Ca\textsuperscript{2+}-dependent Translocation of Calcybin Binding Protein in Neurons and Neuroblastoma NB-2a Cells

Anna Filipek\textsuperscript{1,*}, Beata Jastrzebska\textsuperscript{1}, Marcin Nowotny\textsuperscript{1}, Katarzyna Kwiatkowska\textsuperscript{1}, Michal Hetman\textsuperscript{2}, Liliana Surmacz\textsuperscript{1}, Elzbieta Wyroba\textsuperscript{1}, Jacek Kuznicki\textsuperscript{1,2}

\textsuperscript{1}Nencki Institute of Experimental Biology, 3 Pasteur Street, 02-093 Warsaw, Poland
\textsuperscript{2}International Institute of Molecular and Cell Biology, 4 Trojdena Street, 02-109 Warsaw, Poland

Running title: Localization of CacyBP in neuronal cells

*Corresponding author: Anna Filipek, E-mail: anfil@nencki.gov.pl; Fax: +48-22-822 53 42; Tel: +48-22-659 31 43

SUMMARY

CacyBP binds calcybin (S100A6) at a physiological range of [Ca\textsuperscript{2+}] and is highly expressed in brain neurons. Subcellular localization of CacyBP has been examined in neurons and neuroblastoma NB-2a cells at different [Ca\textsuperscript{2+}]i. Immunostaining indicated that CacyBP was present in cytoplasm of unstimulated cultured neurons, in which resting [Ca\textsuperscript{2+}]i is known to be \sim 50 nM. When [Ca\textsuperscript{2+}]i was increased to above 300 nM by KCl treatment, the immunostaining was mainly seen as a ring around nucleus. Such perinuclear localization of CacyBP was observed in untreated neuroblastoma NB-2a cells in which [Ca\textsuperscript{2+}]i is \sim 120 nM. Additional increase in [Ca\textsuperscript{2+}], to above 300 nM by thapsigargin treatment did not change CacyBP localization. However, when [Ca\textsuperscript{2+}]i in NB-2a cells dropped to 70 nM, due to BAPTA/AM treatment, perinuclear localization was diminished. Ca\textsuperscript{2+}-induced translocation of CacyBP was confirmed by immunogold electron microscopy and by fluorescence of NB-2a cells transfected with an EGFP-CacyBP vector. Recombinant CacyBP can be phosphorylated by protein kinase C \textit{in vitro}. In untreated neuroblastoma NB-2a cells, CacyBP is phosphorylated on serine residue(s), but exists in a dephosphorylated form in BAPTA/AM treated cells. Thus, phosphorylation of CacyBP occurs in the same range of [Ca\textsuperscript{2+}], that leads to its perinuclear translocation.

KEY WORDS: CacyBP, primary culture of neurons, neuroblastoma NB-2a, [Ca\textsuperscript{2+}]-imaging; EGFP fluorescence; immunocytochemistry, electron microscopy, phosphorylation, the perinuclear region.

ABBREVIATIONS: AM, acetoxymethyl ester; BME, basal medium Eagle; CacyBP, Calcybin (S100A6) Binding Protein; CMV, cytomegalovirus; [Ca\textsuperscript{2+}], intracellular calcium concentration; EGFP, Enhanced Green Fluorescent Protein; EGTA, ethylene glycol bis (\beta-aminoethyl ether)-N,N,N'N'-tetraacetic acid; MEM, minimal essential medium; PBS, phosphate buffered saline; PAGE, polyacrylamide gel electrophoresis; PMA, phorbol myristate acetate; SDS, sodium dodecyl sulfate; RPMI, RPMI 1640 medium.
INTRODUCTION

Calcyclin (S100A6) is a calcium binding protein of the EF-hand type belonging to the S100 family (1-3). As in the case of the prototypical “EF-hand” protein calmodulin, S100 proteins act as calcium sensors and modulate activity of enzymes or other target proteins (4). For instance, S100A1 activates twichin kinase (5), S100B activates the nuclear Ndr kinase (6), interacts with p53 (7, 8), binds to the giant protein AHNAK (9). It has also been shown that S100 proteins are associated with cell cycle progression, differentiation, metabolism (3, 10, 11), and with the induction of metastatic phenotype (12, 13). In contrast to calmodulin, S100 proteins exhibit cell and tissue specific expression.

Calcyclin Binding Protein (CacyBP) was originally discovered in the cytosolic fraction of Ehrlich ascites tumor cells, mouse brain and spleen (14). The cDNA clone of CacyBP was isolated from mouse brain library and sequenced (15). The recombinant CacyBP interacts with calcyclin in vitro at micromolar Ca\(^{2+}\) concentration indicating that this interaction may indeed occur physiologically. The region of CacyBP (amino acids 178 - 229), which binds calcyclin, has been identified and the dissociation constant of the complex has been measured (16). Northern and western blot showed that CacyBP is expressed at the highest level in mouse and rat brain and immunohistochemistry performed on rat brain slices revealed that CacyBP is mainly present in neurons of the cerebellum, hippocampus and cortex (17).

When first elucidated, the sequence of CacyBP displayed no apparent similarity to any other known protein sequence, thus an assignment of its function was not possible. Two independent reports showed that the level of CacyBP is increased upon erythropoietin receptor activation (18, 19) and that this increase is parallel to an increase in c-myc and dpp-1 transcriptional activity via the JAK2 pathway (19). These observations suggest that CacyBP might be involved in signalling pathway(s) activated by erythropoietin in erythroid cells and in neuronal cells in which high level of erythropoietin receptor was also found. Recently, a protein called SIP (Siah-1 Interacting Protein), a component of the β-catenin ubiquitin degradation pathway has been reported (20). The predicted human SIP protein shows 93% sequence identity with mouse CacyBP suggesting that it is the human homolog of CacyBP. Since Ca\(^{2+}\)-dependent phenomena related to the activity of ubiquitin/26S proteasome complex were described (21), interaction of CacyBP with an EF-hand calcium binding protein raises the possibility that CacyBP may function via Ca\(^{2+}\)-dependent interactions in ubiquitination and protein degradation process. Therefore, in this work we studied intracellular localization
of CacyBP under different [Ca\textsuperscript{2+}]. We used primary culture of rat cortical neurons and cultured neuroblastoma NB-2a cells. Using both immunostaining and transfection of NB-2a cells with an EGFP-CacyBP vector, we show that cellular localization of CacyBP is modulated by changes in [Ca\textsuperscript{2+}]. We also found that phosphorylation of this protein on serine residue(s) occurs within the same range of [Ca\textsuperscript{2+}], as that which stimulates its perinuclear translocation.
MATERIALS AND METHODS

Chemicals
MEM, RPMI, newborn calf serum, fetal bovine serum, horse serum, trypsin-EDTA 1x solution, penicillin and streptomycin were purchased from Gibco BRL; leupeptin, aprotinin, phenylmethylsulfonyl fluoride, soybean trypsin inhibitor, dithiothreitol, thapsigargin, bovine serum albumin, DABCO (1,4-diazobicyclo-[2.2.2.]octane, 4-chloro-1-naphthol), anti-rabbit IgG conjugated with horseradish peroxidase, anti-rabbit IgG conjugated to colloidal gold, glutaraldehyde and paraformaldehyde were purchased from Sigma; restriction enzymes were from Promega. Other chemicals used were purchased from companies as indicated in the text.

Primary culture of cortical neurons and KCl treatment
Cortical neurons were prepared from newborn Harlan Sprague-Dawley rats and cultured at a density 1500-2000 cells/mm² in BME supplemented with 10% heat inactivated bovine calf serum (HyClone, Logan, UT). Cytosine arabinoside (2.5 µM) was added on the second day after seeding to inhibit the proliferation of non-neuronal cells.

KCl treatment of rat cortical neurons was performed on cells cultured for 5-6 days. Neurons were incubated for 10 min with KCl, which was added to the medium to a final concentration of 30 mM. After that time cells were fixed and applied for immunocytochemistry. In other experiments cells treated with KCl as above were next incubated in the medium without KCl for 2 h, and then fixed and used for immunocytochemistry.

Culture of neuroblastoma NB-2a and PC12 cells and extract preparation
Mouse neuroblastoma cells NB-2a were maintained in MEM supplemented with 10% foetal bovine serum, 25 mM bicarbonate, penicillin (100 µg/ml) and streptomycin (100 µg/ml). PC12 cells were grown in RPMI 1640 containing 10% horse serum, 5% foetal bovine serum, penicillin (50 U/ml) and streptomycin (50 µg/ml). All cultures were maintained in the presence of 5% CO₂ at 37°C. The media were changed every three days and cells were passaged when confluent.

To prepare the protein extracts for western blot, cells were harvested and washed twice with PBS. Next cells were homogenized mechanically using a syringe with a needle (G 26; 0.45 x 12) twenty times in PBS containing 1 mM EDTA and protease inhibitors: leupeptin (10 mg/l), aprotinin (5 mg/l), soybean trypsin inhibitor (20 mg/l) and phenylmethylsulfonyl fluoride (1 mM). The extracts were centrifuged for 45 min at 4°C at 12 000 rpm in an Eppendorf centrifuge. Protein concentration was estimated by Bradford’s procedure (BioRad reagent, Hercules, CA) with bovine serum albumin as a standard.

Construction of EGFP- CacyBP expression vector
PCR was carried out with CacyBP cDNA in pBluescript vector (16) as a template with forward primer: 5′-GGATCCGGATCCATGGCTTCCGTTTTGGAAGAG-3′ and reverse primer: 5′-GAGACGAATTCATCTCAATCAAAAATTCCGTGTTTC-3′ (restriction enzyme recognition sites in bold). Pfu DNA polymerase was used in 30 cycles of PCR with each cycle consisting of 1 min at 94°C; 1 min at 50°C and 2 min at 72°C. The PCR products were digested with BamHI and EcoRI restriction enzymes, purified and ligated with pEGFP-C1 vector (Clontech) linearized with BglII and EcoRI restriction enzymes. The ligation reaction mixture was used to transform E. coli cells (TOP10F’, Invitrogen). Potential clones were screened by colony PCR and the presence of the insert was confirmed by restriction analysis. The sequence of the insert was verified by DNA automatic sequencing.
Transfection of neuroblastoma NB-2a cells

Two constructs were used for transfection experiments: one encoding the EGFP-CacyBP fusion protein and a second encoding only EGFP. Both genes were under the control of the CMV promoter. Plasmids were purified from *E. coli* using a QIAGEN Plasmid Kit MIDI according to the manufacturer’s protocol. DNA transfection in NB-2a cells was performed by the calcium phosphate precipitation technique according to standard procedure (22). Cells grown on coverslips were exposed for 16 h to 2 µg of plasmid DNA per coverslip.

Loading the cells with thapsigargin or BAPTA/AM

Neuroblastoma NB-2a cells were plated onto poly-L-lysine-coated coverslips and cultured for two days in MEM at 37°C in 5% CO2. Cells were washed twice for 5 min in PBS and incubated with 0.2 µM thapsigargin dissolved in buffer containing 20 mM Hepes pH 7.4, 137 mM NaCl, 2.7 mM KCl, 1 mM Na2HPO4, 25 mM glucose, 1 mM MgCl2, 1% bovine serum albumin and 2 mM CaCl2 for 2 min at RT or with 5 µM BAPTA/AM and 1 mM EGTA for 30 min at 30°C. Cells treated with BAPTA/AM were next washed twice and incubated in buffer described above containing 1 mM EGTA for 30 min at 30°C. Cells transfected with CacyBP-EGFP were treated in the same way with thapsigargin or BAPTA/AM and EGFP fluorescence was analysed under a Nikon Optiphot-2 microscope.

Measurement of [Ca2+]i

The cytoplasmic level of Ca2+ was examined in neuroblastoma NB-2a cells (untreated, treated with thapsigargin or BAPTA/AM) using a video imaging system (MagiCal, Applied Imaging, data processing using Tardis V8.0 (Joyce Loebl)) as described by Barańska et al. (23). [Ca2+]i values were calculated according to Grynkiewicz et al. (24).

Immunocytochemistry

Immunocytochemistry experiments were performed on neurons (unstimulated and stimulated with KCl) and neuroblastoma NB-2a cells (untreated, treated with thapsigargin or BAPTA/AM). Neurons were plated onto laminine-coated coverslips whereas neuroblastoma NB-2a cells were plated onto poly-L-lysine-coated coverslips. Cells were fixed with 3% paraformaldehyde in PBS (pH 7.4) for 20 min at RT. The coverslips were washed with PBS, incubated with 50 mM NH4Cl in PBS for 10 min to quench the remaining aldehyde groups (25) and permeabilized for 4 min with 0.1% Triton X-100 in PBS. The cells were washed twice with PBS, incubated with 1% bovine serum albumin in PBS for 1 h and after that incubated with CacyBP antibodies (1:800). After washing (three times for 10 min in PBS), cells were incubated with FITC-conjugated anti-rabbit antibodies (1:200) (Jackson ImmunoResearch Laboratories, Inc.) and mounted on glass slides with a mixture of glycerol and polyvinyl alcohol containing DABCO (1,4-diazobicyclo-[2,2,2,]-octane). For control experiments cells were incubated with pre-immune serum. Cells were analysed under a Nikon Optiphot-2 microscope.

Quantitative analysis of CacyBP redistribution

Digitalized images of cells were analyzed with Quantity One software (BioRad). In each cell, 3 lines of 3 pixels in width were drawn across the center of the cell in a constant distance of 0.5 µm from each other (see also Fig. 1D, E, F), and line intensity profiles of CacyBP labeling were collected. The fluorescence intensity values were within a linear range. Next, the fluorescence intensities in the perinuclear region (I₀) and in the cytoplasm (Ic) at a constant distance from the perinuclear region (2.7 µm) were determined. Such distance was chosen since at this point a maximal decrease in fluorescence intensity in the cytoplasm was observed. The ratio I₀/Ic was calculated to express the changes in CacyBP concentration in the
perinuclear region. At least 10 cells from two independent experiments were analyzed in each variant.

Electron microscopy

Neuroblastoma NB-2a cells were fixed for 1 h at RT in 3% paraformaldehyde/0.5% glutaraldehyde in PBS buffer (26). Next cells were rinsed in PBS four times (5 min) followed by centrifugation for 5 min (350xg). Cells were dehydrated in ethanol and propylene oxide, embedded in LR White (Polyscience) and polymerized for 72 h at 56°C.

After thin sectioning, samples were collected on the carbon-formavir–coated nickel grids and incubated for 30 min in 1% BSA/0.1% Tween/PBS as a non-specific blocking agent and labeled with antibodies against CacyBP (1:250) overnight at RT. After washing with 1% BSA/0.1% Tween/PBS, the cells were incubated in the anti-rabbit IgG (1:20) conjugated to colloidal gold (5 nm) for 1 h at RT. After extensive washing (BSA/Tween/PBS 6x5 min, PBS 4x5 min, H2O 2x5 min) sections were stained with uranyl acetate for 30 min at RT. The sections were observed in a JEM 1200 EX electron microscope.

Phosphorylation of CacyBP

Phosphorylation of CacyBP in vitro and in vivo

Phosphorylation of CacyBP in vitro was performed on purified, recombinant protein expressed in E. coli (16). The reaction mixture for phosphorylation by protein kinase C (final volume 50 µl) contained 10 mM Hepes pH 7.5, 5 mM MgCl2, 50 mM NaCl, 0.05 mM CaCl2, 0.7µg/µl L-α-phosphatidil-L-serine, 80 nM PMA, 0.1 mM dithiotreitol, 70 ng of kinase preparation (Calbiochem) and 0.1 mM ATP. Preincubation was carried out for 20 min at 30°C. After that the reaction was initiated by the addition of the substrate (approximately 2 µg of CacyBP) and [γ-32P]ATP at a final concentration of 0.2 mM. The reaction was carried out for 30 min at 37°C. The reaction mixture for protein kinase A (catalytic subunit from Sigma) contained 50 mM Hepes pH 7.5, 10 mM MgCl2, 0.1 mM ATP and 300 ng of kinase preparation. In this case preincubation and reaction was carried out for 30 min at 30°C. Reactions were terminated by the addition 5 µl of 4 x SDS sample buffer. Phosphorylated proteins were separated on 15% (w/v) SDS gels. Gels were dried and subjected to autoradiography for 18-34 h with an Amersham film at -70°C. Authophosphorylation of kinase preparations was performed as described above but without adding the substrate.

Phosphorylation of CacyBP in NB-2a cells was examined in untreated and BAPTA/AM treated NB-2a cells. Cells were washed in PBS, harvested and sonicated (Branson ultrasonicator) in buffer containing 20 mM Tris-HCl pH 7.5, 8 mM MgCl2, 150 mM NaCl, 0.2 mM EGTA, 1% Nonidet P-40. Extracts were centrifuged for 15 min in 4°C at 12 000 rpm in Eppendorf centrifuge. Supernatants were used for immunoprecipitation assay after adding the protease inhibitors (10 mg/l leupeptin, 5mg/l aprotinin, 20 mg/l soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (0.3 mM okadaic acid, 200 µM Na3VO4, 5 mM NaF). First, the solutions were incubated with protein A-Sepharose for 1 h at 4°C (pre-clearance). The unbound fractions were incubated with serum containing antibodies against CacyBP for 1.5 h at 4°C and then for 1 h at 4°C with a new portion of protein A-Sepharose. The resin was washed three times in a buffer containing 20 mM Tris-HCl pH 7.5 and 150 mM NaCl, twice in a buffer containing 20 mM Tris-HCl pH 7.5 and 500 mM NaCl, and finally in 20 mM Tris-HCl at pH 7.5. All buffers used were supplemented with protease and phosphatase inhibitors. The resin containing bound proteins was solubilized in SDS sample buffer, boiled for 5 min at 95°C and applied on the SDS polyacrylamide gel. Phosphorylation of CacyBP was analysed using western blot technique with monoclonal antibodies against phosphorylated serine residue (Alexis Corporation) as described in the original protocols. When effect of ATP was examined, supernatants were preincubated for 20 min at 37°C with PMA and CaCl2 at a final concentration 8 nM and 0.5
mM, respectively. Then ATP was added to a final concentration of 300 μM and solution was incubated for 30 min at 37°C. Reaction was inhibited by adding the SDS and Nonidet P-40 to final concentration of 0.1%. Protease inhibitors were next added and immunoprecipitation assay was performed as described above.

Other methods

Polyclonal antibodies, northern blots, electrophoresis and immunoblotting were performed as described by Jastrzebska et al (17) with minor modifications described below. New polyclonal antibodies were prepared against recombinant CacyBP cloned in the pET30 vector (16). Control experiments performed on NB-2a cells using preimmune serum, or anti-CacyBP serum saturated with CacyBP, confirmed the high quality of this serum (not shown). For northern blots total RNA was prepared from cells using an RNA purification kit (QIAGEN). For western blots antibodies against CacyBP were diluted 1: 800.
RESULTS

Localization of CacyBP in cultured neurons at different [Ca\(^{2+}\)]

To establish the effect of [Ca\(^{2+}\)] on intracellular distribution of CacyBP, we analyzed primary cultures from rat cortical neurons before and after stimulation. Unstimulated cells, KCl activated cells, and cells in which KCl was washed out after stimulation were probed by immunocytochemistry using CacyBP antibodies. In unstimulated neurons CacyBP was distributed throughout the cytoplasm (Fig. 1A, D); this staining pattern was observed in majority of the cells (Table 1). The level of [Ca\(^{2+}\)] in rat cortical neurons has been established previously to be \(~50\) nM in resting cells, and above 300 nM upon KCl activation (27). We observed that activation of cultured cortical neurons by KCl induces CacyBP translocation mainly to the perinuclear region (Fig. 1B, E). The KCl effect seems to be physiologically significant since about 85% of the cells showed increased CacyBP immunostaining in this region (Table 1). The Ca\(^{2+}\)-induced translocation of CacyBP to the nuclear envelope was reversible. After removing KCl from the medium, the localization of CacyBP in the cytoplasm was restored in the majority of cells (Fig. 1C, F and Table 1).

We next performed quantitative analysis of CacyBP distribution within the cell. For this purpose, line intensity profiles of CacyBP across the cell were obtained (Fig. 1D, E, F). Along these lines the fluorescence intensities in the perinuclear region (I\(_p\)) and in the cytoplasm (I\(_c\)) at a constant distance from the nuclear envelope were determined. In unstimulated neurons the ratio I\(_p\)/I\(_c\) was 1.09 (Fig. 1D, Table 2), indicating lack of significant enrichment of the CacyBP in the perinuclear region. After stimulation of neurons and increase in [Ca\(^{2+}\)], the ratio I\(_p\)/I\(_c\) was 1.58 (Fig. 1E, Table 2), indicating the accumulation of CacyBP in the perinuclear region. After removing of stimulus, the ratio was 1.18 showing that Ca\(^{2+}\)-dependent translocation of CacyBP is reversible (Fig. 1F, Table 2).

Localization of CacyBP in neuroblastoma NB-2a cells at different [Ca\(^{2+}\)]

To learn more about the effect of Ca\(^{2+}\) on CacyBP intracellular localization, we searched for cell lines expressing high levels of CacyBP. The level of this protein was examined in two cell lines of neuronal origin: PC12 cells and neuroblastoma NB-2a cells. Northern blotting with a full length cDNA probe (Fig. 2A, B) showed that CacyBP mRNA was present at much higher level in neuroblastoma NB-2a than in PC12 cells. Also, much more intense immunoreactive protein band representing CacyBP was detected by western blotting in the extracts of neuroblastoma NB-2a than PC12 cells (Fig. 2C). Thus, the NB-2a cells were chosen for further studies.

Neuroblastoma NB-2a cells were treated with different agents affecting [Ca\(^{2+}\)], fixed and then stained with antibodies against CacyBP. In resting cells, in which [Ca\(^{2+}\)], was shown to be 120 nM (Fig. 3), immunostaining was observed mainly in the perinuclear region, similarly to activated neurons (Fig. 4A). As shown in Table 3 more than 80% of the cells exhibited increased immunostaining in the perinuclear region. The treatment of cells with thapsigargin, which led to an increase of [Ca\(^{2+}\)] to above 300 nM (Fig. 3), did not affect the staining pattern (Fig. 4B), nor did it affect the percentage of cells showing such staining pattern (Table 3).

Additional evidence for changes in CacyBP distribution was obtained from experiments in which neuroblastoma NB-2a cells were transfected with an EGFP-CacyBP expression vector. In these cells the fluorescence of EGFP-CacyBP fusion protein was visible mainly in the perinuclear region (Fig. 4D) and its distribution was not affected by thapsigargin treatment (Fig. 4E). Percentage of untreated cells containing high level of CacyBP in the perinuclear region detected by EGFP-CacyBP fluorescence was about 94%, and of thapsigargin treated cells - about 86% (Table 3).
Treatment of NB-2a cells with cell-permeant Ca\textsuperscript{2+}-chelator BAPTA/AM lowered [Ca\textsuperscript{2+}]\textsubscript{i} to approximately 70 nM (Fig. 3), reduced immunostaining of endogenous CacyBP (Fig. 4C), and decreased EGFP-CacyBP fluorescence in the perinuclear region (Fig. 4F). Moreover, CacyBP was no longer seen as a ring around the nuclei but rather was relocated into the cytoplasm. Only about 5\% of the cells exhibited the immunostaining and EGFP-CacyBP fluorescence in the perinuclear region after BAPTA/AM treatment (Table 3).

To confirm these observations, quantitative analysis of CacyBP distribution in NB-2a cells was performed using the same approach as for cortical neurons. In untreated NB-2a cells, the ratio \textit{I}_c/\textit{I}_i was 1.44 and 1.32 for cells stained with anti-CacyBP and cells expressing EGFP-CacyBP, respectively (Table 4). After treatment with BAPTA/AM the ratio \textit{I}_c/\textit{I}_i was 0.96 and 0.99 for cells stained with anti-CacyBP and cells expressing EGFP-CacyBP, respectively (Table 4). These data confirm the accumulation of CacyBP in the perinuclear region of untreated NB-2a cells and translocation of CacyBP toward the cytoplasm after decreasing [Ca\textsuperscript{2+}]\textsubscript{i}.

Subcellular localization of CacyBP by immunogold labelling in electron microscopy

Untreated and BAPTA/AM treated neuroblastoma NB-2a cells were analyzed by the post-embedding immunogold technique. Cells were fixed, probed with antibodies against CacyBP and processed for detection using secondary antibodies conjugated to gold particles (Fig. 5). The specificity of immunolocalization of CacyBP was checked in the control experiment in which primary antibodies were omitted: no gold particles were seen (Fig. 5A). A positive reaction was observed in the cytoplasm and on both sides of the nuclear envelope of untreated neuroblastoma cells (Fig. 5B and C). In BAPTA/AM treated cells, CacyBP was seen in the cytoplasm but immunolocalization in the perinuclear region was significantly decreased (Fig. 5D).

Phosphorylation of CacyBP in neuroblastoma NB-2a cells

One possible mechanism for Ca\textsuperscript{2+}-dependent localization of CacyBP in the perinuclear region is protein phosphorylation. Analysis of the CacyBP sequence indicated several potential sites that can be phosphorylated by different kinases. To verify that CacyBP can be phosphorylated \textit{in vitro}, recombinant purified CacyBP was incubated with commercially available preparations of protein kinase C and protein kinase A. As shown in Fig. 6A, CacyBP was phosphorylated by protein kinase C (lane 1), but not by protein kinase A (lane 3). Other bands seen on the autoradiogram come from autophosphorylation of kinase preparations (lanes 2 and 4).

To examine if CacyBP might exist in a phosphorylated form \textit{in vivo}, the extracts of untreated (Fig. 6B, lane 1 and 2) and BAPTA/AM treated NB-2a cells (Fig. 6B, lane 3 and 4) were immunoprecipitated with antibodies against CacyBP, and the precipitated proteins were analysed by anti-phosphoserine antibodies. In the case of untreated NB-2a cells, CacyBP phosphoserine immunoreactivity was seen (Fig. 6B, lane 1). This immunoreactivity was not affected by incubation of the extracts with ATP (Fig. 6B, lane 2). These results indicate that CacyBP is phosphorylated on serine residue(s) in untreated cells. In cells treated with BAPTA/AM, which exhibit lower levels of [Ca\textsuperscript{2+}]\textsubscript{i}, the immunoprecipitated CacyBP contained no phosphoserine immunoreactivity (Fig. 6B, lane 3). However, when ATP and Ca\textsuperscript{2+} was added to the extract of BAPTA/AM treated cells prior to immunoprecipitation, the serine residue(s) of CacyBP reacted with anti-phosphoserine antibodies to a similar extent as that observed in untreated cells (Fig. 6B, lane 4). These results show that CacyBP is not phosphorylated at low [Ca\textsuperscript{2+}]\textsubscript{i}. Thus, we find that CacyBP phosphorylation occurs within the same range of [Ca\textsuperscript{2+}]\textsubscript{i}, at which it is translocated to the perinuclear region.
DISCUSSION

In resting cultured cortical neurons CacyBP was distributed throughout the cytoplasm, but after stimulation that led to increase of [Ca\(^{2+}\)], the immunoreactivity was mainly visible as a ring around the nucleus. The intracellular Ca\(^{2+}\) level in cultured rat cortical neurons is \(~50\) nM, and upon KCl stimulation raises to above 300 nM (27). Similar low level of resting [Ca\(^{2+}\)], was measured in hippocampal neurons (80 nM) (28), and hypothalamic neurons - (54 or 69 nM) (29, 30). Thus, an increase in [Ca\(^{2+}\)], to above 300 nM upon neuron activation induces the translocation of CacyBP to the perinuclear region. Ca\(^{2+}\)-dependent change of CacyBP localization was also observed in neuroblastoma NB-2a cells. Transformed cells often have higher basic [Ca\(^{2+}\)], than normal cells (31). We confirmed this by establishing that in untreated neuroblastoma NB-2a cells [Ca\(^{2+}\)], is about 120 nM. In these cells CacyBP was mainly present in the perinuclear region. However, in cells treated with BAPTA/AM, in which [Ca\(^{2+}\)], dropped to 70 nM, a case similar to the [Ca\(^{2+}\)], of resting neurons, CacyBP immunoreactivity was no longer visible as a ring around the nucleus. All these data indicate that CacyBP is present throughout the cytoplasm at low [Ca\(^{2+}\)], and is translocated into the perinuclear region when [Ca\(^{2+}\)], is increased.

What mechanism might be responsible for Ca\(^{2+}\)-dependent translocation of CacyBP? There is a possibility that some posttranslational modifications of CacyBP might regulate its localization within the cell as is the case with some other proteins. It has been shown, for instance, that localization of AHNAK, a giant protein originally identified in neuroblastoma, changes upon the phosphorylation by protein kinase B (32). Another example is a protein named TFAF2/SNX6. This protein was translocated from the cytoplasm to the nucleus by Pim-1 kinase phosphorylation (33). Also, nuclear translocation and accumulation was described for wild type p53. In this case nuclear localization was dependent on protein kinase C activity (34).

Theoretical analysis of the CacyBP sequence showed that this protein has potential phosphorylation sites for protein kinase C. In agreement with this prediction we showed that recombinant CacyBP is indeed phosphorylated by protein kinase C *in vitro*. Moreover, we found that CacyBP is phosphorylated on serine residue(s) in untreated neuroblastoma NB-2a cells and exists in a dephosphorylated form in the cells treated by BAPTA/AM. In other words, reversible serine phosphorylation occurs within the same [Ca\(^{2+}\)], range, in which CacyBP translocation to the perinuclear region takes place. Since calcyclin was found to be associated with the nuclear envelope in a calcium dependent manner (35), it was interesting to see if calcyclin might regulate phosphorylation of CacyBP by direct interaction with this protein. It has been reported that S100B inhibits phosphorylation of p53 by direct interaction and not by influencing the kinase activity (36) and that S100C inhibits actin-activated myosin ATPase in the same way (37). Results obtained from experiments *in vitro* show that calcyclin has no influence on CacyBP phosphorylation by protein kinase C (A. Filipek, B. Jastrzebska, J. Kuźnicki, unpublished results). The results presented in this paper show that CacyBP distribution within the cell is modulated by changes in [Ca\(^{2+}\)], and that this phenomenon might be stimulated or regulated by a phosphorylation process.

Our electron microscopy studies showed immunogold staining of CacyBP in the nucleus of NB-2a cells. In fact, Fig. 5C shows CacyBP immunogold reactivity within and on both sides of the nuclear envelope, which suggests that the CacyBP transfer to nucleus has been trapped in this preparation. In fact, nuclear localization is not surprising since analysis of the CacyBP sequence performed using the BLAST server software identified a nuclear localization signal (NLS) between amino acids 144 and 160. The relationship between the putative NLS-mediated localization and Ca\(^{2+}\)-dependent translocation of CacyBP remains an unresolved issue, as does the physiological significance of these observations.
Some insight into the function of CacyBP could be obtained from a recent studies showing that a human protein termed SIP, which is 93% identical to mouse CacyBP, serves as a molecular bridge between Siah-1 and Skp1 proteins, components of a β-catenin ubiquitin ligase system. It has been known that different regulatory complexes and subpopulations of proteasomes have different distribution within mammalian cells (38), for instance 20S proteasomes and their 19S regulatory complexes were found in nuclear, cytosolic and microsomal fractions. Our results are fully consistent with these observations. Together, these data provide evidence for function of CacyBP in a novel ubiquitinylation pathway.

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FIGURE LEGENDS

Fig. 1. Immunofluorescent localization of CacyBP in cultured cortical neurons. Neurons were fixed and immunostained using anti-CacyBP antibodies. (A, D), unstimulated cells; (B, E), cells stimulated with 30 mM KCl for 10 min; (C, F), cells treated with 30 mM KCl for 10 min and next incubated in a medium without KCl for 2 h. In Fig. D, E, F quantitative analysis of CacyBP redistribution is shown. Three vertical lines were placed across the center of the cell to obtain line profiles of the intensity of CacyBP fluorescent labeling. The intensities of the fluorescence in the perinuclear region (I,) and in the cytoplasm (I,) were determined at sites marked for the middle line with white dots. The plot on the left shows intensity profile obtained along the middle vertical line. Labeling of the perinuclear region is seen in KCl stimulated neurons. In A, B, C scale bar is 15 µm and in D, E, F - 5 µm.

Fig. 2. mRNA and protein levels of CacyBP in PC12 (lane 1) and NB-2a (lane 2) cells. (A), Northern blot of total RNA hybridized with specific CacyBP cDNA probe; (B), Total RNA staining; (C), Western blot of protein extracts probed with antibodies against CacyBP – chemiluminescent detection. Each lane contained the same amount of total RNA (5 µg), or protein (100 µg).

Fig. 3. Changes in [Ca^{2+}] in neuroblastoma NB-2a cells analyzed by Fura-2 imaging. Black curve, [Ca^{2+}] in untreated cells (n=40); grey curve, [Ca^{2+}] in cells (n=29) treated with 5 µM BAPTA/AM; dotted line, [Ca^{2+}] in cells (n=8) stimulated with 200 nM thapsigargin. Arrow shows when thapsigargin was added.

Fig. 4. Localization of CacyBP in neuroblastoma NB-2a cells. (A, B, C), endogenous protein identified by immunocytochemistry. (D, E, F), fluorescence of EGFP-CacyBP fusion protein. (A, D), untreated cell; (B, E), cells treated with thapsigargin; (C, F), cells treated with BAPTA/AM. Scale bar is 15 µm.

Fig. 5. Immunogold electron microscopy of neuroblastoma NB-2a cells probed with anti-CacyBP antibodies. A, control without primary antibodies; B and C, untreated NB-2a cells; D, NB-2a cells treated with BAPTA/AM. Positive reaction is seen as black dots. Nuclei are indicated as Nuc. Scale bar is 100 nm.

Fig. 6. Phosphorylation of CacyBP
A, Phosphorylation of recombinant CacyBP with exogenous protein kinases and [γ-32P]ATP. Autoradiogram of the SDS gel. Lanes 1 and 2 show the CacyBP incubated with protein kinase C (PKC), lanes 3 and 4 – with protein kinase A (PKA), respectively. Lanes 2 and 4 show the control experiment in which CacyBP was omitted. Lanes 1 and 3 show the phosphorylation of CacyBP by PKC and PKA, respectively. Arrow indicates the band of CacyBP.
B, Study of CacyBP phosphorylation in NB-2a cells. Western blot after chemiluminescent detection. Endogenous CacyBP was immunoprecipitated from the extract of untreated NB-2a cells (lane 1 and 2) or BAPTA/AM-treated cells (lane 3 and 4) using anti-CacyBP antibodies. Lanes 2 and 4 show how adding of ATP, PMA and Ca^{2+} before immunoprecipitation effected CacyBP phosphorylation. Immunoprecipitated proteins were separated on 15% SDS gel, blotted onto nitrocellulose filter and probed with antibodies against phosphoserine.
TABLE LEGENDS

Table 1. Percentage of neurons containing high level of CacyBP in the perinuclear region. Neurons were unstimulated (none), stimulated with KCl (KCl) or stimulated with KCl and incubated for 2 h in medium without KCl (KCl washed out) and next stained with antibodies against CacyBP. Values shown are mean +/- SE from two experiments. In each experiment 100 cells were counted from two slides. Cells exhibiting a clear ring around the nucleus were considered as those containing a high level of CacyBP in the perinuclear region (see Fig. 1).

Table 2. CacyBP fluorescence intensity in perinuclear region in relation to its value in the cytoplasm in neuronal cells. Neurons were unstimulated (none), stimulated with KCl (KCl) or stimulated with KCl and incubated for 2 h in medium without KCl (KCl washed out) and next stained with antibodies against CacyBP. Results are the mean +/- SE from \( n \) number of cells.

Table 3. Percentage of neuroblastoma NB-2a cells containing high level of CacyBP in the perinuclear region. Cells were stained with antibodies against CacyBP (anti-CacyBP) or transfected with EGFP-CacyBP vector (EGFP-CacyBP). In the last case 100% corresponds to transfected cells. In each experiment 100 cells were counted from two slides. Neuroblastoma NB-2a cells were untreated (none), treated with thapsigargin, or BAPTA/AM. Cells exhibiting a clear ring around the nucleus were considered as those containing a high level of CacyBP in the perinuclear region (see Fig. 4). Values shown are mean +/- SE from two experiments.

Table 4. CacyBP fluorescence intensity in the perinuclear region in relation to its value in the cytoplasm of neuroblastoma NB-2a cells. Quantitative analyses were performed for cells stained with anti-CacyBP antibodies and cells expressing EGFP-CacyBP fusion protein. Since fluorescence intensity around the nucleus for untreated cells (none) and cells treated with thapsigargin was similar, quantitative analysis was performed only for untreated NB-2a cells and cells treated with BAPTA/AM. Results are the mean +/- SE from \( n \) number of cells.
### Table 1

| Treatment Method | None | KCl | KCl wash out |
|------------------|------|-----|--------------|
| Anti-CacyBP Staining | 2.5 ± 0.5 | 85.1 ± 3.9 | 8.0 ± 2.0 |

### Table 2

| Treatment Method | None | KCl | KCl wash out |
|------------------|------|-----|--------------|
| Anti-CacyBP Staining | 1.09 ± 0.04 \(n=11\) | 1.58 ± 0.04 \(n=10\) | 1.18 ± 0.05 \(n=10\) |

### Table 3

| Treatment Method | None | Thapsigargin | BAPTA/AM |
|------------------|------|--------------|----------|
| Anti-CacyBP Staining | 83.5 ± 2.1 | 83.3 ± 6.4 | 5.0 ± 1.4 |
| EGFP-CacyBP Fluorescence | 94.0 ± 1.4 | 86.0 ± 2.8 | 4.5 ± 2.1 |

### Table 4

| Treatment Method | None | BAPTA/AM |
|------------------|------|----------|
| Anti-CacyBP Staining | 1.44 ± 0.05 \(n=13\) | 0.97 ± 0.03 \(n=11\) |
| EGFP-CacyBP Fluorescence | 1.32 ± 0.03 \(n=10\) | 0.99 ± 0.02 \(n=12\) |
A

| PKC | PKA |
|-----|-----|
| +   | +   |
| -   | -   |

CacyBP

CacyBP

1  2  3  4

B

ATP, PMA, Ca^{2+}

CacyBP

1  2  3  4
Ca\textsuperscript{2+}-dependent translocation of calcyclin binding protein (CacyBP) in neurons and neuroblastoma NB-2a cells
Anna Filipek, Beata Jastrzebska, Marcin Nowotny, Katarzyna Kwiatkowska, Michal Hetman, Liliana Surmacz, Elzbieta Wyroba and Jacek Kuznicki

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