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Inositol 1,4,5 Trisphosphate Receptor and Chromogranin B Are Concentrated in Different Regions of the Hippocampus

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Abstract

Calcium (Ca^{2+}) release from intracellular stores plays a crucial role in many cellular functions in the brain. These intracellular signals have been shown to be transmitted within and between cells. We report a non-uniform distribution of proteins essential for Ca^{2+} signaling in acutely prepared brain slice preparations and organotypic slice cultures, both made from rat hippocampus. The Type I inositol-1,4,5 trisphosphate receptor ($InsP_3R1$) is the main $InsP_3R$ subtype in neurons. Immunohistochemistry experiments showed a prominent expression of $InsP_3R1$ in the CA1 region of the hippocampus whereas the CA3 region and dentate gyrus (DG) showed only moderate immunoreactivity. In contrast, chromogranin B (CGB), a protein binding to the $InsP_3R1$ on the luminal side of the endoplasmic reticular membrane was enriched in the CA3 region whereas DG and the CA1 region showed only faint CGB signals. The neuronal kinases leading to the formation of inositol-1,4,5 trisphosphate ($InsP_3$), phosphatidylinositol-4-kinase (PI4K), and phosphatidylinositol-4-phosphate-5-kinase (PIPK), showed strong immunoreactivity throughout all hippocampal cell fields with differences in the subcellular distribution. Moreover, a distinct band of strong CGB and PIPK immunoreactivity was observed in the CA3 region that coincides with the mossy fiber tract (stratum lucidum). These data show differential expression of the components of the signaling toolkit leading to $InsP_3$-mediated Ca^{2+} release in cells of the hippocampus. The regulation of these differences may play an important role in various neuropathologic conditions such as Alzheimer's disease, epilepsy, or schizophrenia.

Keywords

rat; intracellular signaling; calcium; immunohistochemistry; organotypic slice culture

Calcium (Ca^{2+}) as an intracellular second messenger plays a crucial role in a variety of neuronal functions like development, excitability, neurotransmitter release, synaptic plasticity, gene transcription, and neurodegeneration (Berridge, 1998). It can either enter from the extracellular

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side through voltage-gated, ligand-gated, and possibly store-operated channels (Berridge, 1998) or from internal stores through Ca^{2+} channels in the endoplasmatic reticulum (ER), the Golgi apparatus, and the mitochondria (Rizzuto, 2001). The ER is the main intracellular calcium store and it is found throughout the cell (Spacek and Harris, 1997) forming a complex network (Terasaki et al., 1994; Berridge, 1998).

Different Ca^{2+} signals exhibit distinct temporal and spatial patterns. An increase of intracellular Ca^{2+} can either be restricted to the site of Ca^{2+} entry or spread throughout the neuron (Augustine et al., 2003). Because an increase in cytosolic Ca^{2+} can cause a plethora of cellular effects, all Ca^{2+} signals have to be regulated accurately (Usachev and Thayer, 1999). For this purpose, all neurons use a variety of channels, receptors, enzymes, and associated proteins that form a complex Ca^{2+} signaling toolkit (Berridge et al., 2003). The constitution of this toolkit affects strongly the shape of Ca^{2+} signals in neuronally-derived cell lines and hippocampal neurons.

Growing evidence shows that the components involved in Ca^{2+}-dependent signaling cascades show a distinct differential distribution within neuronal cells (Finch and Augustine, 1998; Hanson and Smith, 2002; Johenning et al., 2002). In hippocampal neurons, inositol 1,4,5-trisphosphate ($InsP_3$) produced via post-synaptic Group I metabotropic glutamate receptors (mGluR) has been identified as the main intracellular messenger for Ca^{2+} waves (Yeckel et al., 1999; Nakamura et al., 2000; Kapur et al., 2001). Therefore, comprehension of the kinetics and initiation sites of intracellular Ca^{2+} signals requires an insight into the distribution of the diverse components of the $InsP_3$-mediated signaling cascade.

The synthesis of the second messenger $InsP_3$ starts with the membrane lipid phosphatidylinositol (PI) that is phosphorylated two times and then, when a stimulus appears, the lipid is cleaved into diacylglycerol (DAG) and inositol 1,4,5 trisphosphate. The first phosphorylation step is undertaken by phosphatidylinositol-4-kinase (PI4K) leading to the production of phosphatidylinositol-4 phosphate (PIP). Two groups of PI4 kinases, PI4K II and PI4K III, that show different sensitivity to the inhibitors Wortmannin, phenyl arsine oxide, and 5′-*p*-fluorosulfonylbenzoyladenosine (Barylko et al., 2002; Heilmeyer et al., 2003), are known to date. High levels of PI4K III mRNA can be found in various regions of the brain, among them the hippocampus (Zolyomi et al., 2000). PIP in turn is phosphorylated a second time by phosphatidylinositol-4 phosphate-5 kinase (PIPK) forming phosphatidylinositol-4,5 bisphosphate (PIP2). Two PIPK subtypes, PIPK I and PIPK II, have been described (Anderson et al., 1999) with the PIPK Iγ isoform being the major enzyme in neurons (Wenk et al., 2001).

After a final cleavage of PIP2 and the formation of DAG and $InsP_3$, the second messenger $InsP_3$ diffuses through the cytosol and it binds to an ER transmembrane receptor. The family of $InsP_3$-sensitive receptors consists of three subtypes that differ in their affinity toward $InsP_3$ (Patel et al., 1999; Taylor et al., 1999), their Ca^{2+}-dependent inactivation (Bezprozvanny et al., 1991; Hagar et al., 1998), their distribution in various tissues (Sharp et al., 1999), their regulation by phosphorylation (Tang et al., 2003), and modulating molecules (Hagar and Ehrlich, 2000; Thrower et al., 2001).

Besides the regulatory factors that bind to the $InsP_3$ receptor from the cytosolic side the ER proteins chromogranin A (CGA) and B (CGB) have been shown to increase the $InsP_3R1$ open probability by binding from the luminal side of the ER (Thrower et al., 2003; Thrower et al., 2001). Those high-capacity, low-affinity Ca^{2+} storage proteins have been found in a variety of endocrine and also neuronal tissue (Fischer-Colbrie et al., 1985; Iacangelo et al., 1986; Mahata et al., 1991; Winkler and Fischer-Colbrie, 1992).

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For a variety of experiments on neurons, organotypic slices have emerged as a powerful model (Gahwiler et al., 1997). They can be kept in culture for several weeks and keep differentiating and maturing still resembling the cytoarchitecture and tissue morphology of in-vivo tissue (Zimmer and Gahwiler, 1984; Muller et al., 1993; De Simoni et al., 2003). During the culturing period organotypic slices tend to flatten out considerably allowing microscopic examinations and micromanipulations. Because of their relatively long survival time of several weeks, the slices can be used as a model allowing long-term observations and experiments requiring chronic treatments (Bahr, 1995).

We have used immunohistochemistry and immunoblotting to investigate the distribution of the different components of the signaling toolkit in hippocampus that contribute to the InsP3-mediated Ca^{2+} release from intracellular stores. We have used acutely prepared and organotypic slices from rat to compare the distribution of $InsP_3R1$, PI4K, PIPK, and CGB in those two brain models. We found that PI4K and PIPK were distributed throughout the hippocampus, whereas the $InsP_3R1$ and CGB were distributed in a reciprocal manner in the CA1 and CA3 regions. The CA3 region of hippocampus has been linked to functions such as spatial pattern association, novelty detection, and short-term memory. In contrast, CA1 contributes to temporal pattern processing and intermediate-term memory (Kesner et al., 2004). CA3 seems capable of a more non-linear transformation of sensory information compared to CA1 (Guzowski et al., 2004). The distribution of the proteins described has been shown to be disrupted in various neuropathologic conditions, suggesting that these differences in protein distribution have important functional consequences.

MATERIALS AND METHODS

Acutely Prepared Brain Slices

Rats between 18–21 days old (P18–P21) were anesthetized by intraperitoneal injection of an anesthetic consisting of ketamine (125 mg/kg), xylazine (6.25 mg/kg), and acepromazine (1.25 mg/kg). This anesthesia cocktail was injected (0.1 ml/20 g of body weight. The animals were perfused intracardially with 0.1 M phosphate-buffered saline (PBS), pH 7.4 containing 4% paraformaldehyde for 20 min. Brains were removed from the skulls and then postfixed in the same fixative overnight at 4°C. Transversal serial sections, 60-μm thick, were cut using a Leica vibratome and collected in PBS.

Organotypic Slice Cultures

Hippocampal organotypic slice cultures were prepared using a modification of a protocol published previously (Stoppini et al., 1991). Briefly, after inducing a deep anesthesia (for acute slice preparation) rats between P10–P12 were decapitated. Brains were removed and placed in ice-cold dissecting solution containing NaCl (87 mM), KCl (2.5 mM), $CaCl_2$ (0.5 mM), $MgCl_2$ (7 mM), $NaHCO_3$ (25 mM), NaH_2PO_4 (1.25 mM), D-glucose (10 mM), and sucrose (75 mM). Horizontal brain sections 250-μm thick were cut using a Vibratome (Vibratome, St. Louis, MO) and the hippocampal area was dissected out and incubated in warm oxygenated dissecting solution for 30 min. The slices were then transferred onto Millicell-CM 0.4-μm biophore membranes (Millipore, Billerica, MA) in preincubated culture medium containing 50% DMEM, 25% HBSS, 25% heat-inactivated horse serum, B27 supplement, 100 U/ml penicillin, 100 μg/ml streptomycin (all Gibco, Carlsbad, CA), and 30 mM glucose. The medium was exchanged every second day. On light microscopic examination, the slices showed a well-preserved organotypic appearance with no extensive morphologic changes and no significant signs of cell death throughout the culturing period.

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Antibodies, Immunohistochemistry, and Western Blot Analysis

Anti $InsP_3R1$ antibody was raised against the 19 C-terminal residues of the mouse $InsP_3R1$ and the polyclonal antibody was affinity purified (custom-produce by Research Genetics, Huntsville, AL). It has been described extensively (Koulen et al., 2000; Johenning et al., 2002) and was used at a concentration of 1:2,000. Rabbit polyclonal anti PI4Kβ was used at a concentration of 1:1,000 (Upstate Biotechnology, Lake Placid, NY) and rabbit polyclonal PIPK1γ at a concentration of 1:1,000 (gift from Dr. G. DiPaolo, Yale University). A mouse monoclonal antibody produced to recognize chromogranin B (BD Transduction Laboratories, San Diego, CA), was used at 1:1,000. Primary antibodies were visualized with fluorochrome-coupled secondary antibodies, Alexa Fluor 488 coupled to goat anti-rabbit and goat anti-mouse IgG (Molecular Probes, Eugene, OR).

Immunohistochemistry of organotypic slices was carried out after 8 days in culture. Slices were fixed with 4% (w/v) paraformaldehyde in PBS containing 4% (w/v) sucrose (PBSS), pH 7.4, for 90 min at room temperature and permeabilized with 1% (v/v) Triton X-100 overnight. Nonspecific binding sites were blocked by incubating the cells with PBSS containing 10% normal goat serum (NGS) and 2% BSA for 48 hr. The slices were incubated with the primary antibodies for 48 hr and secondary antibodies for 4 hr in a PBSS solution supplemented with 1% goat serum and 0.1% Triton X-100.

Acutely prepared slices were permeabilized with 1% (v/v) Triton X-100 overnight, unspecific binding sides were blocked by an overnight incubation in PBSS with 10% normal goat serum (NGS) and 2% BSA. Acutely prepared slices were probed with the primary antibodies for 48 hr and secondary antibodies for 4 hr in a PBSS solution containing 1% goat serum and 0.1% Triton-X 100. Control experiments were carried out by incubating the organotypic and acutely prepared slices with only the secondary antibodies and no staining was observed.

For Western blot analysis, proteins from acutely dissected tissue were resolved by SDS-PAGE, transferred to a PVDF membrane, and immunoreactive bands were visualized using standard methods. Membranes were stripped of antibodies and reprobed with the other antibody. The following primary antibodies and their respective dilutions were used: anti-β actin (1:2,500; Abcam), anti-SERCA (1:500), anti-$InsP_3R1$ (1:2,000) (Johenning et al., 2002), anti-Chromogranin B (BD 1:1,000; BD Transduction Laboratories), and PI4K (1:1,000; Upstate Biotechnology).

Immunofluorescence

Labeled cells were visualized on a Zeiss LSM510 META inverted confocal microscope (Zeiss, Oberkochen, Germany) using a 10× and a 40× water immersion objective. Averaging four frames reduced noise. Fluorochromes were excited with an argon laser at 488 nm (Alexa488); appropriate emission filters (505–530 band pass) were used for fluorescence detection. No specific staining was observed when the primary antibody was omitted. Overview pictures were created by merging six single pictures using the Zeiss LSM 5 image browser software (Zeiss, Oberkochen, Germany).

Data Analysis

The relative fluorescence intensities of the stained brain slices were analyzed using Image J software (NIH, Bethesda, MD). Regions of interest (ROI) were drawn over the complete cell body layers and adjacent proximal dendritic shafts of dentate gyrus, CA3, and CA1 regions. Signals in those ROI were compared to the background fluorescence of each slice. A minimum of three slices was taken from each staining and fluorescence values were averaged. An unpaired Student's *t*-test was carried out to evaluate significant intensity differences between the ROI.

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RESULTS

Distribution of $InsP_3R1$

The distribution of $InsP_3R1$ in acutely prepared and organotypic hippocampus samples was observed using immunohistochemical techniques on P10–P12 tissue kept in culture for 1 week and on acutely made tissue slices from hippocampus of P18 rats. All images were done using confocal microscopy. Merged overview pictures of the acutely prepared slices showed only moderate $InsP_3R1$ staining in the granule cell layer of dentate gyrus and the CA3 pyramidal cell layer (Fig. 1A). In contrast, the $InsP_3R1$ signal in the CA1 pyramidal layer was showing progressively increased staining as the region moved from the CA3/CA1 border to the CA1/subicular border, where it showed a maximal intensity at the CA1-subicular border.

In images taken with higher magnification (Fig. 1A), the signal intensity seemed uniform throughout the cell bodies with clear staining of the proximal apical dendritic shaft. In organotypic slices, the primary neuronal layers spread out during the culturing period thus broadening the cell body regions. When compared to the staining observed in the acutely prepared slices, similar patterns were obtained in the organotypic slices. CA1 showed much stronger fluorescence than dentate gyrus and CA3. In close-up images, staining of the proximal apical dendrites could be observed throughout the CA cell fields (Fig. 1A).

Distribution of Chromogranin B

We next investigated the distribution of chromogranins in the acutely prepared brain slices (Fig. 1B). Although we stained the slices for CGA, no specific staining was detected. In contrast, intense staining for CGB was observed. The dentate gyrus seemed to be stained only faintly, whereas there was strong immunofluorescence signals in the hilus of the hippocampus. In the CA3 and CA1 regions, we observed moderate staining particularly in only the stratum oriens and stratum radiatum, and less intense staining in the pyramidal cell layers. In contrast, the region coinciding with the CA3 mossy fiber tract (stratum lucidum) was stained heavily and increased in fluorescence intensity toward the CA3–CA1 border. Images taken with higher magnification show chromogranin-like immunofluorescence signals in the somata of CA3 pyramidal neurons, whereas CA1 neurons seem to be less well stained than even the background, as seen by the pale, dark signals. The staining patterns obtained with organotypic slice preparations were similar to those seen with the acutely prepared slices of hippocampus. Staining in the hilus of organotypic tissue seemed to be more prominent in comparison with the acutely prepared slice preparations. At higher magnification the stratum pyramidale of CA3 and CA1 was strikingly similar, although the organotypic slice cultures displayed an expanded pyramidal layer (Fig. 1B).

Distribution of PI4 Kinase

In the stainings prepared from both acutely prepared and organotypic slices, PI4K immunoreactivity was observed throughout hippocampus (Fig. 2A). Especially intense immunofluorescence was seen in the granule cell layer of the dentate gyrus and the cell body portions of the CA regions. Higher magnification views of the CA3 and CA1 pyramidal neurons showed strong signals throughout the cell body.

Distribution of PIP Kinase

In acutely prepared slices stained for PIPK, we observed relatively strong staining throughout the dendritic regions of the hippocampus (Fig. 2B). The cell body regions of the dentate gyrus and the CA3 and CA1 regions showed faint signals only, with most of the staining in the periphery of the soma. In addition, a strong signal was observed in the CA3 stratum lucidum area in addition to staining in stratum radiatum and stratum moleculare-lacunosum. Both the

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strong signals throughout the CA subfields and the prominent staining in stratum lucidum of the CA3 region were found in the organotypic slices. Higher resolution images showed a staining pattern in the organotypic slices similar to those in the acutely prepared slices where there was a higher signal intensity around the cell borders.

Protein Distribution Around the CA3–CA1 Border

Whereas the expression levels of $InsP_3R1$ increase from dentate gyrus throughout the CA subregions and show a signal maximum in the CA1 region, the immunofluorescence stainings for CGB and PIPK were found at only moderate levels in the CA3-CA1 border region (Fig. 3). In contrast, the mossy fiber tract (stratum lucidum layer) of the CA3 region appeared strongly stained for the modulating proteins CGB and PIPK Iγ (Fig. 3). In comparison with the organotypic slices, the acutely prepared tissue mossy fiber tract appears more intense for both CGB and PIPK Iγ.

Statistical Analysis

To quantitate the signal intensity of the staining, regions of interest were drawn over the cell body layer and adjacent proximal apical dendrites of dentate gyrus, CA3, and CA1 regions (Fig. 4). The statistical significance was tested using the unpaired Student's *t*-test at $P < 0.05$ significant) and at $P < 0.01$ (highly significant). The $InsP_3R1$ distribution showed significant differences between dentate gyrus and the CA3 region in comparison with the CA1 region in both acutely prepared and organotypic slices. However, the CGB signal was significantly higher in the CA3 region when compared to dentate gyrus and the CA1 regions. The kinases showed a much more homogenous distribution in hippocampus. PI4K levels in all subregions were comparable, whereas PIPK stainings showed only lower signal intensity in dentate gyrus compared to CA3 (Fig. 4).

Western Blot Analysis

Acutely prepared tissue slices were divided into three regions (Fig. 5A) and analyzed by antibody staining to determine the relative protein levels in tissue. Consistent with our immunocytochemistry findings, there was a reciprocal distribution of CGB protein (Fig. 5B) and $InsP_3R1$ protein (Fig. 5C) in the CA1 subfield and the CA3 subfield. More specifically, there was a significantly greater quantity of $InsP_3R1$ protein in the CA1 subfield than was found in CA3, and there was significantly less CGB found in CA1 than in CA3. PI4K levels were similar in all three regions (Fig. 5D). The distributions of the loading controls (actin and SERCA) were uniform throughout the samples.

Treatment of Organotypic Slices With Modulating Agents

In several neuropathologic conditions, components of the intracellular Ca^{2+} signaling pathway are altered (Nowakowski et al., 2002; Koh et al., 2003). To assess the plasticity of the protein distribution, the organotypic slices were treated with agents that modulate specific components of the signaling toolbox, or that had been shown to alter $InsP_3R1$ signaling or distribution in other tissues.

The first compounds tested were rapamycin and cyclosporin A, inhibitors of FKBP12, a co-factor of the ryanodine receptor that modifies ryanodine receptor function. In addition, $InsP_3R1$ expression was altered in cultured neurons treated with calcineurin blockers for 7 days (Taylor et al., 1999). Organotypic slices were treated with several concentrations of rapamycin and cyclosporin A for a duration of 1 week. For cyclosporin A the concentrations used were 0.3, 1, and 3 uM; for rapamycin 1, 3, and 10 uM were used. No changes in the distribution of the immunofluorescence for the $InsP_3R1$, PI4K, PIPK, and CGB was detected for either compound.

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The compound selected that modulates intracellular signaling was nicotine. Organotypic slices were treated with nicotine (3 uM) for 1 week. Again, no changes in the distribution of the immunofluorescence for the $InsP_3R1$, PI4K, PIPK and CGB was detected for either compound. The difficulty in finding a treatment that will alter the protein localization suggests that the distribution patterns of these proteins are quite robust.

DISCUSSION

We have investigated the distribution of proteins involved in the $InsP_3$-dependent Ca^{2+} release in acutely prepared and organotypic brain tissue of rat. We show that essential components of the Ca^{2+} signaling toolkit are not distributed uniformly throughout hippocampus. We found only weak immunostaining signals of the $InsP_3R1$ in the dentate gyrus and CA3 area of both acutely prepared and organotypic slice preparations whereas the signal intensity increased strongly throughout the CA1 region. Previous studies showed similar distribution patterns for $InsP_3R1$ mRNA (Furuichi et al., 1993) and protein distribution (Fotuhi et al., 1993) in rat and human hippocampal tissue. Furthermore, the low-threshold $InsP_3R1$ has been shown to be localized throughout the cell in neuron-like PC12 cells (Johenning et al., 2002) and hippocampal pyramidal neurons (Seymour-Laurent and Barish, 1995; Jacob et al., 2005).

In contrast, the distribution of the kinases leading to the formation of $InsP_3$ shows a much more homogenous pattern in hippocampus. Whereas PI4K could be found throughout the pyramidal cell layers of CA3 and CA1, PIPK was identified especially in the periphery of hippocampal pyramidal cells, in stratum oriens and stratum radiatum. PI4K mRNA analyses have shown comparable kinase distributions in brain tissue (Zolyomi et al., 2000).

Immunohistochemical analysis of the hippocampal CGB distribution showed weak signals in dentate gyrus and CA1 and comparatively strong signals in CA3, especially within the stratum lucidum region. Those findings are consistent with data dealing with the CGB mRNA distribution and protein distribution in human and rat tissue (Mahata et al., 1991; Marksteiner et al., 2000). In previous studies, the addition of CGB strongly altered the $InsP_3R1$ open probability in single channel experiments (Thrower et al., 2003) and neuronally-differentiated PC12 cells (Choe et al., 2004). It was concluded that CGB is an essential part of the $InsP_3R1$ channel complex.

The existence of “microdomains” containing components of the signaling toolkit required for intracellular Ca^{2+} release through $InsP_3R$ has been shown in sympathetic ganglion cells (Johenning et al., 2002). Linking of receptors, G proteins, enzymes, and $InsP_3R$ through scaffolding proteins like Vesl/Homer seems to generate specificity of signal transduction and to prevent unintentional crosstalk between different signaling pathways (Delmas et al., 2002). Our finding of a band of strong CGB and PIPK signals in the stratum lucidum subregion of CA3 where there is a low density of the $InsP_3R1$ is consistent with this concept of signaling microdomains.

It has been shown in previous studies that CGB and PIPK Iγ could be found co-located closely within neuronal cells (Jacob et al., 2005). Preventing the kinase from phosphorylating $InsP_3$ precursors by applying Wortmannin and blocking CGB binding to the $InsP_3R1$ had similar effects on the Ca^{2+} release pattern (Choe et al., 2004). In addition, high levels of PIPK (Wenk et al., 2001) and CGB (Zhai et al., 2001) have been shown to be present in central nervous synapses.

Our findings suggest that the proteins regulating the $InsP_3R1$ open probability from the cytosolic and luminal side of the ER by an increase in the $InsP_3$ production (PIPK) and the receptor sensitization (CGB) are expressed especially in certain subfields of hippocampus

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where they may act synergistically to compensate for the differences in the $InsP_3R1$ density and this way shape the region-specific spatial and temporal Ca^{2+} release pattern.

To investigate the usability of organotypic slices as a model system for neuronal Ca^{2+} experiments, we compared the distribution patterns of the components of the Ca^{2+} signaling toolkit with those obtained from acutely prepared slice preparations.

In general, organotypic slice cultures tend to flatten out considerably thus causing a spreading of the cellular layers after 8 days in culture (Gahwiler et al., 1997). The basic morphology, however, was well preserved after the culturing period and the immunostaining patterns of the analyzed proteins were highly similar to those in slice preparations fixed immediately after cutting (Figs. 1,2). Previous studies have dealt with the preservation of basic circuitry in organotypic hippocampal cultures. Although hippocampal cytoarchitecture is well maintained, changes in the mossy fiber tract due to a lack of innervation by the perforant pathway have been described (Zimmer and Gahwiler, 1984). In our slice culture experiments, we could see a decreased level of the proteins found in the mossy fiber tract: both CGB and PIPK Iγ levels were reduced after the culturing period. In contrast, the protein distribution in dentate gyrus, CA3, and CA1 primary neuronal layers seemed highly comparable to the in vivo situation as elucidated by acutely prepared slice stainings.

Overall, the similarity in the expression patterns of key proteins involved in Ca^{2+} release from intracellular stores between acutely prepared and organotypic slice preparations make the cultures a valid tool for long-term neuronal Ca^{2+} experiments in a model system in which the basic circuitry remains intact.

Evidence has been presented in recent years suggesting that the chromogranin proteins are involved in the development of certain neurologic diseases. Immunohistochemical studies showed significant losses in the hippocampal CGA (Iwazaki et al., 2004) and CGB (Nowakowski et al., 2002) concentration of brain slices taken from patients with schizophrenia. Distinct polymorphisms in the CGB gene could be assigned to schizophrenic individuals (Zhang et al., 2002; Iijima et al., 2004). In addition, other diseases like Alzheimer's disease (Marksteiner et al., 2000; Lechner et al., 2004) or temporal lobe epilepsy (Pirker et al., 2001) have been associated with changes in the cerebral CGB levels.

Chromogranin losses in those diseases may not only show a loss in synaptic vesicles and therefore in the amount of synapses in affected brain areas but may also be a sign for altered Ca^{2+} signaling in those parts of the brain. We used organotypic slice cultures from rat to introduce a model for long-term studies of Ca^{2+} signaling in hippocampal neurons, and we investigated the properties of the components of the Ca^{2+} signaling toolkit. In past experiments organotypic slices have proven to be a feasible and powerful tool for electrophysiology, cell transfection (Thomas et al., 1998; Rathenberg et al., 2003), and modeling experiments dealing with physiologic (Yamaguchi et al., 2003) and pathologic conditions (Mulholland et al., 2003; Fan and Tenner, 2004).

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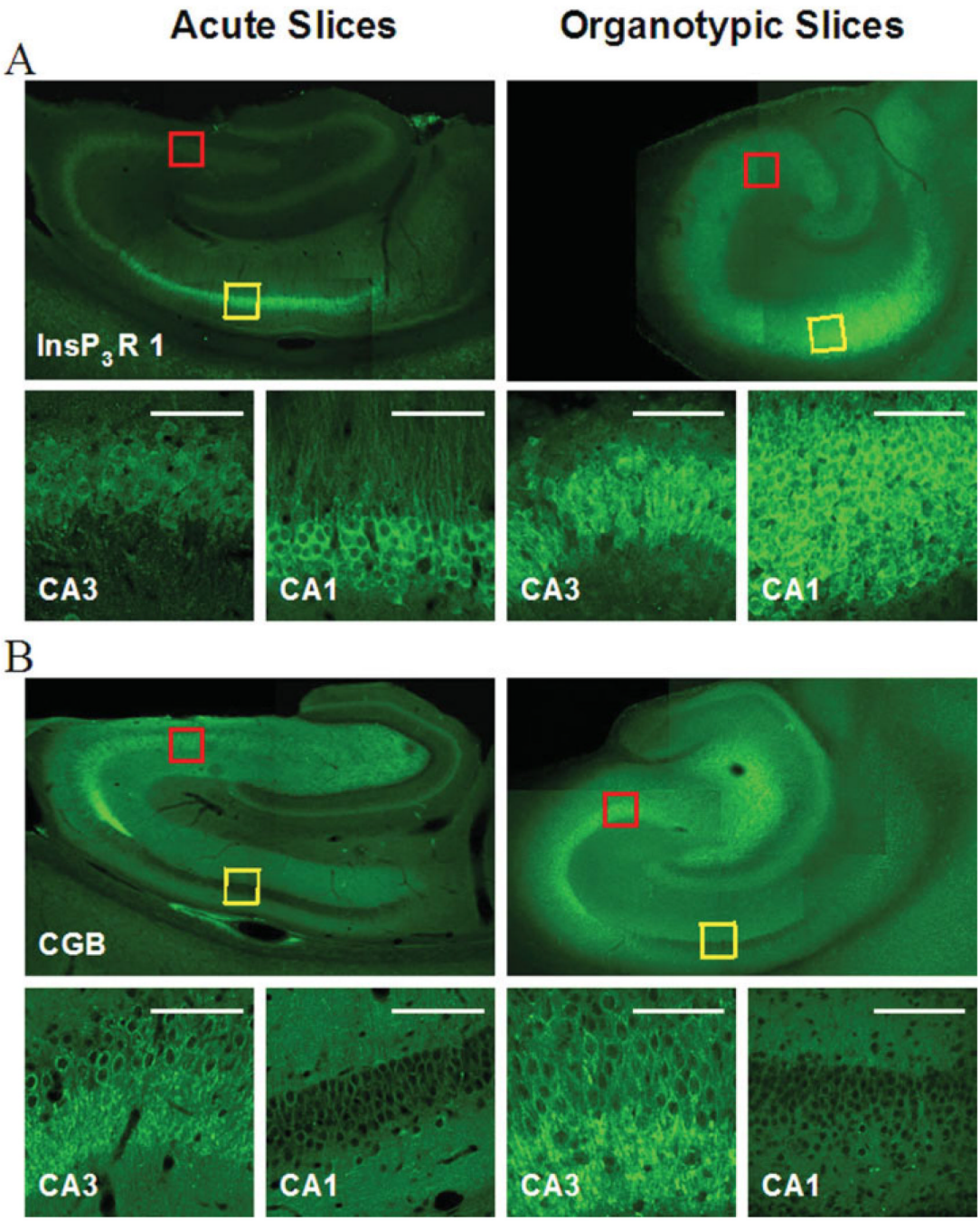

Fig. 1.

Components of the $InsP_3$ pathway are distributed non-uniformly in hippocampus. Immunohistochemistry of $InsP_3R1$ and CGB was carried out by using indirect immunofluorescence on acutely prepared slice preparations (**left panels**) and organotypic hippocampal slice cultures (**right panels**). **A:** $InsP_3R1$ showed only faint signals in dentate gyrus (DG) and CA3 subfields whereas signal intensity increased throughout the CA1 field. Images obtained with higher magnification showed the $InsP_3R1$ signal throughout the pyramidal cell layer and the apical dendrites. Similar $InsP_3R1$ levels were obtained for acutely prepared and organotypic slices with a broader band of pyramidal neurons in the cultured tissue. **B:** In contrast, staining for CGB showed strong signals in the hippocampal hilus and the CA3

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stratum lucidum region whereas DG and CA1 did not show a notable signal. Images obtained with higher magnification show a strong band of CGB signals in the CA3 mossy fiber tract and moderate signals in the CA3 pyramidal cell layer; the CA1 pyramidal neurons did not stain for CGB. Acutely prepared and organotypic hippocampal tissue showed comparable signal distribution. A,B (top panels): Regions magnified. Red boxes: CA3 regions. Yellow boxes: CA1 regions. Scale bar = 50 μm. One of at least three similar experiments.

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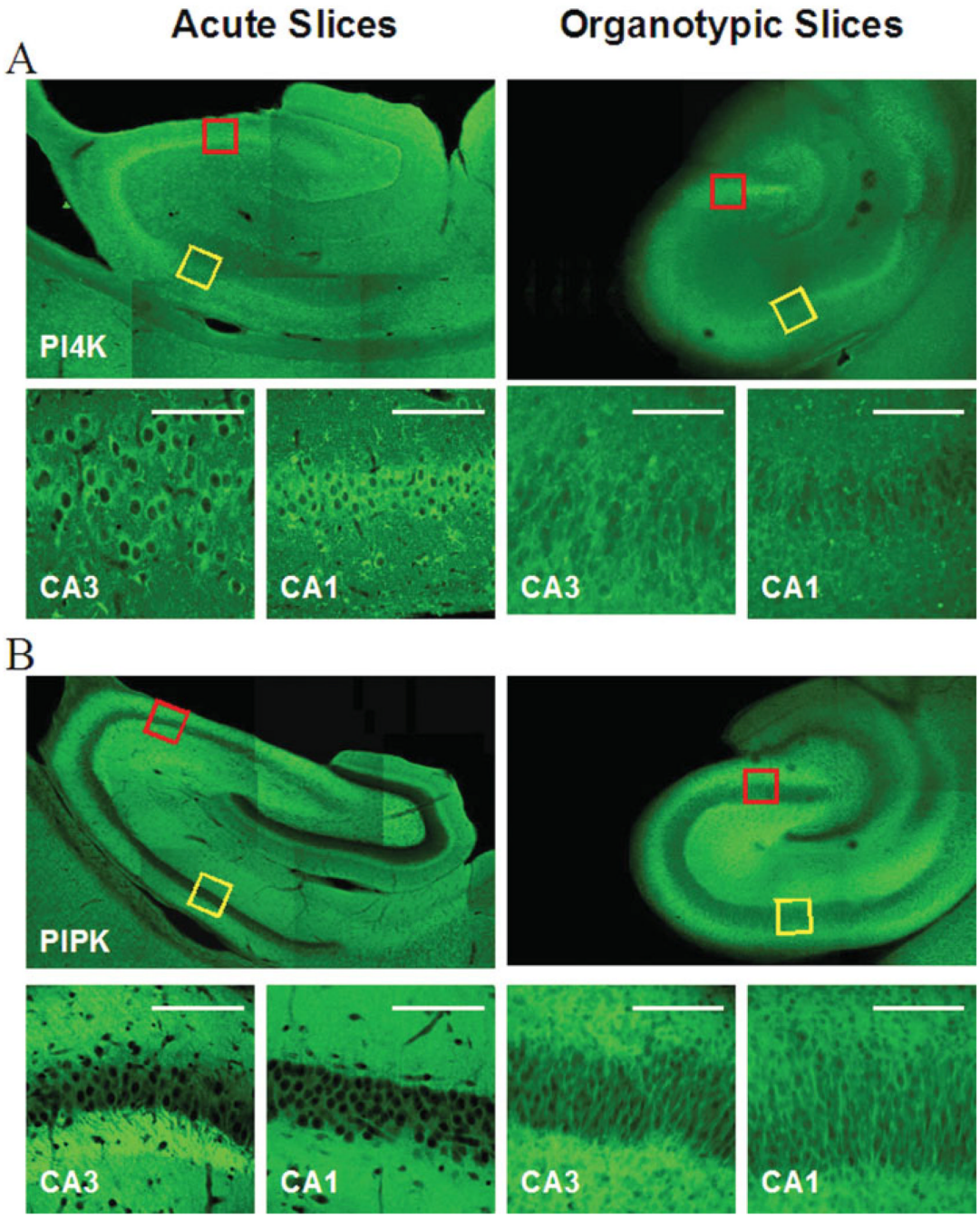

Fig. 2.

Kinases involved in the production of InsP3 show specific distribution patterns in hippocampus. The localization of the kinases PI4Kβ and PIPK Iγ was determined by indirect immunofluorescence on acutely prepared slice preparations (**left panels**) and organotypic hippocampal slice cultures (**right panels**). **A:** PI4Kβ showed strong staining throughout the hippocampus, especially in the pyramidal cell fields of CA3 and CA1. In higher-magnification images the signal was seen throughout the soma. The pyramidal cell field in the acutely prepared slices appears more strongly stained for PI4Kβ than in the organotypic cultures but nonetheless, the protein distribution throughout the hippocampus seems highly similar. **B:** In comparison, PIPK Iγ has a strong signal in CA3 and CA1 regions whereas DG only has

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moderate PIPK Iγ staining. The pyramidal cell layer itself shows weak immunostaining signals only; the adjacent dendritic layers (stratum oriens and stratum radiatum) have strong PIPK Iγ signals. In addition, a band of intense signal can be found in the CA3 stratum lucidum layer. This band is much stronger in the acutely prepared slices even though the overall distribution of PIPK Iγ is comparable in both systems. A,B top panels: Regions magnified. Red boxes: CA3 regions. Yellow boxes: CA1 regions. Scale bar = 50 μm. One of at least three similar experiments.

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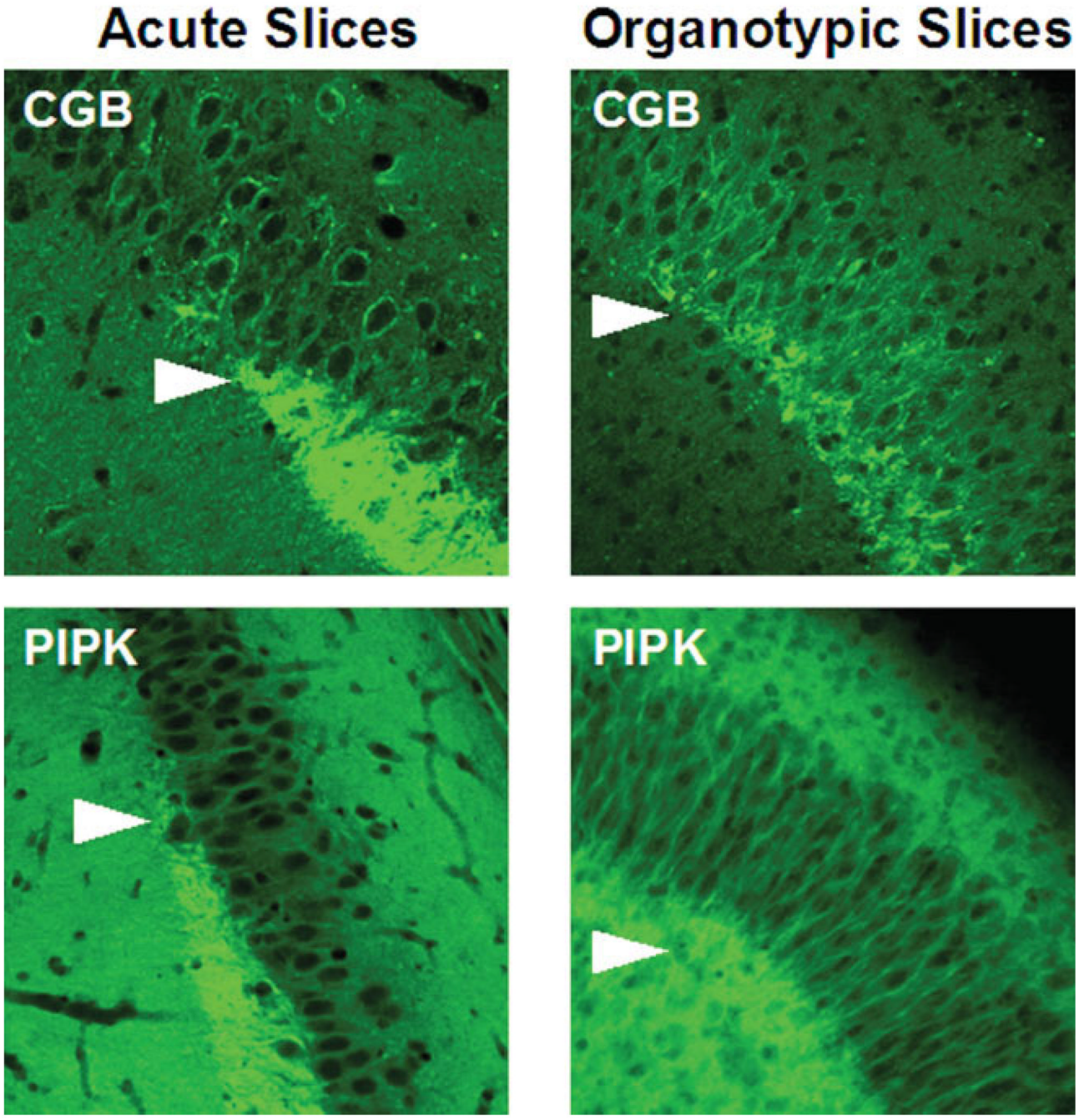

Fig. 3.

Stratum lucidum region of hippocampus shows a strong band of CGB and PIPK Iγ immunoreactivity. The mossy fiber tract in the stratum lucidum region of CA3 shows high levels of CGB and PIPK Iγ (arrows indicate the top most portion of the region under discussion with high intensity staining). Acute slices fixed and processed immediately after cutting appear stained more intensely than the organotypic tissue samples that were kept in culture for 8 days. One of at least three similar experiments.

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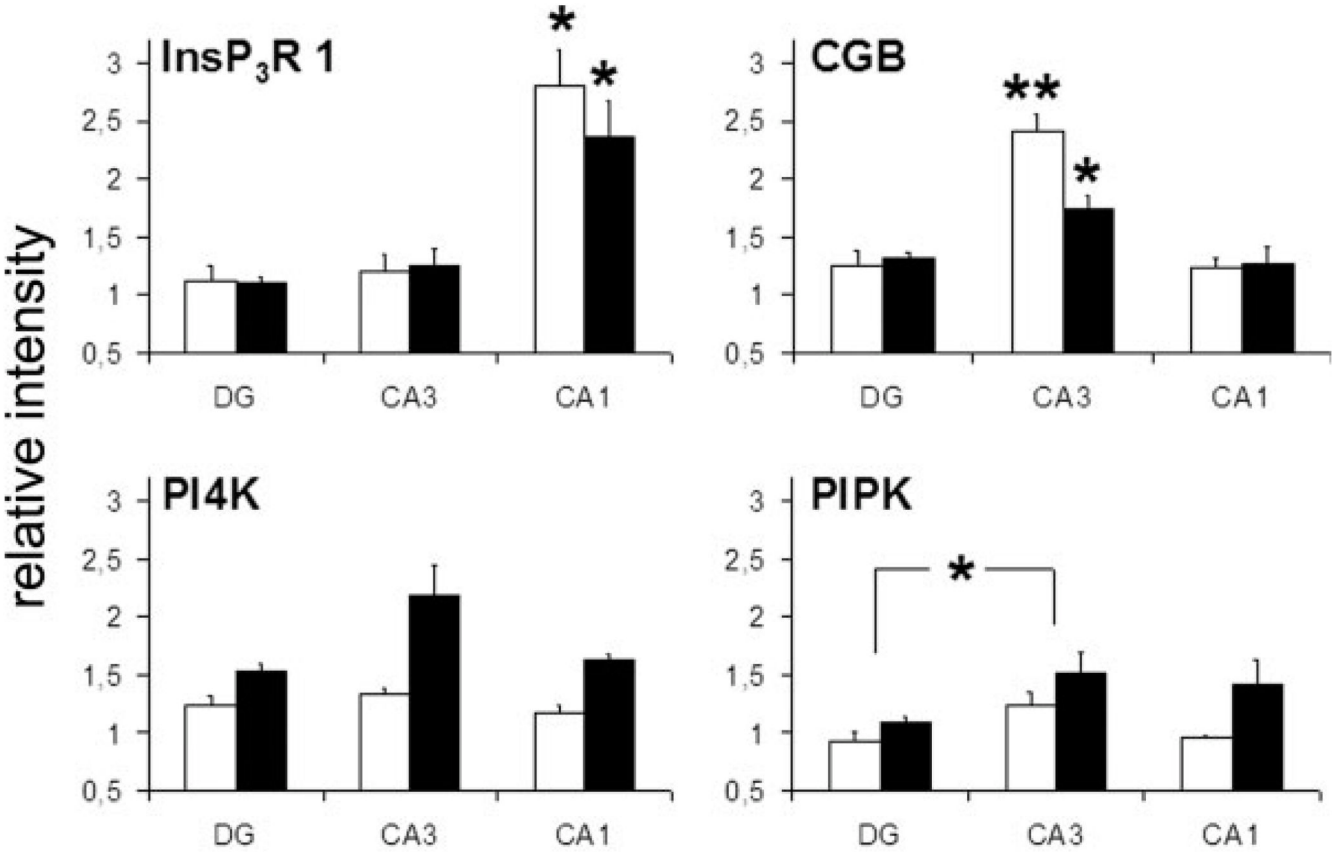

Fig. 4.

Differences in the protein distribution of the components of the Ca^{2+} signaling toolkit. The magnitude of the fluorescent signal was measured and compared; the y axis represents the ratio of arbitrary fluorescence units (relative intensity). The values shown in the white bars are from the acutely prepared brain slices and those in the black bars are from the organotypic slices. The significance of the differences observed was calculated using a unpaired Student's *t*-test. A significantly higher signal intensity for the $InsP_3R1$ was found in CA1 compared to DG and CA3. In contrast, CGB levels were highest in CA3 region. The kinases show a homogenous distribution throughout hippocampus. $*P < 0.05$; $**P < 0.01$.

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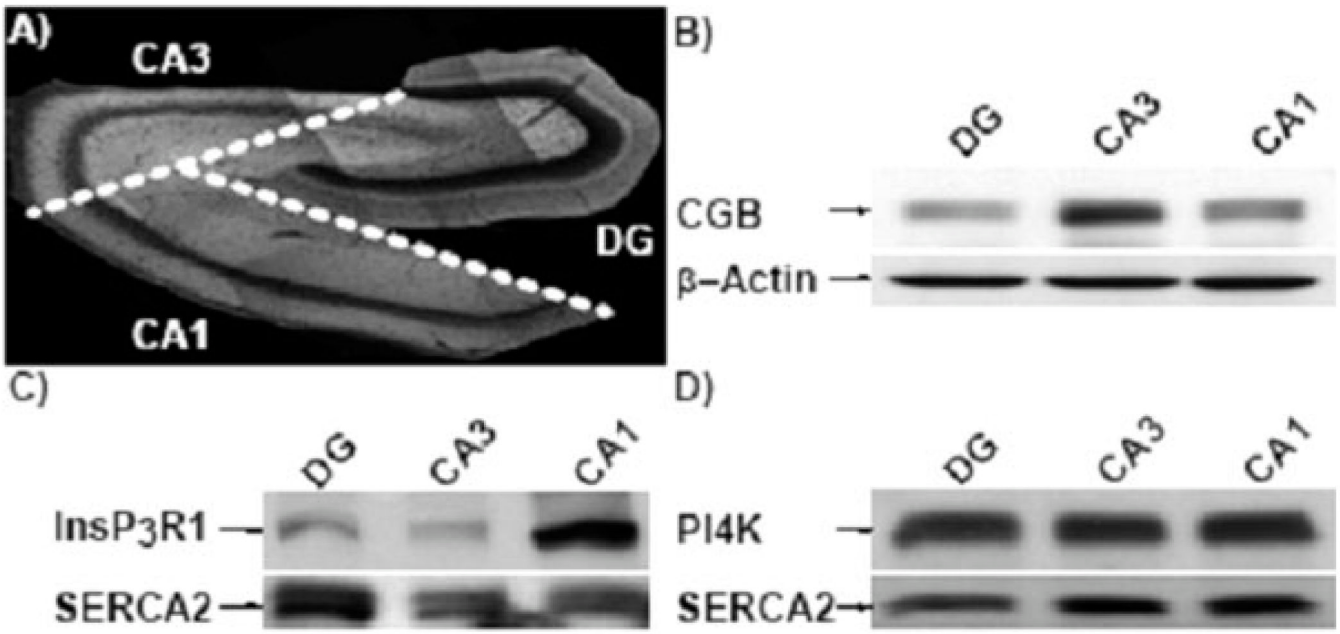

Fig. 5.

Western blot analysis of acutely prepared hippocampal slices. The relative density of the $InsP_3R1$, CGB, and PI4Kβ was determined in acutely prepared brain slices. **A:** Lines through the hippocampal slice define the regions used. The density of CGB (91 kDa) (**B**), $InsP_3R1$ (250 kDa) (**C**), and PI4K (91 kDa) (**D**) was compared to the density of actin (42 kDa) (B) or SERCA (100 kDa) (C,D). Duplicate samples were run in a single Western blot for each protein. The relative density in the three regions of the hippocampus correlates with the immunocytochemistry shown in Figures 1–3.