Adducin Regulation
DEFINITION OF THE CALMODULIN-BINDING DOMAIN AND SITES OF PHOSPHORYLATION BY PROTEIN KINASES A AND C* (Received for publication, May 25, 1996, and in revised form, July 15, 1996)

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Adducin promotes association of spectrin with actin and caps the fast growing end of actin filaments. Adducin contains N-terminal core, neck, and C-terminal tail domains, is a substrate for protein kinases A (PKA) and C (PKC), and binds to Ca2+/calmodulin. Ser-728 and Ser-713 in the C-terminal MARCKS-related domains of α- and β-adducin, respectively, were identified as the major phosphorylation sites common for PKA and PKC. PKA, in addition, phosphorylated α-adducin at Ser-408, -436, and -481 in the neck domain. Phosphorylation by PKA, but not PKC, reduced the affinity of adducin for spectrin-F-actin complexes as well as the activity of adducin in promoting binding of spectrin to F-actin. The myristoylated alanine-rich protein kinase C substrate-related domain of β-adducin was identified as the dominant Ca2+-dependent calmodulin-binding site. Calmodulin-binding was inhibited by phosphorylation of β-adducin and of a MARCKS-related domain peptide by PKA and PKC. Calmodulin in turn inhibited the rate, but not the extent, of phosphorylation of β-adducin, but not α-adducin, by PKA and that of each subunit by PKC. These findings suggest a complex reciprocal relationship between regulation of adducin function by calmodulin binding and phosphorylation by PKA and PKC.

Adducin is a membrane-skeletal protein localized at spectrin-actin junctions (1) that was first purified from human erythrocytes based on calmodulin binding activity (2). Adducin also is a substrate for protein kinase C (PKC),1 and protein kinase A (PKA) (3−5). Adducin associates preferentially with spectrin-actin complexes compared with spectrin alone and promotes association of spectrin with actin (6, 7). Adducin also caps the fast growing end of actin filaments (8). Adducin capping activity may target spectrin-actin complexes to the ends of actin filaments and help stabilize the short actin filaments that comprise the spectrin-actin network of erythrocytes. Adducin is expressed at an early stage in erythropoiesis (normoblast stage) (9) prior to expression of protein 4.1, a protein that also binds to spectrin-actin complexes. These features have led to the hypothesis that adducin participates in the assembly of the spectrin-actin network of erythrocytes and possibly other cells.

Erythrocyte adducin is composed of α and β subunits closely related in amino acid sequence and domain organization (10, 11). α-adducin is expressed in most tissues, while β-adducin has a more restricted pattern of expression (11). 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 (12). Each adducin subunit has three distinct domains: a 39-kDa N-terminal globular protease-resistant head domain, connected by a 9-kDa “neck” domain to a carboxyl-terminal protease-sensitive tail domain (10−12). C termini of all three subunits contain a highly basic stretch of 22 amino acids with sequence similarity to the myristoylated alanine-rich protein kinase C substrate (MARCKS) (11, 13). Erythrocyte adducin in solution is a mixture of heterodimers and tetramers with α and β subunit head domains in contact to form a globular core, and interacting α- and β-adducin tails extended away from the core (14). Tail domains of both the α- and β-adducin subunits are responsible for binding to spectrin-actin complexes (14) and have been proposed to form lateral contacts involving several actin subunits and the β subunit of spectrin (15).

Ca2+/calmodulin preferentially associates with β-adducin (2) and inhibits the recruitment of additional spectrin molecules to adducin-spectrin-F-actin ternary complexes by adducin (6), as well as the ability of adducin to cap actin filaments (8). A potential calmodulin-binding site has been localized to residues 425−444 in the neck domain of β-adducin (16). However, the 18-mer peptide corresponding to the MARCKS-related domain of adducin, which includes residues 718−734 of the α tail and residues 705−721 of the β tail, also binds to calmodulin.2 This study defines the major phosphorylation sites for PKA and PKC in erythrocyte adducin and presents evidence that the dominant site for binding to calmodulin is located in the MARCKS-related domain of adducin. Although adducin is a substrate for PKA and PKC in vitro and in vivo, neither functional consequences nor the site(s) of phosphorylation have been elucidated. Functional consequences of phosphorylation reported here include modulation of adducin interactions with spectrin and actin by PKA and inhibition of calmodulin binding by PKA and PKC. These findings suggest a complex reciprocal relationship between regulation of adducin function by calmodulin binding and phosphorylation by PKA and PKC.

EXPERIMENTAL PROCEDURES

Purification of Proteins—Rabbit muscle actin, bovine brain spectrin, bovine brain calmodulin, and erythrocyte adducin were isolated essentially as described (14). Prior to phosphorylation, adducin and recombinant adducin constructs were dialyzed against 10 mM HEPES-NaOH, 2 mM Na-EGTA, 0.05% Tween 20, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, pH 7.3, overnight at 4 °C. The catalytic subunit of PKA was prepared from bovine heart (18), and PKC was prepared from rat brain (19).

Expression and Purification of Recombinant Adducin Constructs—cDNAs encoding the full-length human α- and β-adducin

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1 The abbreviations used are: PKC, protein kinase C; PKA, cAMP-dependent protein kinase; MARCKS, myristoylated alanine-rich protein kinase C substrate; DTT, dithiothreitol; HPLC, high pressure liquid chromatography; MOPS, 4-morpholinepropanesulfonic acid; CaM-DANS, 5-dimethylaminonaphthalene-1-sulfonyl-(dansyl)-calmodulin.

2 P. J. Blackshear, unpublished data.
genes were cloned into the pGEMEX expression vector, a pET plasmid with a T7 promoter (20). A unique NheI restriction site was generated by polymerase chain reaction. The construct was inserted into the NheI site immediately 3' to the AUG codon so that the expressed polypeptide has the additional amino acid sequence Met-Ala-Ser on the amino terminus (14). The α- and β-adducin polypeptides were isolated from inclusion bodies and solubilized in a buffer containing 8 M urea, 10 mM sodium phosphate, 1 mM NaBr, 1 mM NaEDTA, 10 mM glycine, 0.05% Tween 20, pH 7.4. Soluble protein (140,000 × g supernatant) was applied to a Superose 12 column equilibrated with 4 M urea, 10 mM sodium phosphate, 1 mM NaBr, 1 mM Na-EDTA, 10 mM glycine, 0.05% Tween 20, pH 7.4. Fractions were dialyzed against the 4 M urea column

Fig. 1. Adducin phosphorylation by PKA and PKC. Erythrocyte adducin (1.25 μM) was phosphorylated by incubation with 20 μg/ml of the catalytic subunit of PKA (A) or 10 μg/ml PKC (B) as described under “Experimental Procedures.” The sample was processed as described under “Experimental Procedures” and subjected to SDS-polyacrylamide gel electrophoresis. α- (○) and β- (□) adducin bands were separately excised and counted for 32P in a Beckman liquid scintillation counter. In the kinetic studies, 0.47–1.56 μM of adducin was incubated with PKA (2.8 μg/ml) (C) or PKC (14.4 μg/ml) (D) for 10 min at 25 °C. Data are averages of triplicate determinations.

Fig. 2. Identification of major PKC-phosphorylation sites in erythrocyte adducin. A, radioactivity profile of reverse phase HPLC for V8-digested phosphoadducin. Adducin phosphorylated by PKC was digested successively with V8 protease. An aliquot of the reaction mixture was fractionated as described under “Experimental Procedures.” B, phosphoamino acid analysis of adducin phosphorylated by PKC. The positions of the origin (×), phosphoserine (P-Ser), phosphothreonine (P-Thr), phosphotyrosine (P-Tyr), and inorganic phosphate (P) are indicated.
buffer with no salt, applied to a Mono S column, and eluted with a linear gradient of 0–0.5 M NaBr in 4 M urea buffer. Both \(\alpha\) - and \(\beta\) -adducin eluted at 0.35–0.4 M NaBr and were renatured over several days by dialysis against buffer containing 10% sucrose, 10 mM HEPES, 50 mM NaCl, 1 mM Na-EGTA, 0.05% Tween20, 1 mM DTT, pH 7.4. Recombinant human \(\alpha\) - (residues 430–737) and \(\beta\) - (residues 409–726) adducin neck/tail constructs were expressed as soluble proteins and purified as described (14).

Peptide Synthesis and Purification—A purified 31-amino acid synthetic peptide corresponding to residues 696–726 of human \(\beta\) -adducin (GPILSKSKKKKKFRTPSLKKSKKKEKVES) was greater than 95% pure as determined by C18 reverse phase column HPLC. The sequence of the peptide was confirmed by amino acid sequence analysis, and the concentration of the peptide was determined by amino acid composition analysis.

Phosphorylation of Adducin and Peptide—Erythrocyte adducin (1.25 \(\mu\)M), recombinant \(\alpha\) - and \(\beta\)-adducin (2.5 \(\mu\)M), and recombinant \(\alpha\)- and \(\beta\)-adducin neck/tails (5.9 \(\mu\)M) were phosphorylated by incubation with 20 \(\mu\)g/ml PKA, 0.1 mM \(\gamma\) -32P\(\text{ATP}\), 5 mM MgCl2, 25 mM HEPES-NaOH, pH 7.3, at 25°C. For PKC phosphorylation, each polypeptide was incubated with 10 \(\mu\)g/ml PKC, 0.1 mM \(\gamma\) -32P\(\text{ATP}\), 5 mM MgCl2, 50 \(\mu\)g/ml phosphatidylserine, 0.1 \(\mu\)g/ml 12-O-tetradecanoylphorbol-13-acetate, 25 mM HEPES-NaOH, pH 7.3, at 25°C. The reaction was terminated by adding an equal volume of a stock solution containing 2% SDS, 80 mM DTT, 10% sucrose, 2 mM EDTA, 20 mM Triton X-100, pH 8. The sample was then subjected to SDS-polyacrylamide gel (3.5–17%) electrophoresis (21). The gels were stained with Coomassie Blue, and the band of each polypeptide was excised and counted for 32P in a Beckman liquid scintillation counter. In some experiments, the phosphorylation reaction was carried out under different conditions as indicated.

The 31-mer \(\beta\)-adducin peptide (0.1 mM) was phosphorylated by PKA and PKC under the same conditions as above except using 0.5 mM

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**FIG. 3.** Reverse phase HPLC and phosphoamino acid analysis for PKA-phosphorylated recombinant \(\alpha\)-adducin. A, radioactivity profile of reverse phase HPLC for V8 fragments of PKA-phosphorylated \(\alpha\)-adducin. HPLC was carried out under the same conditions as that for PKC-phosphorylated adducin. No significant amount of radioactivity was detected during the first 20 min. B, phosphoamino acid analysis for the major V8 fragments of \(\alpha\)-adducin separated by HPLC. The positions of phosphoserine (P-Ser), phosphothreonine (P-Thr), phosphotyrosine (P-Tyr), and inorganic phosphate (Pi) are indicated.

**FIG. 4.** Reverse phase HPLC and phosphoamino acid analysis for PKA-phosphorylated recombinant \(\beta\)-adducin. A, reverse phase HPLC for V8 fragments of PKA-phosphorylated \(\beta\)-adducin was carried out under the same condition as that for \(\alpha\)-adducin. No significant amount of radioactivity was detected during the first 20 min. B, phosphoamino acid analysis for the major V8 fragments of \(\beta\)-adducin separated by HPLC. The positions of phosphoserine (P-Ser), phosphothreonine (P-Thr), phosphotyrosine (P-Tyr), and inorganic phosphate (Pi) are indicated.
Adducin Sites of Phosphorylation and Calmodulin Binding

**TABLE I**

| Peptide a | Amino acid sequence b | Relative amount % of phosphate in peptide |
|----------|-----------------------|------------------------------------------|
| MARCKS-related domain | Arg-Thr-Pro-Ser-Phe-Leu (residues 723–728) | 26 |
| α 1       | Arg-His-Ser-Phe-Gln-Lys-Gln-Gln-Arg-Glu (residues 434–443) | 13 |
| α 2        | Ser-Asp-Val-Glu-Val-Pro-Ala-Ser-Val-Thr-Gly-Tyr (residues 408–419) | 15 |
| α i        | Asp-Gly-His-Arg-Thr-Thr-Ser-Thr-Ala-Val-Pro-Asn (residues 476–487) | 23 |
| Head domain | Gln-Lys-Lys-Arg-Val-Ser-Met-Ile-Leu-Gln-Ser-Pro-Arg-Phe-Arg-Glu-Glu (residues 54–70) | 11 |

a Peptides α 1, 2, and 4 were derived from chymotryptic digestion of peptides α 1, 2, and 4, respectively.

b Residue numbers correspond to human a-adducin (11).

determined from radioactivity in the HPLC analysis as shown in Fig. 3. Total is not 100% because the amount of minor peaks is excluded.

d No amino acid was detected at the 15th cycle because of the presence of a cysteine residue.

**TABLE II**

| Peptide a | Amino acid sequence b | Relative amount % of phosphate in peptide |
|----------|-----------------------|------------------------------------------|
| MARCKS-related domain | Arg-Thr-Pro-Ser-Phe-Leu (residues 710–715) | 30 |
| β 1       | Gln-Lys-Lys-Arg-Val-Thr-Met-Ile-Leu-Gln-Ser-Pro-Arg-Phe-Arg-Glu-Glu (residues 50–66) | 17 |

a Peptide β 1 was derived from chymotryptic digestion of peptide β 1.

b Residue numbers correspond to human β adducin (11).

c Determined from radioactivity in the HPLC analysis as shown in Fig. 4. Total is not 100% because the amount of minor peaks is excluded.

[32P]-ATP for 6 h at 15 °C. Stoichiometry of the phosphorylation was assessed following 10% SDS-polyacrylamide gel electrophoresis (22).

The sample was diluted with 1 volume of 0.2% trifluoroacetic acid, and chromatographed with the same linear gradient.

The sample was diluted with 1 volume of 0.2% trifluoroacetic acid, and chromatographed with the same linear gradient.

Modification of Phosphoserine Residue—The purified radioactive fragments (1–3 nmol) were treated with 100 mM NH4HCO3, chromatography as described above, and were digested with V8 protease (1:50 (w/w), Pierce) for 3 h at 30 °C. Samples were applied to a C18 reverse phase column (2.1 x 220 mm, ABI), which was eluted with a linear gradient of 0–50% acetonitrile in 100 min followed by a further linear gradient of 50–100% acetonitrile over 20 min at a flow rate of 0.2 mL/min. After lyophilization, the 32P-labeled fragments were dissolved in 0.5 mL of 100 mM NH4HCO3, pH 8.4, and digested with α-chymotrypsin (1 μg, Sigma) for 3 h at 30 °C. The sample was diluted with 1 volume of 0.2% trifluoroacetic acid, applied to the same column, and chromatographed with the same linear gradient.

Determination of the Major Phosphorylation Sites of Adducin—Adducin phosphorylation sites were determined by automated Edman degradation and confirmed with the amino acid sequences derived from the cDNAs of α- and β-adducin.

Phosphorylation of Erythrocyte Adducin by PKA and PKC—Stoichiometries and Ko values of adducin phosphorylation were measured in Fig. 1. Time courses of phosphorylation of α- and β-adducin subunits by PKA and PKC are shown in Fig. 1, A and B. PKA phosphorylated the α and β subunits of adducin at a 2:1 ratio, and maximal stoichiometries of 2 mol of P/mol of α subunit, 1 mol of P/mol of β subunit. Thus, 3 mol of P/mol of adducin dimer was observed (Fig. 1A). Protein kinase C incorporated 0.8 mol of phosphate into 1 mol of each subunit at the end of 2 h (Fig. 1B). Using a higher concentration of the kinase, approximately 1 mol of phosphate was incorporated per mol of each subunit.

The Kao values of PKA and PKC for adducin were determined based on initial rates of phosphorylation as a function of adducin concentration (Fig. 1, C and D). Fig. 1, C and D, shows double-reciprocal plot analysis for adducin phosphorylation by PKA and PKC as a function of adducin concentration; these curves are representative of three separate experiments. The Kao and Vmax values of PKA for adducin were 7.0 μM and 333 nmol/min/mg, respectively, compared with 0.8 μM and 3.8 nmol/min/mg for PKC at 25 °C. For comparison, PKA utilizes histone H2B as a substrate with a Kao of 32 μM (26), troponin with a Kao of 21 μM (27), and kemptide (LRRASLG) with a Kao of 8 μM (28). A PKC mixture isolated from rat brain utilizes MARCKS with a Kao of 0.4–0.8 μM (29). Adducin, therefore, is a good substrate for PKA and an excellent substrate for PKC.

Determination of the Major Phosphorylation Sites of Adducin for PKA and PKC—Ser-726 and Ser-713 in the C-terminal MARCKS-related domains of α- and β-adducin, respectively, were identified as the major phosphorylation sites for PKC. PKC-phosphorylated native adducin (0.8 mol of P/mol of adducin subunit) was first digested with V8 protease. One major radioactive peak was obtained in the HPLC procedure (Fig. 2A). This major V8 fragment was further digested by α-chymotrypsin, and chymotryptic fragments were again separated by the same HPLC procedure. One major radioactive peak was separated, and the sequence obtained from this fraction was RPSFL, corresponding to residues 723–728 and residues 710–715 of the C-terminal tail domains of human α- and β-adducin, respectively. The relative amount of 32P in the second major peak was estimated as 57% of the total radioactivity. Phosphoamino acid analysis of adducin phosphorylated by PKC showed only the presence of phosphoserine as the phos-
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Ser-703 (β-adducin) and, by inference, Ser-716 (α-adducin) were identified as secondary sites of phosphorylation by analysis of PKC-phosphorylated recombinant β-adducin. A V8 protease/chymotrypsin digest of the phospho-β-adducin yielded two radioactive peaks. Both peptides contained phosphoserine as the only phosphorylated amino acid (data not shown). The major peak contained the sequence R703RTPSFL, corresponding to the phosphopeptide identified above in native adducin. The second, less abundant peptide had the sequence GSPSKSPSKK, with four serine residues. Ser-703 of β-adducin was identified as the phosphorylation site, since S-ethylcysteine converted from phosphoserine by ethanethiol treatment was only detected at position 8 in this peptide (data not shown). Since Ser-703 of β-adducin is located in the region where the amino acid sequence is identical to that of α-adducin, Ser-716 of α-adducin is also a probable phosphorylation site for PKC.

Ser-726 and Ser-713 in the C-terminal MARCKS-related domains of α and β-adducin, respectively, were identified as the major phosphorylation sites common for protein kinases A and C. Protein kinase A, in addition, phosphorylated α-adducin in the neck domain at Ser-408, -436, and -481. Minor sites for PKA include Ser-59 and Thr-55 in the α and β N-terminal head domains, respectively. Recombinant α- and β-adducin constructs were employed to identify the phosphorylation sites for PKA in each subunit due to difficulty in resolving phosphopeptides derived from the combined α and β subunits of erythrocyte adducin. α-adducin (2.5 mol of Pi/mol of subunit) and β-adducin (1.2 mol of Pi/mol of subunit) phosphorylated by PKA were first digested by V8 protease. Three and two major radioactive peaks were obtained from the α- and β-adducin, respectively, in the HPLC procedure (Figs. 3A and 4A). Each of the isolated fragments was further digested by a-chymotrypsin, and peptides were separated by the same HPLC procedure. The amino acid sequences from major radioactive peaks were as given in Tables I and II. Phosphoamino acid analysis of the V8 fragments from PKA-phosphorylated adducin showed the presence of only phosphoserines except fragment β2 in which phosphothreonine was the phosphoamino acid (Figs. 3B and 4B). Since phosphopeptides a1, a2-1, and β1 and phosphopeptide β2 contained single serine residues and a single threonine residue, respectively, Ser-726 and Ser-436 of α-adducin and Ser-713 and Thr-55 of β-adducin were identified as the PKA phosphorylation sites (Tables I and II).

Since phosphopeptides a2-2, a3, and a4 had more than two serine residues, the exact phosphorylation sites were defined by treating the phosphopeptides with ethanethiol in an alkaline condition to convert specifically phosphoserine to S-ethylcysteine (30). The high release of S-ethylcysteine was observed at the 6th cycles for both peptides a3 and a4+, indicating that the phosphates were located on Ser-59 and Ser-481 of α-adducin (data not shown). S-ethylcysteine was not detected for peptide a2-2 treated with ethanethiol. It has been reported that if a phosphoserine residue possesses a free amino or carboxyl terminus, no transformation into S-ethylcysteine will occur (23). Since this peptide had a serine residue (Ser-408) at the amino-terminal position, Ser-408 was predicted to be the phosphoserine residue. This was finally confirmed by the release of S-ethylcysteine at the 6th cycle for V8 fragment a2-2 treated with ethanethiol (data not shown).

All of the identified phosphorylation sites of adducin for PKA and PKC conform to the known recognition sequences of both kinases (Arg/Lys-Arg/Lys-X-Ser/Thr or Arg/Lys-X-Ser/Thr for PKA and Arg/Lys-X-X-Ser/Thr/X-Arg/Lys for PKC) (31, 32).
The lysine residue at the carboxyl-terminal side to the major PKC-phosphorylated serine in adducin (RTPSFLK) is displaced by one residue from the consensus sequence for PKC. However, several other examples that have a basic residue at the same position analogous to the adducin site have been reported (33–35). An adjacent hydrophobic amino acid (especially Leu and Phe) on the carboxyl-terminal side of the phosphorylation site is very important for a high affinity interaction of PKC with the substrate (33, 36).

Adducin-Spectrin-Actin Ternary Complex Formation Is Inhibited by PKA but Not PKC Phosphorylation—PKA-phosphorylation of adducin reduced activity of adducin in association with spectrin-actin complexes and in promoting binding of spectrin to F-actin. PKC phosphorylation, in contrast, had no measurable effect on adducin activities (Figs. 5 and 6). PKA-phosphorylated adducin (3 mol of PI/mol of dimer) displayed a 3–4-fold lower affinity for spectrin-actin complexes (\(K_D = 570\) nM) than that of the unphosphorylated adducin (\(K_D = 150\) nM). In contrast, the affinity (\(K_D = 200\) nM) of PKC-phosphorylated adducin (2 mol of PI/mol of dimer) was the same as that of the unphosphorylated adducin. Neither PKA nor PKC phosphorylation affected the capacity of adducin bound to spectrin-actin complexes (Fig. 5B). PKA-phosphorylated adducin also displayed a 3-fold lower activity in promoting binding of spectrin to F-actin (half-maximal stimulation at 240 nM) than the unphosphorylated adducin (half-maximal activation at 80 nM). The final amount of spectrin recruited to F-actin was the same in both cases (Fig. 6, A and B). In contrast, the activity of the unphosphorylated and the PKC-phosphorylated adducin were the same (both with half-maximal activation at 100 nM), and

Fig. 6. Promotion of spectrin binding to F-actin by nonphosphorylated and phosphorylated adducin. 125I-labeled bovine brain spectrin (3 nM, \(7.41 \times 10^5\) cpm/pmol) was incubated with 3 \(\mu\)M F-actin and increasing concentrations of nonphosphorylated adducin (○), PKA-phosphorylated adducin (3 mol of PI/mol; □), and PKC-phosphorylated adducin (2 mol PI/mol; △). The conditions for the cosedimentation assay with spectrin, actin, and adducin were described in the legend for Fig. 5. Saturation binding curves (A and C) and double reciprocal plots (B and D) are shown. Binding data are averages of triplicate determinations, and these are representatives of three separate experiments.
equivalent amounts of spectrin were recruited (Fig. 6, C and D). Phosphorylation of adducin by PKA and PKC did not affect binding of adducin to F-actin (data not shown).

Reciprocal Relationship between Calmodulin Binding and Phosphorylation of MARCKS-related Domains of Adducin by PKA and PKC—Recombinant \( \alpha \) (residues 430–737) and \( \beta \) (residues 409–726) adducin neck/tail domains and a 31-residue MARCKS-related domain peptide corresponding to residues 696–726 of human \( \beta \)-adducin were evaluated for the ability to bind to CaM-DANS (Fig. 7). The \( \alpha \) - and \( \beta \)-adducin constructs and the MARCKS-related domain peptide all exhibited calcium-dependent enhancement of fluorescence of CaM-DANS (67 nM) with maximal increases of 1.6-fold for \( \alpha \)-adducin neck/tail and 1.8-fold for \( \beta \)-adducin neck/tail (estimated from double-reciprocal plots) (Fig. 7, A and B). The MARCKS-related peptide was the most active, with half-maximal enhancement of fluorescence at 55 nM. The \( \beta \)-adducin construct expressed half-maximal effect at 85 nM compared with 120 nM for the \( \alpha \) construct. The \( \alpha \) - and \( \beta \)-adducin constructs were equivalently pure based on SDS-polyacrylamide gel electrophoresis. The concentration of the MARCKS-related domain peptide was based on amino acid composition analysis, and concentrations of the adducin constructs were determined by absorbance (\( A_{280} \)) using an extinction coefficient of 0.42 calculated based on the amino acid composition (37).

Calmodulin binding activities of the adducin recombinant polypeptides and the MARCKS-related domain peptide were inhibited by PKA and PKC phosphorylation. The \( \beta \) neck/tail (1 mol of P/mol), and the MARCKS-related domain peptide (1 mol of P/mol) phosphorylated by PKC exhibited greater than 80% reduction in binding to CaM-DANS compared with nonphosphorylated forms (Fig. 7, B and C). PKC-phosphorylated \( \alpha \)-adducin neck/tail (2 mol of P/mol), in contrast, was only 50% reduced in calmodulin binding activity (Fig. 7A). The recombinant \( \beta \)-adducin has been reported to contain a calmodulin-binding site at residues 425–461 (16). Since the \( \beta \) polypeptide and MARCKS-related domain peptide phosphorylated by PKC lost most of the calmodulin binding activity, the MARCKS-related domain is most likely the primary calmodulin-binding domain in \( \beta \)-adducin. The inhibitory effect of PKC phosphorylation on the \( \alpha \) polypeptide activity was only partial. The \( \alpha \) neck/tail domain, therefore, may have a second calmodulin-binding site with a lower affinity than that of the MARCKS-related domain. Similar inhibition of calmodulin binding was observed when the adducin polypeptides and MARCKS-related domain peptide phosphorylated by PKA were used in the fluorescence assay (data not shown).

Binding of calmodulin to native adducin, as monitored by photoaffinity labeling with radiolabeled azidocalmodulin, also was inhibited by phosphorylation with protein kinases A and C.
Fig. 8. The migration of calmodulin-labeled adducin polypeptides is diffuse in this experiment, and it is not possible to clearly distinguish between \( \alpha \) - and \( \beta \) -adducin polypeptides, which differ in mobility equivalent to only 6 kDa. The major effect of PKC phosphorylation on calmodulin binding indicates that the MARCKS-related domain is the dominant site of calmodulin binding in native adducin as well as the isolated recombinant polypeptides.

Activity of calmodulin in inhibiting phosphorylation of native adducin was evaluated in Fig. 9. In the case of PKA phosphorylation, calmodulin inhibited the rate of phosphorylation of \( \beta \)-adducin but not the \( \alpha \) subunit (Fig. 9A). However, the rate of PKC phosphorylation of both \( \alpha \)- and \( \beta \)-adducin was inhibited by calmodulin (Fig. 9B). For both PKA and PKC phosphorylation of adducin, the inhibitory effects of calmodulin were limited to a reduction in rate with no effect on the final extent of phosphorylation. No calmodulin effect was observed when adducin was phosphorylated by either PKA or PKC in the presence of 3 \( \mu \)M of calmodulin for longer than 60 min (data not shown).

DISCUSSION

This report identifies the MARCKS-related domain of adducin as a major focus for regulatory signals involving phosphorylation and calcium-dependent binding of calmodulin. The MARCKS-related domain comprises the principal target for PKC, provides the dominant binding site for calmodulin, and also is one of the targets for PKA (Fig. 10). Calmodulin binding of native adducin and recombinant adducin polypeptides is inhibited by phosphorylation with PKC and by PKA. Other activities of adducin in association with spectrin-actin complexes were not affected by phosphorylation with PKC, raising the possibility that the primary impact of phosphorylation by PKC is through modulation of effects of calmodulin. MARCKS-related domains are nearly identical between \( \alpha \) - and \( \beta \)-adducins of humans and rats (11, 38) as well as the recently defined \( \gamma \) subunit (12). The activities defined in this study in the context of such a high level of conservation indicate a fundamental role of the MARCKS-related domain in regulation and function of forms of adducin containing this domain. Alternatively spliced forms of both \( \alpha \) (39) and \( \beta \) (40) adducin have been reported that lack this MARCKS-related domain. Presumably, these forms of adducin are not subject to the levels of regulation shown in this study.

Several observations suggest that the calmodulin binding activity of the MARCKS-related domain is modulated by adjacent peptide sequence and is more active in \( \beta \)-adducin than in \( \alpha \)-adducin. \( \alpha \)-Adducin associated with calmodulin with a lower affinity and produced a lower final extent of fluorescence change than either \( \beta \)-adducin or the isolated peptide (Fig. 7). The difference in half-maximal binding between \( \alpha \)- and \( \beta \)-adducin was approximately 40%, which was reproducible in at least three experiments. Phosphorylation by PKC resulted in an almost complete loss of calmodulin binding for \( \beta \)-adducin.
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and the isolated peptide but only a 50% reduction of binding of α-adducin (Fig. 7). Finally, calmodulin reduced the rate of phosphorylation of β-adducin by PKA but had no effect on phosphorylation of α-adducin (Fig. 9). Preference of calmodulin for β-adducin is consistent with the previous observation of β-adducin being selectively photoaffinity-labeled by radiola-beled azidocalmodulin (2). These considerations suggest that in cells, under limiting conditions of low calcium, calmodulin prefer-entially associates with β-adducin, whereas at higher levels of calcium, calmodulin occupies binding sites of α- as well as β-adducin.

Protein kinase A phosphorylates α-adducin at three sites in the neck domain (Ser-408, -436, and -481) in addition to the MARCKS-related domain of both subunits (Fig. 10). Phospho-rylation by PKA, in contrast to PKC, reduced affinity of ery-throcyte adducin for spectrin-F-actin complexes as well as activity of adducin in promoting binding of spectrin to F-actin. The PKA-unique sites of α-adducin are not present in β- and γ-adducin (12) and represent a specialized feature of the α subunit. Patterns of expression of adducin subunits suggest heteromers composed of an α subunit in association with either a β or a γ subunit depending on the tissue and cell type (11, 12). Phospho-rylation of α-adducin by PKA and modulation of interaction with spectrin-F-actin complexes could therefore represent a general feature of adducin regulation.

Binding sites on adducin for spectrin-actin complexes have been mapped to both the α and β C-terminal domains, which include the MARCKS-related domain and sites of phosphorylation by PKA (14). The spectrin/actin binding site has recently been localized to the C-terminal 100 residues of α- and β-adducin. Inhibition of adducin binding by phosphorylation with PKA is thus not likely to result from direct interference with contact between adducin and spectrin-F-actin complexes. One possibility is that phosphorylation modulates association be-tween adducin subunits, which could result in differences in apparent affinity. The role of the MARCKS-related domain in mediating contact with spectrin-F-actin complexes remains to be determined.

Polybasic domains with mutually exclusive calmodulin binding and PKC phosphorylation sites were first described for the MARCKS family of proteins (13, 41). Adducin, based on the present study, and the cytoplasmic domain of the NR1 subunit of the N-methyl-D-aspartate receptor (42) share these features. The MARCKS-related domains of adducin, MARCKS, and the N-methyl-D-aspartate receptor, although related functionally and in overall physical properties, exhibit limited sequence identity. These polybasic domains, therefore, could have evolved by convergent evolution, rather than from a common ancestral protein. In support of the possibility of parallel evo-lution for MARCKS-related domains, convergence of PKC phosphorylation and Ca2+/calmodulin-binding sites has been noted in several unrelated proteins including myosin light chain kinase (43, 44), neurmodulin (GAP43) (45, 46), and membrane Ca2+-ATPase (47). Functional roles in addition to sites of regulation shared by MARCKS-related polybasic domains have yet to be clearly defined. It is of interest that MARCKS, adducin, and the cytoplasmic domain of the N-methyl-D-aspartate receptor are all localized on the cytoplasmic surface of the plasma membrane and interact with F-actin-based structures (6, 48, 49).

Adducin is expressed at high levels in brain (7, 11), and α-adducin has been localized at synapses as well as in giall cells (50).4 The role of adducin as a regulated assembly factor for spectrin/F-actin-based structures could have an important role in neural activities related to synaptic plasticity and regulation of neurosecretion. In support of a role for adducin in neurosecreteion, adducin has recently been reported to bind to tetanus and botulinum toxins in a ganglioside-dependent manner (51) and to interact with rabphilin-3A, a peripheral membrane protein of synaptic vesicles (17). It will be important to determine if adducin is a substrate for PKA and PKC in vivo under physiological stimulation related to neurosecretion.

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