A Phosphatidylinositol 4,5-*bis*phosphate–sensitive Casein Kinase Iα Associates with Synaptic Vesicles and Phosphorylates a Subset of Vesicle Proteins

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Abstract. In interphase cells, α-casein kinase I (α-CKI) is found associated with cytosolic vesicular structures, the centrosome, and within the nucleus. To identify the specific vesicular structures with which α-CKI is associated, established cell lines and primary rat neurons were immunofluorescently labeled with an antibody raised to α-CKI. In nonneuronal cells, α-CKI colocalizes with vesicular structures which align with microtubules and are partially coincident with both Golgi and endoplasmic reticulum markers. In neurons, α-CKI colocalizes with synaptic vesicle markers. When synaptic vesicles were purified from rat brain, they were highly enriched in a CKI, based on activity and immunoreactivity. The synaptic vesicle–associated CKI is an extrinsic kinase and was eluted from synaptic vesicles and purified. This purified CKI has properties most similar to α-CKI. When the activities of casein kinase I or II were specifically inhibited on isolated synaptic vesicles, CKI was shown to phosphorylate a specific subset of vesicle proteins, one of which was identified as the synaptic vesicle-specific protein SV2. As with α-CKI, the synaptic vesicle CKI is inhibited by phosphatidylinositol 4,5-*bis*phosphate (PIP_2). However, synthesis of PIP_2 was detected only in plasma membrane–containing fractions. Therefore, PIP_2 may spatially regulate CKI. Since PIP_2 synthesis is required for secretion, this inhibition of CKI may be important for the regulation of secretion.

CELLS transport proteins and lipids throughout their cytoplasm using small vesicles containing specific integral membrane proteins (2–5, 13, 24, 35, 38, 52, 58, 62, 72–74). This transport system is microtubule dependent and requires motor proteins (16, 19, 31). In this process, vesicles bud from donor membranes and are shunted to specific acceptor membranes where the vesicles fuse and release their contents. These general events can be divided into constituitive and regulated pathways (13, 17, 24, 35, 41, 65, 72–74). Although distinct, the two pathways appear to have similar components in common. A typical constitutive pathway would be the shuttling of vesicles within and between the endoplasmic reticulum, the Golgi organelle, and the plasma membrane. The movement and Ca^{2+}-dependent fusion of synaptic vesicles provides an example of a regulated secretory pathway.

Südhof and Jahn (74) have divided synaptic vesicle transport, docking, exocytosis, and recycling into seven discrete stages. Many of the factors involved in these processes and their respective roles have now been identified. For example, two vesicle-associated proteins, synaptophysin and synaptobrevin, and two proteins integral to the plasma membrane, syntaxin and neurexin are involved in docking (4, 5, 10, 11, 26, 62, 68, 75). Synaptotagmin, another vesicle integral membrane protein, is thought to act in both the positioning of vesicles at the plasma membrane and the Ca^{2+}-dependent step of the fusion process (12, 22, 34, 37, 55, 60, 61). Finally, the SNAPs (*N*-ethylmaleimide–sensitive factor-associated proteins) have been linked to vesicle targeting and fusion (11, 15, 35, 71, 77).

Within each of these stages, the myriad of processes involved are extensively regulated. Evidence exists showing that G-proteins (16–18, 23, 41, 42), phosphoinositide kinases (23, 45–48, 69), phospholipase D (23, 30) and protein kinases (9, 12, 14, 27, 33, 34, 36, 51, 52, 59, 70) play regulatory roles in vesicular trafficking, exocytosis, and long term potentiation. However, these mechanisms are not well defined. Moreover, it is likely that many of the kinases and their respective substrates remain to be defined. A growing number of protein kinases have been implicated in the regulation of neuronal transmission. Evidence demonstrates that α-CaM kinase II is involved in regulation of long-term potentiation and learning (8, 9, 27, 51, 57, 70). In addition, a number of other protein kinases have been localized to the synaptosome such as cAMP-dependent protein kinase, protein kinase C, *src* tyrosine kinase, a proline-directed protein kinase, and casein kinase II (12,

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27, 34, 51–54, 74). Direct association of these protein kinases with synaptic vesicles is less defined, but evidence suggests that CAM kinase (9) and an *src* tyrosine kinase (53) directly associate with synaptic vesicles. As mentioned above, protein phosphorylation events appear to be involved in regulating synaptic transmission (8, 14, 27, 57, 70). Therefore, the identification and characterization of additional protein kinases associated with synaptic vesicles could provide insight into not only the regulatory cascade involved in vesicle movement and fusion, but potentially also the constituitive vesicular trafficking pathways because of the functional conservation between the two. The association of a protein kinase with secretory vesicles suggests a role in vesicular trafficking. We describe here the localization and biochemical characterization of a casein kinase I which is associated with synaptic vesicles.

Materials and Methods

Antibodies

The 36-kD α-casein kinase I (α-CKI)[1] antibodies (21) and the type I and II PIP 5-kinase antibodies (7, 50) were prepared as previously described. The following antibodies were kind gifts: SV-2 mouse monoclonal antibody from Dr. Erik Schweitzer (UCLA, Los Angeles, CA), syntaxin polyclonal and synaptotagmin monoclonal antibodies from Dr. Richard Scheller (Stanford University, Stanford, CA), syntaxin monoclonal antibodies from Dr. Colin Barnstable (Yale University, New Haven, CT), synaptophysin monoclonal antibody from Dr. Pietro DeCamilli (Yale University, New Haven, CT). BiP monoclonal antibody was from Dr. David Bole (University of Michigan, Ann Arbor, MI). β-COP mouse monoclonal was from Dr. Thomas E. Kreis (EMBL, Heidelberg, Germany). N-CAM monoclonal antibody was purchased from Sigma Chem. Co. (St. Louis, MO). Synaptophysin antibody was also purchased from Boehringer-Mannheim Corp. (Indianapolis, IN). Secondary antibodies were purchased from Jackson Immunoresearch (West Grove, PA).

Cell Culture and Immunofluorescence Labeling

CHO and LLC-PK (porcine kidney) cells were grown in Ham's F-10 or DME, respectively, with 10% FBS. PC-12 cells were grown in DME with 10% FBS and 5% horse serum. All cells were grown at 37°C with 5% CO_2. PC-12 cells were differentiated by addition of nerve growth factor and dibutyl-cyclic adenosine monophosphate. After 2 d of treatment, growth cones were readily apparent and, after 6 d, extensive axonal arrays had developed. For immunofluorescence, cells were grown on glass coverslips and fixed with −20°C methanol. Nonspecific sites were then blocked with 3% BSA in PBS (pH 7.4) for 60 min at room temperature or overnight at 4°C. Coverslips were incubated with primary antibodies for 60 min at room temperature or overnight at 4°C and then washed with 1% BSA/PBS. Texas-red donkey anti–mouse IgGs and fluorescein isothiocyanate–conjugated goat anti–rabbit IgGs (Jackson Immunoresearch) at a dilution of 1:1,000 in 3% BSA/PBS were then applied to the coverslips and incubated for 60 min at room temperature. Coverslips were washed as before, and then mounted on slides with 90% glycerol/PBS supplemented with 1 mg/ml *p*-phenylenediamine and diazobicyclo-[2,2,2]-octane (DABCO) at a concentration of 100 mg/ml. Sympathetic ganglia were isolated from newborn rat pups and grown as previously described (1). For immunofluorescence, the neurons were grown on lamin-coated glass coverslips and fixed in one of two ways: cold methanol, as described above, or paraformaldehyde fixation. For paraformaldehyde fixation, neurons were initially rinsed in 37°C PBS, incubated with 4% paraformaldehyde/4% sucrose/PBS for 10–20 min at 23°C and extracted with 1% Triton X-100 in PBS for 10 min. These neurons were treated for immunofluorescence as above.

Primary antibodies were prepared for immunofluorescence at the following concentrations in 3% BSA/PBS: α-CKI rabbit polyclonal, 10 μg/ml; β-tubulin mouse monoclonal (Amersham, Corp. Arlington Heights, IL), 1:1,000 dilution of ascites; β-COP mouse monoclonal (Kreis), 1:100 dilution of ascites; BiP rat monoclonal (Bole), 1:25 dilution of ascites; SV-2 (Schweitzer), 1:20 dilution of ascites; synaptophysin rabbit polyclonal, 10 μg/ml; Synaptophysin mouse monoclonal (deCamilli), 1:25 dilution from ascites; Synaptophysin mouse monoclonal (Boehringer-Mannheim), 2 μg/ml.

As negative controls, 10 μg/ml of CKI antibody in 3% BSA/PBS was preincubated with CKI-Sepharose beads or pure, denatured CKI and then used for immunofluorescence as above. Cells were observed with a Zeiss Axiovert 135, or a Nikon Optiphot equipped with an MRC Lasersharp confocal imaging system (Bio-Rad Labs., Hercules, CA).

Synaptic Vesicle Isolation

Synaptic vesicles were isolated from rat cerebral cortex by the method of Huttner et al. (49), except for the following changes. Fresh rat brains were homogenized in cold "buffered sucrose" (320 mM sucrose, 4 mM Hepes-NaOH, pH 7.3, 1 mM EDTA, 0.1 mM EGTA, 10 mg/ml leupeptin, 0.1 mM PMSF, 0.1 mM diisopropylfluorophosphate [DFP]). The remaining methods were identical to that of Huttner et al. (49) except that all buffers contain the following: 1 mM EDTA, 0.1 mM EGTA, 10 μg/ml leupeptin, 0.1 mM PMSF, and 0.1 mM DFP. The resulting fractions were defined using the Huttner et al. nomenclature (49). In some preparations, the extensively washed, crude synaptosomal fraction (P2') was further purified by discontinuous Ficoll density gradient sedimentation. For Ficoll density gradient sedimentation, 5 ml of the P2 fraction (30 mg protein) was applied to a 25-ml discontinuous Ficoll gradient consisting of 10 ml of 13%, 5 ml of 9%, and 10 ml of 4% Ficoll (wt/vol). This was centrifuged for 50 min at 23,000 rpm in a SW28 rotor (Beckman Instrs., Fullerton, CA). Intact synaptosomes banded in the 9% Ficoll layer and were isolated by fractionating with a pipette. The 4% Ficoll fraction (F_1) and the 9–13% Ficoll fraction (F_2) were assayed for CKI and synaptophysin by Western blotting. The synaptic vesicles were released from the purified, washed synaptosomes by low osmolarity lysis and Dounce homogenization. The crude synaptic vesicles (LP_2 fraction) were isolated by differential centrifugation (49). The LP_2 fraction (4 ml) was further purified using continuous sucrose (50–800 mM, 25 ml) velocity gradient sedimentation. The gradient was centrifuged for 5 h at 65,000 *g*. The synaptic vesicles band at ~400 mM sucrose while most membranes sediment at the bottom of the gradient (sucrose gradient pellet, SGP). The band of synaptic vesicles (fractions 4–9) was pooled and concentrated by centrifugation. This synaptic vesicle fraction was further analyzed by Nycodenz equilibrium sedimentation. The synaptic vesicle pool in 4 ml of 5% (wt/vol) Nycodenz was loaded onto a 10–30% 25-ml Nycodenz gradient. This was centrifuged for 18 h at 22,000 rpm in a Beckman SW28 rotor, and 20 fractions of 1.5 ml each were collected. The SGP was washed by centrifugation and further homogenized with ten up-and-down strokes of a glass-on-glass Dounce homogenizer. This was followed by ten passages through a 27-gauge needle. This homogenate was centrifuged at 10,000 *g* for 15 min. The supernatant was analyzed by sucrose velocity gradient sedimentation, as above, or by equilibrium density gradient sedimentation on a 7–28% (wt/vol) Nycodenz gradient, centrifuged 18 h in a SW28 rotor at 22,000 rpm and fractionated as above.

Electron Microscopy

Subcellular fractions were fixed in suspension by addition of glutaraldehyde to a final concentration of 2%. The fixed membranes were sedimented and prepared for transmission electron microscopy according to Huttner et al. (49).

Western Blotting

For blotting, cells were lifted from culture dishes with PBS containing 2 mM EDTA, and lysed in hot Laemmli SDS-sample buffer. Protein concentration was determined as before (21). Proteins were then separated on a 7–15% SDS-PAGE and transferred to nitrocellulose. The membrane was blocked in PBS containing 5% nonfat dry milk and 0.05% Tween-20. Next, the membrane was incubated with 10 μg/ml affinity-purified CKI antibody in PBS with 3% BSA and 0.05% Tween-20 for 2 h at 23°C. Bound antibody was detected by incubating the membrane for 1 h with ^{125}I-protein A (Amersham, 2×10^5 cpm/ml) in PBS, 3% BSA and 0.05% Tween-20. The nitrocellulose was subsequently exposed to X-ray film.

Purification of the 36-kD CKI from Synaptic Vesicles

The 36-kD CKI was purified from the LP_2 membrane fraction of 200 rat

1. *Abbreviations used in this paper*: α-CKI, α-casein kinase I; DFP, diisopropylfluorophosphate; PIP_2, phosphatidylinositol 4,5-*bis*phosphate; PIP, phosphatidylinositol 4-phosphate; SGP, sucrose gradient pellet.

brains (530 mg of protein). The LP_2 membrane fraction in 40 mM buffered sucrose (49) was extracted by addition of dry KCl to a final concentration of 1 M. This solution was gently stirred for 30 min on ice and then centrifuged for 2 h at 230,000 *g*. The supernatant was dialyzed against 0.15 M NaCl, 10 mM sodium phosphate, pH 7.5, 2 mM dithiothreitol, 1 mM EDTA, 0.1 mM PMSF and 0.1 mM DFP (buffer A). This was applied to a 15-ml phosphocellulose column, washed with two column volumes of buffer A containing 250 mM NaCl, and eluted with a 0.25–1.0 M NaCl gradient. The column fractions were assayed using phosvitin and casein as substrates. Two peaks of kinase activity eluted at 0.54 and 0.72 M NaCl, respectively. The first peak of kinase activity corresponded to the 36-kD CKI by Western blotting and was pooled, concentrated, and applied to a Superose 12 column (Pharmacia LKB Bi), equilibrated with buffer A containing 0.5 M NaCl at a flow rate of 0.5 ml/min. Fractions were collected every 2 min starting 60 min after injection of sample. The column was standardized by the elution of calibration markers shown in Fig. 7. The peak of the 36-kD CKI activity was pooled and dialyzed into 50 mM KCl, 10 mM Tris, pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, and 0.1 mM PMSF (buffer B).

Kinase Assays and Inhibition of CKI Activity

CKI was assayed as described previously using α-casein or phosvitin as substrates (6, 20, 21). Briefly, protein phosphorylation was initiated with the addition of 50 mM Tris, pH 7.5, 5 mM $MgCl_2$, 50 mM [γ-^{32}P]ATP (30 Ci/mol; final concentrations). When inhibitors were used, they were added before ATP. For determination of kinase activity towards endogenous substrates on purified synaptic vesicles, the vesicles were dialyzed into buffer B and the above ATP-containing buffer was added to initiate the reaction. For antibody inhibition, affinity-purified CKI antibody in buffer B was combined with synaptic vesicles and incubated 12 h on ice. After addition of ATP, the reactions proceeded for 5–15 min (linear range of phosphorylation) and were then quenched with four times concentrated Laemmli sample buffer or 500 mM EDTA to 5 mM (for subsequent immunoprecipitation experiments, see below). Proteins were separated by 7–15% SDS-PAGE and bands corresponding to casein, phosvitin or other substrates were excised and scintillation counted using the ^{32}P channel. Phosphatidylinositol 4-phosphate 5-kinase assays were done as previously described (7, 50).

Immunoprecipitation of Small Synaptic Vesicles

Immunoprecipitation protocol was adapted from Schweitzer et al. (67). 100 μg of synaptic vesicles were used for immunoprecipitation reactions. Each reaction was supplemented with either 50 μg normal mouse sera (negative control, Jackson Immunoresearch) or 50 μg SV2 ascites and placed on a rotator at 4°C for 1 h. 10 μg of rabbit anti–mouse IgG was then added to the reactions and incubated on rotator for an additional 1 h at 4°C. Finally, the reactions were each supplemented with 50 mg Pansorbin (Calbiochem-Novabiochem Corp., La Jolla, CA) and incubated as before. The Pansorbin was then pelleted with a 1-min spin at 16,000 *g* in a microfuge and in some instances the resultant pellet was washed twice with 500 μl PBS or Hepes-buffered saline as detailed in the text or figure legend. Immunoprecipitate pellets were resuspended in Hepes-buffered saline in a volume equivalent to their respective supernatants. All the samples were then supplemented with 4× SDS Laemmli sample buffer and boiled for 10 min. In certain instances, synaptic vesicles were first phosphorylated as described above for subsequent immunoprecipitation experiments.

In the studies identifying SV2 as a substrate, both phosphorylated and unphosphorylated synaptic vesicles were immunoprecipitated as described above but in the presence of 0.5% Triton X-100 during the actual immunoprecipitation or immediately before the pellet wash steps. The resultant immunoprecipitate pellets and supernatants were then analyzed by SDS-PAGE and autoradiography or Western blotting with the SV2 antibodies.

Results

Immunofluorescent Localization of CKI in Nonneuronal and Neuronal Cells

In all nonneuronal cells stained with the α-CKI antibodies, the kinase is found associated with cytosolic vesicular structures (21). The vesicular staining in flat, adherent cells was often distinctly oriented and aligned with microtubule arrays. Fig. 1, *A* and *B* depict confocal indirect immunofluorescence images of α-CKI and tubulin antibody staining of an LLC-PK cell, respectively. The vesicular staining of α-CKI could be colocalized with microtubules by image overlay (Fig. 1 *C*). Because CKI had previously been shown to associate with membranes as well the detergent-insoluble cytoskeleton, it was not clear whether these punctate structures were membraneous vesicles or other components. Therefore, CKI antibodies were used to double-label cells with additional antibodies specific for different internal membrane markers. As shown in Fig. 1, *D–F*, CKI was coincident with β-COP, a Golgi-specific marker. However, this colocalization was apparent only at the cell periphery. When cells were stained with BiP antibodies, an ER-specific marker, as in Fig. 1, *G–I*, the kinase appeared to be associated in part with the ER. This limited association occurred on a substructure of the ER and was more vesicular in morphology. Taken together, these images suggested that CKI associates with a select membrane population which has markers derived from both the ER and Golgi organelles.

To determine whether CKI was associated with a specific type of membrane vesicle, newborn rat primary neurons derived from sympathetic ganglia were double labeled with antibodies specific for CKI and the synaptic vesicle-specific proteins, synaptophysin, and SV-2 (Fig. 2). Colocalization of synaptic vesicle markers with CKI was most apparent in the axon and growth cone (Fig. 2, *A–C*, *small arrows*). The association of CKI with synaptic vesicle markers in primary neurons was less evident in the Golgi region (Fig. 2, *A–C*, *large arrow*). In Fig. 2, *G* and *I*, primary neurons were double labeled with α-CKI and SV-2 antibodies. The colocalization of CKI in the axon and growth cone was one-to-one with both synaptophysin and SV-2 (see Fig. 2, *G–I*). Differentiated PC-12 cells were also labeled for CKI and synaptic vesicle markers. In these cells, CKI appeared to be coincident with synaptic vesicle markers, particularly along axons and in the growth cone (data not shown).

The specificity of the α-CKI antibodies was previously established by Western blotting in all nonneuronal cells assayed (21). A Western blot (Fig. 3) of undifferentiated (lane *A*) and differentiated (lane *B*) PC-12 cells demonstrated that the α-CKI antibody specifically detected the 36-kD CKI. The visible minor band with an apparent molecular mass of 40 kD was likely the αL-CKI alternatively spliced isoform (Zhang, J., and R. A. Anderson, manuscript in preparation). To demonstrate that CKI staining in primary neurons particularly in the axon, was specific for the 36-kD CKI, neurons were Western blotted with the α-CKI antibody (Fig. 3). Lane *C* was a lysate of rat primary sympathetic ganglia, and lanes *E* and *F* were blots of the ganglia cell bodies and axons, respectively. For this experiment, ganglia were grown as an explant (cell mass) from which the axons radiate outward. Growing the neurons in this fashion permitted the separation of cell bodies from their axons. The cell bodies and axons were then lysed in SDS-containing sample buffer, separated by SDS-PAGE and Western blotted with CKI antibodies. Western blots of both axons and cell bodies with α-CKI antibody only

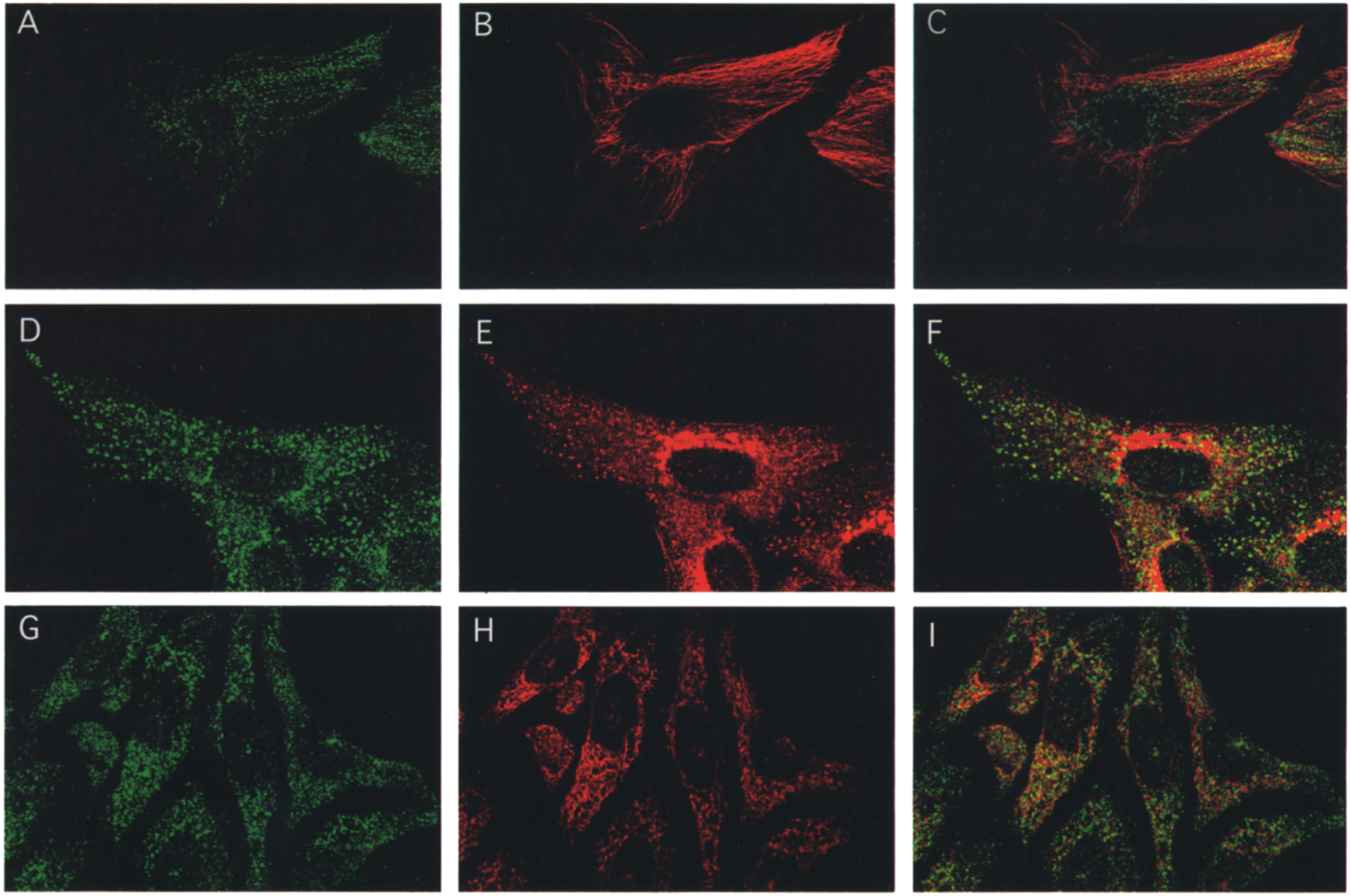

Figure 1. Subcellular distribution of CKI in nonneuronal cells to cytosolic vesicular structures which were partially contiguous with the ER and the Golgi Apparatus. CKI (*A*) associated with vesicular structures in LLC-PK cells that appeared to align with microtubule fibers (*B*). Image overlay (*C*) of CKI and mictrotubule staining illustrates the alignment. These CKI-positive punctate structures colocalized with the Golgi Apparatus–specific marker β-COP at the cell periphery as shown in *D–F*. The vesicular structures with associated CKI in *G* were coincident with the border of the ER as identified by BiP antibodies shown in *H* and by overlay in *I*. Cells were grown on coverslips, fixed in methanol and stained as described in Materials and Methods. Paraformaldehyde-fixed cells gave a similar staining pattern (not shown). The confocal images in *A–F* are 0.2 μm focal sections. *G–H* are 0.5 μm focal sections.

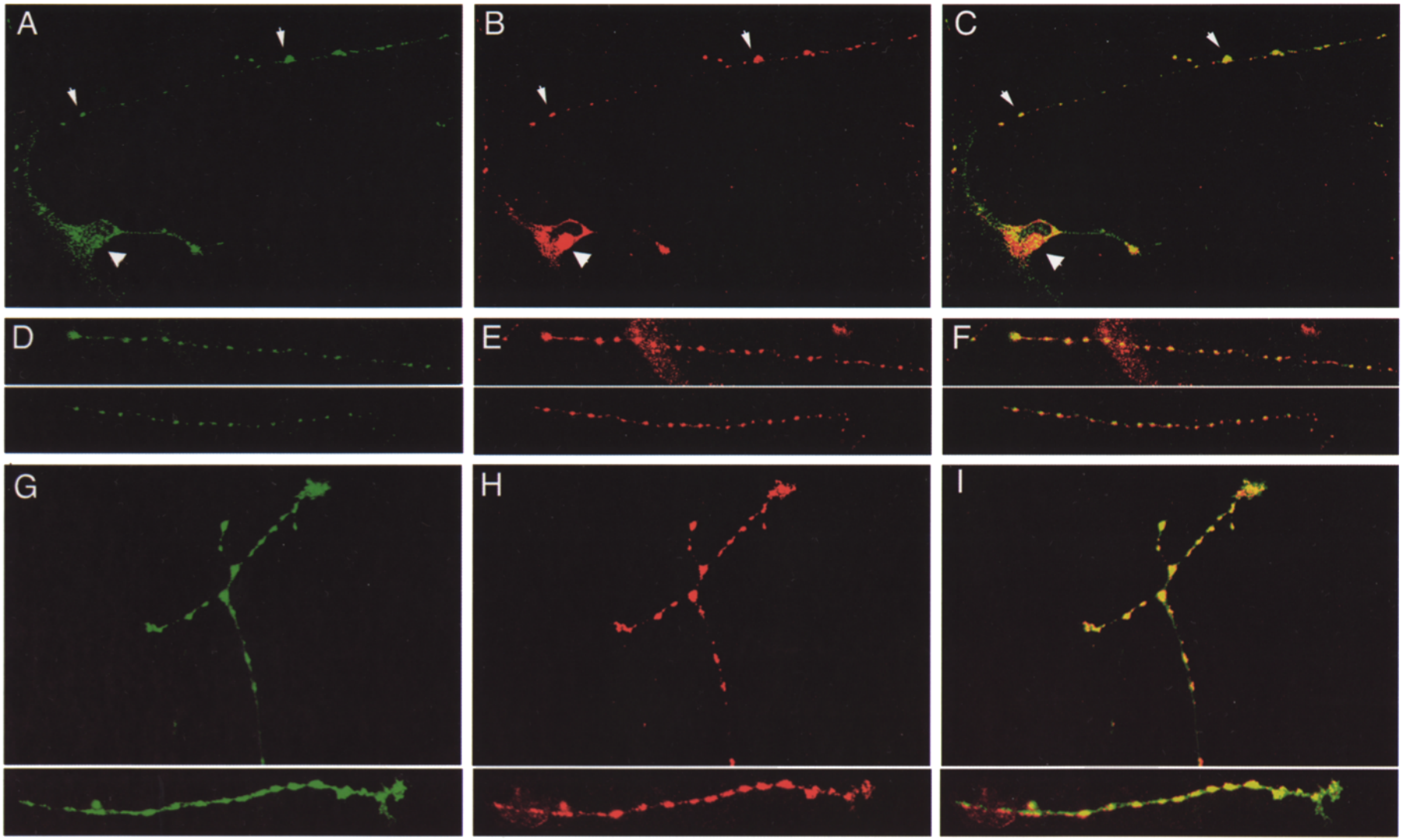

Figure 2. Immunofluorescent localization of CKI in primary rat neurons to structures containing synaptic vesicle-specific antigens. *A–F* depict axonal arrays immunolabeled for CKI and synaptophysin. *C* and *F* are image overlays of *A* and *B*, and *D* and *E*, respectively, depicting the one-to-one correspondence of the two signals (shown in yellow). Note the large accumulation of synaptophysin in a perinuclear distribution indicative of the Golgi apparatus which is not readily apparent for CKI (*A–C*, *large arrows*). The alignment of the two signals was most apparent within the axon itself (*A–C*, *small arrows*). CKI (*G*) and the synaptic vesicle–specific protein SV-2 (*H*) also colocalized within axons as displayed by image overlay (*I*). Neuronal structures displayed in *A–F* were fixed in methanol and the axons depicted in *G–I* were fixed in paraformaldehyde as described in Materials and Methods. The micrographs in *A–F* are 1 μm laser confocal sections whereas *G–I* are immunofluorescence micrographs using a Zeiss Axiovert 135.

detected a 36-kD protein, consistent with observations in nonneuronal cells (21).

The 36-kD CKI Was Associated with Rat Brain Synaptic Vesicles

The fact that CKI and synaptic vesicle proteins were distributed within the cell in a similar fashion did not establish a direct association of the kinase with synaptic vesicles. Therefore, rat brain synaptic vesicles were isolated using the method of Huttner et al. (49). During the purification, the content of CKI in the various membrane fractions was assessed by Western blotting equal protein loads with CKI- and synaptophysin-specific antibodies. As shown in Fig. 4, the 36-kD CKI immunoreactivity did copurify identically with synaptic vesicles. In Fig. 4, lane *D*, the cell body and nuclei (P_1) contained little synaptophysin but had an amount of CKI equivalent to that in lane *C* (S_1). These results were consistent with previous observations that CKI is present in the nucleus (76). After high speed centrifugation, little CKI was detected in the cytosolic fractions (lanes *G* and *P*). However, the microsomal fraction (P_3) was enriched for CKI. This result was consistent with the immunofluorescence data shown in Fig. 2, *A–C*, where CKI was observed in the cell body but was not coincident with synaptophysin staining. After lysis, the CKI in Ficoll-purified synaptosomes was enriched in synaptic vesicle–containing fractions. In addition, CKI cosedimented with the (LP_2) high speed pellet which was enriched for synaptic vesicles as determined by synaptophysin Western blotting. Trace 36-kD CKI immunoreactivity was detected in the LP_1 fraction which largely consisted of plasma membrane.

To determine the extent of CKI's association with synaptic vesicles, vesicles were further purified by velocity sucrose density gradient sedimentation. CKI from the LP_2 fraction cosedimented with small synaptic vesicles through the gradient as determined by Western blotting equal volumes of the gradient fractions for both CKI and SV-2 (Fig. 5, *top panel*). The peak of synaptic vesicles was pooled and further purified by equilibrium sedimentation on a Nycodenz gradient. CKI and SV-2 cosediment identically to a density of 1.12 g/cc (Fig. 5, *bottom panel*). The kinase activity towards phosvitin and a 52-kD synaptic vesicle–associated protein also correlated with CKI immunoreactivity.

Fig. 6 is a comparison of the content of the 36-kD CKI on synaptic vesicles isolated by the above methods with the content of 36 kD CKI associated with synaptic vesicles isolated by the methods of Floor et al. (39, 40). Lanes *A* and *B* are Western blots with CKI antibody of two preparations of synaptic vesicles isolated by the methods outlined in Materials and Methods and lane *C* shows a Western blot of synaptic vesicles which were >85% pure, prepared by the methods of Floor et al. (40). When the ratio of CKI to SV-2 was assessed by quantitative Western blotting, these synaptic vesicles contained 2–4-fold more 36 kD CKI than did vesicles prepared by procedures outlined in Materials and Methods. The difference in CKI content of the two vesicle preparations may have been due to differences in vesicle population or in retention of kinase by the vesicles. However, the observation that CKI was retained by synaptic vesicles prepared using two different methods further supported the contention that the 36-kD CKI was associated with synaptic vesicles both in vitro and in vivo.

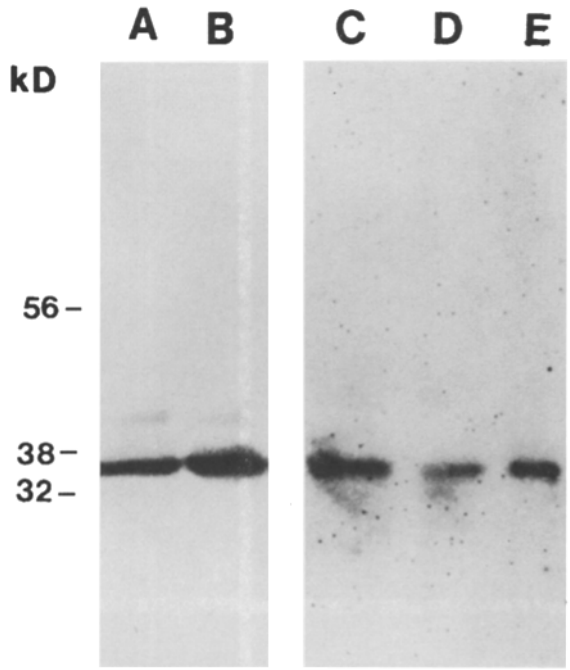

Figure 3. Western blot analysis with α-CKI antibodies demonstrated that the antibodies were specific for the 36-kD α-CKI. Lanes *A* and *B* are 50 μg of lysate from undifferentiated and differentiated PC-12 cells, respectively. Lanes *C*, *D*, and *E* are 15 μg of lysates from a total sympathetic ganglion neurons in culture, separated cell bodies, and separated axons, respectively.

In the sucrose gradient (Fig. 5, *top*), a large fraction of the total protein, including CKI and synaptic vesicle markers, was found in the sucrose gradient pellet, which contained N-CAM, an integral plasma membrane protein (32, 56). The CKI in the gradient pellet was not trapped in intact synaptosomes as the ratio of CKI to synaptic vesicle marker proteins was 3–5-fold greater (depending on the preparation) in the sucrose gradient pellet than in the isolated synaptosomes. Rehomogenization of the pellet, followed by sedimentation on a second sucrose gradient, released only a minor fraction of the SV2 or CKI. Further analysis by Nycodenz gradient centrifugation demonstrated that CKI, SV2, and N-CAM immunoreactivity cosedimented identically. Moreover, transmission electron microscopic analysis of the peak of CKI, SV2, and N-CAM immunoreactivity from the sucrose gradient indicated that there were intact synaptic vesicles present which were not trapped within sealed membrane structures. Furthermore, 80–90% of the 36-kD CKI in the sucrose gradient pellet was solubilized by high ionic strength extraction indicating that the CKI was not trapped.

Isolation and Characterization of the Synaptic Vesicle Associated 36-kD CKI

Three isoforms of CKI, with molecular masses in the 36–39-kD range, have been identified in brain; two alternatively spliced isoforms of α-CKI (α and αL) and a highly

homologous β-CKI isoform (79% sequence identity) (66, Zhang, J., and R. A. Anderson, unpublished results). All three isoforms only differ in size by 2–3 kD. In addition, our CKI antibody, which was prepared against the erythrocyte α-CKI (21), immunocrossreacts with the β-CKI isoform as determined by Western blotting both recombinant proteins (unpublished results). Therefore, the kinase was purified from synaptic vesicles and characterized to establish the identity of the synaptic vesicle-associated CKI.

The synaptic vesicle–associated CKI was eluted from the LP_2 vesicle fraction with 1 M KCl, removing ~90% of the 36-kD CKI as quantified by Western blotting. The extracted CKI was then purified by phosphocellulose ion exchange, as described previously (21, 66). Two peaks of casein kinase activity eluted at 0.54 M NaCl and 0.72 M NaCl, respectively. Western blotting demonstrated that the first peak of activity was the 36-kD CKI which eluted at a similar NaCl concentration as the erythroid and thymus α-CKI (21, 66). The second peak of casein kinase activity possessed properties indistinguishable from casein kinase II (76). The 36-kD CKI peak of activity was pooled and applied to a Superose 12 column. As shown in Fig. 7, two peaks of casein kinase activity eluted from the column with apparent masses of 230 kD and 45–50 kD. Both kinase activities phosphorylated phosvitin in preference to casein but only the 45–50-kD peak of kinase activity was immunoreactive with the α-CKI antibody. Because of its ability to use GTP, the 230-kD casein kinase peak was indistinguishable from casein kinase II, which has been reported in synaptosomes (27, 34, 76).

Inhibitors of CKI and casein kinase II were used to biochemically define the activity of the purified synaptic vesicle CKI. When [γ-^{32}P]ATP was used as a phosphate donor, GTP did not inhibit activity indicating that it cannot be used by this kinase in the phosphotransfer reaction. Fig. 8 demonstrates that this protein kinase is inhibited by both CKI-7 and CKI-8, specific inhibitors of CKI activity (28). The IC_{50} value for CKI-7 inhibition of the synaptic vesicle CKI is 12 μM, somewhat lower than the value reported for α-CKI (78). CKI-8 inhibits with an IC_{50} of 80 μM which closely approximates that reported for α-CKI (78). The CKI was also inhibited by heparin although this effect was substrate-specific (Fig. 8 *B*). Heparin inhibition has also been described for the thymus and erythroid α-CKI (78). Like the erythroid α-CKI (6, 20), the synaptic vesicle CKI was potently inhibited by PIP_2. Inhibition by PIP_2 is substrate-dependent as shown in Fig. 8 *C* for casein and phosvitin. However, PIP_2 inhibited the synaptic vesicle CKI more potently than the erythroid α-CKI (6, 20).

When assayed with common CKI substrates, the synaptic vesicle kinase phosphorylated human erythrocyte spectrin (not shown), and preferred phosvitin as a substrate over casein (Fig. 8 *C*). This substrate specificity appeared unique to α-CKIs, considering that α-CKI expressed in *E. coli* phosphorylates α-casein and prefers phosvitin to casein as well (78, Zhang, J., and R. A. Anderson, unpublished results). In contrast, bacterially expressed β-CKI prefers both the α- and β-caseins over phosvitin (data not shown). In combination, these data indicated that the synaptic vesicle associated 36-kD CKI is most similar to α-CKI.

Because α-CKI was associated with synaptic vesicles and inhibited by CKI-7 and CKI-8, the phosphorylation of synaptic vesicle proteins by this kinase was determined. Using synaptic vesicles isolated by Nycodenz gradient sedimentation (Fig. 9), the ATP-dependent phosphorylation of vesicle proteins is shown (lane *A*). Under these phosphorylation conditions, described in Materials and Methods, the two major protein kinases likely to be active on the vesicles are casein kinases I and II. To mask casein kinase II activity, [γ-^{32}P]ATP was used as the phosphate donor (Fig. 9), in the presence of a 10-fold molar excess of GTP (lane *B*). Although GTP masked activity towards some minor substrates, the majority of phosphorylated proteins were not affected. When CKI-7 was used at 20 and 80 μM, the phosphorylation of specific proteins was inhibited. Addition of CKI-8 at 80 and 320 μM inhibited phosphorylation of the same protein bands. However, heparin, a potent inhibitor of casein kinase II in membrane fractions (34), had no apparent effect on intrinsic synaptic vesicle protein phosphorylation under these conditions.

To further demonstrate the specificity of these CKI inhibitors, the CKI antibody was used to inhibit kinase activ-

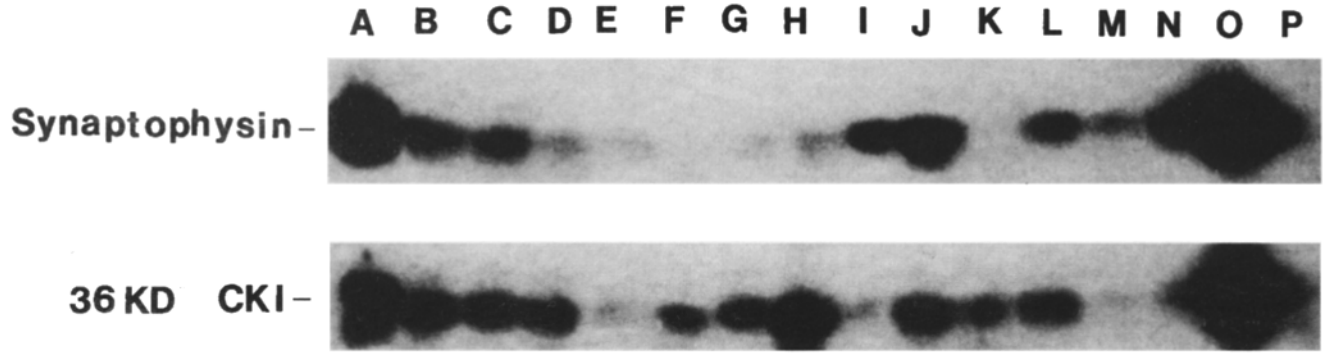

Figure 4. An autoradiogram of a Western blot of various subcellular fractions during the isolation of rat brain synaptic vesicles probed with rabbit antisera to synaptophysin (*top*) and affinity purified antibodies to α-CKI (*bottom*). For each brain fraction, 50 μg of protein was loaded unless otherwise noted and Western blotted according to the Materials and Methods. Lanes *A* are partially purified synaptophysin (0.2 μg) and purified human erythroid α-CKI (50 ng); lanes *B–P* correspond to the following fractions from a synaptic vesicle purification using the terminology in reference 49 and the Materials and Methods: *B*, H; *C*, S_1; *D*, P_1; *E* (15 μg protein), S_2; *F* (15 μg protein), P_2; *G*, S_3; *H*, P_3; *I*, S_2'; J (Ficoll-purified synaptosomes), P_2'; *K*, F_1; *L*, F_2; *M*, LP_1; *N*, LS_1; *O*, LP_2; *P*, LS_2.

ity (Fig. 10). Although the CKI antibodies poorly inhibit kinase activity towards soluble substrates, the antibody does inhibit erythrocyte α-CKI activity towards membrane substrates (Brockman, J. L., and R. A. Anderson, unpublished results). In this assay, the antibodies were preincubated with purified synaptic vesicles and the phosphorylation was assayed as in Fig. 9. The left panel shows an autoradiogram of the SDS-PAGE after phosphorylation with [γ-^{32}P]ATP with increasing concentrations of CKI antibody; the right panel shows a corresponding autoradiogram densitometry scan of phosphorylation in the absence of antibody (*B*) and in the presence of 40 μg/ml of CKI antibody (*A*). Nonspecific IgG had no effect on the phosphorylation of synaptic vesicle proteins. When compared with the results in Fig. 9, the CKI antibody inhibited phosphorylation of the same synaptic vesicle proteins as did CKI-7 and CKI-8, demonstrating that these substrates were phosphorylated by the 36-kD CKI.

Immunoprecipitation of Small Synaptic Vesicles Demonstrates That CKI Is on Synaptic Vesicles, the Major Substrates Are Associated with Synaptic Vesicles, and That SV2 Is a Substrate for CKI

Even though CKI colocalizes and copurifies with synaptic vesicle-specific markers, this did not eliminate the possibility that other membranes might be contributing to all or part of the observed immunoreactivity and kinase activity. In addition, some of the observed substrates for CKI could be associated with membranes whose source might be other than synaptic vesicle in origin. To address these questions, synaptic vesicles were immunoprecipitated using increasing amounts of a mouse monoclonal antibody to the synaptic vesicle integral membrane protein SV2. Initially, unlabeled synaptic vesicles were immunoprecipitated in an effort to establish whether CKI was actually associated with synaptic vesicles. The resultant washed immunoprecipitate pellets and corresponding supernatants were Western blotted for CKI and SV2 using ^{125}I-labeled Staph A protein as a means of detection. The signals were quantified by densitometry and γ-counting, respectively. As displayed in Fig. 11 *A*, both SV2 and CKI were equally depleted from the synaptic vesicle sample. In addition, the immunoprecipitate pellets were enriched for both CKI and SV2 (data not shown).

Next, synaptic vesicles were phosphorylated using [γ-^{32}P]ATP under conditions where CKI should be the predominant kinase activity on vesicles (see Materials and Methods). All kinase activity was quenched with the addition of EDTA to 5 mM and the vesicles were immunoprecipitated with SV2 antibodies. The resultant immunoprecipitate pellets and supernatants were analyzed by SDS-PAGE and subsequent autoradiography. As shown in Fig. 11 *B*, all but two of the substrates were immunoprecipitated with the SV2 antibody and no phosphorylated proteins were precipitated with normal mouse sera. These two substrates of 18 and 21 kD were still visible in the SV2 immunoprecipitate pellet but with significantly reduced signal. Moreover, their signal was not visible in the SV2 immunoprecipitate supernatant. Subsequent experimentation suggested that the loss of ^{32}P from these two substrates is likely due to a dephosphorylation event occurring during immunoprecipitation. Supporting this contention, phosphorylated synaptic vesicles quenched with EDTA and incubated in parallel with the immunoprecipitates also exhibited reduced phosphate incorporation into these 18- and 21-kD substrates.

Based upon the observation that one of the major substrates for CKI on synaptic vesicles was a diffuse 80-kD

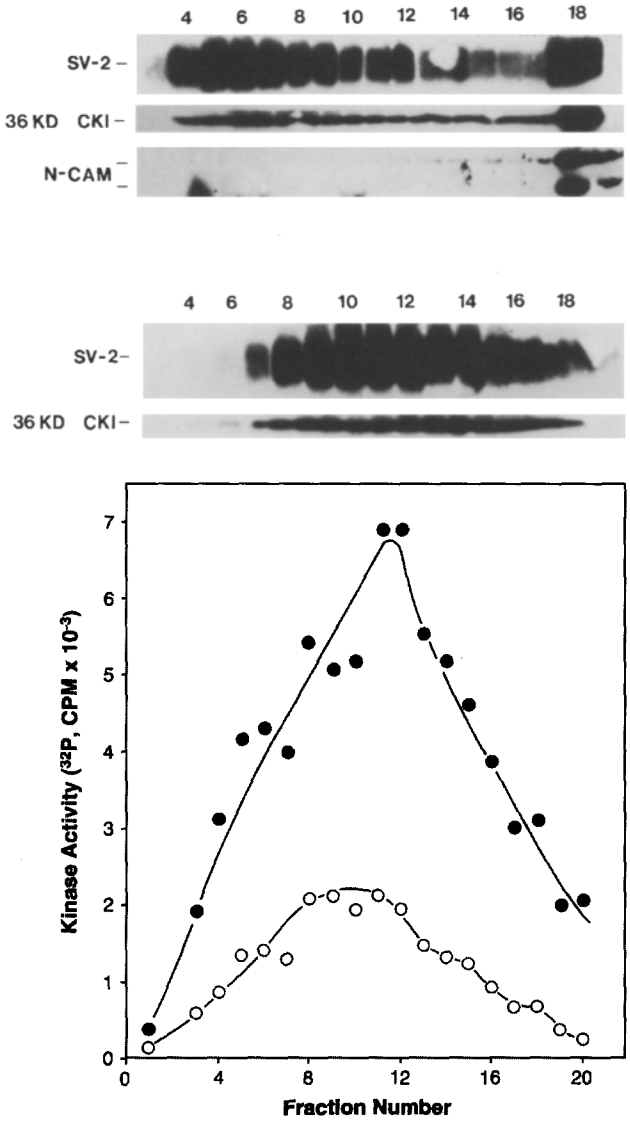

Figure 5. CKI is associated with small synaptic vesicles when analyzed by sucrose and then Nycodenz density gradient sedimentation. The LP_2 fraction from rat brain, as in Fig. 4, was washed by centrifugation and loaded on a sucrose gradient as outlined in the Materials and Methods. After sedimentation, 80 μl of the resulting fractions were assayed for α-CKI, SV-2, and N-CAM by Western blotting. Fractions 4–9 were pooled and concentrated by centrifugation. This synaptic vesicle fraction was further analyzed by Nycodenz equilibrium sedimentation and the resulting fractions were assayed by Western blotting with SV-2 and α-CKI antibodies (*middle panel*). These fractions were also assayed for protein kinase activity towards phosvitin (*closed circles*), an excellent α-CKI substrate (see Fig. 9) and the intrinsic phosphorylation (see Fig. 11) of the 52-kD major phosphorylated synaptic vesicle protein (*open circles*) according to the Materials and Methods.

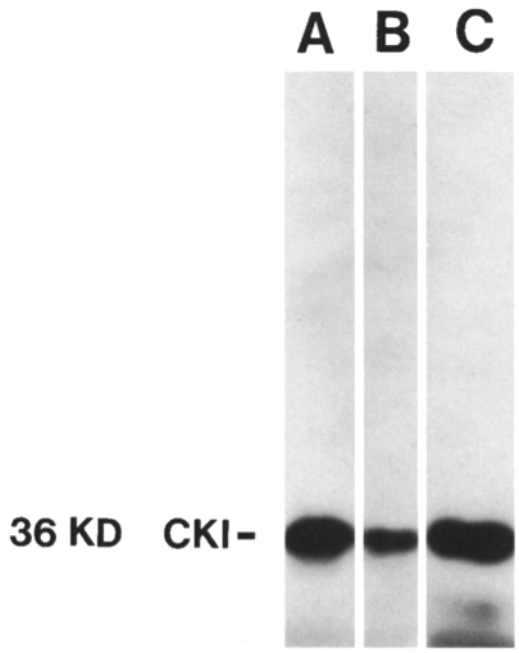

Figure 6. The 36-kD CKI was found on small synaptic vesicles purified by two distinct methods. Lanes *A* and *B* show Western blots with the α-CKI antibody of two synaptic vesicle preparations purified according to the Materials and Methods. Lane *C* are synaptic vesicles prepared by the method of Floor et al. (38, 39). Lanes *A–C* are adjacent lanes on the same Western blot with the same exposure time. By immunodot blotting, all three synaptic vesicle preparations contained equal amounts of SV-2 protein but had a different content of the 36-kD CKI. The ratio of the 36 kD CKI to SV-2 in lanes *A* and *B* is 2–4-fold less, respectively, than that associated with the vesicles in lane *C*.

band similar to that of SV2 in both size and appearance (3), we examined whether SV2 was a substrate for CKI. ^{32}P-phosphorylated and unlabeled synaptic vesicles were treated with 0.5% Triton X-100 to disperse the vesicle membranes and then immunoprecipitated with normal mouse sera or SV2 antibodies. The resultant immunoprecipitates were analyzed by SDS-PAGE and autoradiographed. As shown in Fig. 11 *C* lane *1*, no detectable phosphorylated proteins were being immunoprecipitated with normal mouse sera. In contrast, a protein of the size of SV2 is readily apparent in the immunoprecipitate using SV2 antibodies (Fig. 11 *C*, lane *3*). Additionally, immunoprecipitates with normal mouse sera or SV2 antibodies were conducted in parallel on unlabeled vesicles for subsequent Western blotting with the SV2 antibody. As depicted in Fig. 11 *C*, lane *5*, SV2 was immunoprecipitated with the SV2 antibodies. No immunoreactive proteins of this mobility were observed in the normal mouse sera immunoprecipitate (data not shown). The possibility existed, however, that the Triton solubilization rendered SV2 accessible to another kinase or to CKI itself, whereas, under more "normal" conditions (i.e., intact synaptic vesicle membranes) it would not. Therefore, the identical procedure was undertaken in the absence of detergent. However, immediately before SDS-PAGE preparation, the immunoprecipitates were treated with Triton as before with no observed change in the outcome.

PIP_2 Is Produced on the Plasma Membrane but Not on Synaptic Vesicles

Previously, α-CKI has been shown to be inhibited on native membranes by PIP_2 (6, 20). Additionally, when the membrane content of PIP_2 was increased, the affinity of α-CKI for membranes was enhanced (6, 20). Since the synaptic vesicle 36-kD CKI was also inhibited by PIP_2 the sucrose gradient fractions were assayed for phosphatidylinositol 4-phosphate (PIP) 5-kinase activity and the type I and II PIP 5-kinases by Western blotting (7, 50). As shown in Fig. 12, all of the intrinsic PIP kinase activity and the majority of type I and II PIP kinase immunoreactivity were found in the gradient pellet. PIP 5-kinase activity and immunoreactivity were not detected on purified synaptic vesicles. This result demonstrated that in vitro PIP_2 production occurred in plasma membrane but not synaptic vesicle fractions. This spatial segregation of PIP_2 production may account for the increased ratio of CKI to synaptic vesicle markers in the plasma membrane–containing fractions compared to purified synaptic vesicles.

Discussion

Here we have shown that a PIP_2-sensitive CKI was associated with synaptic vesicles. In addition, PIP_2 production was restricted to specific cellular membranes and may therefore modulate α-CKI activity only when the kinase is situated close to these membranes. The spatial synthesis of PIP_2 in the nerve terminal could be a mechanism to selec-

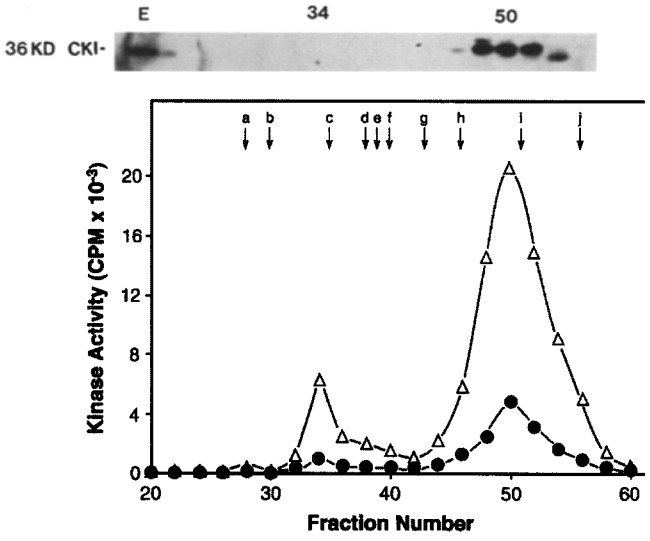

Figure 7. Molecular sieve chromatography of the synaptic vesicle 36 kD CKI. The 36-kD CKI was eluted from the rat brain LP_2 fraction (Fig. 4) with 1 M KCl, fractionated by phosphocellulose, and then applied to a Superose 12 column. As two peaks kinase activity eluted from the Superose 12 column, the elution was Western blotted with α-CKI antibodies (*upper panel*). Lane *E* is the purified erythroid α-CKI and the peaks of kinase activity are noted above the Western blot. The size markers are: α, urease (540 kD); *b*, apoferrin (443 kD); *c*, β-amylase (200 kD); *d*, catalase (240 kD); *e*, alcohol dehydrogenase (150 kD); *f*, phosphorylase B (97 kD); *g*, BSA (66 kD); *h*, ovalbumin (45 kD); *i*, carbonic anhydrase (29 kD); and *j*, cytochrome C (12.4 kD).

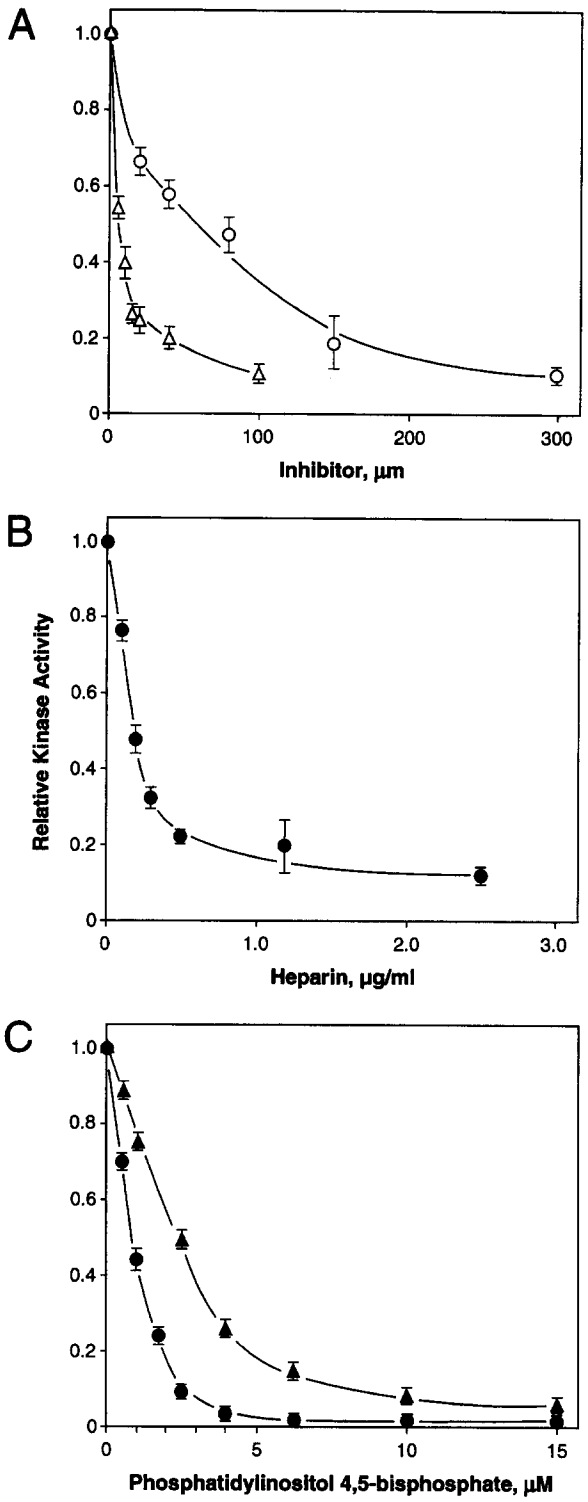

Figure 8. Characterization of the purified 36-kD casein kinase I by inhibition with CKI-7, CKI-8, heparin, and PIP_2. The upper panel shows inhibition of kinase activity toward casein by CKI-7 (*triangles*) and CKI-8 (*circles*). The middle panel shows inhibition of kinase activity toward casein by heparin. The lower panel shows inhibition of kinase activity toward phosvitin (*closed circles*) and casein (*closed triangles*) by increasing amounts of pure PIP_2. The data shown is the average of duplicate determinations with the variation shown.

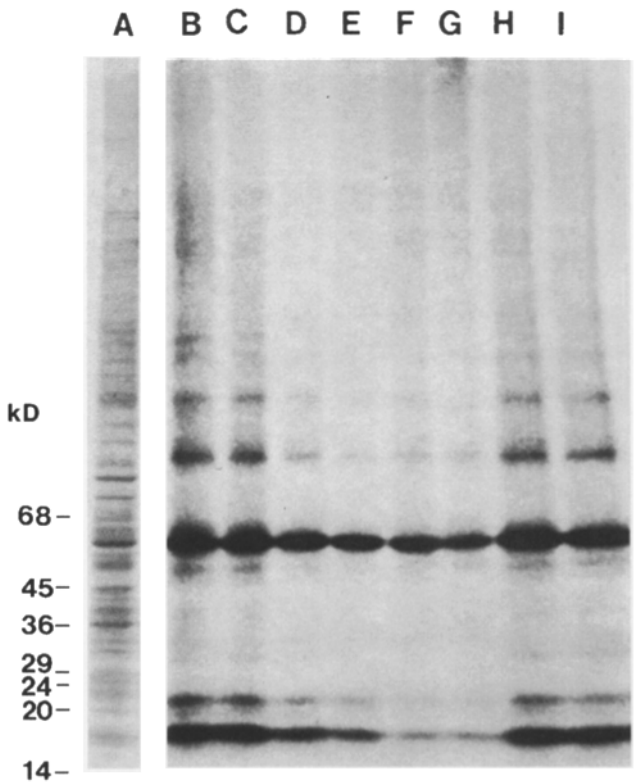

Figure 9. Inhibition of intrinsic phosphorylation of synaptic vesicle proteins by casein kinase I inhibitors. Synaptic vesicles purified by Nycodenz equilibrium sedimentation (see lane *A* for Coomassie blue–stained SDS-PAGE) were incubated (lane *B*) with 50 μM [γ-^{32}P]ATP as described in Materials and Methods in the absence of additions. In lane *C*, 500 μM GTP was added to block any casein kinase II activity and this was added in all of the remaining lanes. In lanes *D* and *E*, 20 and 80 μM CKI-7 was added, in lanes *E* and *F*, 80 and 320 μM of CKI-8 was added, in lanes *H* and *I*, 1 and 10 μg/ml heparin was added. This result was duplicated three times with two different synaptic vesicle preparations.

tively modulate CKI activity. Thus, cytosolic vesicles may possess high CKI activity whereas vesicles docked at the plasma membrane would have a lower level of activity as a result of PIP_2 inhibition.

Recent reports indicate that PIP_2, as well as other products of the PI cycle, play essential roles in the general process of vesicular trafficking (25, 45–48). For example, in *S. cerevisiae*, a phosphatidylinositol 3-kinase, VPS34, has been genetically linked to vesicular trafficking, particularly as concerns vacuolar sorting (45–48, 69). Another yeast gene product, SEC14, was originally isolated as a mutant defective in Golgi secretory function (25, 29). It has since been established that this gene codes for a phosphatidyl-inositol transfer protein, the mammalian homolog being required for the ATP-dependent priming of regulated neurotransmitter secretion in PC-12 cells (43–45). Additionally, a recent report by Brown et al. (23) describes a role for PIP_2 in vesicular trafficking. They established that PIP_2 is required for significant ADP-ribosylation factor (ARF)-mediated stimulation of phospholipase D activity. ARF activation of phospholipase D activity, the authors speculate, could then alter the topology of the membranes in such a way as to allow for critical interac-

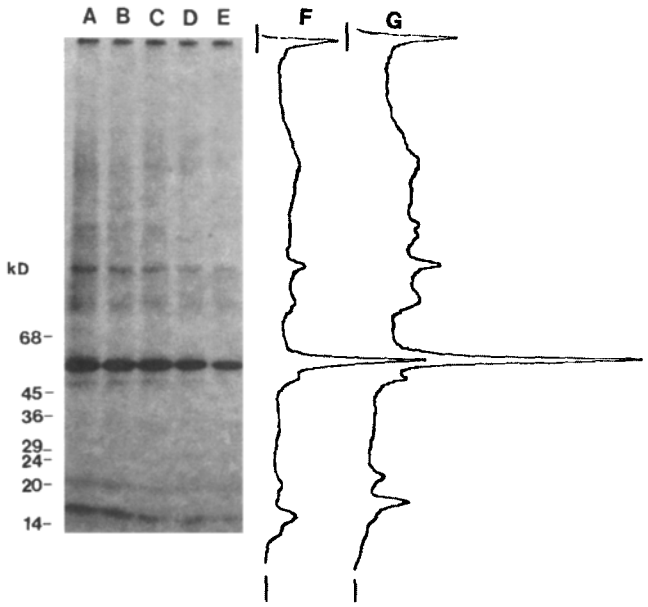

Figure 10. Inhibition of CKI phosphorylation of synaptic vesicle proteins by antibodies specific for the α-CKI. Affinity-purified α-CKI antibody (IgG) was combined with 50 μg of synaptic vesicles and then incubated on ice for 10 h. The phosphorylation was initiated by addition of 50 μM [γ-^{32}P]ATP in the presence of 500 μM GTP. The lanes contain the following: *A*, none; *B*, 5 μg/ml; *C*, 10 μg/ml; *D*, 20 μg/ml; and *E*, 40 μg/ml. Panels *F* and *G* show the laser densitometric scans of lanes *A* and *E* of the autoradiogram, respectively.

tions involved in the budding, targeting, and/or fusion process. PIP_2 may serve as a binding intermediary for ARF, phospholipase D or other factors which might actually mediate their functional interaction (23). Additionally, Hay et al. have recently shown that a type I PIP kinase activity is required for the ATP-dependent step of Ca^{2+}-regulated exocytosis in semi-intact PC12 cells (45, 50). The production of PIP_2 appears not to be involved in the typical phospholipase C-dependent pathways (45). Consequently, PIP_2 production could be altering the fusogenic properties of the vesicles as Brown et al. (23) have speculated or, alternatively, modulating the activity of regulatory enzymes such as CKI.

In nonneuronal cells, CKI is associated with vesicular structures that align with microtubules and overlap with a portion of both the endoplasmic reticulum and Golgi apparatus. These results suggest that CKI is associated with actively transported vesicles. To identify a specific population of vesicles with which CKI was associated, primary neurons were double labeled with antibodies against both CKI and synaptic vesicle-specific markers. Results indicated that there was a one-to-one correspondence between CKI and synaptic vesicle markers within the axon. However, CKI was not coincident with these synaptic vesicle markers within the Golgi region, similar to nonneuronal cells (Fig. 2). In addition, CKI copurified with synaptic vesicles as they were purified from rat brain. Finally, CKI's interaction with synaptic vesicles appeared to occur by a direct association since two different synaptic vesicle purification protocols yielded vesicle fractions enriched in the 36-kD CKI.

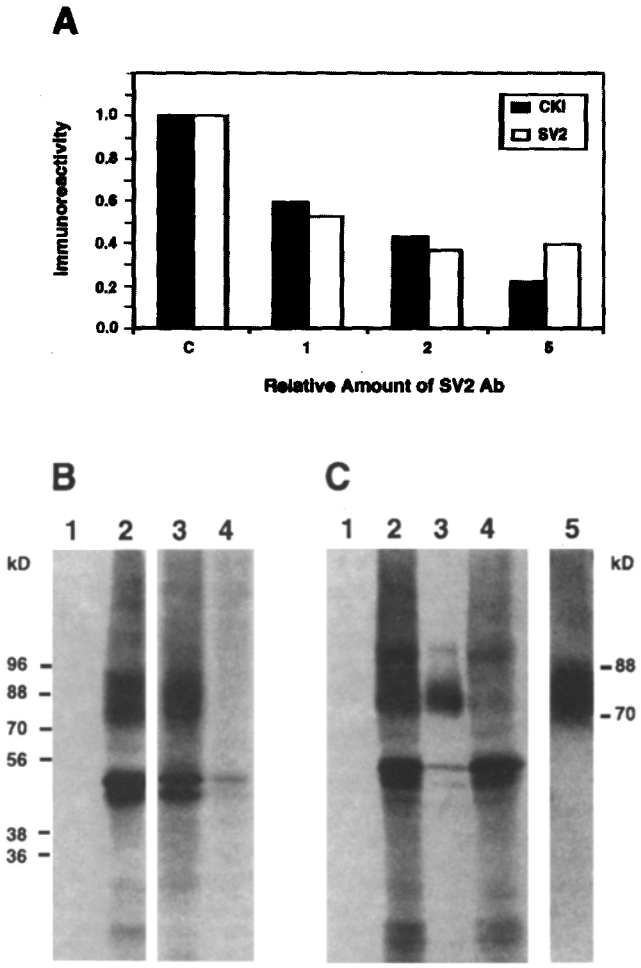

Figure 11. Immunoprecipitation of synaptic vesicles established CKI's association with synaptic vesicles, confirmed major substrates as being synaptic vesicle in origin, and identified SV2 as a substrate for CKI. (*A*) Increasing amounts of SV2 antibody in the immunoprecipitation reaction equally immunodeplete both SV2 and CKI from the synaptic vesicle preparation by an increasing degree. SV2 immunoreactivity was measured by γ-counting, and CKI immunoreactivity was determined by autoradiographic densitometry. All results were normalized to the control immunoprecipitate which contained no primary antibody. (*B*) Lanes *1* (immunoprecipitate pellet) and *2* (immunoprecipitate supernatant) depict the resultant autoradiogram generated by the immunoprecipitation of a ^{32}P-labeled synaptic vesicle preparation using normal mouse sera. Lane *1* demonstrates that no phosphorylated proteins were detected in the pellet. Lanes *3* (immunoprecipitate pellet) and *4* (immunoprecipitate supernatant) represent the immunoprecipitation of a ^{32}P-labeled synaptic vesicle sample using the SV2 antibody. Lane *3* contains all of the observed phosphorylated proteins indicating that the major substrates are all associated with synaptic vesicles. (*C*) Lanes *1–2* and *3–4* represent the immunoprecipitate pellets and supernatants of ^{32}P-labeled synaptic vesicles using normal mouse sera or SV2 antibodies, respectively. As shown in lane *3*, a phosphorylated protein is precipitated with the SV2 antibodies possessing an apparent molecular mass of 80 kD and SV2 immunoreactivity, as determined by Western blotting the immunoprecipitate pellet (lane *5*). No phosphorylated protein of that size was detected in the immunoprecipitate pellet using normal mouse sera (lane *1*).

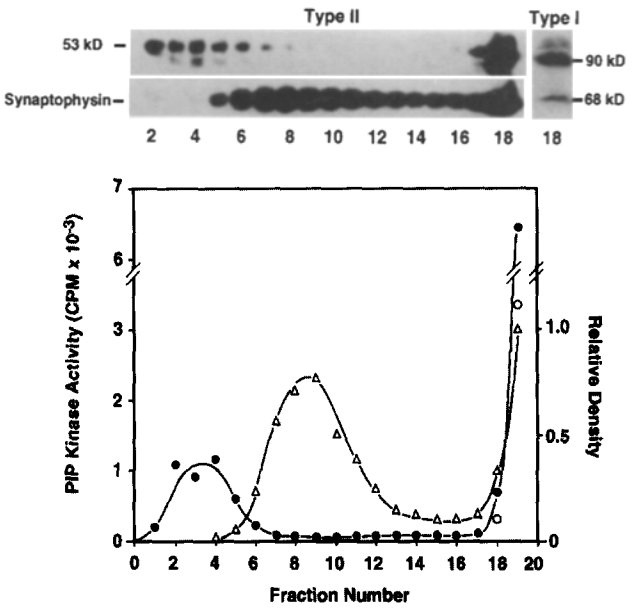

Figure 12. PIP 5-kinase activity is present in the synaptic vesicle/plasma membrane fraction but not on synaptic vesicles. The top panel depicts a Western blot of fractions from the sucrose gradient sedimentation of the LP_2 membrane fraction with the type II PIP 5-kinase antibody and a corresponding blot of the same fractions with the synaptophysin antibody. A portion of the type II PIP 5-kinase is soluble and remained at the top of the gradient. The remainder sedimented with the synaptosomal plasma membrane to the bottom. Western blots of the same fractions using the type I PIP 5-kinase antibodies elicited a signal only in the sucrose gradient pellet (*top, right panel*). The lower panel demonstrates the PIP kinase activity in the sucrose gradient fractions with (*closed circles*) and with out (*open circles*) exogenous PIP as substrate. The open triangles show a densitometric measure of the synaptophysin from the Western blot autoradiogram. The type I and II PIP 5-kinases and PIP kinase activity were not detected in the synaptic vesicle fractions but were detected in the pellet fractions which contained both plasma membrane and synaptic vesicle markers.

The biochemical properties of the synaptic vesicle 36 kD CKI were most similar to α-CKI. It was an extrinsic protein which eluted from synaptic vesicles with high ionic strength. The CKI gel-filtered with an apparent mass of 45–50 kD close to that expected for the monomer. In addition, the purified synaptic vesicle CKI was inhibited using known inhibitors of α-CKI. However, there exist differences. Rat brain synaptic vesicle CKI was more potently inhibited by CKI-7, heparin, and PIP_2 than the human erythroid α-CKI as compared in parallel assays. However, the α-CKI antibodies did not distinguish between α-CKI and the synaptic vesicle CKI. Thus, the kinetic differences between the synaptic vesicle 36-kD CKI and α-CKI could be due to posttranslational modifications or associated components, although differences in protein sequence can not be discounted.

When associated with synaptic vesicles, CKI appeared to be the major protein kinase activity detected on the synaptic vesicles under our assay conditions. Furthermore, a limited number of substrates were phosphorylated by the intrinsic synaptic vesicle CKI activity. These assay conditions were selective for the casein kinases. In the presence of excess GTP, five major protein bands were phosphorylated. ^{32}P-labeled, immunoprecipitated synaptic vesicles contained all these observed substrates. The phosphorylation of these proteins was inhibited by CKI-7 and CKI-8 at concentrations that inhibit α-CKI in solution. Moreover, α-CKI antibodies inhibited kinase activity towards the same substrates, whereas nonspecific IgGs had no effect.

In an effort to identify these substrates, we Western blotted ^{32}P-labeled vesicles (see Materials and Methods) with antibodies to VAMP, rab3a, synapsin I, and synaptotagmin using an alkaline phosphatase detection methodology (data not shown). None of these proteins comigrated with synaptic vesicle–associated proteins phosphorylated by CKI. However, immunoprecipitation studies on labeled vesicles identified SV2, a glycosylated synaptic vesicle-associated integral membrane protein, as being one of the five major substrates for CKI described above. As demonstrated in both Figs. 10 and 11, respectively, a phosphorylated protein with a mobility between 70 and 88 kD is prevented from being phosphorylated by known inhibitors of CKI and it can be immunoprecipitated by SV2 antibodies. Although cloned and sequenced, the function of SV2 remains unknown (3). Sequence analysis suggests that the protein may transport small molecules or ions across membranes due to its putative twelve membrane-spanning regions, common to known fungal and bacterial transporters (3). If the proposed structure of SV2 is correct, there is only a limited amount of the protein that would be available as substrate to an extrinsically associated synaptic vesicle kinase (3). However, at least three potential CKI phosphorylation sites do exist in SV2, residing in the cytoplasmically exposed amino terminus of the protein and within the cytoplasmic loop between putative membrane-spanning domains 6 and 7 (3, 64, 76). SV2 has also been detected in a number of different secretory cells suggesting that its putative role in the secretory process might be a more general one. CKI is a highly conserved, essential activity from yeast to humans indicating that its function might be more universal as well (63, 76). While intriguing, no activity has yet been ascribed to SV2 and therefore no readily assayable affect of CKI's modification of this protein is possible (3). Consequently, such an endeavor remains for future investigation.

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