Adducin Is an In Vivo Substrate for Protein Kinase C: Phosphorylation in the MARCKS-related Domain Inhibits Activity in Promoting Spectrin–Actin Complexes and Occurs in Many Cells, Including Dendritic Spines of Neurons

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Abstract. Adducin is a heteromeric protein with subunits containing a COOH-terminal myristoylated alanine-rich C kinase substrate (MARCKS)-related domain that caps and preferentially recruits spectrin to the fast-growing ends of actin filaments. The basic MARCKS-related domain, present in α, β, and γ adducin subunits, binds calmodulin and contains the major phosphorylation site for protein kinase C (PKC). This report presents the first evidence that phosphorylation of the MARCKS-related domain modifies in vitro and in vivo activities of adducin involving actin and spectrin, and we demonstrate that adducin is a prominent in vivo substrate for PKC or other phorbol 12-myristate 13-acetate (PMA)-activated kinases in multiple cell types, including neurons. PKC phosphorylation of native and recombinant adducin inhibited actin capping measured using pyrene-actin polymerization and abolished activity of adducin in recruiting spectrin to ends and sides of actin filaments. A polyclonal antibody specific to the phosphorylated state of the RTPS-serine, which is the major PKC phosphorylation site in the MARCKS-related domain, was used to evaluate phosphorylation of adducin in cells. Reactivity with phosphoadducin antibody in immunoblots increased twofold in rat hippocampal slices, eight- to ninefold in human embryonal kidney (HEK 293) cells, threefold in MDCK cells, and greater than 10-fold in human erythrocytes after treatments with PMA, but not with forskolin. Thus, the RTPS-serine of adducin is an in vivo phosphorylation site for PKC or other PMA-activated kinases but not for cAMP-dependent protein kinase in a variety of cell types. Physiological consequences of the two PKC phosphorylation sites in the MARCKS-related domain were investigated by stably transfecting MDCK cells with either wild-type or PKC-unphosphorylatable S716A/S726A mutant α adducin. The mutant α adducin was no longer concentrated at the cell membrane at sites of cell–cell contact, and instead it was distributed as a cytoplasmic punctate pattern. Moreover, the cells expressing the mutant α adducin exhibited increased levels of cytoplasmic spectrin, which was colocalized with the mutant α adducin in a punctate pattern. Immunofluorescence with the phosphoadducin-specific antibody revealed the RTPS-serine phosphorylation of adducin in postsynaptic areas in the developing rat hippocampus. High levels of the phosphoadducin were detected in the dendritic spines of cultured hippocampal neurons. Spectrin also was a component of dendritic spines, although at distinct sites from the ones containing phosphoadducin. These data demonstrate that adducin is a significant in vivo substrate for PKC or other PMA-activated kinases in a variety of cells, and that phosphorylation of adducin occurs in dendritic spines that are believed to respond to external signals by changes in morphology and reorganization of cytoskeletal structures.

Key words: membrane skeleton • cytoskeleton • actin binding protein • synapse • synaptic plasticity

Adducin is a ubiquitously expressed calmodulin-binding protein (Gardner and Bennett, 1986; Bennett et al., 1988) and substrate for protein kinase C (PKC)[1] (Palfrey and Waseem, 1985; Cohen and Foley, 1986; Ling et al., 1986; Kaiser et al., 1989). Adducin was first purified from human erythrocyte membrane skeletons (Gardner and Bennett, 1986) and from brain membranes (Bennett et al., 1988). Adducin is localized at spectrin–actin junctions in erythrocyte membrane skeletons (Derick et

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1. *Abbreviations used in this paper*: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; HA, hemagglutinin; IBMX, 3-isobutyl-1-methylxanthine; MARCKS, myristoylated alanine-rich C kinase substrate; NMDA, *N*-methyl-D-aspartate; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PKM; the catalytic domain of protein kinase C; PMA, phorbol 12-myristate 13-acetate.

al. 1992) and colocalizes with spectrin at sites of cell–cell contact in epithelial cells (Kaiser et al., 1989; Hu et al., 1995) and in dendritic spines of hippocampal neurons (Seidel et al., 1995). Adducin exhibits in vitro activities of promoting association of spectrin with actin (Gardner and Bennett, 1987; Bennett et al., 1988), association with sides of actin filaments (Mische et al., 1987; Taylor and Taylor, 1994), and capping the fast-growing ends of actin filaments (Kuhlman et al., 1996). Recently, adducin has been demonstrated to preferentially cap and recruit spectrin to the fast-growing ends of actin filaments (Li et al., 1998).

Adducin is a heteromeric protein comprised of either α and β or α and γ subunits (Gardner and Bennett, 1986; Bennett et al., 1988; Dong et al., 1995; Hughes and Bennett, 1995). α adducin is expressed in most tissues, while β adducin has a more restricted pattern of expression (Joshi et al., 1991). γ adducin, which is similar in sequence to α and β adducin, is a likely companion for α adducin in cells lacking the β subunit (Dong et al., 1995). Adducin subunits are closely related in amino acid sequence and domain organization (Joshi and Bennett, 1990; Joshi et al., 1991; Dong et al., 1995). Each adducin subunit has three distinct domains: a 39-kD NH_2-terminal globular protease-resistant head domain, connected by a 9-kD "neck" domain to a COOH-terminal protease-sensitive tail domain (Joshi and Bennett, 1990; Joshi et al., 1991; Dong et al., 1995). Adducin isolated from erythrocytes is a mixture of heterodimers and tetramers with NH_3-terminal head domains in contact to form a globular core and with interacting tails extended away from the core (Hughes and Bennett, 1995).

COOH termini of all three subunits of adducin contain a highly basic stretch of 22 amino acids with sequence similarity to a domain in the myristoylated alanine-rich C kinase substrate (MARCKS protein) (Joshi et al., 1991; Dong et al., 1995). The MARCKS-related domain is required for interactions of adducin with actin and spectrin, which is consistent with the possibility that the MARCKS-related domain mediates contact with actin (Li et al., 1998). The MARCKS-related domain also has a major phosphorylation site, the RTPS-serine, for PKC as well as cAMP-dependent protein kinase (PKA), and contains the primary calmodulin-binding site of adducin (Matsuoka et al., 1996). Ca^{2+}/calmodulin inhibits actin-capping and spectrin recruitment activities of adducin (Gardner and Bennett, 1987; Kuhlman et al., 1996). Although phosphorylation of the MARCKS-related domain by PKC inhibits calmodulin binding (Matsuoka et al., 1996), effects of phosphorylation on the other activities of adducin have not been resolved.

The MARCKS family of proteins has been studied extensively as in vivo substrates of PKC (Aderem, 1992; Blackshear, 1993). A 25–amino acid basic domain is the site for both PKC phosphorylation and calmodulin binding of the MARCKS protein and is similar in sequence to the MARCKS-related domain of adducin (Aderem, 1992; Blackshear, 1993). The MARCKS protein binds and cross-links actin in in vitro assays, and its capacity to cross-link actin is regulated by PKC phosphorylation and calmodulin-binding to the phosphorylation site domain (Hartwig et al., 1992). A COOH-terminal (C1) region in the NR1 subunit of *N*-methyl-D-aspartate (NMDA) receptor, a glutamate receptor of neuron, has sequence similarity to the PKC phosphorylation/calmodulin-binding domain of the MARCKS protein (Tingley et al., 1993; Ehlers et al., 1996). The C1 region also contains PKC phosphorylation sites and is a high-affinity binding site for calmodulin (Tingley et al., 1993; Ehlers et al., 1996). Moreover, phosphorylation and calmodulin-binding to the C1 region may regulate interaction of the NR1 subunit of NMDA receptor with the actin cytoskeleton (Ehlers et al., 1995, 1996). The PKC phosphorylation site domain of the MARCKS protein is also involved in its cell membrane binding through the interaction between a cluster of basic residues and acidic phospholipids, such as phosphatidylserine (Kim et al., 1994; McLaughlin and Aderem, 1995). Phosphorylation of the serine residues in the poly-basic domain reduces its electrostatic interaction with the phospholipids and has been proposed to provide an electrostatic switch mechanism for the reversible binding of the MARCKS protein to the cell membrane.

This report presents evidence that adducin is an in vivo substrate for PKC or other phorbol 12-myristate 13-acetate (PMA)-activated kinases, and that activities of adducin in capping and recruiting spectrin to actin filaments are regulated by PKC phosphorylation of the MARCKS-related domain. Mutation of the two PKC phosphorylation sites in the MARCKS-related domain of α adducin produced striking effects on distribution of the mutant adducin as well as of spectrin in MDCK cells. Adducin phosphorylated at the major PKC site in the MARCKS-related domain was specifically localized in dendritic spines of cultured neurons. Adducin is a candidate molecule to mediate downstream consequences of PKC activation in postsynaptic sites in neurons as well as other dynamic cellular domains.

Materials and Methods

Proteins

Actin was purified from acetone powder of rabbit skeletal muscle (Pardee and Spudich, 1982) with a modification (Li and Bennett, 1996). Brain spectrin was isolated by high salt extraction from bovine brain membranes (Davis and Bennett, 1983; Li and Bennett, 1996). Erythrocyte adducin and a recombinant β adducin ($\beta_{335–726}$) were purified as previously described (Hughes and Bennett, 1995; Li et al., 1998). The catalytic domain of PKC was purchased from Calbiochem (San Diego, CA).

Antibodies

Phosphoadducin-specific rabbit polyclonal antibody was raised against a synthetic phosphopeptide (FRTPphosphoSFLKK) (Andrews et al., 1991) corresponding to the major PKC phosphorylation site of adducin (Matsuoka et al., 1996) and affinity-purified as described (Matsuoka et al., 1992). This antibody was preincubated with the unphosphorylated form of the peptide and used in this study. Polyclonal antibodies against α adducin and the MARCKS-related domain were generated using a recombinant human α adducin (residues 536–737) and a synthetic peptide corresponding to the residues 696–726 of human β adducin, respectively, as antigens. Affinity-purified rabbit polyclonal antibody against brain spectrin was reported previously (Davis and Bennett, 1983). An anti–spectrin β_G monoclonal antibody was raised against a synthetic peptide corresponding to the residues 2096–2122 of human β spectrin. Monoclonal anti-hemagglutinin (HA) epitope antibody (HA.11) was purchased from Berkeley Antibody Co. (Richmond, CA), monoclonal antisynaptophysin antibody was from Boehringer Mannheim (Indianapolis, IN), anti-GluR2/4 antibody (clone 3A11) was from PharMingen (San Diego, CA), and FITC- and TRITC-conjugated goat secondary antibodies were from Pierce Chemical Co. (Rockford, IL).

Phosphorylation of Adducin and Protease Digestion

Erythrocyte adducin (2.5 μM monomer) and $\beta_{335-726}$ (5 μM monomer) were phosphorylated by incubation with 0.4 μg/ml catalytic domain of PKC (PKM), 0.1 mM ATP, 5 mM $MgCl_2$, 2 mM sodium EGTA, 25 mM Tris-HCl, pH 7.5 at 15°C for 12–14 h. The reaction was terminated by adding 50 nM (final) bisindolylmaleimide (Calbiochem). ATP was added to the unphosphorylated adducin sample after quenching the reaction. To measure stoichiometry of the phosphorylation, adducin was phosphorylated in parallel using 0.1 mM [γ-^{32}P]ATP. The sample was processed as described previously (Matsuoka et al., 1996). In some experiments, [^{32}P]phosphoadducin (50 μg) was digested with *Staphylococcus aureus* V8 protease (1:50 wt/wt; Pierce Chemical Co.) for 3 h at 30°C as described previously (Matsuoka et al., 1996). After quenching the digestion by adding 2 mM PMSF, the sample was applied to an S-Sepharose column (0.5 ml bed volume; Pharmacia Biotech, Piscataway, NJ) equilibrated with 20 mM Tris-HCl, 2 mM sodium EGTA, 1 mM DTT, 1 mM PMSF, pH 8.0 (column buffer). Fractions (0.2 ml) were collected by eluting the column with a step gradient of NaBr (0.1, 0.2, 0.3, and 0.5 M in the column buffer) after wash with the column buffer and subjected to scintillation counting and SDS-PAGE (3.5–17%).

Actin Polymerization and Depolymerization Assays

Pyrene-labeled actin was prepared according to the method modified by Weber et al. (1987). G buffer (5 mM Tris-HCl, 0.2 mM Na_2ATP, 0.5 mM 2-mercaptoethanol, 0.2 mM $CaCl_2$, 0.005% sodium azide, pH 8.0, at 25°C) was used in all assays. The assay used to quantitate inhibition of actin polymerization at the barbed ends used the method of Pollard (1983), in which rapid polymerization was initiated using F-actin nuclei.

Spectrin Recruitment Assay

A full description of this assay system is presented in Li et al. (1998).

Immunoblotting

Hippocampal slices (220 μm) were prepared from 5–6-wk-old Sprague-Dawley rats according to Garver et al. (1995). Slices were immediately immersed in an ice-cold preincubation buffer (Garver et al., 1995) and incubated with 40 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; Tocris Cookson, Ballwin, MO) in the buffer at 37°C for 30 min. Buffer was replaced with the fresh one without CNQX before further manipulations. Human erythrocytes were separated and resuspended in the modified Hepes-Tyrode buffer (Matsuoka et al., 1994) at a final concentration of 20%. Human embryonal kidney cell line (HEK 293; American Type Culture Collection, Rockville, MD) was maintained in 5% FCS/DME and serum-starved overnight before the following treatments. Human erythrocytes (1 ml suspension), HEK 293 cells (ϕ 6-cm dish), and rat hippocampal slices (5–10 slices) were treated either with 50 μM 3-isobutyl-1-methylxanthine (IBMX; Calbiochem)/50 μM forskolin (Calbiochem) or 1 μM PMA (Calbiochem) at 37°C for 15 min. After the treatments, erythrocytes were lysed, and the membrane fractions were collected. Erythrocyte membranes, HEK 293 cells, and rat hippocampal slices were solubilized in a minimum volume of 50 mM Tris-HCl, pH 7.5, 7 M urea, 1% SDS, 5 mM sodium EDTA, 1 mM DTT, 10 μg/ml leupeptin, 1 mM PMSF with brief sonication. Proteins (10 μg) were separated by SDS gel electrophoresis with buffers of Fairbanks et al. (1971) on 5% gels in 0.2% SDS. The amounts of phosphoadducin and α adducin were determined from scans of the autoradiographs using a densitometer as described elsewhere (Matsuoka et al., 1994). The amount of phosphoadducin in each lane was normalized by the amount of α adducin loaded and was used to calculate the change of phosphoadducin level after treatment.

Construct Generation and Transfection of MDCK Cells

An HA epitope–tagged wild-type α adducin cDNA in pGEMEX vector was used as a template to mutate the PKC phosphorylation sites in MARCKS-related domain (Ser716 and Ser726) to alanine residues. Sense (5′-CCGGCCTTTCTTAAGAAGAGCAAGAAGAAGAGTGACTCC-3′) and antisense (5′-GGTACGAAACTTCTTCTTCTTTTTGGCTGG-GGACTTGC-3′) primers were used to mutate the sites with the Exsite PCR-based mutagenesis kit (Stratagene, La Jolla, CA). Mutation of the construct was confirmed by DNA sequencing. Both HA epitope–tagged wild-type and PKC sites–mutated α-adducin cDNA were subcloned into pCMV vector (Garver et al., 1997) at NheI-XhoI sites. Transfection of MDCK cells was performed using lipofectamine (GIBCO-BRL, Gaithersburg, MD) as described elsewhere (Garver et al., 1997).

Rat Hippocampal Cell Culture

Isolation of hippocampal formations from newborn (P2) Sprague-Dawley rats and the dissociation of neurons was performed as described (Brewer et al., 1993) with a modification. Briefly, hippocampi from newborn rats were first incubated with 0.027% trypsin in HBSS without Ca^{2+} and Mg^{2+}, 0.035% sodium bicarbonate, 1 mM pyruvate, 10 mM Hepes, pH 7.4 (HBSS), in a 95% O_2, 5% CO_2 incubator for 20 min at 35°C. Hippocampi were washed with HBSS and triturated through a 1-ml plastic pipette tip in HBSS. 1 ml of cell suspension was diluted with 2 ml HBSS with Ca^{2+}, Mg^{2+}. The cells were collected by centrifugation for 1 min at 200 *g*, resuspended in Neurobasal medium (GIBCO-BRL) containing 0.5 mM glutamine, 25 μM glutamate, and 1% B-27 supplement (GIBCO-BRL), and plated at a concentration of 160 cells/mm^2. Half of the culture medium was replaced by the same medium without glutamate every 4 d. Primary culture of hippocampal neuron was maintained for at least 7 d before the following treatment. Neurons were incubated with fresh Neurobasal medium (no glutamate) containing 15 mM $MgCl_2$ without B-27 supplement for 90 min, and with 40 μM CNQX for a further 30 min before activation. The medium was changed to the new one (0.8 mM $MgCl_2$), and either 10 μM glutamate or 1 μM PMA was applied to the neurons at 37°C for 6 min.

Immunofluorescence

P15 rat brain was fixed and processed as described elsewhere (Lambert and Bennett, 1993). Cells were fixed with 4% formaldehyde (freshly made from paraformaldehyde) in 0.1 M phosphate buffer, pH 7.4, containing 0.32 M sucrose for 10 min at room temperature. After fixation, cells were permeabilized and blocked with 0.05% Triton X-100 (1% for MDCK cells) in a blocking buffer (PBS with 10% goat serum, 1% BSA, and 5% sucrose) for 15 min at room temperature. Cells were then incubated with primary antibodies diluted in the blocking buffer followed by FITC- or TRITC-conjugated goat secondary antibodies (1.5 μg/ml; Pierce Chemical Co.).

Results

PKC Phosphorylation of the MARCKS-related Domain of Adducin Modulates Activities of Actin Capping and Promoting Spectrin–Actin Complexes

In a previous study, we did not observe an effect of PKC phosphorylation on the activity of adducin in promoting binding of spectrin to F-actin (Matsuoka et al., 1996). In these experiments, phosphatidylserine and a phorbol ester were included to activate PKC isolated from rat brain, which was used at a concentration of 125 nM (Matsuoka et al., 1996). It is possible that either PKC itself, a contaminant in the PKC preparation, or phosphatidylserine obscured effect of the phosphorylation in the assay, even though in some experiments the phosphoadducin was isolated by cation exchange chromatography. In the present study, PKM, which lacks the regulatory domain and does not require phosphatidylserine or PMA for its activity, was used at 15-fold lower concentrations than used previously to phosphorylate adducin. Kinase activity was quenched with bisindolylmaleimide, and adducin samples in the phosphorylation mixture were directly used for actin capping and spectrin recruitment assays in this study. Controls included the kinase and inhibitor, with ATP added after quenching the phosphorylation reaction.

The MARCKS-related domain of adducin was also the major phosphorylation site for PKM. 75% of the radioactivity in V8 protease digests of ^{32}P-labeled adducin phosphorylated by PKM (0.9 mol phosphate/mol subunit) asso-

ciated with fragments migrating at the same position as the MARCKS-related domain of β adducin (Fig. 1 *A*). In addition, these fragments were highly positively charged, since the polypeptides tightly bound to a cation-exchange resin at pH 8 (>0.3 M NaBr was required to elute the fragments) (Fig. 1 *A*), as did the polybasic MARCKS-related domain (Matsuoka et al., 1996). Therefore, the V8 fragments most likely are the MARCKS-related domains of α and β adducin. PKM also phosphorylated the major PKC-site in the MARCKS-related domain based on immunoreactivity with phosphoadducin-specific antibody (see below) (Fig. 3 *C*). Similar results were obtained using the neck–tail domain construct of human β adducin ($\beta_{335-726}$) (data not shown). Taken together, these results support the conclusion that the MARCKS-related domain is the major phosphorylation site of adducin for PKM as well as for PKC.

In a recent study, we have demonstrated that recombinant $\beta_{335-726}$ adducin has comparable activity with that of erythrocyte adducin (Li et al., 1998). Therefore, $\beta_{335-726}$ adducin was first used to investigate phosphorylation effects on its actin capping activity. PKM phosphorylation of the MARCKS-related domain (1.9 mol Pi/mol $\beta_{335-726}$) greatly reduced the capping activity of $\beta_{335-726}$ adducin from 68 to 12% at 0.5 μM of the construct (Fig. 1 *B*). Similar results were obtained at up to 1 μM of the construct (data not shown). The ability of PKM phosphorylation to eliminate the barbed end capping activity of adducin was also seen in actin depolymerization experiments, as evidenced by the return of the depolymerization rate in the presence of adducin phosphorylated by PKM to that of pure actin (Fig. 1 *C*). PKM phosphorylation of the MARCKS-related domain (0.9 mol Pi/mol subunit) also greatly reduced the capping activity of native adducin (Fig. 1 *D*). The average reduction of capping activity by phosphorylation was 64 ± 14%. Similar results were obtained using $\beta_{335-726}$ (data not shown).

Spectrin recruiting activity of adducin was also found to be a target of regulation through phosphorylation of the MARCKS-related domain. Phosphorylation of adducin by PKM almost completely abolished spectrin recruiting activity at up to 600 nM of the phosphorylated adducin (Fig. 2). Since adducin promotes binding of spectrin to the fast-growing ends of actin filaments in addition to filament sides (Li et al., 1998), recruitment of spectrin to both the ends and sides of actin filaments is inhibited by PKM phosphorylation. PKM phosphorylation showed a stronger effect on spectrin recruitment activity than actin capping, although the basis for this difference is not known. This is the first demonstration that actin filament capping

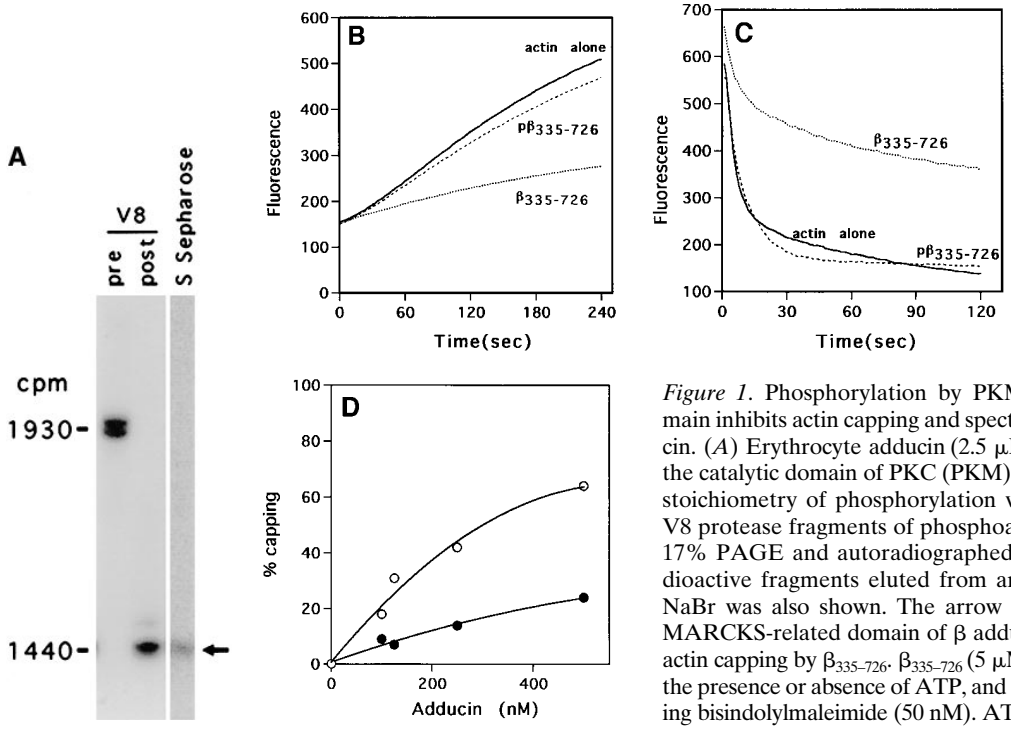

Figure 1. Phosphorylation by PKM of the MARCKS-related domain inhibits actin capping and spectrin recruitment activities of adducin. (*A*) Erythrocyte adducin (2.5 μM monomer) was incubated with the catalytic domain of PKC (PKM) in the presence of [^{32}P]ATP. The stoichiometry of phosphorylation was 0.9 mol Pi/mol subunit. The V8 protease fragments of phosphoadducin were separated with 3.5–17% PAGE and autoradiographed. An autoradiography of the radioactive fragments eluted from an S-Sepharose column by 0.5 M NaBr was also shown. The arrow indicates the position where the MARCKS-related domain of β adducin migrates. (*B*) PKM effect on actin capping by $\beta_{335-726}$. $\beta_{335-726}$ (5 μM) was preincubated with PKM in the presence or absence of ATP, and the reaction was quenched by adding bisindolylmaleimide (50 nM). ATP was added to unphospho$\beta_{335-726}$ after terminating the reaction. Elongation was initiated by the addition of F-actin nuclei (final 0.25 μM) and salts (final 50 mM KCl and 1 mM $MgCl_2$) to a 4 μM G-actin solution (5% pyrenyl actin) with or without 0.5 μM of each $\beta_{335-726}$. The stoichiometry of phosphorylation was 1.9 mol Pi/mol $\beta_{335-726}$. (*C*) Effect of PKM phosphorylation on the ability of $\beta_{335-726}$ to inhibit the rate of actin depolymerization. The same samples used in the polymerization assay (*B*) were used. F-actin (10 μM) was diluted into G buffer containing 0.5 μM of $\beta_{335-726}$ or phospho$\beta_{335-726}$ to a final concentration of 0.5 μM. Data are representative of three measurements. (*D*) Percentage of inhibition of elongation (percent capping) plotted against increasing concentrations of unphospho- (*open circles*) or phospho- (*filled circles*) adducin. The polymerization rate was calculated as a percentage of the rate of polymerization in the absence of adducin 30–150 s after initiation and was used to calculate the capping rate. The concentrations were expressed as monomer of adducin. Data are averages of at least three measurements. K_{cap} value (concentration required to produce 50% inhibition of polymerization) of unphosphorylated adducin was calculated as 125 nM monomer.

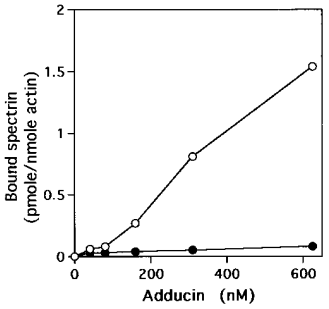

Figure 2. PKM effect on spectrin recruitment by adducin. ^{125}I-labeled brain spectrin was incubated with biotinylated actin (0.4 μM) coupled onto avidin microspheres in 60 μl of a reaction mixture (30 mM Hepes, pH 7.0, 50 mM KCl, 2 mM $MgCl_2$, 1 mM NaEGTA, 10% sucrose, 0.05% Tween-20, 2 mg/ml BSA, 0.5 mM ATP, 0.2 mM DTT, 0.5 mM NaN_3) in the presence of increasing concentrations of unphospho- (*open circles*) or phospho- (*filled circles*) adducin for 1 h at 4°C. Actin-bound spectrin was separated from unbound by layering the mixture onto 200 μl of 20% sucrose barrier dissolved in the same buffer and centrifuging for 10 min at 5,000 *g*. Data are averages of at least two measurements.

and spectrin recruiting activities of adducin can be regulated by phosphorylation of the MARCKS-related domain. The MARCKS-related domain is thus a site of regulation for actin filament capping and spectrin recruiting activities of adducin as well as the primary functional domain for these activities.

Phosphorylation of the MARCKS-related Domain of Adducin Occurs In Vivo

Antibodies specific to phosphorylated sites of a substrate are particularly useful tools to evaluate the extent of in vivo phosphorylation and to determine the cellular localization of phosphorylated proteins (Nishizawa et al., 1991; Dent and Meiri, 1992; Matsuoka et al., 1992; Liao et al., 1995). We produced an antibody against a peptide, FRTP-phosphoSFLKK, containing the phosphorylated form of the RTPS-serine, which is the major site of phosphorylation by PKC of adducin (Matsuoka et al., 1996). The RTPS-serine is also phosphorylated by PKA, although the K_m of PKA for adducin is 10-fold higher than that of PKC (Matsuoka et al., 1996).

Immunoblots revealed that the RTPS-phosphoserine–specific antibody has negligible reactivity towards adducin in unstimulated human erythrocytes (Fig. 3 *A*, *a*), but at least a 10-fold increase in signal occurred after activation of PKC by addition of phorbol ester (PMA) (Fig. 3 *A*, *c*). The phosphoadducin antibody was specific for adducin based on lack of reaction with other proteins in crude homogenates or with recombinant human MARCKS protein (data not shown). Moreover, the antibody reaction was displaced completely by a 10-fold molar excess of the phosphopeptide (data not shown). An eight- to ninefold increase in reactivity with the phosphoadducin-specific antibody occurred after PMA treatment of human embryonal kidney (HEK 293) cells (Fig. 3 *A*, *d* and *f*), and a twofold increase occurred in rat hippocampal slices (Fig. 3 *A*, *g* and *i*). In contrast to PMA, forskolin (50 μM), an adenylyl cyclase activator, even in combination with a phosphodiesterase inhibitor (IBMX, 50 μM), resulted in only a 1.4-fold increase of the phosphorylation in HEK 293 cells (Fig. 3 *A*, *b*, *e*, and *h*). Thus, the RTPS-serine in the MARCKS-related domain of each adducin subunit is an in vivo phosphorylation site for PKC or other PMA-activated kinases but not PKA. A 90-kD adducin polypeptide detected in HEK 293 cells may correspond to the human counterpart of rat γ adducin (Dong et al., 1995) (Fig. 3 *A*, *f*).

Recombinant human α adducin (residues 536–737) was used to produce an anti–α adducin antibody. The affinity-purified antibody specifically recognized α adducin in human erythrocytes (Fig. 3 *B*, *a–c*). The amount of α adducin was used, as a representative of adducin subunits, to normalize the amount of phosphoadducin in each sample. α and β adducin in HEK 293 cells and rat hippocampal lysates migrated at the same position as human erythrocyte α adducin, as confirmed by antibodies specific to human α (Fig. 3 *B*, *d–f* and *g–i*) and β (data not shown) adducin.

Specificity of the phosphoadducin antibody was further analyzed using α adducin mutated at Ser716Ala/Ser726Ala and resistant to phosphorylation by PKC (referred to as α adducin$^{\Delta pkc}$). The HA epitope–tagged α adducin and α adducin$^{\Delta pkc}$ expressed in MDCK cells were immunoprecipitated by an anti–HA epitope antibody followed by immunoblot with the phosphoadducin-specific antibody. Reactivity of the phosphoadducin antibody for the HA epitope–tagged α adducin was increased about threefold after PMA treatment (0.1 μM, 15 min) of the cells, whereas the antibody showed no reactivity toward α adducin$^{\Delta pkc}$ (Fig. 3 *C*). Lack of reactivity of the phosphoadducin antibody with α adducin$^{\Delta pkc}$ provides additional support for its specificity toward the RTPS-phosphoserine of adducin.

The RTPS-Phosphoserine Adducin and Spectrin Accumulate in the Cytoplasm in MDCK Cells after PMA Treatment

α adducin and spectrin were concentrated at the cell membrane of cell–cell contact sites in serum-starved MDCK cells as reported previously (Fig. 4 *A* and Kaiser et al., 1989). The anti–α adducin antibody also consistently showed weak nuclear staining (Fig. 4 *A*, *b* and *e*). Very low levels of the RTPS-serine phosphorylation was detected in the cytoplasm, while none was detected at the cell–cell contact sites of the cells using the phosphoadducin antibody (Fig. 4 *B*, *b*). When the cells were stimulated with PMA, levels of α adducin slightly but significantly increased in the cytoplasm (Fig. 4 *A*, *e*). Increase of the RTPS-phosphoserine adducin was clearly observed in the cytoplasm (Fig. 4 *B*, *e*). The increases of α adducin and the phosphoadducin were accompanied by redistribution of spectrin from the cell–cell contact sites into the cytoplasm (Fig. 4, *A* and *B*). Phosphoadducin and spectrin were distributed in a punctate pattern in the cytoplasm after PMA treatment (Fig. 4 *B*, *d* and *e*). Redistribution of adducin and spectrin from the cell membrane into the cytoplasm has been reported in several types of epithelial cells treated with PMA (Kaiser et al., 1989; Dong et al., 1995). Similar results were also obtained using HEK 293 cells (data not shown). Therefore, phosphorylation of the RTPS-serine in adducin appears to correlate closely with redistribution of the protein and spectrin from the cell membrane into the cytoplasm. This is consistent with the biochemical data showing that phosphorylation of the MARCKS-related domain by PKC interferes with interactions of adducin with spectrin and actin.

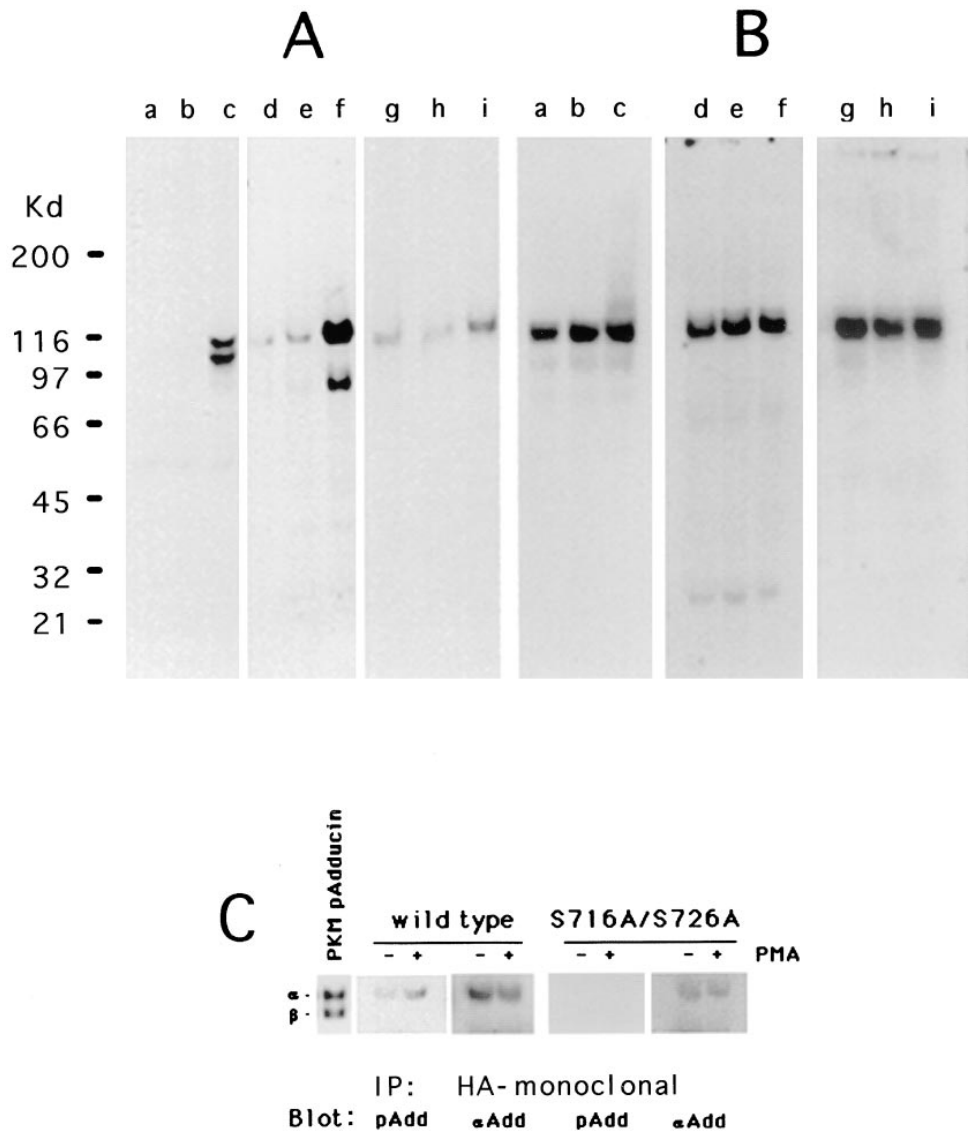

Figure 3. Specificity of the antiphosphoadducin and anti–α adducin antibodies and effect of forskolin and PMA on adducin phosphorylation in vivo. Western blot analysis for the antiphosphoadducin antibody (3.5 μg/ml) (*A*) and the anti–α adducin antibody (1 μg/ml) (*B*). Lanes *a–c*, human erythrocyte membrane; lanes *d–f*, human embryonal kidney (HEK 293) cells; lanes *g–i*, rat hippocampal slices. Lanes *a*, *d*, and *g*, unstimulated conditions; lanes *b*, *e*, and *h*, 50 μM forskolin/50 μM IBMX treatment; lanes *c*, *f*, and *i*, 1 μM PMA treatment. Both α and β adducin in HEK 293 cells and rat hippocampal lysates migrated at the same position of human erythrocyte α adducin (lanes *d–f* and *g–i*). Each lane was loaded with 10 μg total protein. (*C*) Confluent cultures of stable MDCK cells expressing HA epitope–tagged full-length human α adducin and the PKC sites mutant were serum starved overnight and were either untreated or treated with 0.1 μM PMA for 15 min. After washing with ice-cold PBS containing 2 mM PMSF, the cells were lysed with an ice-cold lysis buffer (1 ml/ø10-cm dish) consisting of 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM sodium EGTA, 2 mM sodium EDTA, 50 mM NaF, 100 nM caliculin A, 1% Triton X-100, 1% NP-40, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 1 mM bebzamidine, and 1 mM PMSF using a glass-Teflon pestle homogenizer. The lysate was cleared by centrifugation at 15,600 *g* for 10 min. The HA epitope–tagged α adducin was immunoprecipitated for 3 h at 4°C from 1 ml of cell lysate using an HA-specific mouse IgG preadsorbed on protein G–Sepharose beads (50 μl; Pharmacia Biotech). The beads were washed twice with lysis buffer and then twice with the same buffer without Triton and NP-40 before addition of 100 μl SDS-sample buffer. Erythrocyte adducin phosphorylated by PKM was used as a positive control for the RTPS-serine phosphorylation.

Phosphorylation State of the MARCKS-related Domain Determines Adducin Distribution in MDCK Cells

Roles of the phosphorylation state of the MARCKS-related domain in determining distribution of adducin and spectrin were evaluated by transfecting cells with α adducin$^{\Delta pkc}$, which was resistant to phosphorylation by PKC at the MARCKS-related domain. MDCK cell lines were generated that stably expressed the HA epitope–tagged human α adducin (MDCK/α add) and the mutant human α adducin (MDCK/α add$^{\Delta pkc}$) (see Materials and Methods).

The HA epitope–tagged human α adducin and spectrin in MDCK/α add cells were concentrated at the cell membrane at sites of cell–cell contact (Fig. 5, *a–c*) as observed for the native proteins in nontransfected cells (Fig. 4 *A*). We assumed that a PKC-unphosphorylatable version of adducin would stay at the cell membrane even after PMA treatment if phosphorylation of the PKC sites in MARCKS-related domain was responsible for the redistribution of adducin. Surprisingly, the mutant α adducin was not localized at the cell–cell contact sites of the PMA-untreated cells, but instead exhibited a punctate distribution in the cytoplasm (Fig. 5, *d* and *f*). Moreover, cells expressing the mutant α adducin exhibited increased levels of cytoplasmic spectrin in a punctate pattern, which was in some cases colocalized with the mutant α adducin (Fig. 5, *e* and *f*). This result clearly showed involvement of the PKC phosphorylation sites in controlling subcellular distribution of adducin as well as spectrin. The nature of the punctate structures containing the mutant adducin and spectrin

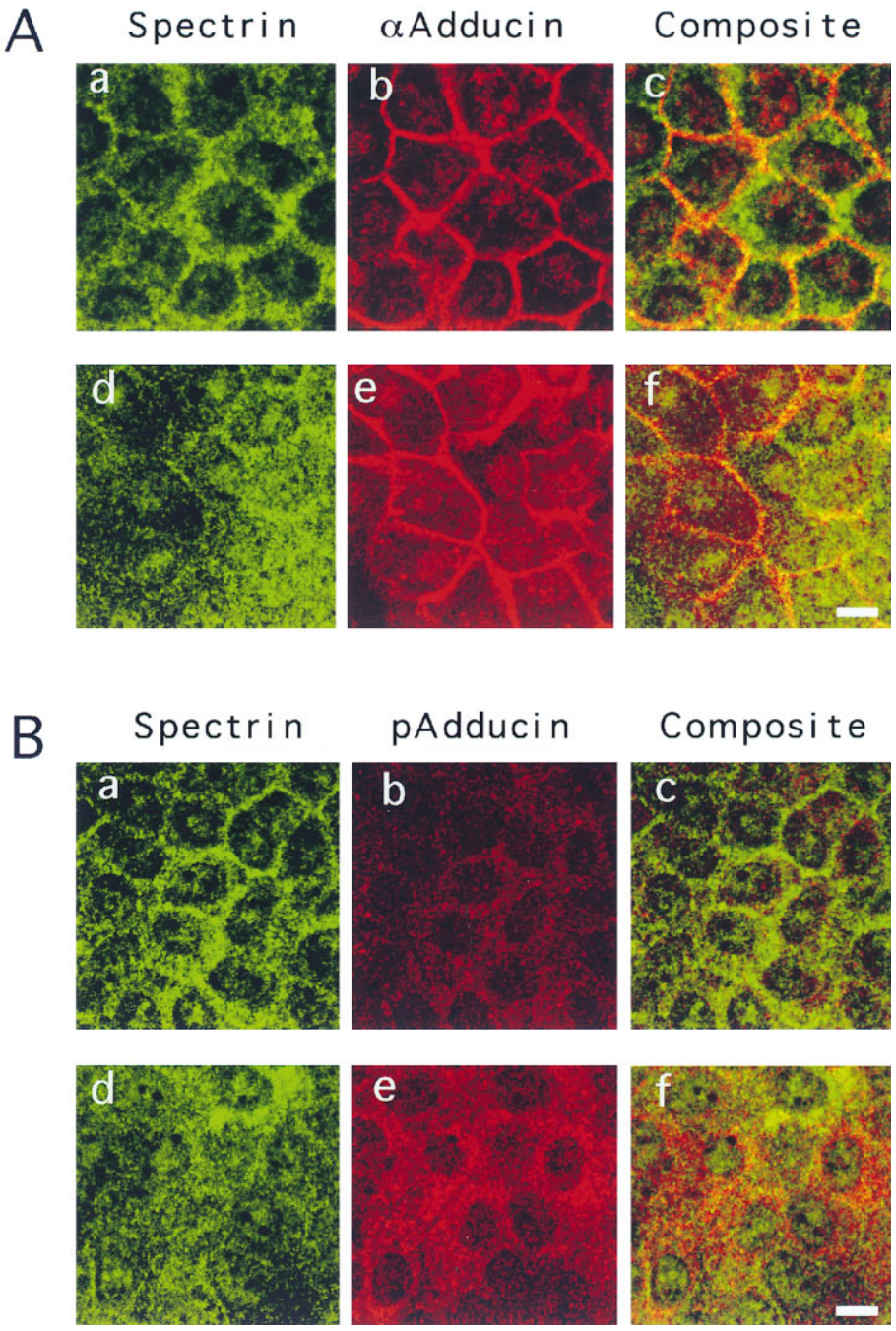

Figure 4. Redistributions of the RTPS-phosphoserine adducin and spectrin after PMA treatment in MDCK cells. Confluent cultures of MDCK cells were treated without (*a–c*) or with (*d–f*) 0.1 μM PMA for 30 min followed by double staining with spectrin β_G- (*a* and *d*) and α adducin- (*b* and *e*) specific antibodies (*A*) or with spectrin β_G- (*a* and *d*) and phosphoadducin- (*b* and *e*) specific antibodies (*B*). Composite images show colocalization of signals (*yellow*; *c* and *f*). Primary antibodies were used at the indicated concentrations: anti–spectrin β_G (5 μg/ml), anti–α adducin (3 μg/ml), and antiphosphoadducin (3.5 μg/ml) antibodies. All images were taken at the nucleus level. Fluorescent images were visualized by a confocal laser microscope (model LSM 410; Carl Zeiss, Inc., Thornwood, NY). Bars, 10 μm.

has not been resolved, but they could represent vesicles en route to or returning from the plasma membrane.

Localization of the RTPS-Phosphoserine Adducin in the Hippocampus

High levels of the phosphoadducin were detected in the dendritic fields, i.e., *stratum radiatum* and *stratum oriens*, of CA1 region in P15 rat hippocampus (Fig. 6 *a*). In the dentate gyrus, the staining intensity for phosphoadducin was higher in the hilus region than in the molecular layer (Fig. 6 *a*). In the dendritic fields of CA3 region, phosphoadducin staining was slightly weaker than CA1 region (Fig. 6 *b*). Confocal microscopy for *stratum radiatum* of CA1 region at high magnification revealed phosphoadducin antibody staining of round and elliptical structures with diameters <1 μm (Fig. 6 *c*). Double labeling with antibodies against synaptophysin and the phosphoadducin revealed that some of the phosphoadducin-stained structures were immediately adjacent to presynaptic endings defined by synaptophysin staining. (Fig. 6 *c*, *arrows*). Synaptophysin is a marker for neurosecretory vesicles, which are concentrated at presynaptic endings (Wiedenmann and Franke, 1985). Therefore, at least a subpopulation of the phosphoadducin-positive structures may correspond to dendritic spines, which are specialized postsynaptic structures.

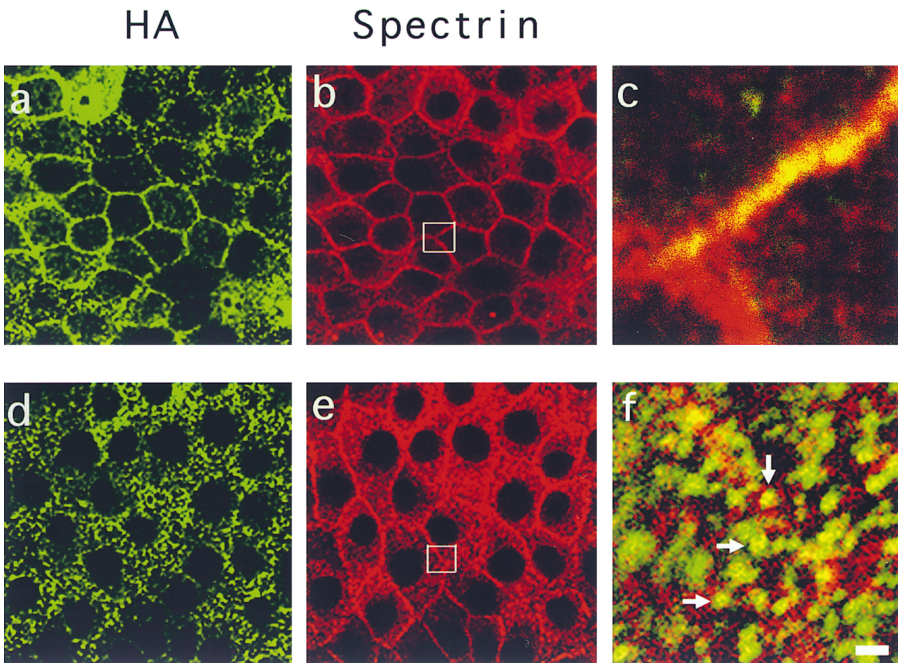

Figure 5. Effects of the mutation at PKC phosphorylation sites of α adducin on distributions of α adducin and spectrin in MDCK cells. Distributions of HA epitope–tagged α adducin and spectrin were detected by anti-HA epitope (1:1,000) and antispectrin (1.5 μg/ml) antibodies, respectively. (*a–c*) MDCK/α add cells. (*d–f*) MDCK/α add$^{\Delta pkc}$ cells. (*c* and *f*) Higher-magnification composite images of the boxed areas. Note that α adducin$^{\Delta pkc}$ showed a diffuse punctate staining pattern in the cytoplasm of MDCK/α add$^{\Delta pkc}$ cells. Arrows indicate structures where α adducin$^{\Delta pkc}$ and spectrin colocalize. All images were taken at the nucleus level. Bar: (*a*, *b*, *d*, and *e*) 10 μm; (*c* and *f*) 1 μm.

Localization of the Phosphoadducin in Dendritic Spines of Cultured Hippocampal Neurons

Resolution of dendritic spines is difficult in brain tissue sections, but these structures can be visualized in cultured hippocampal neurons. Distributions of the RTPS-phosphoserine adducin and total adducin were compared in cultured hippocampal neurons using the phosphoadducin-specific and the MARCKS-related domain–specific antibodies, respectively. Phosphoadducin was highly concentrated in spots flanking dendrites, which we interpret to be dendritic spines based on colocalization with AMPA receptors (a non-NMDA glutamate receptor) detected using antibody against the GluR2/4 subunits (Fig. 7, *a–c*, *arrowheads*). At least 80% of the spots contained both proteins, although in some cases the phosphoadducin-positive spots did not contain GluR2/4 and vice versa (Fig. 7, compare *a* and *b*). This result strongly supports the interpretation that the small structures containing high levels of the phosphoadducin observed in brain sections (Fig. 6 *c*) are dendritic spines.

Neither glutamate nor PMA treatment caused further increase in the level of adducin phosphorylation in spines (the ratio of the signals, phosphoadducin/glutamate receptor, was used to assess changes of adducin phosphorylation, $n = 20$ each) (Fig. 7, *d–i*). The cultures were preincubated with glutamate-free medium in the presence of high Mg^{2+} (15 mM) and an AMPA receptor antagonist (CNQX) to block NMDA and non-NMDA receptors, respectively (see Materials and Methods). Adducin therefore already was fully phosphorylated in the dendritic spines of hippocampal neurons under resting conditions in culture. However, PMA treatment did strongly enhance levels of the phosphoadducin located at the plasma membrane of cell body, the cytoplasm and dendrites (Fig. 7 *h*). The membrane-associated phosphoadducin was unanticipated. This result suggests involvement of additional domains, such as the head domain, for adducin to associate with the cell membrane.

Total adducin staining using antibody against the MARCKS-related domain, in contrast to the phosphoadducin, was observed along the entire cell membrane and in the cytoplasm of cell body and dendrites as well as in the dendritic spines (Fig. 8, *a–c*). No significant changes of adducin distribution were observed after glutamate and PMA treatments of the neurons (Fig. 8, *d–f* and *g–i*). However, the MARCKS-related domain–specific antibody has weak reactivity with the RTPS-phosphoserine adducin and underrepresents phosphoadducin (data not shown). These results combined with Fig. 7 demonstrate that adducin is uniformly distributed along the plasma membrane and that dendritic spines are the most active sites for adducin phosphorylation in hippocampal neurons.

Spectrin β_G was located mostly in a punctate pattern associated with dendrites that were not labeled with the phosphoadducin antibody (Fig. 9). These findings suggest that spectrin and phosphoadducin exist in distinct sites in neurons.

Discussion

This study presents the first evidence that phosphorylation of the RTPS-serine in the MARCKS-related domain of adducin occurs in vivo and has an important role in regulating distribution of adducin in cells. Furthermore, phosphorylation by PKC of adducin at the MARCKS-related domain inhibits adducin activities of promoting spectrin–actin interactions and capping the fast-growing ends of actin filaments. PMA-activated phosphorylation of the RTPS-serine was demonstrated, using a phosphoadducin-specific antibody, in erythrocytes, HEK 293 cells (human embryonic kidney origin), brain slices, and MDCK cells. A PKC-unphosphorylatable S716A/S726A mutant α adducin expressed in MDCK cells was distributed in a cytoplasmic punctate pattern and was no longer concentrated at the cell membrane

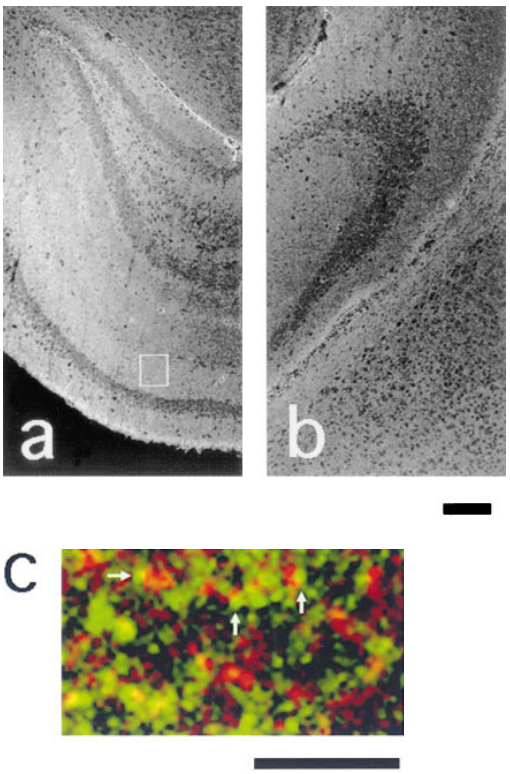

Figure 6. Distribution of the RTPS-phosphoserine adducin in rat hippocampus. (*a* and *b*) Low-magnification confocal images of P15 rat hippocampus stained with the phosphoadducin-specific antibody. The boxed area was examined with higher magnification. (*c*) High-magnification image of CA1 region. A composite confocal image for the phosphoadducin (*green*) and synaptophysin (*red*) was shown. Arrows indicate putative dendritic spines stained with the phosphoadducin antibody. Bars: (*a* and *b*) 250 μm; (*c*) 2.5 μm.

at sites of cell–cell contact. Moreover, cells expressing the mutant α adducin exhibited increased levels of cytoplasmic spectrin, which was colocalized with the mutant adducin in a punctate pattern. Finally, high levels of the RTPS-serine phosphorylation were visualized in sections of rat hippocampus and in the dendritic spines of cultured hippocampal neurons.

Dendritic spines are dynamic structures that receive the majority of exitatory synaptic connections in the mammalian central nervous system (Harris et al., 1992; Papa et al., 1995; Ziv and Smith, 1996). Dendritic spines change in shape during neuronal development as well as concurrently with long-term potentiation, a widely studied experimental model of learning (Fifkova and Van Harreveld, 1977; Schuster et al., 1990; Geinisman et al., 1991; Hosokawa et al., 1995). Actin and actin-binding proteins, including spectrin, adducin, and myosin, are the major cytoskeletal components in dendritic spines, where microtubules and neurofilaments are virtually absent (Westrum et al., 1980; Landis and Reese, 1983; Morales and Fifkova, 1989; Seidel et al., 1995). The actin filaments of the spine neck are longitudinally situated, whereas those in the head are organized into a lattice (Fifkova and Delay, 1982). This organization suggests that actin filaments provide the basic structural scaffolding of the spine (Harris and Kater, 1994). Thus, changes in the spine actin network through modulation of the activities of actin-regulating proteins have been proposed as a basis for activity-dependent structural changes in spine morphology (Coss and Perkel, 1985; Fifkova and Morales, 1991).

Synaptic stimulation of dendritic spines induces an increase in Ca^{2+} up to micromolar levels (Holmes, 1990; Gold and Bear, 1994). Probable targets for Ca^{2+} in dendritic spines include calmodulin (Malenka et al., 1989; Ehlers et al., 1996; Wyszynski et al., 1997), PKC (Malinow et al., 1989; Abeliovich et al., 1993; Hrabetova and Sacktor, 1996), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) (Silva et al., 1992; Kennedy, 1993; Lledo et al., 1995), and Ca^{2+}/calmodulin-dependent protein phosphatase 2B or calcineurin (Mulkey et al., 1994). Among substrates for these enzymes are several cytoskeletal proteins, including microtubule-associated protein 2, (Quinlan and Halpain, 1996), myosin (Kawamoto et al., 1989), and adducin. However, adducin is the first cytoskeletal protein directly demonstrated to be phosphorylated in dendritic spines of living hippocampal neurons. Given the biochemical activities of adducin in promoting assembly of spectrin–actin complexes and that these activities of adducin are regulated by PKC and calmodulin, adducin is a logical candidate to participate in activity-dependent shape changes of dendritic spines. In addition, the striking localization of phosphoadducin in the spines further supports the idea that dendritic spines are distinct biochemical and Ca^{2+} compartments (Guthrie et al., 1991; Müller and Connor, 1991; Koch and Zador, 1993; Yuste and Denk, 1995).

The identity of adducin kinase(s) and phosphatases responsible for regulating the phosphorylation state of adducin in dendritic spines remains to be determined. Since CaMKII does not phosphorylate α or β adducin in vitro (Matsuoka, Y., unpublished results) and PKA does not in vivo (Fig. 1 *A*), an isoform of PKC is the most likely candidate. It is noteworthy that at least one isoform of PKC, PKCγ, is localized in dendritic spines by immunoelectron microscopy (Tsujino et al., 1990; Saito et al., 1994). Phosphoadducin is likely to be a substrate for protein phosphatase(s) in dendritic spines, which could represent an important aspect of activity-dependent regulation of adducin functions. Calcineurin, a Ca^{2+}/calmodulin-dependent protein phosphatase, is present at high levels in hippocampal neurons (Steiner et al., 1992) and is a good candidate for an adducin phosphatase.

Kaibuchi and colleagues have found that adducin is an in vivo substrate for Rho-associated kinase (Rho-kinase) and myosin phosphatase, which are involved in the regulation of actin cytoskeleton in the cells (Amano et al., 1997; Kimura et al., 1998). Phosphorylation by Rho-kinase occurs at a different site from PKC and increases the affinity of α adducin for F-actin (Kimura et al., 1998). It will be of interest to determine localization of Rho-kinase–phosphorylated adducin in neurons and possibly dendritic spines.

The basic MARCKS-related domain of adducin has recently been demonstrated to be required for association of adducin with spectrin and actin (Li et al., 1998). Moreover, activity of adducin in promoting association of spectrin with actin is inhibited by high ionic strength, consistent with an electrostatic origin of binding energy between adducin and actin (data not shown). These findings considered together with the evidence in this study that phosphorylation of a site in the MARCKS-related domain inhibits

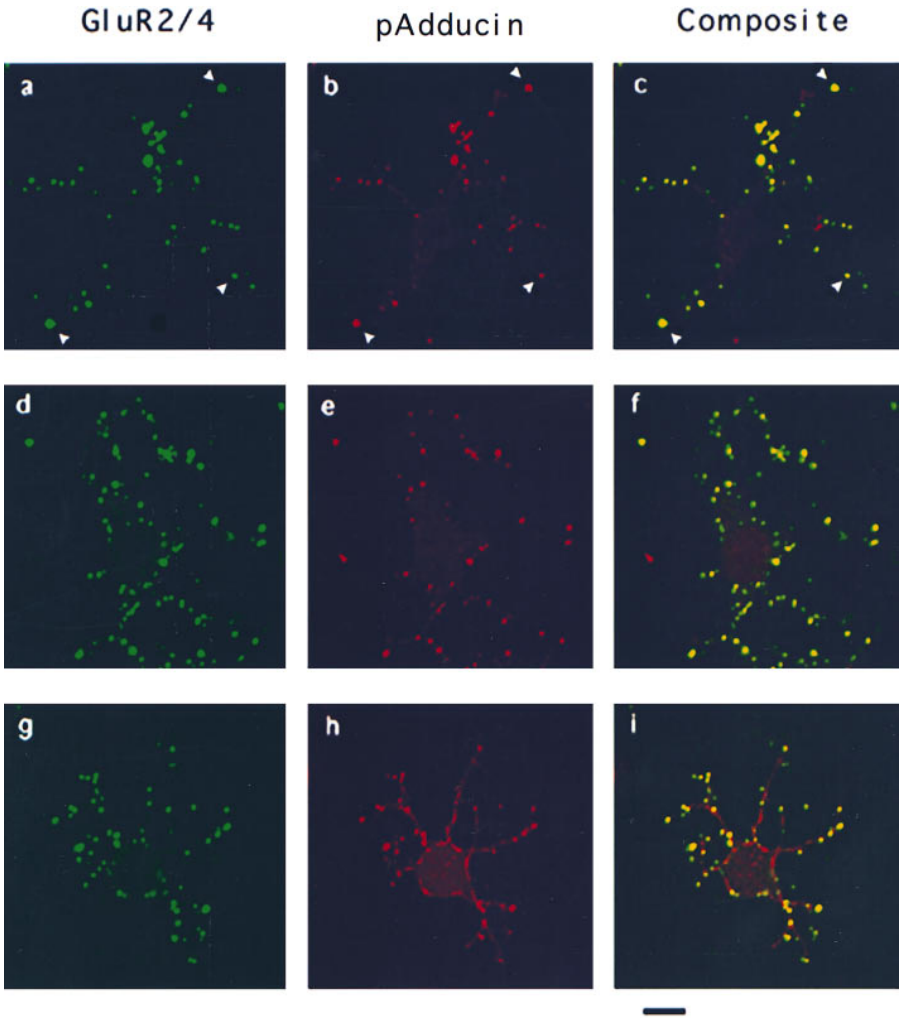

Figure 7. Colocalization of phosphoadducin with GluR2/4 and specific phosphorylation of adducin in dendritic spines of cultured hippocampal neurons. Colocalization of GluR2/4 (*green*; *a*, *d*, and *g*) and phosphoadducin (*red*; *b*, *e*, and *h*). (*a–c*) Neurons before stimulation; (*d–f*) after glutamate (10 μM) stimulation; (*g–i*) after PMA (1 μM) stimulation. Composite images show colocalization of signals (*yellow*; *c*, *f*, and *i*). Arrowheads represent dendritic spines containing both phosphoadducin and AMPA-receptor (*a–c*). Neither glutamate nor PMA treatment caused further increase in the level of adducin phosphorylation in spines (ratio of the signals, phosphoadducin/glutamate receptor, was used to assess changes of adducin phosphorylation, $n = 20$ each) (*d–i*). Primary antibodies were used at the indicated concentrations: antiphosphoadducin (3.5 μg/ml) and monoclonal anti–GluR2/4 3A11 (5 μg/ml) antibodies. Fluorescent images of hippocampal neurons were visualized by a confocal laser microscope (model LSM 410; Carl Zeiss, Inc.). Bar, 10 μm.

actin interactions suggest that this domain provides a direct contact with actin. Incorporation of phosphate with its bulk and negative charge into the basic MARCKS-related domain would be anticipated to interfere with electrostatic interactions dependent on positively charged residues. These considerations suggest that the actin contact sites for adducin will include negatively charged residues. Negatively charged residues (aspartate or glutamate) are exposed on the lateral surface of rabbit skeletal actin in positions 1–4, 24, 25, 99, 100, 360, 361, 363, and 364, while residues 167, 288, and 292 are located at the fast-growing ends of actin filaments (Holmes et al., 1990; Kabsch et al., 1990). The negatively charged residues exposed on the lateral surface of actin have been implicated in association with a positively charged lysine-rich loop in the head domain of myosin (Rayment et al., 1993; Schröder et al., 1993) and make up a possible actin–myosin interface (Holmes et al., 1990; Kabsch et al., 1990; Johara et al., 1993). It will be of interest to determine if adducin can inhibit actin-activated Mg^{2+}-ATPase activity of myosin and if such effects are controlled by phosphorylation of adducin.

Nonphosphorylatable mutant adducin (α adducin$^{\Delta pkc}$) and spectrin were colocalized as a punctate pattern in the cytoplasm of MDCK/α add$^{\Delta pkc}$ cells (Fig. 5 *f*). The identity of structures containing the mutant adducin and spectrin is not known but could represent vesicles in transit between the Golgi and plasma membrane. It is interesting in this regard that adducin and spectrin associate with dynactin, which is a multiprotein complex including an actin-related protein Arp1 or centractin and promotes dynein-mediated vesicle motility along microtubules (Schafer et al., 1994; Holleran et al., 1996). It will be of interest to determine if the structures with the mutant adducin contain other members of the dynactin complex.

We propose adducin as a candidate for a Ca^{2+}/calmodulin- and phosphorylation-sensitive modulator of the organization of spectrin–actin complexes in a variety of dynamic cellular domains. PKC has been demonstrated to be involved in reorganization of the actin cytoskeletons coupling with membrane ruffling in neutrophils (Downey et al., 1992), spreading of platelets (Haimovich et al., 1996), and integrin clustering in lymphocytes (Pardi et al., 1992). Adducin, based on results of this study, is a candidate downstream effector of PKC in these cells and possibly in dendritic spines. Since homologues of mammalian adducin have been identified in *Caenorhabditis elegans* (Moorthy, S., L. Chen, and V. Bennett. 1997. *Mol. Biol. Cell.* 8:274a), adducin may have a fundamental role conserved during evolution in regulation of assembly of spectrin–actin complexes in neurons as well as other types of cells.

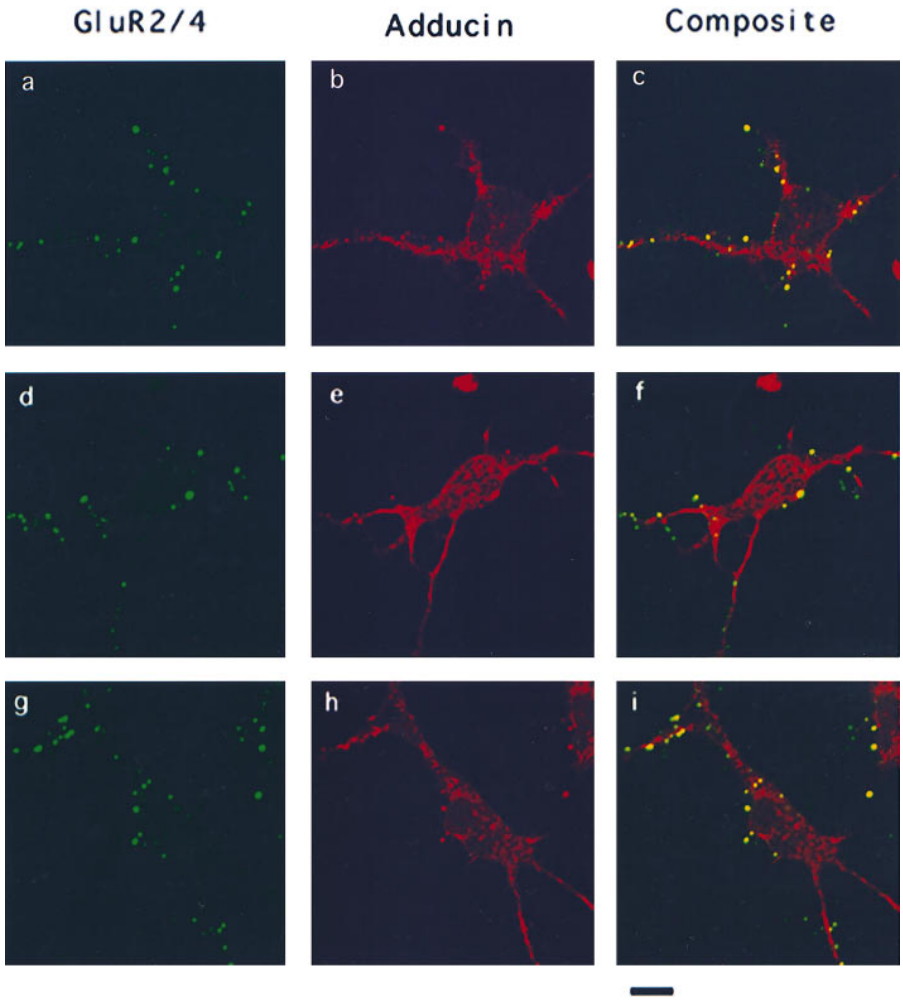

Figure 8. Colocalization of adducin with GluR2/4 in cultured hippocampal neurons. Colocalization of GluR2/4 (*green*; *a*, *d*, and *g*) and whole adducin (*red*; *b*, *e*, and *h*). (*a–c*) Neurons before stimulation; (*d–f*) after glutamate (10 μM) stimulation; (*g–i*) after PMA (1 μM) stimulation. Composite images show colocalization of signals (*yellow*; *c*, *f*, and *i*). Note that the cell membrane staining of cell body becomes weaker after PMA treatment because the anti–MARCKS-related domain antibody has much less reactivity toward the phosphoadducin than unphosphoadducin. Primary antibodies were used as at the indicated concentrations: anti–MARCKS-related domain (1.6 μg/ml) and monoclonal anti–GluR2/4 3A11 (5 μg/ml) antibodies. Fluorescent images of hippocampal neurons were visualized by a confocal laser microscope (model LSM 410; Carl Zeiss, Inc.). Bar, 10 μm.

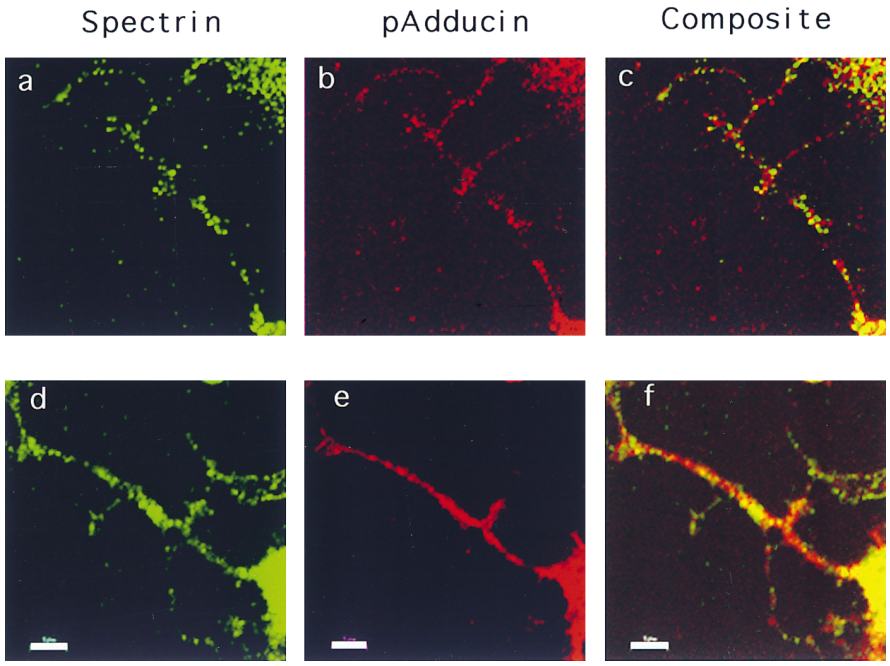

Figure 9. Localization of spectrin β_G and the phosphoadducin in cultured hippocampal neurons. Localization of spectrin β_G (*green*; *a* and *d*) and the RTPS-phosphoserine adducin (*red*; *b* and *e*) in dendrites. Composite images show colocalization of signals (*yellow*; *c* and *f*). The neurons were treated without (*a–c*) or with (*d–f*) PMA. Primary antibodies were used at the indicated concentrations: anti–spectrin β_G (5 μg/ml) and antiphosphoadducin (3.5 μg/ml) antibodies. Fluorescent images of hippocampal neurons were visualized by a confocal laser microscope (model LSM 410; Carl Zeiss, Inc.). Bar, 5 μm.

We are grateful to Dr. P.J. Blackshear for providing recombinant human MARCKS protein and Dr. T. Garver for technical assistance for rat hippocampal slice experiments. We also thank Suraj Moorthy for preparing and providing an HA-tag α adducin construct and α and β adducin–specific antibodies, and Mildred McAdams and Judith Phelps for peptide synthesis and analysis.

Received for publication 6 November 1997 and in revised form 10 June 1998.

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