control (CONT) rats were sacrificed and decapitated. Brain sections were hybridized with oligonucleotide probes specific for SytIV and SytI. Competition with excess unlabeled oligonucleotides blocked all hybridization. (*Left*) Sections from individual animals. (*Right*) The optical densities of radioautographs from brain regions in the five experimental and five control animals were determined as described in the text. Data are expressed as ratios of optical densities for kainate-treated animals divided by optical density for control animals. CA1 and CA3, hippocampal pyramidal cell layers; CTX, parietal cortex; DG, dentate gyrus; PIR, piriform cortex. Error bars indicate standard errors. Asterisk indicates statistically significant differences at a P value of 0.01 for kainate-treated and control animals. Solid bars, SytIV; open bars, SytI.

Kainic Acid-Induced Seizures Are Followed by Accumulation of SytIV Message in Rat Brain. We utilized the kainate seizure model to determine whether SytIV induction occurs in brain. Five male rats received kainate (10 mg/kg); five animals were used as controls. All kainate-treated animals exhibited seizures. Animals were sacrificed 4 hr after seizure onset and brain sections were hybridized to SytI- or SytIV-specific oligonucleotide probes (Fig. 4). SytIV mRNA levels were substantially elevated in the whole limbic system following depolarization. The piriform cortex and the hippocampus showed strong increases in SytIV hybridization, with highest stimulation observed in the CA1 pyramidal cells (Fig. 4), as well as the dentate granule cells and hilar cells. In the piriform cortex, layer II showed the greatest increase in SytIV message. We saw no elevation in SytI mRNA following seizure, in agreement with Mahata *et al.* (19). In fact, a small but statistically significant decrease in SytI mRNA occurred in the dentate gyrus, in hippocampal pyramidal cells, and in the piriform cortex following seizure.

DISCUSSION

Synaptotagmins are present in small, clear synaptic vesicles and large, dense-core vesicles (20, 21). They contain four domains (22): (*i*) an N-terminal region within the synaptic vesicle, (*ii*) a hydrophobic transmembrane region, (*iii*) a spacer region, and (*iv*) a C-terminal region that contains two calcium-binding C2 domains involved in the formation of a ternary complex of protein, phospholipid, and calcium (23, 24). The two SytIV C2 regions share strong sequence conservation with other synaptotagmins (18, 22–25). In contrast, there is no sequence similarity between the spacer region of SytIV and any of the other synaptotagmins. Amino acids 14–39 of SytIV satisfy the criteria of the MOMENT program (26) for a transmembrane region but differ significantly from the transmembrane sequences of other synaptotagmins. Finally, the C-terminal 24 aa of SytIV share sequence similarities with the region of SytI responsible for binding to neurexin, a presynaptic membrane-spanning protein (27).

One hypothesis for calcium-regulated synaptic activation suggests that the synaptotagmins act, through their C2 regions, as the calcium sensor to initiate vesicle fusion with the presynaptic plasma membrane. Another hypothesis suggests that the synaptotagmins serve as negative barriers to fusion of synaptic vesicles with the presynaptic membrane. In this latter model, the inhibitory role of synaptotagmin is relieved by increased calcium, following depolarization, and vesicle-plasma membrane fusion follows. Synaptotagmin C2 peptides and antibodies to synaptotagmins block synaptic vesicle fusion, suggesting the synaptotagmins do indeed play key roles in calcium-dependent exocytosis (28). Synaptotagmin null mutations have profound effects on synaptic function in nematodes and *Drosophila* (29–32). The most recent models based on these mutational data suggest that synaptotagmins both promote the docked vesicle state and inhibit vesicle/membrane fusion and neurotransmitter release (30, 32).

SytII is most prevalent in phylogenetically older regions of the brain (25). SytIII is more abundant in the cerebellum than in the cortex (18). The strong overlap of SytIV and SytI mRNAs in hippocampus and cortex and the presence of SytIV and SytI mRNAs in clonal PC12 cells suggest that the two genes are expressed in common neurons in brain. If SytIV is indeed a synaptic vesicle component, it is likely that changing populations of SytI and SytIV proteins are present in common populations of synaptic vesicles.

How might depolarization-dependent alterations in synaptic efficacy be brought about? If newly synthesized SytIV is incorporated into recycling synaptic vesicles following depolarization, the response to subsequent stimulation of this recycled vesicle subpopulation containing new SytIV molecules could be altered relative to the unrecycled vesicle population. A gene expression-dependent event regulated by stimulus-induced neuronal depolarization could, in this way, be directly linked to subsequent alterations in synaptic structure and thus to synaptic function. It is now necessary to demonstrate that the induction of SytIV mRNA following depolarization is accompanied by an increase in SytIV protein synthesis and that incorporation of SytIV protein into synaptic vesicles alters their biochemical properties.

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