Brain Adducin: A Protein Kinase C Substrate That May Mediate Site-directed Assembly at the Spectrin-Actin Junction*

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Erythrocyte adducin is a membrane skeletal protein that binds to calmodulin, is a major substrate for protein kinase C, and associates preferentially with spectrin-actin complexes. Erythrocyte adducin also promotes association of spectrin with actin, and this activity is inhibited by calmodulin. This study describes the isolation and characterization of a brain peripheral membrane protein closely related to erythrocyte adducin. Brain and erythrocyte adducin have at least 50% antigenic sites in common, each contains a protease-resistant core of $M_r = 48,000-48,5000$, and both proteins are comprised of two partially homologous polypeptides of $M_r = 103,000$ and $97,000$ (erythrocytes) and $M_r = 104,000$ and $107,000-110,000$ (brain). Brain and erythrocyte adducin associate preferentially with spectrin-actin complexes as compared to spectrin or actin alone, and both proteins also promote binding of spectrin to actin. Brain adducin binds calmodulin in a calcium-dependent manner, although the $K_d$ of 1.3 mM is weaker by 5-6-fold than the $K_d$ of erythrocyte adducin for calmodulin. Brain adducin is a substrate for protein kinase C in vitro and can accept up to 2 mol of phosphate/mol of protein. Adducin provides a potential mechanism in cells for mediating site-directed assembly of additional spectrin molecules and possibly other proteins at the spectrin-actin junction. Brain tissue contains 12 pmol of adducin/mg of membrane protein, which is the most of any tissue examined other than erythrocytes, which have 50 pmol/mg. The presence of high amounts of adducin in brain suggests some role for this protein in specialized activities of nerve cells.

Spectrin in association with actin forms a membrane skeleton that lines the inner surface of the human erythrocyte plasma membrane and most likely certain membrane regions of most eukaryotic cells (Bennett, 1985; Marchesi, 1985). Potential functions for the spectrin-based membrane skeleton and its associated proteins include physical support of the lipid bilayer in erythrocytes and participation in other cells in activities such as organization of integral membrane proteins into specialized domains on cell surfaces (Drenckhahn et al., 1985; Nelson and Veshnock, 1986), regulation of access of secretory vesicles to the inner surface of the plasma membrane (Perrin et al., 1987), and movement of membrane proteins (Levine and Willard, 1981, 1983; Nelson et al., 1983).

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1. Bourguignon et al., 1985). Spectrin-actin complexes in erythrocyte membranes have been visualized in the electron microscope as a regular two-dimensional network of spectrin molecules interconnected by short actin filaments with five to seven spectrin molecules clustered about each actin filament (Byers and Branton, 1985; Shen et al., 1986; Liu et al., 1987). These images support the concept of the membrane skeleton as a well-organized discrete structure and raise new questions about how spectrin and actin assemble in cells. Spectrin associates with actin and cross-links actin filaments in vitro, but the isolated proteins do not form a network. Presumably additional protein(s) are required to shorten actin filaments and to bring together multiple spectrin molecules at localized regions along actin filaments.

A new protein named adducin has been isolated from erythrocyte membranes that may play an important role in assembly of spectrin with actin and in regulation of this process. Adducin binds to calmodulin (Gardner and Bennett, 1986), is a major substrate for protein kinase C (Palfrey and Waseem, 1985; Cohen and Foley, 1986; Ling et al., 1986), and associates preferentially with spectrin-actin complexes compared to spectrin or actin alone (Gardner and Bennett, 1987). Adducin also promotes association of spectrin with actin, and this activity is inhibited by calmodulin. These features have led to the hypothesis that adducin is involved in an assembly pathway beginning with binding of spectrin to actin, followed by association of adducin with spectrin-actin complexes and finally by recruitment of additional spectrin molecules to the spectrin-actin-adducin ternary complex. The activity of adducin is likely to have relevance beyond the erythrocyte since polypeptides cross-reacting with adducin have been detected in brain membranes (Gardner and Bennett, 1986). This report describes purification of an immunoreactive form of adducin from brain and characterization of physical and functional properties that are closely related to those of erythrocyte adducin.

EXPERIMENTAL PROCEDURES

Materials

Carrier-free Na<sup>125</sup>I was from Amersham Corp., and <sup>125</sup>I-labeled Bolton-Hunter reagent and [γ-<sup>32</sup>P]ATP were from ICN. Diisopropyl fluorophosphate, leupeptin, pepstatin A, dithiothreitol, phenylmethylsulfonyl fluoride, EGTA, sodium bromide, Tween 20, and Triton X-100 were from Sigma, Mono Q, and Superose 6 fast pressure liquid chromatography columns, cyanoen bromide-activated Sepharose CL-4B, phenyl-Sepharose, and protein A were from Pharmacia LKB Biotechnology Inc. Hydroxylapatite (high resolution), biotin-X-N-hydroxysuccinimide

1. The abbreviations used are: EGTA, [ethylenebis(oxyethylene-nitrilo)]tetraacetic acid; MES, 4-morpholinolinesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate.
Brain Adducin

ester, streptavidin, and phorbol ester 12-O-tetradecanoylphorbol 13-acetate were from Calbiochem Brand Biochemicals. Ethylene glycol bis(succinimydyl succinate) and N-hydroxysuccinimyd 4-azidobenzoate were from Pierce Chemical Co. Sucrose, ammonium sulfate, and urea were from Schwarz/Mann, and &-chymotrypsin (45 units/mg) was from Worthington. Nitrilotriacetic acid, electrophoresis reagents, and Affi-Gel 721 beads were from Bio-Rad. Bovine brains were obtained from freshly slaughtered animals and washed in 0.52 M sucrose, 2 mM NaEGTA and the meninges were removed, frozen in liquid nitrogen, and stored at -80°C for up to 3 weeks. Calmodulin was purified from bovine brain as described (Gopalakrishna and Anderson, 1982). Actin was isolated from an acetone powder of rabbit skeletal muscle (Pardee and Spudich, 1982) and was further purified by gel filtration on Superose 12. Bovine brain spectrin was isolated as immunoadsorbent as described (Bennett and Davis, 1982) except that a Mono Q anion-exchange column was substituted for DEAE-cellulose (Bennett et al., 1986). Bovine brain protein kinase C was isolated from cytosol prepared in the absence of calcium by DEAE chromatography, followed by adsorption to and elution from immobilized anti-erbB1 polyacrylamide beads as described (Hall and Bennett, 1987). Negative staining and electron microscopy were performed as described (Gardner and Bennett, 1986). Physical properties were determined using known proteins as standards (Gardner and Bennett, 1986) with gel filtration on a Superose column for estimations of Stokes radius and migration on linear 5–20% sucrose gradients for the sedimentation coefficient (Martin and Ames, 1981). Sedimentation analysis was performed by David Viskup (Department of Pediatri, Johns Hopkins School of Medicine).

Preparation of Antibody against Brain Adducin—Brain adducin purified as described below was electrophoresed on a 3-mm thick SDS-polyacrylamide slab gel (0.5 mg/slab), which was stained lightly with Coomassie Blue. The bands were cut out, homogenized with volumes of normal saline and 1 volume of Freund’s adjuvant (complete for the first injection and incomplete for subsequent injections), and used to immunize rabbits subcutaneously with 100 µg/injection (four injections); and antisera were prepared as described (Bennett and Davis, 1982). Affinity-purified antibodies were isolated using erythrocyte adducin coupled to CNBr-activated Sepharose CL-4B as an immunoadsorbent as described (Bennett and Davis, 1982) except that the antibody was eluted with 4 M MgCl2 and stored in 30% sucrose, 150 mM NaCl, 10 mM sodium phosphate, 1 mM NaEDTA, 1 mM NaNt.

Purification of Brain Adducin—All procedures were performed at 2–4°C unless otherwise stated. Frozen bovine brain (390 g) was homogenized with a Polytron in 1.5 liters of 0.32 M sucrose, 2 mM NaEGTA, 1 mM NaNO3, pH 7.4, with the following protease inhibitors: 0.5 mM disopropyl fluorophosphate, 200 µg/ml phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, and 10 µg/ml pepstatin A. The homogenate was centrifuged for 5 min at 9000 × g, and the supernatant was centrifuged for 30 min at 30,000 × g in a JA-14 rotor. The 30,000 × g pellets were denatured by resuspension with 1 M sucrose, 2 mM NaEGTA, 1 mM NaNO3, 50 µg/ml phenylmethylsulfonyl fluoride, pH 7.4, followed by centrifugation for 45 min at 30,000 × g and aspiration of the supernatant. Denatured brains were then washed with 1 liter of 10 mM sodium phosphate, 1 mM NaEDTA, 0.5 mM dithiothreitol, 50 µg/ml phenylmethylsulfonyl fluoride, pH 7.4, followed by a second wash with 0.5 M NaCl dissolved in the same buffer. The washed membranes were then extracted for 30 min with 0.8 M NaBr, 0.15% Tween 20, 10 mM sodium pyrophosphate, 10 mM sodium phosphate, 1 mM NaEDTA, 1 mM NaNt, 0.5 mM dithiothreitol, 5 µg/ml leupeptin, 5 µg/ml pepstatin A, 100 µg/ml phenylmethylsulfonyl fluoride, pH 7.4, and centrifuged 2 h at 30,000 × g. The supernatant was dialyzed overnight against 20 liters of 9.2 M NaCl, 10 mM sodium phosphate, 1 mM NaEDTA, 0.5 mM dithiothreitol and centrifuged for 2 h at 30,000 × g. The purpose for the dialysis and repeated centrifugation is to remove a population of membranes that is buoyant in extracts of erythrocyte plasma membrane and interferes with the next step of ammonium sulfate precipitation. Solid ammonium sulfate was added to the supernatant at 50% saturation (291 g/liter), followed by addition of 1 liter of 40% saturated ammonium sulfate dissolved in 10 mM sodium phosphate, 1 mM NaEDTA. The purpose of addition of the 40% ammonium sulfate solution is to prevent flotation of the precipitated proteins which can occur with extracts that contain Tween 20.

The precipitated protein was collected by centrifugation for 20 min at 15,000 × g, resuspended with 45 ml of Superose 6 buffer (1 M NaBr, 10 mM sodium pyrophosphate, 10 mM sodium phosphate, 1 mM NaEDTA, 1 mM NaNt, 1 mM dithiothreitol, 0.05% Tween 20, pH 7.4), and dialyzed for 3 h against this buffer with addition of 15% sucrose. The purpose of the sucrose is to reduce the volume of the sample. The dialyzed material was centrifuged 16 h at 35,000 rpm in a Ti-60 rotor, and the supernatant was applied to a column (5 × 90 cm) packed with Superose 6 and eluted at a flow rate of 60 ml/h. The fractions were monitored by SDS-polyacrylamide gel electrophoresis and immunoblotting with anti-brain adducin antibody. It was not possible at this stage to rely simply on Coomassie Blue staining to detect adducin since other polypeptides co-migrated on SDS gels. Fractions containing adducin (typically, V, 1.65–1.85) were pooled, dialyzed against Mono Q buffer (10 mM sodium phosphate, 0.5 mM NaEDTA, 1 mM NaNt, 0.5 mM dithiothreitol, 0.05% Tween 20, pH 7.4), and centrifuged 16 h at 35,000 rpm in a Ti-45 rotor. This centrifugation step removed some adducin but also a substantial amount of other polypeptides. The supernatant was applied to a Mono Q HR 10/10 anion-exchange column and eluted with the following program: flow rate, 2 ml/min; fraction size, 3 ml; and elution with 70% buffer A, 30% buffer B for 30 min, followed by a linear gradient of 70% buffer A, 30% buffer B, 40% buffer A, 60% buffer B over 90 min, where buffer A was dialysis buffer and buffer B was 0.5 M NaBr dissolved in buffer A. Fractions containing adducin (now visible as a Coomassie Blue-stained band in SDS-polyacrylamide gels) were pooled; dialyzed against pH 6 buffer containing 10 mM MES, 1 mM NaEDTA, 1 mM NaNt, 0.5 mM dithiothreitol; and centrifuged for 60 min at 50,000 × g. The supernatant was dialyzed for 2 h against 0.5 M NaBr, 10 mM sodium phosphate, 1 mM NaNt, 0.5 mM dithiothreitol, 0.05% Tween 20, pH 7.4, and applied to a 0.5 × 7-cm hydroxyapatite column equilibrated in this buffer. The hydroxyapatite column was eluted at room temperature with sodium phosphate at concentrations of 25, 50, 75, and 100 mM dissolved in 0.5 M NaBr, 0.05% Tween 20, 1 mM NaEDTA. Adducin typically eluted with 75 mM sodium phosphate and was 60–80% pure as judged by Coomassie Blue staining of SDS gels. The protein was dialyzed against 10% sucrose, 10 mM sodium phosphate, 0.5 mM NaEGTA, 1 mM NaNt, 0.5 mM dithiothreitol, pH 7.4; frozen rapidly in dry ice, and stored at −80°C. Yields are summarized in Table I, and samples at various stages of purification are analyzed by SDS electrophoresis and immunoblotting in Fig. 1.

RESULTS

Isolation of an Immunoreactive Form of Erythrocyte Adducin from Brain—Polypeptides in brain of M. = 104,000–109,000 that cross-react with antibodies raised against erythrocyte adducin (Gardner and Bennett, 1986) have been purified and immunoblotting with anti-erythrocyte adducin antibody as an assay (see “Methods,” Fig. 1). The cross-reactivity is not due to contaminating erythrocytes in the brain preparations since 1) antibody against band 3, an erythrocyte-specific protein, reveals the presence of less than 0.1% erythrocyte membrane protein in brain (not shown); and 2) the amount of adducin present in brain (12 pmol/ml of brain membrane protein; see below) is 25% of the amount.
either the gels were stained with Coomassie Blue

... were analyzed by SDS-polyacrylamide electrophoresis, and the polypeptides were electrophoretically transferred to nitrocellulose for exchange chromatography;

... stages of purification of brain adducin (see "Experimental Procedures") were assuming a combination of high salt (0.8 M NaCl, followed by extraction with 0.1% NaBr supernatant; lane 3, 0.5 M NaCl supernatant; lane 4, membranes following extraction with 0.5 M NaCl; lane 5, 0.8 M NaBr extract; lane 6, membranes following extraction with 0.8 M NaBr; lane 7, Superose 6 starting sample; lane 8, fractions pooled following Superose 6 chromatography; lane 9, starting sample for Mono Q anion-exchange chromatography; lane 10, fractions pooled following Mono Q chromatography; lane 11, starting sample for hydroxylapatite chromatography; lane 12, sample after hydroxylapatite chromatography.

**FIG. 1. Purification of brain adducin.** Samples from various stages of purification of brain adducin (see "Experimental Procedures") were analyzed by SDS-polyacrylamide electrophoresis, and the polypeptides were electrophoretically transferred to nitrocellulose for exchange chromatography;

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**TABLE I**

| Fraction* | Protein mg | pmol/mg | Purification -fold | Yield % |
|-----------|------------|---------|--------------------|---------|
| 30,000 x g supernatant | 7,440 | 3.7 |
| Demyelinated membrane | 17,800 | 12.5 | 1 | 100 |
| Washed membranes | 15,750 | 9.3 |
| Extracted membranes | 13,650 | 5.6 |
| NaBr extract | 2,640 | 25.4 | 2.0 | 30 |
| Superose 6 start | 1,064 | 54.6 | 4.4 | 26 |
| Mono Q start | 86.4 | 377 | 30 | 14 |
| Hydroxylapatite start | 14.7 | 1,080 | 85 | 7.1 |
| Hydroxylapatite pool | 0.64 | 3,780 | 300 | 1.1 |

*Fractions are described under "Methods."

**Fig. 2. Two-dimensional maps of 125I-labeled chymotryptic peptides of subunits of brain adducin.** Brain adducin (260 μg/ml) was reduced and alkylated by incubation for 10 min at 50°C with 0.1% SDS, 1 mM dithiothreitol, followed by 30 min at 24°C with 5 mM N-ethylmaleimide. The protein (10 μl) was radioiodinated with 1 mCi of Na125I using chloramine T as an oxidant and electrophoresed on an SDS-polyacrylamide gel. Adducin polypeptides were visualized by staining with Coomassie Blue and cut out, and α-chymotryptic peptide maps were prepared (Davis and Bennett, 1983). Shown are maps of the M1 = 109,000 polypeptide (A), M1 = 107,000 polypeptide (B), M1 = 104,000 polypeptide (C) and mixture of peptides from M1 = 109,000 and 104,000 polypeptides (D).
value representing the mean of triplicate values. The ratio of adducin to spectrin is relatively higher in brain than in erythrocytes, which have about 50 pmol/mg of adducin and 200 pmol/mg of spectrin tetramer.

Brain and erythrocyte adducin share a substantial degree of immunological cross-reactivity that is distributed in multiple domains of these proteins (Fig. 3). In radioimmunoassays, human erythrocyte adducin displaced over 50% of binding of radiolabeled bovine brain adducin to antisera raised against bovine brain adducin, although with a reduced affinity compared to bovine brain adducin itself (note that in this experiment, the antibody was not affinity-purified). At least 50% of the antigenic sites of these proteins thus are partially conserved between human and bovine species as well as in different tissues. Limited proteolysis of erythrocyte and brain adducin, followed by SDS-polyacrylamide electrophoresis, reveals that both proteins contain a protease-resistant domain of $M_r = 48,000$ for erythrocyte and $M_r = 48,500$ for brain adducin. The digests of these proteins were immunoblotted with antibodies cross-reacting with both brain and erythrocyte adducin. These antibodies were prepared using antisera raised against brain adducin and affinity-purified using erythrocyte adducin coupled to agarose as an immunoadsorbent (see “Methods”). Multiple cross-reacting bands occur in both proteins including the $M_r = 48,000$ resistant domain of erythrocyte adducin. The pattern of cross-reacting polypeptides differed between erythrocyte and brain adducin since the $M_r = 48,500$ domain of brain adducin is considerably less reactive than the $M_r = 48,000$ domain of erythrocyte adducin. Thus, brain and erythrocyte adducin share antigenic sites over extended regions of their polypeptide chains and differ in the location of antigenic sites.

A potential concern, in view of the lack of immunoreactivity of the $M_r = 48,500$ polypeptide in digests of brain adducin, is that the antibody actually is directed against a minor component that copurified with the major brain adducin polypeptides. This possibility is ruled out by the fact that the antibody can immunoprecipitate at least 80% of radiolabeled brain adducin (not shown).

Affinity-purified antibody cross-reacting with both erythrocyte and brain adducin was used to examine other tissues for related polypeptides (Fig. 4). Polypeptides of the $M_r$ of erythrocyte adducin are present in membrane fractions of kidney, lung, and testes, and a small amount is present in liver. Lens membranes contain cross-reacting polypeptides which co-migrate with brain adducin. It is unlikely that the cross-reacting polypeptides in tissues other than lung are due to erythrocyte contamination since these tissues were obtained from a rat perfused to remove blood cells. The effectiveness of perfusion in removing erythrocytes has been demonstrated using antibodies against the erythrocyte-specific protein, band 3, with no detectable band 3 in brain or kidney (Drenckhahn and Bennett, 1987). The perfusion of lung, as opposed to the other tissues, was not complete; and cross-reacting polypeptides in lung tissue may, in part, be due to contaminating erythrocyte membranes. Brain and erythrocyte membranes contain the most adducin, normalized with respect to membrane protein; and brain has the most adducin relative to membrane spectrin. The antibody utilized in this study appears to react with only a single polypeptide in kidney, testes, and lens; whereas it recognizes both subunits of brain and erythrocyte adducin. The other adducin subunit may either be absent in these tissues or not cross-react with this antibody.

**Physical Properties of Brain Adducin**—Brain adducin in dilute solution has a Stokes radius of 7.3 nm, a sedimentation coefficient of 7 S, a partial specific volume of 0.72 cm$^3$/g, and a frictional ratio of 1.62 (Table II). These determinations were made with adducin, radiolabeled with $^{125}$I-labeled Bolton-Hunter reagent, at concentrations of less than 10 nM. The molecular weight calculated from these parameters is 207,000, which is quite close to the values of 206,000–220,000 expected for hetero- or homodimers of adducin. The high frictional ratio indicates that brain adducin has considerable asymme-
Bovine eye lens and tissues removed from a rat perfused with 0.15 M NaCl, 5 mM sodium phosphate, 2.5 mM NaEDTA, 10 mM diisopropyl fluorophosphate, pH 7.5, were homogenized with a Brinkmann Polytron in 10 volumes of 0.32 M sucrose, 2 mM NaEDTA, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM diisopropyl fluorophosphate, 100 μg/ml phenylmethylsulfonyl fluoride, pH 7. The homogenates were pelleted for 5 min at 9000 × g to remove nuclei and tissue fragments, and the supernatants were pelleted for 30 min at 40,000 × g. The 40,000 × g pellets were resuspended to the original volume with homogenization buffer, and samples of pellets (lanes p) and supernatants (lanes s) were analyzed by SDS-polyacrylamide gel electrophoresis. Rat erythrocyte ghosts were prepared by hypotonic lysis (Bennett, 1983). Gels were either stained with Coomassie Blue (C, Blue, left) or the polypeptides were transferred electrophoretically to nitrocellulose and incubated with affinity-purified antibody against brain adducin (see "Experimental Procedures") (right). Samples are: pellet of rat erythrocyte ghosts (lanes A), pellets (lanes p) and supernatants (lanes s) of liver (lanes B), kidney (lanes C), lung (lanes D), lung (lanes E), and brain (lanes F), and pellet of lens (lanes G). Lens cytosol contained large amounts of crystallins that interfered with electrophoresis and is not included.

FIG. 4. Identification of polypeptides cross-reacting with erythrocyte and brain adducin in membranes of various tissues. Bovine eye lens and tissues removed from a rat perfused with 0.15 M NaCl, 5 mM sodium phosphate, 2.5 mM NaEDTA, 5 mM diisopropyl fluorophosphate, pH 7.5, were homogenized with a Brinkmann Polytron in 10 volumes of 0.32 M sucrose, 2 mM NaEDTA, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM diisopropyl fluorophosphate, 100 μg/ml phenylmethylsulfonyl fluoride, pH 7. The homogenates were pelleted for 5 min at 9000 × g to remove nuclei and tissue fragments, and the supernatants were pelleted for 30 min at 40,000 × g. The 40,000 × g pellets were resuspended to the original volume with homogenization buffer, and samples of pellets (lanes p) and supernatants (lanes s) were analyzed by SDS-polyacrylamide gel electrophoresis. Rat erythrocyte ghosts were prepared by hypotonic lysis (Bennett, 1983). Gels were either stained with Coomassie Blue (C, Blue, left) or the polypeptides were transferred electrophoretically to nitrocellulose and incubated with affinity-purified antibody against brain adducin (see "Experimental Procedures") (right). Samples are: pellet of rat erythrocyte ghosts (lanes A), pellets (lanes p) and supernatants (lanes s) of liver (lanes B), kidney (lanes C), lung (lanes D), lung (lanes E), and brain (lanes F), and pellet of lens (lanes G). Lens cytosol contained large amounts of crystallins that interfered with electrophoresis and is not included.

try, which could, in general, result from a shape of either an oblate (disc-shaped) or prolate (rod-shaped) ellipsoid of revolution. Visualization of this protein in dilute solution by negative staining and electron microscopy reveals circular forms about 13 nm in diameter (Fig. 5). These images could result either from a disc-shaped molecule with its long axis facing the viewer or from a spherical molecule. A spherical shape for the adducin dimer is not likely since a spherical molecule of 13-nm diameter would have a molecular weight of about 500,000 and a sedimentation coefficient of 15–17 S. In view of the fact that adducin can be cross-linked to a tetramer (see below), it is difficult to exclude the possibility that the images result from spherical tetramers. However, it is clear that brain adducin does not have the shape of a rod. The asymmetry of the molecule (at least in the dimer form) thus is, by default, most likely due to a disc shape. It should be mentioned that a small protrusion or tail from the molecule may be difficult to visualize and could also contribute to the asymmetry.

Brain adducin contains two polypeptides that can be distinguished by peptide mapping (Fig. 2) and, based on its calculated molecular weight in solution, may be a homodimer, a heterodimer, or a mixture of homo- and heterodimers. Erythrocyte adducin is a heterodimer (Gardner and Bennett, 1986); and, by analogy, brain adducin may also be a heterodimer.

Chemical cross-linking suggests that brain adducin can form a complex indicative of a tetramer (Fig. 6). Adducin polypeptides at 150 nM were cross-linked by a 30-min incubation with ethylene glycol bis(succinimidyl succinate) to products of $M_2 = 220,000$ and a major form of $M_2 = 450,000$. These reactions reflect protein-protein associations since cross-linking is abolished by addition of urea at concentrations above 2 M. The characterization of adducin as a dimer based on physical properties thus reflects the behavior of this protein under dilute conditions. Additional work will be required to evaluate the various self-association states of adducin.

**Brain Adducin Associates with Spectrin-Actin Complexes—** Erythrocyte adducin associates weakly with spectrin or actin alone, but binds well to spectrin-actin complexes (Gardner and Bennett, 1987). Association of brain adducin with actin, spectrin, and mixtures of spectrin and actin exhibits similar behavior as for erythrocyte adducin (Figs. 7 and 8). Binding of brain adducin, labeled with $^{125}$I-labeled Bolton-Hunter reagent, to actin filaments was measured by sedimentation of actin through sucrose barrier gradients in T1-42.2 tubes, followed by removing the tips and either analyzing the samples by SDS electrophoresis (Fig. 7) or measuring radioactivity (Fig. 8). Adducin had no detectable effect on the extent of

FIG. 5. Visualization of negatively stained brain adducin by electron microscopy. Brain adducin (4 μg/ml) was negatively stained with uranyl formate and visualized by transmission electron microscopy (see "Experimental Procedures"). Bar = 50 nm.
actin sedimentation under these experimental conditions and thus is not active in capping or severing of actin filaments. Adducin polypeptides, visualized by autoradiography, associated with actin in the absence of brain spectrin; but the association of adducin with actin was enhanced at least 4-fold in the presence of spectrin, although at less than 2.5 times more concentrated than the supernatants. The increased binding of adducin in the presence of spectrin and actin required both spectrin and actin since neither adducin nor spectrin sedimented in the absence of actin, and neither adducin nor spectrin altered recovery of actin filaments, that neither adducin caused negative cooperativity or, more likely, slightly curvilinear, suggesting some interesting possibilities: either adducin caused negative cooperativity or, more likely, adducin increased the affinity of spectrin for actin, with little effect on the overall capacity for spectrin (Fig. 9). Adducin stimulated binding of spectrin in a saturable manner with half-maximal stimulation at 30 nM adducin and nearly maximal stimulation at 100 nM adducin.

The effect of adducin on spectrin binding to actin is most pronounced at low concentrations of spectrin, suggesting that adducin increases the affinity of spectrin for actin. A double reciprocal plot of spectrin associated with actin versus free spectrin in the presence of adducin demonstrates an increased affinity of spectrin for actin, with little effect on the overall capacity for spectrin (Fig. 9). The double reciprocal plot was slightly curvilinear, suggesting some interesting possibilities: either adducin caused negative cooperativity or, more likely, adducin increased the affinity of spectrin for actin (from a $K_d$ of 500 nM in the absence of adducin to a $K_d$ of 70 nM in the presence of adducin), although at less than 5% of the maximal number of sites. It is pertinent in this regard that the amount of adducin available in this experiment was 60 nM, which is considerably less than the concentration of actin of 2.5 µM. Adducin associated with spectrin immobilized on beads at only 5-10% of the level achieved with actin plus spectrin, and this low binding occurred at all concentrations of adducin. These results suggest that brain adducin preferentially binds to spectrin-actin complexes. It is important to emphasize that direct association of adducin with actin also occurs, but is significantly less than when spectrin and actin are present together.

Brain Adducin Promotes Binding of Brain Spectrin to Actin—Erythrocyte adducin promotes binding of spectrin to actin (Gardner and Bennett, 1987), and brain adducin exhibits a similar activity (Fig. 9). Brain adducin increased binding of low concentrations of radiolabeled brain spectrin to actin by 2-3-fold, from a bound/free ratio of 0.18 to 0.50. Adducin stimulated binding of spectrin in a saturable manner with half-maximal stimulation at 30 nM adducin and nearly maximal stimulation at 100 nM adducin.
Brain Adducin

FIG. 8. Association of ¹²⁵I-labeled brain adducin with spectrin-actin complexes as a function of brain adducin (left) and as a function of brain spectrin (right). Left, ¹²⁵I-labeled brain adducin (5 nM, 346,000 cpm/pmol) was incubated in a 100-µl volume under the conditions described for Fig. 7 with the addition of 3 mg/ml bovine serum albumin in the presence of increasing concentrations of unlabeled adducin and actin (1 µM) (○), actin (1 µM) plus brain spectrin (32 nM) (●), or brain spectrin (15 nM) immobilized on biotin beads (see "Experimental Procedures") (■). Free adducin was separated from adducin associated with actin filaments or spectrin beads by sedimentation through sucrose barrier gradients as described for Fig. 7, and the tips of the tubes were cut off and analyzed for ¹²⁵I. Data (mean of duplicate determinations) are expressed as picomoles of brain adducin associated with actin or actin plus spectrin and are corrected for sedimentation of adducin in the absence of these proteins, whereas the control for binding of adducin to spectrin beads was an equal amount of beads lacking spectrin. Values for binding of adducin to spectrin beads have been multiplied by a factor of 2.1 to permit direct comparison with binding of spectrin in the presence of actin. The concentration of spectrin attached to beads was estimated by SDS electrophoresis and staining of gels with Coomassie Blue.

Right, ¹²⁵I-labeled brain adducin (8 nM, 99,000 cpm/pmol) was incubated in a 100-µl volume with increasing concentrations of brain spectrin in the presence (○) or absence (○) of actin (1 µM) and adducin associated with actin or actin plus spectrin determined as described above.

FIG. 9. Brain adducin promotes association of ¹²⁵I-labeled brain spectrin with actin filaments. Left, ¹²⁵I-labeled brain spectrin (1.1 nM, 1.4 × 10⁶ cpm/pmol) was incubated with increasing concentrations of brain adducin in the presence or absence of polymerized actin (2.5 µM), and the samples were sedimented through sucrose barrier gradients as described (see legends to Figs. 7 and 8). Data (mean of duplicate determinations) are expressed as the fraction of spectrin associated with actin filaments and has been corrected for the amount of spectrin that sedimented in the absence of actin. The concentration of free spectrin was determined from an aliquot of the supernatants following sedimentation of actin filaments. Right, ¹²⁵I-labeled brain spectrin (2.6 nM, 1.4 × 10⁶ cpm/pmol) was incubated with increasing concentrations of unlabeled brain spectrin with and without actin (2.5 µM) and in the presence (○) and absence (●) of brain adducin (65 nM). The concentrations of free spectrin and spectrin associated with actin filaments were determined as described above. The data are expressed as a double reciprocal plot of 1/actin-associated spectrin versus 1/free spectrin. The specific activity of ¹²⁵I-labeled spectrin was recalculated for each concentration of unlabeled spectrin, and these values were used to estimate the actual nanomoles of spectrin bound and free.

Brain adducin was initially discovered due to its calmodulin-binding activity (Gardner and Bennett, 1986). Brain adducin also associates with calmodulin in a calcium-dependent manner, as determined by photoaffinity labeling with ¹²⁵I-labeled azido-calmodulin (Fig. 10). The labeling of adducin is abolished in the absence of calcium and is displaced by unlabeled calmodulin. The subunit of adducin labeled by calmodulin is difficult to identify in these experiments. The affinity of adducin for calmodulin was estimated by quantitating the displacement of two concentrations of photoaffinity-labeled
calmodulin with unlabeled calmodulin (Fig. 10). These data can be treated as those for enzyme inhibitors using a Dixon (1953) plot which yields a $K_i$ of 0.2 $\mu$M and thus, at least under these experimental conditions, binds calmodulin with a 5-6-fold higher affinity than brain adducin. Calmodulin inhibits the ability of erythrocyte adducin to promote spectrin binding to actin with half-maximal inhibition at a concentration of calmodulin of about 0.9 $\mu$M (Gardner and Bennett, 1987). Calmodulin at 1 $\mu$M has little effect on binding of brain adducin to spectrin-actin complexes or on the ability of brain adducin to increase binding of spectrin to actin (not shown). Effects of calmodulin at higher concentrations were not examined due to their questionable physiological relevance.

**Brain Adducin Is a Substrate for Protein Kinase C**—Brain adducin is a substrate for protein kinase C in solution and following SDS electrophoresis and transfer of the polypeptides to nitrocellulose paper (Fig. 11). Phosphorylation of adducin in solution with protein kinase C isolated from brain was stimulated 5-7-fold above basal levels in the presence of phorbol ester, phosphatidylycerine, and calcium. All three adducin polypeptides were labeled in amounts proportional to their staining with Coomassie Blue. Phosphorylation of adducin occurs at a maximal stoichiometry of 2 mol of phosphate/mol of adducin and was complete in this experiment within 1 h at 24°C (Fig. 11, lower). The rate of phosphorylation of adducin by protein kinase C increased in a hyperbolic manner with increasing concentrations of adducin and was half-maximal with 0.5 $\mu$M adducin. Brain adducin is an excellent substrate for protein kinase C in comparison to other protein substrates such as caldesmon with a $K_m$ of 9 $\mu$M (UmeKawa and Hidaka, 1985) and myosin light chain kinase with a $K_m$ of 4 $\mu$M (Nishikawa et al., 1985).

Brain adducin is phosphorylated in a phorbol ester-dependent manner following transfer of the polypeptides to nitrocellulose paper. The reaction is relatively specific for adducin since few polypeptides in brain cytosol or membranes were labeled under the same conditions. It is of interest that a polypeptide(s) of the same mass as adducin would not reverse phosphorylation that had already occurred (not shown). It is not known if the inhibitory activity only...
co++ phosphorylation reactions on nitrocellulose were performed following various times with protein kinase C as described above in the presence of decanoylphorbol 8-acetate. Phosphorylated polypeptides were visualized by autoradiography. Phosphate incorporation was determined by addition of 0.9 ml of 1 mg/ml bovine serum albumin, 5 mM NaEDTA, followed by 0.1 ml of 50% trichloroacetic acid to precipitate the protein. The tubes were centrifuged for 15 min at 4000 x g. Supernatants were aspirated, and the protein pellets were assayed for radioactivity. Data are expressed as moles of phosphate incorporated per mol of adducin, and these values have been corrected for phosphate incorporated in the absence of adducin due to autophosphorylation by protein kinase C. The effect of concentration of brain adducin was determined using a 10-min incubation and otherwise identical conditions as described for the time course.

blocks phosphorylation of adducin or if other substrates for protein kinase C are also affected.

**Discussion**

This report describes isolation of a protein from brain membranes that is closely related to adducin, a recently identified membrane skeletal protein of erythrocytes. Brain and erythrocyte adducin have at least 50% antigenic sites in common, each contains a protease-resistant core of $M_\text{r} = 48,000$-$48,500$, both are comprised of two distinct polypeptides that have some peptides in common, and these proteins have nearly identical physical properties. Brain and erythrocyte adducin associate preferentially with spectrin-actin complexes compared to spectrin or actin alone, and both proteins also promote binding of spectrin to actin. Brain adducin binds calmodulin in a calcium-dependent manner, although the affinity of $1.3 \mu M$ is weaker by 5-6-fold than the affinity of erythrocyte adducin for calmodulin. Finally, brain adducin, like erythrocyte adducin, is a substrate for protein kinase C in *vitro* assays and can accept up to 2 mol of phosphate/mol of protein. Brain tissue contains 12 pmol of adducin/mg of membrane protein, which is the most of any tissue examined other than erythrocytes, which have 50 pmol/mg. Neurons are likely to be one cell type in brain that contains adducin since adducin has been visualized by immunofluorescence in primary cultures of neurons. The presence of high amounts of adducin in neuronal cells suggests some role for this protein in specialized activities of nerve cells.

An unusual feature of erythrocyte and brain adducin is that these proteins form ternary complexes with spectrin and actin, but bind relatively weakly to spectrin or actin alone. Erythrocyte protein 4.1 also associates with spectrin-actin complexes but, in contrast to adducin, binds directly to spectrin with high affinity. The basis for recognition of spectrin complexes by adducin could be that adducin has two low affinity binding sites, one for spectrin and one for actin. The apparent affinity of a spectrin-actin-adducin complex would be higher than the affinities for individual proteins, and such a ternary complex with multiple interaction sites would be much more stable than a binary complex dependent on only individual associations. Alternatively, association of spectrin with actin may induce a conformational change in one of these proteins that is recognized by adducin. The site of spectrin involved in binding to adducin has not been addressed in this study. The simplest possibility is that adducin and spectrin interact close to the actin-binding site of spectrin at the ends of spectrin tetramers. If adducin associates with spectrin at the actin-binding region of spectrin, then adducin may provide a potential mechanism in cells for selectively targeting proteins to spectrin-actin complexes.

A working hypothesis for a cellular function for brain and erythrocyte adducin is that these proteins have a role in mediating site-directed assembly of additional proteins at the spectrin-actin junction. One possible protein that could be recruited by adducin is spectrin itself. Experiments with erythrocyte adducin suggest that the adducin-promoted binding of spectrin to actin is due to creation of new sites for spectrin that are distinguished by their inhibition with calmodulin (Gardner and Bennett, 1987). Brain adducin increases binding of spectrin to actin (Fig. 9), which, by analogy to erythrocyte adducin, may also represent creation of new sites for spectrin. Adducin-mediated recruitment of additional spectrin molecules to spectrin-actin complexes could explain how spectrin and actin form the configuration of five to seven spectrin molecules clustered about small oligomers of actin, which, by analogy, may provide a potential mechanism in cells for selectively targeting proteins to spectrin-actin complexes.

V. Bennett, K. Gardner, and J. P. Steiner, unpublished results.
and mediates their interaction with spectrin-actin complexes.

Calmodulin associates with brain adducin with a low affinity ($K_d = 1.3 \mu M$), and the physiological significance of this interaction is not clear. Possible explanations for the low affinity of brain adducin for calmodulin may be that the protein as isolated has been modified by phosphorylation or other post-translational events that could alter binding to calmodulin, that the experimental conditions were not optimal, or that an accessory protein is missing. It also is conceivable that brain adducin has evolved to interact with a calmodulin-related protein and has lost the ability to bind to calmodulin itself.

Brain adducin is most likely a new addition to the list of substrates for protein kinase C and is distinct on the basis of $M_r$ and subcellular and tissue distribution from other identified protein kinase C substrates such as tyrosine hydroxylase (Albert et al., 1984), glycogen synthetase (Ahmad et al., 1984), vinculin (Werth et al., 1983), caldesmon (Umekawa and Hidaka, 1985), and myosin light chain kinase (Nishikawa et al., 1985). An 87-kDa polypeptide has been characterized as a protein kinase C substrate that is similar to adducin in that it is enriched in brain and is associated with membrane fractions (Blackshear et al., 1986; Albert et al., 1986). Adducin differs from the 87-kDa polypeptide in that it contains two polypeptides rather than one and is primarily associated with membrane fractions. It will be important in future work to determine if brain adducin is phosphorylated in vivo by protein kinase C and if the same phosphopeptides are modified in vitro and in vivo.

The functional consequences of phosphorylation of adducin remain to be elucidated. In preliminary assays, phosphorylation by protein kinase C had no effect on association of adducin with spectrin-actin complexes or on stimulation of spectrin binding to actin. It will be important to evaluate a possible effect of phosphorylation on the adducin dimer-tetramer equilibrium (Fig. 6) and on the affinity of adducin for calmodulin, which is modulated by phosphorylation in the case of myosin light chain kinase (Ikebe et al., 1985; Conti and Adelstein, 1981; Nishikawa et al., 1985). Phosphorylation may modify a function or protein association of adducin that has not yet been identified. It is of interest in this regard that brain and erythrocyte adducin closely resemble, in $M_r$, polypeptides that bind protein kinase C following their transfer to nitrocellulose paper (Wolf and Sahyoun, 1986). Adducin thus may play a role in targeting protein kinase C or one of its isoforms to spectrin-actin complexes on the membrane.

These initial studies suggest that adducin has the potential to play an important role in assembly and regulation of the spectrin-based membrane skeleton, although many questions remain to be answered. Information about adducin is limited at this time to simple in vitro assays that have only begun to explore the implications of ternary and higher order protein interactions. Finally, the major challenge for the future will be to eventually understand the function and regulation of adducin in living cells.

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