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Type II Regulatory Subunits of cAMP-dependent Protein Kinase and Their Binding Proteins in the Nervous System of *Aplysia californica**

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Two type II regulatory (R) subunits of cAMP-dependent protein kinase (PKA) of 50 and 47 kDa have been identified in *Aplysia* neurons by several criteria which include phosphorylation by the catalytic subunit of PKA and nanomolar affinity for a peptide fragment of the human thyroid protein Ht 31, properties that in mammals distinguish type II from type I R subunits. The neuronal type II R subunits are differentially localized within cells. For example, the 50-kDa polypeptide is enriched in taxol-stabilized microtubules. In addition, at least seven high molecular mass neuronal RII-binding proteins ranging in mass from 110 to 420 kDa have been demonstrated by a blot overlay technique, which uses ^{32}P-labeled bovine RIIα as a probe. The RII-binding proteins also exhibit discrete patterns of subcellular localization. For example, the 420 kDa species is enriched in taxol-stabilized microtubules and therefore may serve to anchor the 50-kDa RII subunit. The localization of PKA through the association of RII subunits with the binding proteins may anchor the multifunctional kinase close to key substrates and thereby contribute to the spatial organization required to mediate the orderly phosphorylation events that underly neuronal modulation.

Neuronal modulation (1) is the basis of diverse physiological processes including changes in the behavior of organisms. One important component of neuronal modulation, in both invertebrates and vertebrates, is the activation of intracellular signal transduction pathways in target neurons upon binding of neuromodulatory transmitters (2, 3). In the marine invertebrate *Aplysia californica*, presynaptic facilitation at sensorimotor synapses contributes to sensitization of the defensive gill- and tail-withdrawal reflexes, simple forms of learning and memory. cAMP-dependent protein kinase (PKA)[1] plays crucial roles in both short and long term facilitation. In short term facilitation, release of the neuromodulator serotonin (5HT) by facilitatory interneurons near presynaptic sensory cell terminals effects the receptor-mediated activation of adenylate cyclase leading to a rise in intracellular cAMP concentration (2). A major consequence of the subsequent activation of PKA is the phosphorylation of K^+ channels or associated proteins and a decrease in outward K^+ currents (4–8). The resulting prolongation of the action potential and increased excitability of the sensory cells enhances transmitter release at the sensorimotor synapse. Evidence for the involvement of PKA includes direct measurements of cAMP concentrations in sensory cells (9) and their processes (10), as well as measurements of the effects of microinjection into sensory neurons of cAMP (8, 11), bovine catalytic (C) subunit (12), and protein kinase inhibitor peptide (7, 13).

While short and long term facilitation share a number of features including characteristic changes in electrophysiological parameters in sensory neurons (14, 15) and in electrophoretic profiles of abundant phosphoproteins in these cells (16), the two processes can be distinguished. Long term facilitation requires new protein synthesis, which is initiated by renewed transcription mediated by phosphorylation of the cAMP response element-binding protein (CREB) by PKA (17, 18). Treatments that induce long term facilitation cause the down-regulation of the regulatory (R) subunits of PKA (19, 20). Down-regulation of R may serve to maintain the phosphorylation of key substrates by permitting the continued presence of free C subunit. In addition, long term facilitation is accompanied by the formation of new contacts at the sensorimotor synapse (21) and occurs cell wide (22, 23). Hence, the simple view is that short term facilitation is mediated by rapid events at presynaptic sensory cell terminals, while long term facilitation is initiated by phosphorylation in the cell nucleus that is manifested cell wide and relatively slowly in changes at terminals similar to those seen after short term facilitation, as well as additional structural alterations that strengthen the sensorimotor synapse.

To understand the molecular basis for the spatial and temporal separation of the PKA-mediated phosphorylation events involved in presynaptic facilitation in *Aplysia*, it is necessary to define the properties of the neuronal holoenzyme (R_2C_2). A diversity of structures (24–26) is already emerging from studies of *Aplysia* R and C subunits, which may provide neuronal holoenzymes with different substrate specificities, regulation, subcellular locations, and translocation properties. We earlier analyzed cDNAs encoding all known *Aplysia* C subunit isoforms. Through alternative promoter use and alternative RNA splicing, four C subunits are generated from a single gene by

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[1] The abbreviations used are: PKA, cAMP-dependent protein kinase; BP, binding protein for RII subunit; BSA, bovine serum albumin; C subunit, catalytic subunit of PKA; MES, 3-[*N*-morpholino]ethane]sulfonic acid; MOPS, 3-[*N*-morpholino]propane]sulfonic acid; $C_{APL-A}N1A1$, a C subunit from *Aplysia*; 5HT, 5-hydroxytryptamine (serotonin); Ht 31, a human thyroid PKA anchoring protein; MT, microtubule; PIPES, piperazine-*N*,*N*′-bis[2-ethane sulfonic acid]; PKIP, PKA inhibitor peptide; PMSF, phenylmethanesulfonyl fluoride; R subunit, regulatory subunit of PKA; RI, type I regulatory subunit of PKA; RII, type II regulatory subunit of PKA; R_{APL-N4}, type N4 RI subunit from *Aplysia* PKA; PAGE, polyacrylamide gel electrophoresis; cpm, counts/min; DTT, dithiothreitol; Tricine, *N*-tris(hydroxymethyl)methylglycine.

combinatorial expression of two alternative N termini (N1 and N2) with two alternative internal amino acid cassettes (A1 and A2) (25, 26). Recombinant A1 and A2 forms of C have significantly different catalytic properties and affinities for R and may therefore contribute to the fine tuning of neuronal responses (27). For example, holenzymes with different affinities for cAMP (27), located in different parts of the cell (this paper), could modulate different ion channels at different intracellular cAMP concentrations, which has recently been observed in *Aplysia* sensory cells (7). While the functions of the alternative N termini of C are unknown, preliminary experiments are consistent with a role in substrate targeting (28).

In addition to the four C subunits, at least five R subunits ($R_{APL\text{-}N1}$, $R_{APL\text{-}N2}$, $R_{APL\text{-}N3}$, $R_{APL\text{-}N4}$, and $R_{APL\text{-}N5}$) have been identified in extracts of *Aplysia* ganglia by photoaffinity labeling with the cAMP analogue [^{32}P]8-azido-cAMP (24). With the exception of $R_{APL\text{-}N2}$, all of them could be assigned as type I R subunits based on peptide mapping (24) and cDNA sequencing (20). By contrast, type II R subunits have not been demonstrated incontrovertibly in invertebrates, including *Aplysia* (20, 29, 30). In mammals, RII subunits anchor PKA holoenzyme to subcellular structures (31, 32). For example, the N termini of both RIIα and RIIβ dimers associate with the N terminus of the microtubule-associated protein MAP2 (33, 34). Because anchoring may serve to localize PKA close to important substrates such as ion channels (24, 35), we have sought to demonstrate the presence of RII subunits and corresponding RII-binding proteins in *Aplysia*.

In the present report, we provide definitive evidence for the existence of at least two *Aplysia* type II R subunits in neurons, as well as the presence of at least seven RII-binding proteins. Both the RII subunits and the binding proteins exhibit differential subcellular localization. Incorporation of these findings into models of presynaptic facilitation in *Aplysia* will enhance our understanding of how an enzyme with a broad spectrum of activity can nonetheless mediate phosphorylation events that are both spatially and temporally separated.

EXPERIMENTAL PROCEDURES

Protein Assays and Gel Electrophoresis —Protein concentrations in small tissue samples were determined by a solid-phase colloidal gold binding technique (36) using bovine serum albumin (BSA: ultra-pure, lipid-free, BM Biochemica) as the standard. SDS-PAGE was carried out according to Laemmli (37). Molecular weight markers (Bio-Rad) were (M_r): rabbit muscle phosphorylase *b* (97,400), bovine serum albumin (66,200), hen egg white ovalbumin (45,000), bovine carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and hen egg white lysozyme (14,400). Dynein heavy chains from *Strongylocentrotus purpura* provided a marker of M_r ~520,000. ^{14}C-labeled markers (Life Technologies, Inc.) were (M_r): myosin heavy chain (200,000); phosphorylase *b* (97,400); bovine serum albumin (68,000); ovalbumin (43,000); carbonic anhydrase (29,000); β-lactoglobulin (18,400), and lysozyme (14,300). For peptide mapping by limited proteolysis, a Tricine-SDS-PAGE system (38) was used as detailed previously (39). Low molecular weight markers (Sigma) were glucagon and CNBr fragments of horse heart myoglobin (M_r: 17,000, 14,400, 10,600, 8,200, 6,200, 3,500, and 2,500).

Construction of a Baculovirus Containing an $R_{APL\text{-}N4}$ cDNA— Plasmid pN4 (20) was digested with EcoRV and *Bam*HI to liberate a cDNA corresponding to the entire coding region of the type I *Aplysia* R subunit $R_{APL\text{-}N4}$. After the addition of *Bam*HI linkers, the cDNA was inserted into the unique *Bam*HI site of the transfer vector pVL941 (40) to yield pVL-$R_{APL\text{-}N4}$. The orientation of the insert was determined by digestion with *Nde*I and *Eco*RV. Recombinant baculovirus, AcMNPV-RN4, was produced by cotransfecting Sf9 cells with AcMNPV DNA (1 μg) and pVL-$R_{APL\text{-}N4}$ (2 μg, purified on a CsCl gradient). Recombinant virus was purified by three rounds of limiting dilution in 96-well microtiter plates, as previously described (27).

Purification of Aplysia Type I ($R_{APL\text{-}N4}$) Regulatory Subunit—$R_{APL\text{-}N4}$ (20)) was purified from recombinant baculovirus-infected Sf9 cells by affinity chromatography over tandem dye columns. For large scale expression, Sf9 cells (100 ml) were grown in a spinner flask, containing TNM-FH (39) supplemented with 5% fetal bovine serum, to a density of 1–2 × 10^6 cells/ml and then inoculated with AcMNPV-RN4 at a multiplicity of infection of 10 plaque forming units/cell. The baculovirus-infected cells were harvested 72 h post-inoculation by centrifugation at 1,000 × *g* for 10 min at 4 °C, washed with 10 volumes MES-buffered saline (20 mM MES, 150 mM NaCl, pH 6.2) and recentrifuged. Cell pellets were resuspended at 10^7 cells/ml in hypotonic lysis buffer: 10 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and 10 μM leupeptin and placed on ice for 30 min. The resulting lysate was centrifuged at 180,000 × *g* for 1 h at 4 °C. The supernatant was mixed with 1 volume of 2 × Blue-2 binding buffer (BBB: 2 × BBB is 40 mM potassium phosphate (pH 6.6), 50 mM NaCl, 10% glycerol, 1 mM PMSF, 20 μM leupeptin) and then loaded onto a Reactive Yellow 86-agarose column (Sigma, no. R8504: $R_{APL\text{-}N4}$ does not bind) connected in tandem to a Reactive Blue 2-agarose column (Sigma, no. C1660, recently renamed Cibacron Blue 3G-A-agarose: $R_{APL\text{-}N4}$ binds). The dye matrices had been washed and equilibrated in 1 × BBB. One ml of each matrix was used per 10^8 cells of the original harvest. After loading, the columns were rinsed with 2 bed volumes of 1 × BBB and disconnected. The Blue-2 agarose column was washed with 10 bed volumes of 1 × BBB containing 2 × salt (50 mM NaCl). $R_{APL\text{-}N4}$ was then eluted with 10 bed volumes of 1 × BBB containing 100 μM cGMP, which, unlike cAMP, can be readily dissociated from R without urea treatment. The binding, rinsing, and washing steps were carried out at 4 °C, while elution was conducted at room temperature. Bound cGMP was removed by dialysis at 4 °C against 3 × 2 liters of storage buffer lacking glycerol: 10 mM sodium phosphate, 1 mM EDTA, 1 mM DTT (pH 6.8). The dialysate was made 5% (w/v) in glycerol and frozen in aliquots at −70 °C. Yields were typically 7.5 mg of purified R subunit/10^8 cells (50–100 ml of spinner culture). The nucleotide-free, recombinant $R_{APL\text{-}N4}$ inhibits recombinant *Aplysia* C subunits with a subnanomolar K_i.

Purification of Bovine Type II (RIIα) Regulatory Subunit—RIIα was purified from bovine heart employing urea elution from a cAMP-agarose column (C8-linked; Sigma, no. A0144) followed by the rapid removal of urea and renaturation by passage through a Sephadex G-50 column as previously described (27, 41, 42).

Purification of Aplysia PKA Catalytic Subunit—The *Aplysia* PKA catalytic subunit $C_{APL\text{-}A}$N1A1 was purified as described previously from the supernatant medium of recombinant baculovirus-infected Sf9 cells by a two-step procedure involving ion-exchange chromatography followed by affinity chromatography on PKIP-Affi-Gel (27).

Tissue Fractionation: General Points—Aplysia californica (250–350 g; Marinus, CA) were anesthetized by injecting half of the body weight of 0.4 M $MgCl_2$ into the abdominal cavity. The abdominal, pleural-pedal, cerebral, and buccal ganglia were removed and the connective sheaths separated by microdissection to obtain the neuronal components (cell bodies and neuropil) (43). To reduce protease and phosphatase activity in crude tissue preparations, *Aplysia* tissues were homogenized in extraction buffers containing protease inhibitors (PMSF and leupeptin) and phosphatase inhibitors (NaF and Na_3VO_4) and, where possible, manipulations were carried out at 4 °C or on ice. Supernatants and pellets used in this study are designated, respectively, S and P.

Total Tissue Homogenates—Buccal muscle, ovotestis, desheathed nervous tissue, or the sheath (200 mg) was homogenized with 250 μl of 10 mM Tris-HCl (pH 7.4), 1 mM DTT, 1 mM EDTA, 1 mM $MgCl_2$, 1 mM PMSF, 10 μM leupeptin, 5 mM NaF, and 100 μM Na_3VO_4, and stored in aliquots at −70 °C.

Preparation of Detergent-extracted Cytoskeletons—The preparation of detergent-extracted cytoskeletons was carried out essentially as described by Solomon *et al.* (44). Desheathed *Aplysia* nervous tissue from five animals was washed once with 1 ml of extraction buffer without detergent (0.1 M PIPES (pH 6.9, titrated with NaOH) containing 1 M glycerol, 1 mM $MgSO_4$, 2 mM EGTA, 1 mM PMSF, 10 μM leupeptin, 5 mM NaF, 100 μM Na_3VO_4) prior to homogenization in 500 μl of the same buffer containing 0.1% Nonidet P-40. After 15 min on ice, the lysate was centrifuged at 16,000 × *g* and the supernatant stored in aliquots at −70 °C (S). The cytoskeletal pellet was washed twice with 1 ml of extraction buffer (without detergent), resuspended in 250 μl of the same buffer, and stored in aliquots at −70 °C (P).

Membrane Protein-enriched Fraction—Neuronal components from five animals were homogenized in 500 μl of extraction buffer (10 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 1 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 10 μM leupeptin, 5 mM NaF, 100 μM Na_3VO_4) using a disposable polypropylene pestle in a 1.5-ml centrifuge tube (Kimble, no. 95050–99). The homogenate was kept on ice for 5 min. The top layer was pipetted off the settled cellular debris and centrifuged at 150,000 × *g* for 60 min at 4 °C. The resulting cytosolic supernatant, which doubtless includes fragmented cytoskeleton, was stored at −70 °C (S). The pellet

was washed with 1 ml of solubilization buffer (50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM $MgCl_2$, 1 mM DTT, 5 mM NaF, 100 μM Na_3VO_4) without detergent and then resuspended in 250 μl of the same buffer containing 1% Nonidet P-40. The suspension was kept on ice for 15 min and then centrifuged at 16,000 × *g* for 10 min at 4 °C. The resulting supernatant, a solubilized membrane pellet, was stored in aliquots at −70 °C (P).

Taxol-stabilized Microtubules—Microtubules were prepared by the taxol stabilization method described by Chen *et al.* (45) with minor modifications. Microdissected desheathed *Aplysia* nervous tissue from five animals was homogenized in 500 μl of extraction buffer (0.1 M PIPES, pH 6.6 (titrated with NaOH), 1 mM EGTA, 1 mM $MgSO_4$, 10 μM leupeptin, and 1 mM PMSF). The homogenate was centrifuged at 30,000 × *g* for 30 min at 4 °C to yield a cytosolic extract. After the addition of GTP (to 1 mM) and taxol (to 20 μM), the extract was incubated at 37 °C for 15 min and the microtubules pelleted by centrifugation at 30,000 × *g* for 30 min at 37 °C. The supernatant was stored at −70 °C (S). The microtubule pellet was resuspended in the above buffer containing GTP and taxol, recovered by centrifugation at 30,000 × *g* for 30 min at 37 °C and finally resuspended in 150 μl of the same buffer, but without GTP and taxol, prior to storage in aliquots at −70 °C (P).

In Vitro Phosphorylation—In vitro phosphorylation was carried out in a reaction mix (30 μl) containing 50 mM MOPS, pH 6.8 (titrated with NaOH), 15 mM $MgCl_2$, 2.5 mM 1-isobutylmethylxanthine, 0.1% Nonidet P-40, 5 mM NaF, 100 μM Na_3VO_4 containing 3.3 pmol of [γ-^{32}P]ATP (3000 Ci/mmol) in the presence or absence of 2 ng of purified *Aplysia* C subunit ($C_{APL\text{-}A}$N1A1). Reactions were initiated by the addition of 20 μg of protein from total homogenates or subcellular fractions and incubated at 30 °C for 5 min. Reactions were stopped by the addition of gel loading buffer, prior to heating at 100 °C for 5 min. Protein kinase inhibitor peptide (Sigma, no. P0300) was used in some experiments.

Phosphopeptide Mapping—Solubilized membrane fractions from *Aplysia* nervous tissue were phosphorylated *in vitro* as described above. ^{32}P-Labeled polypeptides were separated in a 12.5% SDS-polyacrylamide gel. Labeled polypeptides were excised from the dried gel using an autoradiogram as a template. The excised slices were rehydrated in the wells of a 16.5% Tricine-SDS-polyacrylamide gel and subjected to limited digestion with staphylococcal V8 protease followed by electrophoresis as detailed previously (39).

Detection of cAMP-binding Proteins—A solubilized membrane fraction derived from *Aplysia* nervous tissue or ovotestis was phosphorylated *in vitro* as described above. To analyze ^{32}P-labeled proteins, cAMP-agarose (C8-linked; Sigma, no. A0144) was first equilibrated with binding buffer (10 mM Tris-HCl, 100 mM NaCl (pH 7.4)). The labeled extract (100 μl) was diluted with 2 × binding buffer (100 μl, 20 mM Tris-HCl, 200 mM NaCl (pH 7.4)) and adsorbed onto 50 μl (settled volume) of the matrix on a rotator for 1 h at 4 °C. The matrix was then washed with 2 × 0.5 ml of 10 mM Tris-HCl (pH 7.4), containing 1.5 M NaCl. Salt was removed by an additional wash with 2 × 0.5 ml of 10 mM Tris-HCl (pH 7.4), prior to elution of proteins from the matrix with 10 mM cAMP (200 μl) at room temperature or by heating for 4 min at 100 °C with gel loading buffer (100 μl).

Detection of Ht-31 Binding Proteins—A synthetic peptide DLIEEAASRIVDAVIEQVKAAGAY, which represents the minimum region of Ht 31 (amino acid residues 493–515) required to bind mammalian RII (32), was linked to Affi-Gel-15 essentially as described (Bio-Rad product bulletin 1085). Briefly, Ht 31 peptide (5 mg) was dissolved in 5 ml of 100 mM MOPS (pH 6.8, titrated with NaOH) and coupled at 4 °C for 4 h with gentle rotation to 2 ml of Affi-Gel-15 that had been washed with ice-cold water (5 × 5 ml). Remaining active esters were blocked by the addition of 0.25 ml of 1 M ethanolamine (pH 8.0, titrated with concentrated HCl) to the bead suspension, followed by incubation for 1 h at 4 °C. After washing, the beads were resuspended at 50% in 100 mM MOPS (pH 6.8). The suspension was made 10% (w/v) in glycerol and stored in aliquots at −70 °C.

To analyze Ht 31-binding proteins, a solubilized membrane preparation of desheathed *Aplysia* nervous tissue (20 μg of protein) was phosphorylated *in vitro* with purified $C_{APL\text{-}A}$N1A1 kinase and [γ-^{32}P]ATP, as described above, in a total volume of 30 μl. The mixture was then added to 300 μl of binding buffer: 50 mM MOPS (pH 6.8), 100 mM NaCl, 0.1% Nonidet P-40, 15 mM $MgCl_2$, 1 mM PMSF, 5 mM NaF, 100 μM Na_3VO_4. The diluted sample was divided into three 0.5-ml plastic centrifuge tubes (100 μl in each). Binding buffer (250 μl), with or without Ht 31 peptide (0.5 mM final concentration), was added to each of the tubes, followed by Affi-Gel-Ht 31 (50 μl, settled volume) that had been washed and equilibrated in binding buffer. After gentle rotation at 4 °C for 30 min, the beads were washed with 2 × 0.5 ml of binding buffer containing 2 × salt (200 mM NaCl). Ht 31-binding proteins were eluted either by heating for 4 min at 100 °C with gel loading buffer (100 μl) or by treatment with binding buffer (100 μl) containing 0.5 mM Ht 31 peptide at room temperature.

CNBr Peptide Mapping of Photolabeled, Ht 31 Affinity Purified, 50- and 47-kDa Polypeptides—Photoaffinity labeling and peptide mapping of *Aplysia* neuronal R subunits was carried out as described previously (20, 24). To label the 50- and 47-kDa subunits, a solubilized membrane-enriched fraction of desheathed *Aplysia* nervous tissue from two animals was dispensed into twelve 10-μl aliquots (~3 mg/ml protein) in 1.5-ml centrifuge tubes. Each aliquot was mixed with 10 μl of 2 μM

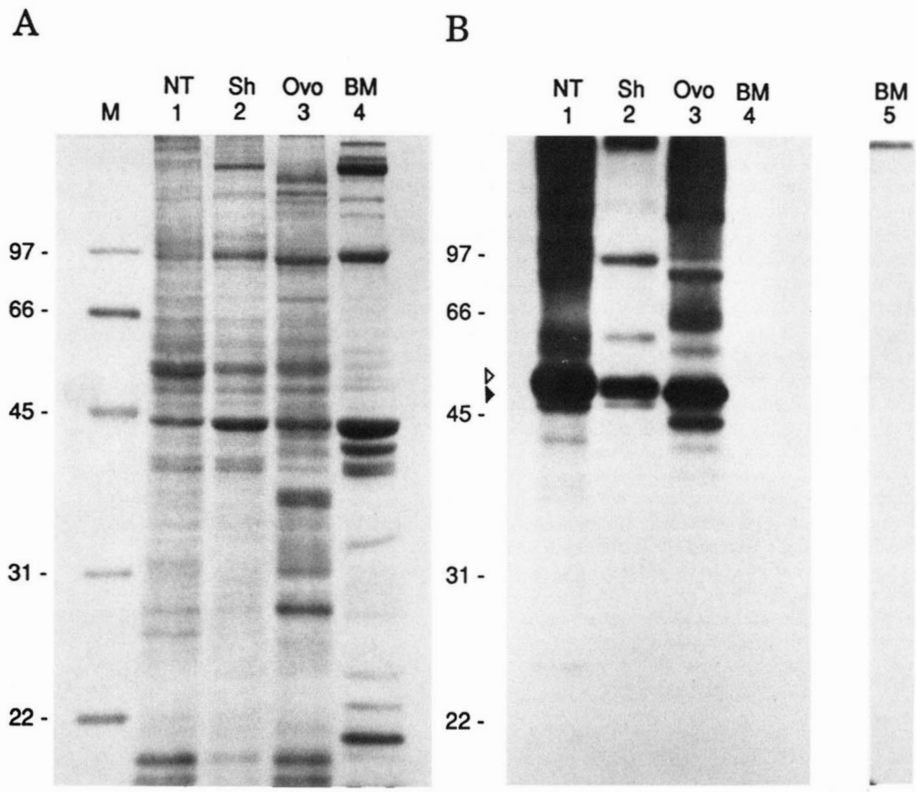

FIG. 1. **Phosphoproteins from various *Aplysia* tissues.** *A*, Coomassie Blue-stained 12.5% SDS-polyacrylamide gel showing polypeptide profiles of unfractionated tissue homogenates from desheathed ganglia (*NT*, *lane 1*), connective sheath (*Sh*, *lane 2*), ovotestis (*Ovo*, *lane 3*), and buccal muscle (*BM*, *lane 4*). *M*, molecular weight markers. The homogenates (40 μl) were phosphorylated with [γ-^{32}P]ATP by treatment with $C_{APL\text{-}A}$N1A1 (2 ng) for 5 min at 30 °C (see "Experimental Procedures"). Equal amounts of protein (20 μg) were run in each lane. *B*, autoradiogram of the same gel. Lanes as in *A*, except that a longer exposure of the buccal muscle polypeptides (*lane 4*) is displayed as *lane 5*. The 50-kDa (*open arrowhead*) and 47-kDa (*filled arrowhead*) phosphoproteins are indicated. The experiment shown here and the experiments displayed in subsequent figures were done at least three times with similar results.

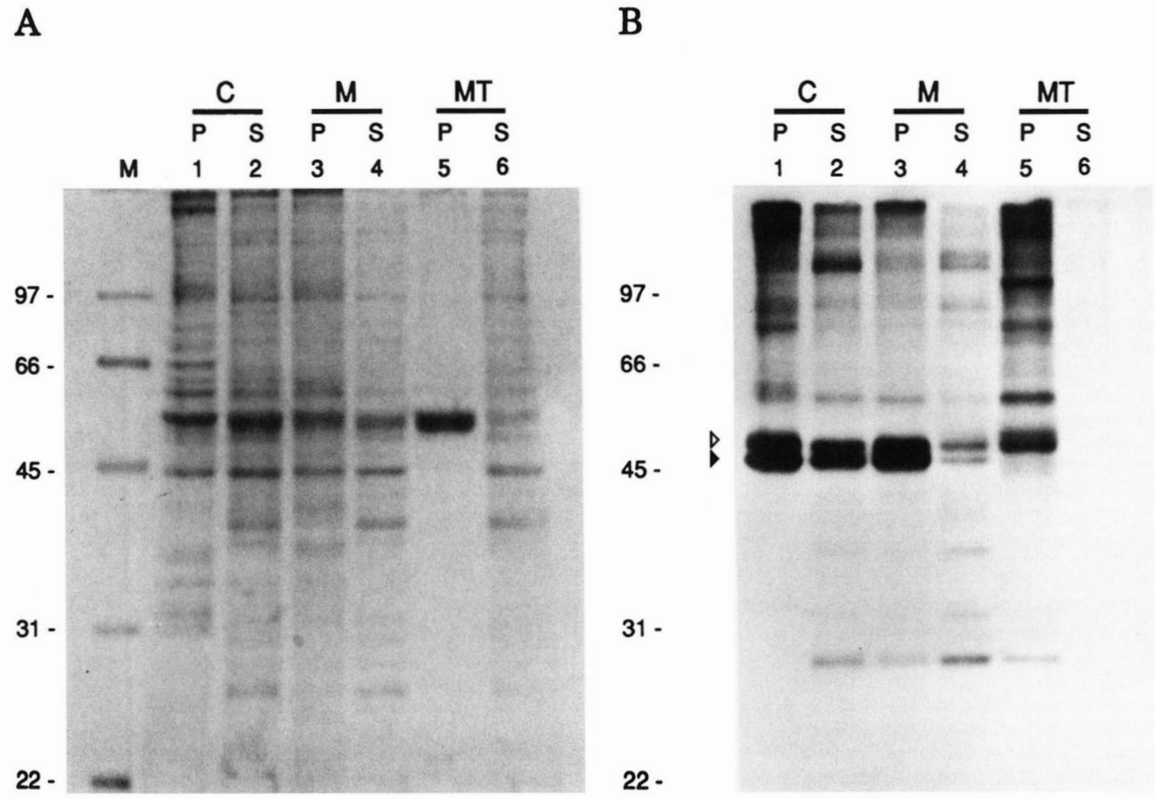

FIG. 2. **Subcellular localization of the 50- and 47-kDa phosphoproteins of *Aplysia* nervous tissue.** *A*, Coomassie Blue-stained 12.5% gel SDS-polyacrylamide gel showing polypeptides from various subcellular fractions: cytoskeleton (*C*) pellet and supernatant (*P* and *S*, *lanes 1* and *2*), membrane (*M*) pellet and supernatant (*lanes 3* and *4*) and taxol-stabilized microtubule (*MT*) pellet and supernatant (*lanes 5* and *6*). Equal amounts of protein (20 μg) were loaded in each lane. *B*, autoradiogram of the same gel showing profiles of the various subcellular fractions after phosphorylation with [γ-^{32}P]ATP by treatment with $C_{APL\text{-}A}$N1A1 (2 ng) for 5 min at 30 °C in a volume of 40 μl (see "Experimental Procedures"). *M*, molecular weight markers. The 50-kDa (*open arrowhead*) and 47-kDa (*filled arrowhead*) phosphoproteins are indicated.

[^{32}P]8-azido-cAMP (50 Ci/mmol; ICN, Costa Mesa, CA). After 10 min on ice, the samples were irradiated with a 254-nm lamp (Mineralight UVG-11) at a distance of 4 cm for 5 min. Type II R subunits were then extracted by Affi-Gel-Ht 31 affinity chromatography, separated in an 8% SDS-polyacrylamide gel and located by autoradiography. Slices containing one or the other of the two labeled polypeptides from the ~50 kDa region of the gel were treated with CNBr for 16 h at room temperature. Labeled fragments were then separated by electrophoresis in a second gel containing 15% polyacrylamide, 6 M urea, and 0.2% SDS in buffer containing 0.1 M sodium phosphate (pH 7.2) and 0.2% SDS as detailed previously (20). The gel was dried and subjected to autoradiography. For comparison, purified recombinant $R_{APL\text{-}N4}$ (0.1 μg in 10 μl) was similarly treated with omission of the affinity chromatography step.

Overlay Detection of RII-binding Proteins—Bovine RIIα was phosphorylated *in vitro* by the addition of 1 ng of purified $C_{APL\text{-}A}$N1A1 kinase to a reaction mix (25 μl) consisting of 50 mM MOPS, pH 6.8 (titrated with NaOH), 15 mM $MgCl_2$, 10 μM cAMP containing 1 μg of RIIα and 4 pmol of [γ-^{32}P]ATP (3000 Ci/mmol). After incubation on ice for 1 h, remaining [γ-^{32}P]ATP was removed by gel filtration on a Bio-Gel P-6DG column (5 ml) equilibrated with 10 mM potassium phosphate (pH 7.4), 150 mM NaCl containing 0.1% BSA. The incorporation of labeled phosphate was typically >2 × 10^7 cpm/μg RII. RII-binding proteins from *Aplysia* tissues were detected by a solid-phase overlay technique (46, 47). Fractions to be analyzed were separated in a 7.5% SDS-polyacrylamide gel and then electroblotted onto nitrocellulose in 25 mM Tris-HCl, 192 mM glycine, 0.1% SDS, and 20% methanol (150 mA for 2 h). Blots were overlayed for 15 h at 4 °C with ^{32}P-labeled RIIα (1–2 × 10^5 cpm/ml) in 10 mM sodium phosphate (pH 7.4), 150 mM NaCl, 0.02% (w/v) NaN_3, containing 5% dry milk powder and 0.1% BSA, in the presence or absence of 1 μM Ht 31 peptide. After washing the blots, RII-binding proteins were detected by autoradiography.

RESULTS AND DISCUSSION

Phosphorylation of Two Neuronal Polypeptides of 50 and 47 kDa by Aplysia PKA—SDS-PAGE analyses of labeled extracts generated by incubating unfractionated homogenates from desheathed ganglia, connective sheath, ovotestis, and buccal muscle with [γ-^{32}P]ATP and a catalytic subunit of *Aplysia* PKA ($C_{APL\text{-}A}$N1A1) reveal the phosphorylation of a number of polypeptides (Fig. 1). Two polypeptides of 50 and 47 kDa are most evident in nervous tissue and are the focus of this work. Coomassie Blue-stained protein profiles of equal masses of protein from the isolated desheathed ganglia and the surrounding connective sheath (Fig. 1*A*), as well as their corresponding phosphorylation patterns (Fig. 1*B*), exhibit several differences, which demonstrates effective removal of the sheath. Interestingly, nervous tissue proteins were phosphorylated to the highest extent compared with proteins from other tissues. Buccal muscle proteins showed relatively low levels of phosphorylation except for a very high molecular mass species (>500 kDa: Fig. 1*B*, *lane 5*), which is perhaps the same polypeptide that undergoes phosphorylation in intact *Aplysia* ARC muscle in response to neuropeptides (48).

To determine whether the 50- and 47-kDa polypeptides are differentially localized in neurons, subcellular fractionation was performed on desheathed ganglia. Equal amounts of protein from both the pellets and supernatants of three major neuronal subcellular fractions (cytoskeleton, membranes, and microtubules) were phosphorylated *in vitro* and analyzed by SDS-PAGE (Fig. 2). Phosphorylation profiles show different amounts of the two polypeptides in the various fractions (Fig. 2*B*). The membrane-enriched pellet fraction (Fig. 2, *A* and *B*, *lanes 3*) shows the highest levels of the two polypeptides, which are also enriched in the cytoskeleton pellet fractions (Fig. 2, *A* and *B*, *lanes 1*). A striking observation is the selective enrichment of the 50-kDa phosphoprotein in the microtubule pellet fraction. The corresponding Coomassie Blue-stained profiles

(Fig. 2*A*) of the various fractions are different, indicating that effective fractionation is achieved.

Phosphorylation by Endogenous PKA—Upon addition of [γ-^{32}P]ATP to *Aplysia* neural extracts, the 50- and 47-kDa polypeptides are also phosphorylated in the absence of exogenous C subunit. The phosphorylation occurs with washed membrane fractions in the absence of added cAMP and in the presence of protein kinase inhibitor peptide (PKIP), suggesting that the endogenous kinase is either the C subunit of PKA bound to the substrates, or a different kinase. The former interpretation would be in keeping with the identification of the 50- and 47-kDa polypeptides as type II R subunits of PKA (see below).

To determine whether PKA phosphorylates the 50- and 47-kDa polypeptides on the same sites as the endogenous kinase, a solubilized neuronal membrane fraction was phosphorylated *in vitro* in the absence (Fig. 3*A*, *lanes 1–5*) or presence (Fig. 3*A*, *lanes 6–10*) of exogenous C subunit. Phosphopeptide mapping was then performed to determine whether the enhanced phosphorylation observed with the exogenous kinase is on the same amino acid residues phosphorylated by the endogenous kinase. The phosphopeptide profiles of the 50-kDa polypeptides, generated by staphylococcal V8 protease treatment after phosphorylation in the presence or absence of exogenous PKA, were very similar (Fig. 3*B*). The profiles of the 47-kDa polypeptides were also closely related to each other (Fig. 3*B*). Again, the phosphopeptide maps obtained after exhaustive trypsin treatment of the pair of 50-kDa polypeptides (or of the 47-kDa polypeptides) were also very similar (data not shown). Hence, phosphorylation occurs on common sites, in the presence or absence of exogenous C subunit, and therefore these data suggest that the endogenous kinase is PKA. However, phosphorylation on the common sites by a different endogenous kinase cannot be strictly eliminated. For example, PKA and Ca^{2+}/calmodulin-dependent kinase phosphorylate the same site in CREB (49, 50).

Because the peptide maps of the 50- and 47-kDa polypeptides diverge from each other (Fig. 3*B*), the data further suggest that these polypeptides are not derived, one from the other, by a post-translational modification such as a proteolytic cleavage. However, the possibility that the phosphorylated bands represent closely related polypeptides phosphorylated on two different sites cannot be ruled out.

The 50- and 47-kDa Phosphoproteins Bind cAMP and Ht31—Rapid phosphorylation by C subunits is a property of mammalian type II R subunits (51). Therefore, experiments were performed to determine whether the 50- and 47-kDa phosphorylated polypeptides might be *Aplysia* RII. Nervous tissue and ovotestis membrane fractions were labeled with [γ-^{32}P]ATP and $C_{APL\text{-}A}$N1A1 kinase, followed by adsorption onto cAMP-agarose. After a high-salt wash, bound proteins were eluted and analyzed by SDS-PAGE. For both tissues, the 50- and 47-kDa polypeptides were depleted in flow-through fractions (Fig. 4*A*, *lanes 3* and *4*), relative to bound fractions (Fig. 4*A*, *lanes 5* and *6*). The 50- and 47-kDa phosphorylated polypeptides are thus cAMP-binding proteins.

Additional evidence for RII subunits in *Aplysia* neural extracts was sought with the aid of an Affi-Gel-Ht 31 affinity matrix. Ht 31 is a human thyroid protein that binds RIIα subunits or type II holoenzyme with nanomolar affinity (32). A synthetic peptide representing residues 493–515, comprising the minimum region of the Ht 31 protein required for RII binding, was linked to Affi-Gel-15 to generate an affinity matrix. The 50- and 47-kDa polypeptides, from ^{32}P-labeled neural membrane extracts, bound to the matrix (Fig. 4*B*, *lane 2*) and could be eluted with Ht 31 peptide (Fig. 4*B*, *lane 3*). Binding was prevented by Ht 31 peptide (Fig. 4*B*, *lane 1*). The other

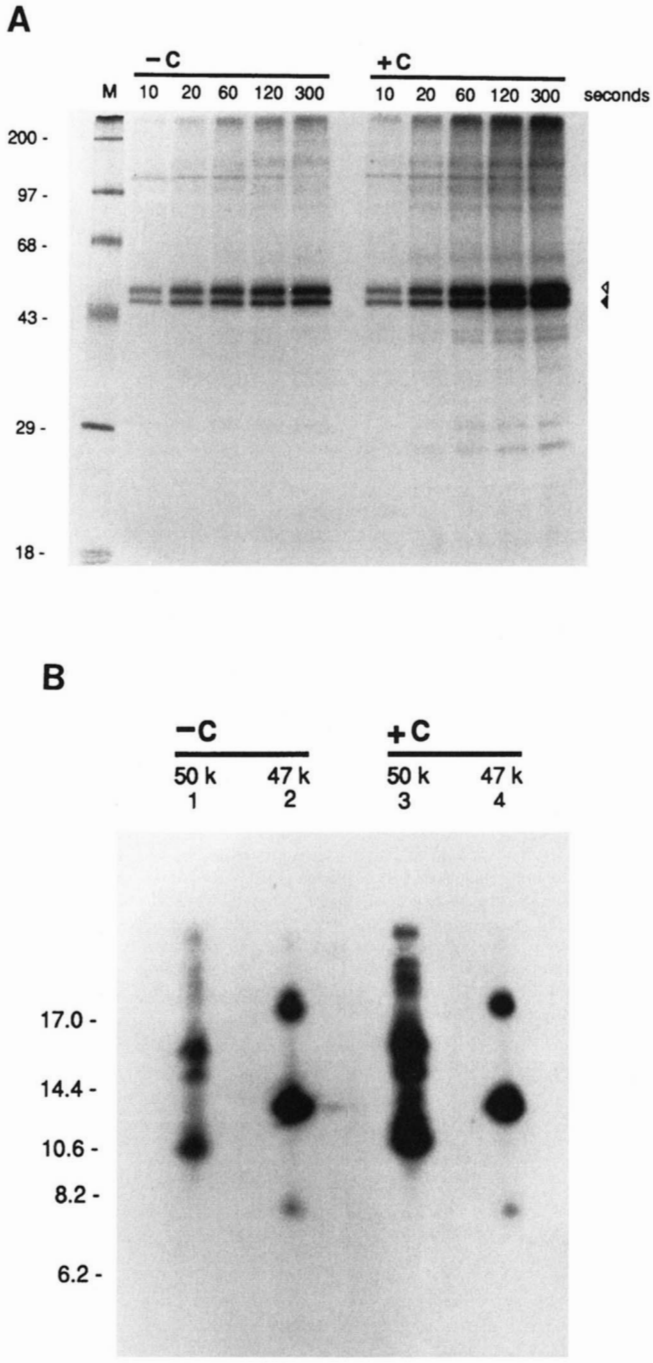

FIG. 3. **PKA is probably responsible for endogenous phosphorylation of the 50- and 47-kDa polypeptides in *Aplysia* nervous tissue.** *A*, exogenous C enhances the phosphorylation of the two polypeptides. Autoradiogram of a 12.5% SDS-polyacrylamide gel showing phosphorylation of polypeptides in a solubilized membrane fraction of desheathed ganglia in the absence (*-C*, *lanes 1–5*) or presence (*+C*, *lanes 6–10*) of exogenous PKA ($C_{APL\text{-}A}$N1A1, 2 ng in 100 µl) after 10, 20, 60, 120, and 300 s at 30 °C. *M*, ^{14}C-labeled molecular weight markers. The 50-kDa (*open arrowhead*) and 47-kDa (*filled arrowhead*) phosphoproteins are indicated. *B*, endogenous protein kinase activity and exogenous C subunit phosphorylate the 50- and 47-kDa polypeptides on the same sites. Comparison of staphylococcal V8 protease phosphopeptide maps of the 50-kDa (*lanes 1* and *3*) and 47-kDa (*lanes 2* and *4*) phosphoproteins. The latter were generated by phosphorylation in the absence (*-C*, *lanes 1* and *2*) or presence (*+C*, *lanes 3* and *4*) of exogenous $C_{APL\text{-}A}$N1A1 kinase.

phosphoproteins in the extract (*e.g.* Fig. 4*A*, *lane 1*) did not bind.

Comparison with $R_{APL\text{-}N4}$—The ability to be phosphorylated by the C subunit of PKA and to bind to Ht 31 are regarded as

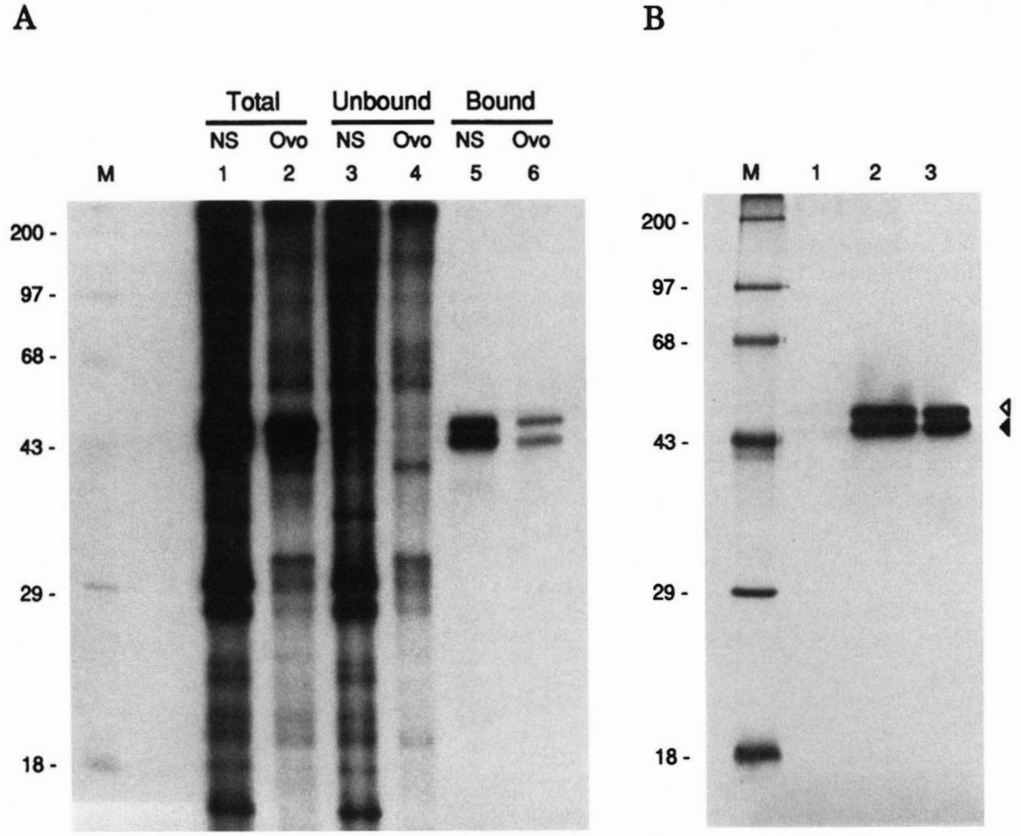

FIG. 4. **The 50- and 47-kDa phosphoproteins bind cAMP and Ht 31.** *A*, autoradiogram of a 12.5% SDS-polyacrylamide gel comparing the phosphorylation profiles of polypeptides from solubilized membranes from complete ganglia (*NS*, *lanes 1*, *3*, and *5*) and ovotestis (*Ovo*, *lanes 2*, *4*, and *6*). Phosphorylated extracts (*lanes 1* and *2*) were applied to cAMP-agarose and the unbound fractions were collected (*lanes 3* and *4*). After a wash with 1.5 M NaCl, cAMP-binding proteins were eluted with Laemmli sample buffer (*lanes 5* and *6*). *B*, autoradiogram of a 12.5% SDS-polyacrylamide gel showing phosphorylated polypeptides from solubilized membranes from desheathed ganglia that bind to Affi-Gel-Ht 31 in the presence (*lane 1*) or absence (*lanes 2* and *3*) of competing Ht 31 peptide (0.5 mM). Ht 31-binding proteins were eluted with hot gel loading buffer (*lanes 1* and *2*) or with binding buffer containing 0.5 mM Ht 31 peptide (*lane 3*). The 50-kDa (*open arrowhead*) and 47-kDa (*filled arrowhead*) phosphoproteins are indicated.

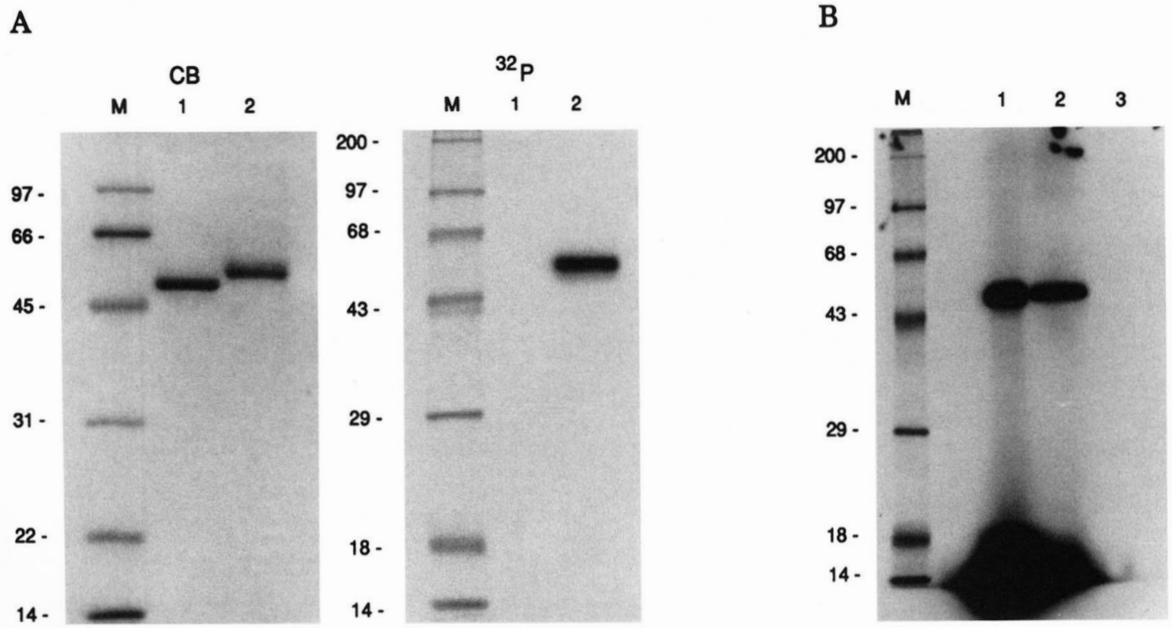

FIG. 5. ***Aplysia* neuronal type I R subunit (R_{APL-N4}) is not phosphorylated by PKA and does not bind Ht 31.** *A*, 12.5% SDS-polyacrylamide gel of purified recombinant R_{APL-N4} (*lane 1*) and purified bovine RIIα (*lane 2*) after phosphorylation with 1 nM [γ-^{32}P]ATP by using C_{APL-A}N1A1 (1 ng) at 0 °C for 20 min. Equal amounts of protein (2 μg) were loaded in each lane. *CB*, Coomassie Blue staining (*M*: unlabeled molecular weight markers). ^{32}P, autoradiogram of the same gel (*M*: ^{14}C-labeled molecular weight markers). Phosphorylated RII has a slightly lower mobility than the unphosphorylated polypeptide. *B*, recombinant R_{APL-N4} does not bind to Affi-Gel-Ht 31. R_{APL-N4} was photoaffinity labeled with [^{32}P]8-azido-cAMP and incubated with Affi-Gel-Ht 31. An autoradiogram of a 12.5% SDS-polyacrylamide gel displays: ^{14}C-labeled markers (*M*); ^{32}P-labeled R_{APL-N4} before application to the matrix (*lane 1*, one-tenth of loaded volume); the column flow-through (*lane 2*, one-tenth of loaded volume); material eluted with 0.5 mM Ht 31 peptide (*lane 3*, half of total eluate).

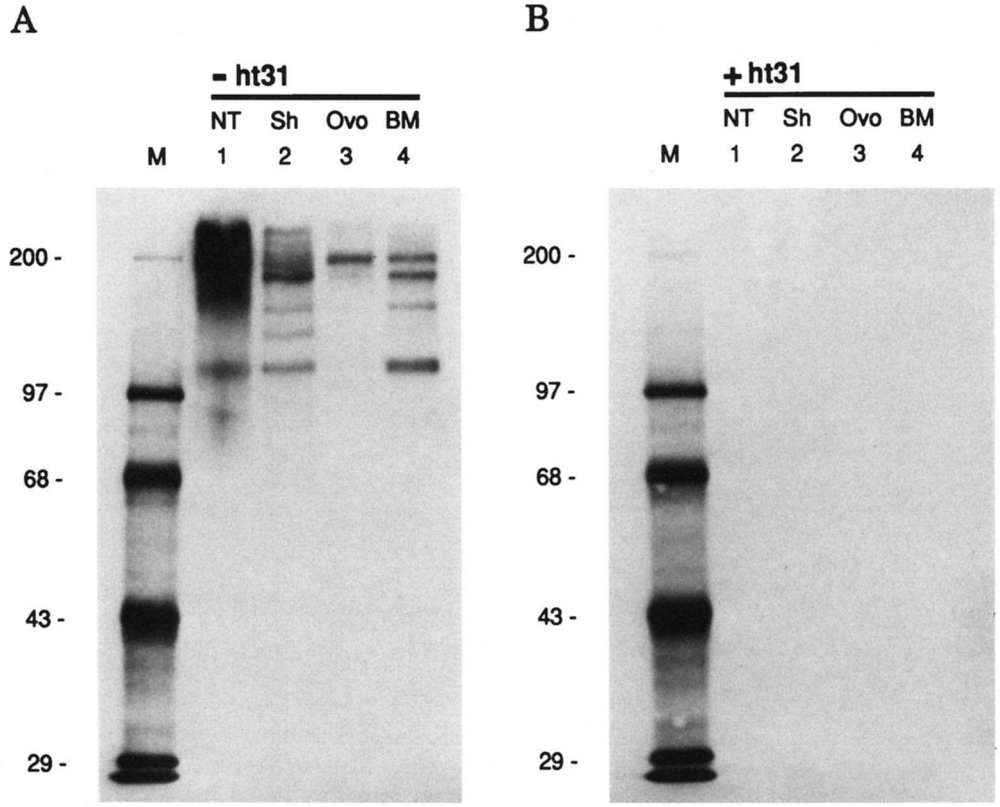

FIG. 6. **RII-binding proteins exist in various *Aplysia* tissues.** *A*, autoradiogram of a nitrocellulose blot showing RII-binding proteins in unfractionated *Aplysia* tissue homogenates from desheathed ganglia (*NT*, *lane 1*), connective sheath (*Sh*, *lane 2*), ovotestis (*Ovo*, *lane 3*), and buccal muscle (*BM*, *lane 4*). The blot was treated with ^{32}P-labeled bovine RIIα. *M*, ^{14}C molecular weight markers. The unlabeled ~500-kDa marker is not visible. Equal amounts of protein (20 μg) were run in each lane of a 7.5% SDS-polyacrylamide gel prior to transfer to nitrocellulose. *B*, duplicate blot treated with ^{32}P-labeled bovine RIIα in the presence of 1 μM Ht 31 peptide.

properties of type II R subunits that are not shared by type I subunits. These criteria were upheld when the previously cloned Aplysia type I subunit, $R_{APL\text{-}N4}$, was examined *in vitro*; recombinant $R_{APL\text{-}N4}$ was not readily phosphorylated by $C_{APL\text{-}A}N1A1$ (Fig. 5*A*), nor did it bind to Affi-Gel Ht 31 (Fig. 5*B*). Moreover, CNBr cleavage of Ht 31-affinity-purified RII subunits from *Aplysia* nervous tissue that had been photoaffinity labeled with [^{32}P]8-azido-cAMP generated peptide fragments of 12 and 9.5 kDa (data not shown), which had previously been derived from the neuronal $R_{APL\text{-}N2}$ subunit (20). These fragments are distinct from the 18-kDa CNBr fragment that is characteristic of the type I neuronal R subunits $R_{APL\text{-}N1}$, $R_{APL\text{-}N3}$, $R_{APL\text{-}N4}$, and $R_{APL\text{-}N5}$ (20). We cannot conclude definitively that either the 50- or the 47-kDa phosphorylated polypeptide is $R_{APL\text{-}N2}$ (47 kDa) because phosphorylation can produce appreciable changes in the electrophoretic mobility of RII subunits (52). However, the data strongly suggest that $R_{APL\text{-}N2}$ is a type II subunit.

We earlier found that the *Aplysia* type I R subunit, $R_{APL\text{-}N4}$, exhibits similar overall sequence identity to mammalian RIα (79%) and RIβ (76%). Therefore, the α and β forms of mammalian RI, which are closely related to each other (83% identity), are likely to have arisen after the phylogenetic branchpoint that led to vertebrates and molluscs. But, because mammalian RIIα and RIIβ are more divergent (66% identity) than the RI forms, it is possible that the 50- and 47-kDa phosphoproteins represent *Aplysia* α and β subunits.

RII-binding Proteins Exist in Various Aplysia Tissues—Because the 50- and 47-kDa phosphorylated polypeptides bind both cAMP and Ht 31, it is reasonable to conclude that they are type II R subunits homologous to those found in mammals. Therefore, the presence of RII-binding proteins in *Aplysia* nervous tissue was examined next. Because sufficient amounts of the two candidate RII proteins could not be obtained from available *Aplysia* nervous tissue (the entire nervous system from one animal contains ~1 mg of protein), RIIα from bovine heart was used as a probe for detecting RII-binding proteins on blots of *Aplysia* proteins separated by SDS-PAGE (46, 47). The success of this approach relies on the evolutionary conservation of the interacting sites on the RII molecule and its corresponding binding proteins, which is demonstrated below.

Bovine heart RIIα was labeled to high specific radioactivity (>2 × 10^7 cpm/μg) with [γ-^{32}P]ATP and *Aplysia* C subunit ($C_{APL\text{-}A}N1A1$) and used to probe a blot of polypeptides from various *Aplysia* tissues (Fig. 6*A*). At least seven RII-binding proteins (BP 1–7, see Fig. 7) of high molecular mass (>100 kDa) are visualized by this procedure. The RII-binding proteins have different distributions in the various tissues (Fig. 6*A*). Interestingly, the bovine RII subunit is sufficiently homologous to the *Aplysia* RII polypeptides to form heterodimers as evidenced by the attachment of the labeled bovine probe to nitrocellulose-bound *Aplysia* R subunits (data not shown). The binding of ^{32}P-labeled RIIα to the *Aplysia* RII-binding proteins was completely blocked by 1.0 μM Ht 31 peptide (Fig. 6*B*).

Aplysia RII-binding Proteins Exhibit Differential Subcellular Localization—To investigate whether the RII-binding proteins identified in *Aplysia* nervous tissue are localized to discrete compartments within the cell, subcellular fractions were examined by the blot overlay technique. The seven high molecular mass neuronal RII-binding proteins (BP1, 430 kDa, BP2, 310 kDa, BP3, 220 kDa, BP4, 190 kDa, BP5, 160 kDa, BP6, 140 kDa, BP7, 110 kDa) exhibit preferential localization to various subcellular compartments (Fig. 7). BP1 and BPs 3 through 7 are enriched in the crude membrane (particulate) fraction (Fig. 7*A*, *lane 3*), which is likely to include remnants of the cytoskeleton, and are largely solubilized by detergent as seen in the

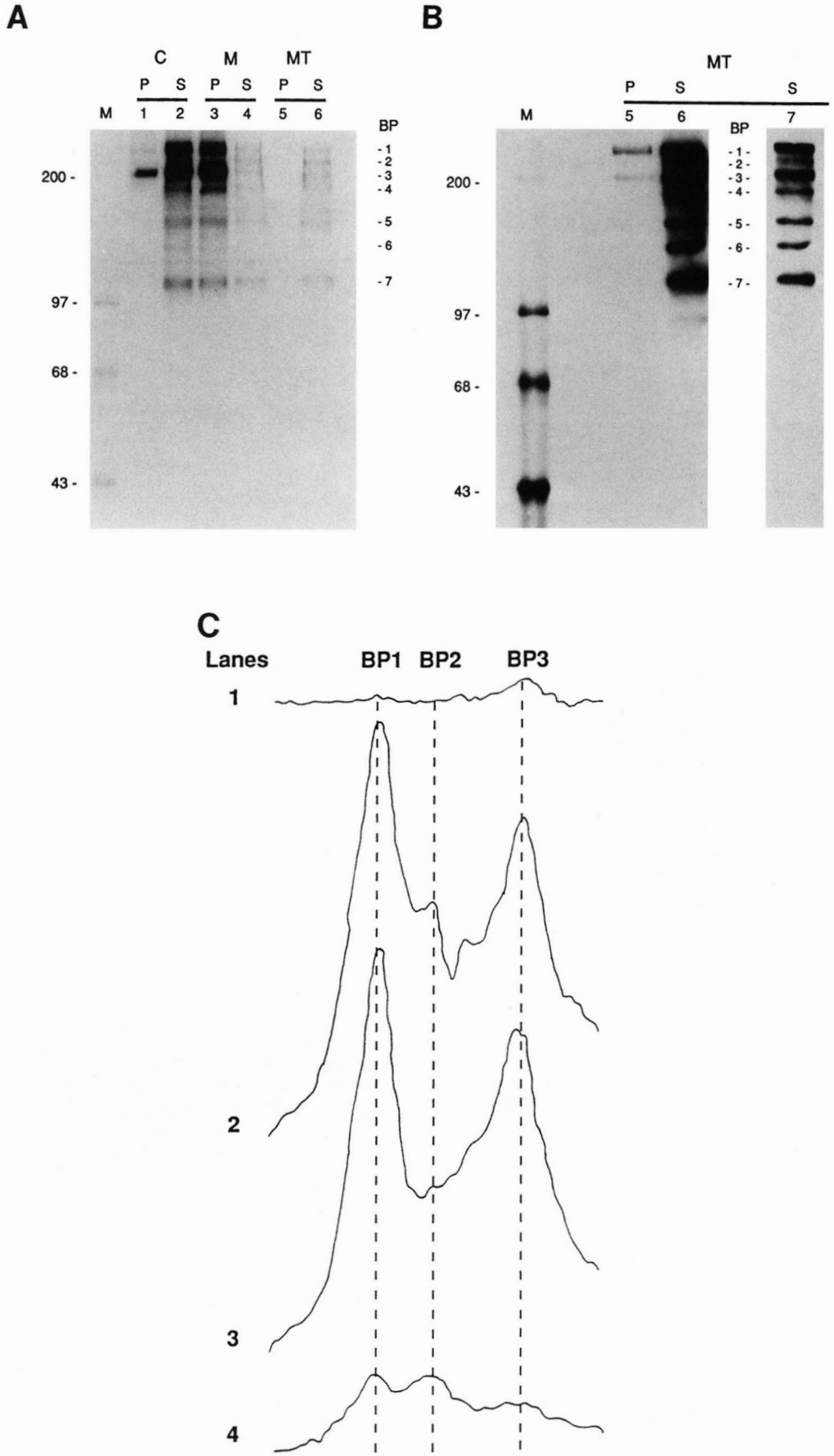

FIG. 7. **Neuronal RII-binding proteins of *Aplysia* exhibit differential subcellular localization.** *A*, autoradiogram of a blot after treatment with ^{32}P-labeled bovine RIIα as a probe for binding proteins in various subcellular fractions: cytoskeleton (*C*) pellet, and supernatant (*P* and *S*, *lanes 1* and *2*), membrane (*M*) pellet and supernatant (*lanes 3* and *4*), and microtubule (*MT*) pellet and supernatant (*lanes 5* and *6*). Equal amounts of protein (20 μg) were run in each lane of a 7.5% SDS-polyacrylamide gel prior to transfer to nitrocellulose. *M*: ^{14}C molecular weight

cytoskeleton supernatant (Fig. 7*A*, *lane 2*). BP2 is found exclusively in the supernatant, in all three fractionation procedures (Fig. 7, *A–C*). BP3 and to a lesser extent BP1 are associated with the cytoskeleton fraction (Fig. 7, *A* and *C*, *lanes 1*), while BP1 is the predominant species in taxol-stabilized microtubules (MTs; Fig. 7*B*, *lane 5*). In both these cases, the RII-binding proteins are also found in the corresponding supernatants. A plausible interpretation is that BP1 and BP3 are membrane proteins with, respectively, attachment sites for MTs and for another component of the cytoskeleton. The presence of BP1 in MTs is interesting because it may serve to anchor the 50-kDa type II R subunit, which is enriched over the 47-kDa species in this fraction.

Conclusions—In short and long term facilitation of sensory neurons in *Aplysia*, PKA carries out several roles that are spatially and temporally separated. Therefore, because the enzyme has broad substrate specificity *in vitro*, its activity must be regulated *in vivo* to prevent chaotic phosphorylation. At least three phenomena may contribute to this regulation.

First, when different regions of a sensory cell are stimulated with the same concentration of 5HT, the elevation of cAMP might not be evenly distributed throughout the cell. Further, the spatial distributions of cAMP might differ after treatments that produce short and long term facilitation. Recent direct evidence for compartmentation of cAMP in *Aplysia* sensory neurons in response to the application of 5HT has been provided by monitoring energy transfer between exogenous fluorescent R and C subunits with confocal microscopy (10). After the application of low concentrations of 5HT, cAMP levels were elevated in the processes of neurons compared with levels found in the cell body. Only strong or repeated stimulation resulted in levels of cAMP in the cell body sufficient to release C subunit from holoenzyme located in this region and thereby allow its translocation into the nucleus, where it can trigger long term changes in neuronal properties (18). The spatial distributions of cAMP are likely to be a consequence of cell geometry (10), although the subcellular distribution of adenylate cyclase may play a role (53).

Second, there may be selective facilitatory neural inputs to different regions of sensory neurons (22, 54). While evidence has been presented for serotonergic inputs at both the cell body of sensory neurons and their presynaptic terminals (55, 56), there is as yet no evidence for selective activation of these pathways *in vivo* (22), although synapse-specific short term facilitation has been demonstrated after activation of facilitatory interneurons that project to specific sets of terminals (54). Nevertheless, it is striking that short term and cell wide long term facilitation can be independently activated by the selective experimental application of 5HT to, respectively, sensory neuron terminals and cell bodies (22). In addition, long term facilitation can be induced by strong 5HT stimulation of peripheral sensory terminals, which suggests the existence of a retrograde signal to the cell nucleus (23), as well as the anterograde signal suggested by the facilitation induced by stimulating cell bodies. The retrograde signal could simply be the diffusion of cAMP into the cell body, which has been observed in cells in culture (10), or more likely, given the distances involved in the animal, the retrograde transport of a protein (perhaps a C subunit of PKA) to the cell nucleus (57).

Third, the response to elevated cAMP may be concentration-dependent. At a given intracellular concentration, cAMP may activate one set of signal transduction pathways, while at a higher concentration additional pathways may be activated even if there is no spatial variation in cAMP concentration. For example, this would occur if PKA holenzymes exist with different affinities for cAMP expressed in combination with different subcellular locations, substrate specificities or translocation properties. A possible example is the modulation of different K^+ channels at different intracellular cAMP concentrations in *Aplysia* sensory cells (7). The present paper and related work by us and others (20, 24–27) lend support for the third possibility because they demonstrate PKA subunits with structural diversity that is translated into functional diversity at the level of affinity for cAMP (27), substrate specificity (27), and cellular location (24). The RII subunits demonstrated here are likely to contribute to the functional diversity of *Aplysia* PKA because they have different distributions of subcellular locations, which are mediated by a diverse set of RII-binding proteins. Further, in mammals, the affinity for C of RII, but not of RI, is modulated by autophosphorylation (58) and may contribute thereby to neuronal modulation (59–61).

Accordingly, it seems likely that a complex interplay of phenomena including compartmented second messengers, local synaptic inputs, and functional diversity within signal transduction pathways will account for the spatial and temporal regulation of PKA in neurons. Further experimentation must be done to define the contributions of each aspect of signaling diversity.

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markers. The unlabeled ~500-kDa marker is not visible. *BP1–7* are RII-binding proteins. A faint band at the position of migration of the *Aplysia* RII subunits is not visible in this exposure. *B*, the overlay with the microtubule pellet and supernatant was repeated with 60 μg of protein/lane and with ^{32}P-labeled RIIα at 10^6 cpm/ml in the overlay. *Lane 7* is a shorter exposure of *lane 6*, which more clearly shows the RII-binding proteins. *C*, densitometric scans of autoradiograms showing *BP1–3* in a cytoskeleton pellet and supernatant (*lanes 1* and *2*) and a membrane pellet and supernatant (*lanes 3* and *4*). The experiment was a duplicate of that displayed in *A*. *BP2* is present only in the supernatants (*lanes 2* and *4*).

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