Phosphorylation of Membrane-Bound Acetylcholine Receptor by Protein Kinase C: Characterization and Subunit Specificity†

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ABSTRACT: Acetylcholine receptor (AChR) from *Torpedo* electric organ in its membrane-bound or solubilized form is phosphorylated by the Ca^{2+}/phospholipid-dependent protein kinase (PKC). The subunit specificity for PKC is different from that observed for cAMP-dependent protein kinase (PKA). Whereas PKC phosphorylates predominantly the δ subunit and the phosphorylation of the γ subunit by this enzyme is very low, PKA phosphorylates both subunits to a similar high extent. We have extended our phosphorylation studies to a synthetic peptide from the γ subunit, corresponding to residues 346–359, which contains a consensus PKA phosphorylation site. This synthetic peptide is phosphorylated by both PKA and PKC, suggesting that in the intact receptor both kinases may phosphorylate the γ subunit at a similar site, as has been previously demonstrated by us for the δ subunit [Safran, A., et al. (1987) *J. Biol. Chem. 262*, 10506–10510]. The diverse pattern of phosphorylation of AChR by PKA and PKC may play a role in the regulation of its function.

Protein phosphorylation plays a key role in the regulation of biological processes (Krebs & Beavo, 1979). It is now evident that receptor phosphorylation is a primary mechanism in both mediating and modulating the responsiveness of cells to extracellular signals (Sibley et al., 1987; Miles & Huganir, 1988). The nicotinic acetylcholine receptor (AChR)[1] mediates synaptic transmission at the postsynaptic membrane of nicotinic cholinergic synapses. This multisubunit complex ($\alpha_2\beta\gamma\delta$) is a prototype of the chemically gated ion-channel receptors that contain the ligand-binding site as well as an intrinsic ion channel responsible for signal transduction (Popot & Changeux, 1984; Hucho, 1986; Changeux et al., 1987). Phosphorylation of muscle AChR has been implicated in playing a role in the regulation of the receptor ion channeling. Both cAMP-elevating agents, which act presumably by activating the cAMP-dependent protein kinase (PKA), and phorbol esters, which activate the Ca^{2+}/phospholipid-dependent protein kinase (protein kinase C; PKC), were shown to enhance receptor desensitization (Eusebi et al., 1985; Middleton et al., 1986; Albuquerque et al., 1986). It was thus postulated that direct phosphorylation of AChR by these two kinases may result in the observed physiological response. Indeed, phosphorylation of the receptor was demonstrated in intact mammalian muscle cells (Miles et al., 1987; Smith et al., 1987; Ross et al., 1987) and this phosphorylation was stimulated by cAMP and Ca^{2+} (Miles et al., 1987; Smith et al., 1987).

The nicotinic AChR from *Torpedo* electric organ was shown to serve as a substrate for phosphorylation by endogenous protein kinases present in *Torpedo* postsynaptic membranes (Gordon et al., 1977; Teichberg et al., 1977). Endogenous PKA (Huganir & Greengard, 1983) and a protein tyrosine kinase (Huganir et al., 1984) were identified and shown to specifically phosphorylate the receptor. Moreover, phosphorylation by either of these endogenous kinases affects receptor function by increasing its rate of desensitization (Huganir et al., 1986; Hopfield et al., 1988). These results indicate that the function of the *Torpedo* AChR, like that of the mammalian receptor, is modulated by various kinases.

We have previously demonstrated that purified *Torpedo* AChR serves as a high-affinity in vitro substrate of PKC. We have mapped the site for phosphorylation in the receptor δ subunit and could demonstrate that it is identical with the site of phosphorylation by PKA (Safran et al., 1987). We have now extended these in vitro studies to examine whether the receptor, when present in its native form in intact postsynaptic membranes, can also serve as a substrate of PKC. In this report we demonstrate that PKC phosphorylates the membrane-bound *Torpedo* AChR with a similar subunit specificity when compared to that of the solubilized receptor. Unlike endogenous PKA, which phosphorylates the receptor γ and δ subunits to a similar high extent, the major target for PKC phosphorylation is the δ subunit, while the phosphorylation of the γ subunit is significantly lower. The physiological significance of this differential subunit specificity is discussed.

Materials and Methods

Materials. [γ-^{32}P]ATP (3000 Ci/mmol) was purchased from Amersham. Protein A coupled Sepharose was from Pharmacia P-L Biochemicals. ATP, CTP, phosphatidylserine, the catalytic subunit of cAMP-dependent protein kinase, phenylmethanesulfonyl fluoride (PMSF), pepstatin, leupeptin, antipain, and aprotonin were obtained from Sigma.

Proteins and Peptides. AChR was purified from the electric organ of *Torpedo californica* as previously described (Aharonov et al., 1977). Protein kinase C was partially purified from rat basophilic leukemia (RBL-2H3) cells as described (Sagi-Eisenberg & Pecht, 1984). Peptide synthesis was carried

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[1] Abbreviations: AChR, acetylcholine receptor; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; SDS, sodium dodecyl sulfate; TPA, 12-*O*-tetradecanoylphorbol 13-acetate.

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out by the solid-phase method of Merrifield (1965) as described (Neumann et al., 1985). The amino acid composition of the peptides was verified by amino acid analysis.

Preparation of Postsynaptic Membranes. Postsynaptic membranes rich in AChR were prepared from frozen dissected electric organs of *T. californica* by the method of Sobel et al. (1977). Briefly, the minced organs were homogenized four times for 30 s each in a Waring blender in 20 mM Tris·HCl, pH 7.4, 10 mM EGTA, 5 mM EDTA, 10 mM 2-mercaptoethanol, 1 mM PMSF, 10 μg of pepstatin/mL, 10 μg of leupeptin/mL, 10 μg of antipain/mL, and 10 μg of aprotonin/mL (buffer A). The homogenate was centrifuged in a Sorvall GSA rotor at 5000 rpm for 10 min, and the supernatant was further centrifuged in a Beckman JA rotor at 7000 rpm at 4 °C for 2 h. The pellet was suspended in a minimal volume of buffer A, which was brought to a final sucrose concentration of 32% (w/w). The homogenized pellet was layered on top of a discontinuous sucrose gradient [25 mL of 41.5% (w/w), 15 mL of 37.5% (w/w), and 10 mL of 35% (w/w)] and centrifuged in a Beckman Ti rotor for 3 h at 32000 rpm. The material at the 35%/37.5% sucrose interface was collected, diluted in buffer A (1:10), and centrifuged in a Beckman 35 Ti rotor at 20000 rpm for 2 h. The membrane pellet was dissolved in buffer A and kept at −70 °C until use.

Phosphorylation Assays. Endogenous phosphorylation by cAMP-dependent protein kinase was measured by incubating 20–50 μg of membrane proteins in 20 mM Tris·HCl, pH 7.4, 20 mM $MgCl_2$, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 0.5% NP-40, and 2 μM [γ-^{32}P]ATP (500 cpm/pmol) in the presence or absence of 1 μM cAMP in a final volume of 50 μL. The reaction was initiated by the addition of ATP and stopped after 5 min by the addition of Laemmli's sample buffer. Samples were subjected to 10% SDS–polyacrylamide gel electrophoresis, and the gels were stained with Coomassie blue, dried, and autoradiographed. Phosphorylation of membrane proteins by exogenously added protein kinase C was measured by incubating 20–50 μg of membrane proteins in 20 mM Tris·HCl, pH 7.4, 20 mM $MgCl_2$, 1 mM CTP, and 2–10 μM [γ-^{32}P]ATP (500 cpm/pmol) in the presence or absence of 2 mM $CaCl_2$ and 0.05 units of protein kinase C partially purified from rat basophilic leukemia cells as described (Safran et al., 1987), in a total volume of 50 μL. Phosphorylation was carried out as described above, and samples were similarly analyzed by gel electrophoresis.

Phosphorylation of affinity-purified AChR and synthetic peptides by the catalytic subunit of cAMP-dependent protein kinase and protein kinase C was performed as previously described (Safran et al., 1987). The incorporation of ^{32}P into receptor subunits was quantitated by cutting the appropriate gel pieces out of the gel and counting by liquid scintillation spectrophotometry. Quantitation of peptide phosphorylation was performed by using phosphocellulose filters (Whatman, P-81) as described (Glass et al., 1978).

Immunoprecipitation. For immunoprecipitation, phosphorylation of membrane proteins was stopped under nondenaturing conditions by the addition of 3× concentrated stopping solution containing NaF (150 mM), $NaPP_i$ (15 mM), EDTA (45 mM), VO_3 (6 mM), ATP (9 mM), NaHepes, pH 7.5 (150 mM), and Triton X-100 (3%). Following solubilization, samples were centrifuged (12000*g*, 15 min), and 250-μL aliquots of the supernatant were incubated with 15 μL of protein A–Sepharose (50% v/v) for 10 min at 4 °C to remove nonspecific binding. Following centrifugation (12000*g*, 2 min), the supernatants were collected and further incubated for 3 h at 4 °C with the appropriate rabbit antibody, diluted 1:50 with stopping solution. Protein A–Sepharose (50 μL of 50% v/v) was subsequently added and incubation continued for 1 h at 4 °C. Immunocomplexes were washed twice in 50 mM NaHepes, pH 7.5, 1% Triton X-100, and 1% SDS and once in 50 mM NaHepes, pH 7.5, 1% Triton X-100, and 150 mM NaCl and resuspended in 100 μL of sample buffer. Following 30 min of incubation at 37 °C, samples were separated by 10% SDS–polyacrylamide gel electrophoresis, and the gels were dried and exposed to autoradiography.

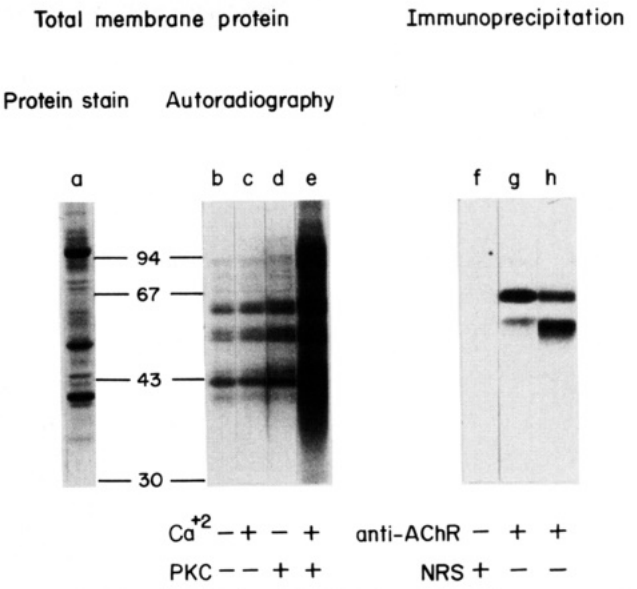

FIGURE 1: Phosphorylation of AChR from *Torpedo* postsynaptic membranes by PKC. *Torpedo* postsynaptic membranes were incubated under phosphorylation conditions (ATP, Mg^{2+}) in the absence (b) or presence (c) of 2 mM Ca^{2+} or with the addition of exogenous PKC in the absence (d) or presence (e) of 2 mM Ca^{2+}. Lane a represents Coomassie staining of membrane proteins. Immunoprecipitation of membrane proteins phosphorylated by PKC in the presence of Ca^{2+} was performed by using specific anti-*Torpedo* AChR antibodies (g) or nonspecific antibodies (NRS) (f). For comparison, postsynaptic membrane proteins were phosphorylated by endogenous PKA as described under Materials and Methods and immunoprecipitated by anti-AChR antibodies (h).

Results

Phosphorylation of Torpedo Postsynaptic Membranes by PKC. Incubation of postsynaptic membranes from *Torpedo* electric organ with [γ-^{32}P]ATP and Mg^{2+} resulted in the phosphorylation of several proteins (Figure 1, lane b). These phosphoproteins did not correspond to the most abundant proteins present in this membrane preparation (Figure 1, lane a). Addition of Ca^{2+} (2 mM) to these membranes (in both the presence and absence of the phorbol ester TPA) did not affect protein phosphorylation (Figure 1, lane c). However, addition of exogenous PKC, in the presence of 2 mM Ca^{2+}, markedly stimulated the phosphorylation of various membrane proteins (Figure 1, lane e). This phosphorylation was dependent on the presence of Ca^{2+} (Figure 1, lane d) and was sensitive to heat inactivation of the enzyme or its inhibition by polymyxin B (data not shown). Therefore, it appears that while our membrane preparation does not contain endogenous PKC, postsynaptic membrane proteins do serve as substrates for phosphorylation by this kinase.

To test whether any of the proteins phosphorylated by PKC are related to the acetylcholine receptor, phosphorylated membranes were solubilized and subjected to immunoprecipitation by anti-receptor antibodies (Figure 1). Only 60- and 65-kDa phosphoproteins specifically immunoprecipitated with these antibodies. On the basis of their molecular weights, these

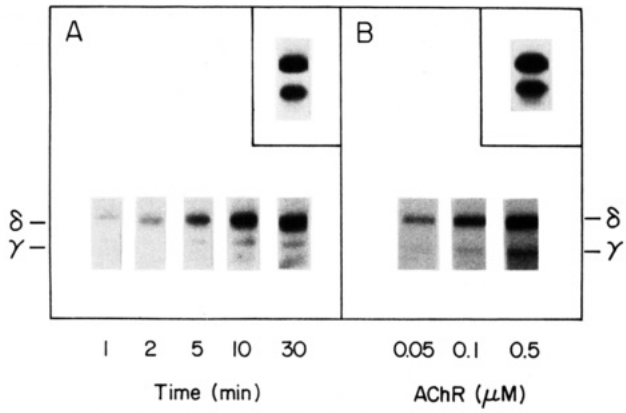

FIGURE 2: Analysis of the phosphorylation of purified AChR by PKC. (A) Affinity-purified AChR (40 pmol) was phosphorylated by PKC for the indicated time periods. Samples were separated by 10% SDS–PAGE, and the gels were dried and exposed to autoradiography. (B) Affinity-purified AChR at the indicated concentrations was phosphorylated for 10 min by PKC. Samples were separated by 10% SDS–PAGE. The phosphorylation of affinity-purified AChR by PKA for 30 min (A) and at a receptor concentration of 0.5 μM (B) is shown in the inserts.

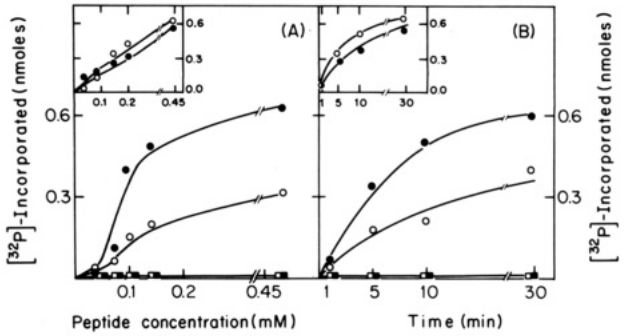

FIGURE 3: Peptide phosphorylation by PKC. (A) Peptide 346–359 from the AChR γ subunit was phosphorylated for 10 min by PKA (●) or by PKC (○) at different peptide concentrations as indicated. Analysis of peptide phosphorylation was performed as described under Materials and Methods. (B) Peptide 346–359 (0.15 mM) from the AChR γ subunit was phosphorylated for the indicated time periods by PKA (●) or by PKC (○). The phosphorylation of the homologous peptide from the δ subunit (residues 354–367) by both kinases is shown in the inserts. ■ and □ represent the phosphorylation of peptide 373–387 from the receptor δ subunit by PKA and PKC, respectively.

proteins represent the γ and δ subunits of AChR, respectively. The δ subunit was the major target for PKC phosphorylation, while the phosphorylation of the γ subunit was significantly lower (Figure 1, lane g). For comparison, activation of endogenous PKA by cAMP resulted in phosphorylation of both the γ and δ subunits to a high extent (Figure 1, lane h; Huganir & Greengard, 1983).

The lower efficiency of phosphorylation of the γ subunit by PKC is evidenced by both time– and dose–response analyses of phosphorylation of affinity-purified AChR. As shown in Figure 2A, phosphorylation of the δ subunit was detectable already following 1 min of incubation with PKC, while that of the γ subunit was detected only after longer (10 min) incubation periods. The maximal stoichiometry of phosphorylation of the δ subunit was 0.6 mol of phosphate/mol of subunit, while that of the γ subunit was less than 0.1. When the affinity-purified receptor was phosphorylated by PKA, the stoichiometries of phosphorylation of the δ and γ subunits were calculated to be 0.8 and 0.6, respectively (Figure 2A, insert). The differential phosphorylation of the two subunits by PKC was reflected also in dose–response analysis, and at all receptor concentrations tested, the phosphorylation of the δ subunit at a 10-min incubation time was 4-fold higher than that of the γ subunit as measured by densitometric scanning (Figure 2B).

Peptide Phosphorylation by PKA and PKC. We have previously shown that PKA and PKC phosphorylate an identical site within the δ subunit of AChR (Safran et al., 1987). We were therefore interested to find out whether the two kinases phosphorylate a similar site also within the γ subunit. For that purpose we have synthesized a 14 amino acid peptide, Lys-Pro-Gln-Pro-Arg-Arg-Arg-Ser-Ser-Phe-Gly-Ile-Met-Ile, corresponding to residues 346–359 of the γ subunit. This peptide contains the identified PKA phosphorylation site within this subunit (Yee & Huganir, 1987) and it is homologous to peptide 354–367 of the δ subunit (Asp-Leu-Lys-Leu-Arg-Arg-Ser-Ser-Ser-Val-Gly-Tyr-Ser-Ile) that has previously been shown to undergo phosphorylation by both kinases (Safran et al., 1987). Furthermore, in the cytoplasmic loop of the γ subunit, this selected peptide contains the most appropriate sequence for phosphorylation by PKC. As shown in Figure 3, the γ peptide is phosphorylated both by PKA and PKC. Control serine-containing peptides derived from the α (residues 330–340) and δ (residues 364–374 and 373–387) subunits were not phosphorylated by either kinase. In analogy to the differential phosphorylation of the γ subunit by PKA and PKC, the γ peptide was a better substrate for PKA than for PKC in both dose– (Figure 3A) and time– (Figure 3B) response analyses. The homologous δ peptide was similarly phosphorylated by the two kinases (Figure 3, inserts).

Discussion

The nicotinic AChR from *Torpedo* postsynaptic membranes has been demonstrated to undergo phosphorylation by several endogenous protein kinases, including a cAMP-dependent protein kinase (Huganir & Greengard, 1983) and a protein tyrosine kinase (Huganir et al., 1984). In this study, we have analyzed the phosphorylation of the receptor by protein kinase C. Our experiments indicate that *Torpedo* postsynaptic membranes are devoid of endogenous PKC activity. This is not surprising, as PKC is a soluble protein that binds to the membranes in a Ca^{2+}-dependent manner (Nishizuka, 1986). Our procedure for preparing the postsynaptic membranes involves the use of high concentrations of EGTA (10 mM) in order to avoid Ca^{2+}-dependent proteolysis of the receptor. Under these conditions, the membranes are likely to be depleted of or, at best, poor in endogenous PKC. However, addition of exogenous PKC in the presence of 2 mM Ca^{2+}, to enable the binding and activation of the kinase, resulted in a marked stimulation of the phosphorylation of various membrane proteins. Using specific antibodies, we could demonstrate that AChR is a major target for PKC phosphorylation in these membranes (Figure 1). Comparing the subunit specificities of PKA and PKC, both kinases were found to phosphorylate both the γ and δ subunits of the membrane-bound AChR. However, while PKA phosphorylated both subunits to a similar extent, PKC phosphorylated predominantly the δ subunit.

The difference in phosphorylation of the γ subunit by the two kinases most probably cannot be attributed to the activation of membranal Ca^{2+}-dependent phosphatases that specifically dephosphorylate the γ subunit. This is illustrated by the finding that in the affinity-purified receptor the two kinases also differ in their ability to phosphorylate the γ subunit (Figure 2). Furthermore, that the different pattern of phosphorylation of the affinity-purified receptor by these two kinases results from intrinsic properties of the receptor itself and not as a result of the γ subunit being prephosphorylated in the PKC phosphorylation site is supported by phosphorylation

experiments of the synthetic peptides (Figure 3).

We have previously demonstrated (Safran et al., 1987) that a distinct portion of the δ subunit of Torpedo AChR, which includes the consensus site for phosphorylation by PKA (Arg-Arg-Ser-Ser-Ser; Kemp et al., 1977), also includes the phosphorylation site for PKC within this subunit. The γ subunit contains a similar yet not identical sequence (Arg-Arg-Arg-Ser-Ser), which was demonstrated to be the site of PKA phosphorylation within the γ subunit (Yee & Huganir, 1987). It was, therefore, of interest to elucidate whether, in analogy to the δ subunit where both kinases phosphorylate the same site, this sequence in the γ subunit also comprises a site of phosphorylation by PKC. Indeed, like peptide 354–367 from the δ subunit, a 14 amino acid peptide corresponding to residues 346–359 of the γ subunit also serves as a substrate for phosphorylation both by PKA and PKC (Figure 3). It should, however, be noted that a difference was observed in the ability of the two kinases to phosphorylate the γ peptide. In both time– and dose–response analyses, the phosphorylation of the γ peptide by PKC was lower when compared to its phosphorylation by PKA. As mentioned above, it seems that the sequence at the putative phosphorylation site of the γ subunit contributes to the difference in the phosphorylation of this subunit within the intact soluble as well as the membrane-bound receptor. It is yet possible that an additional factor, such as the accessibility of this subunit to the two kinases, might also be involved. Indeed, it has been recently demonstrated that the conformation of proteins is crucial for determining their susceptibility to serve as substrates of PKC (Sagi-Eisenberg, 1989; Sagi-Eisenberg et al., 1989).

The sequence requirement of PKC within its protein substrates is not completely resolved, but the common denominator of many substrates studied was the presence of basic residues surrounding the phosphorylation site (Turner et al., 1985; Davis et al., 1986). Several reports describing different protein substrates have indicated that both PKA and PKC phosphorylated a common site which includes the consensus recognition site for PKA (Arg-Arg-X-Ser-Y; Kishimoto et al., 1985; Campbell et al., 1986; Safran et al., 1987; Sibley et al., 1988). Our results demonstrating that sequence 346–359 of the γ subunit of AChR serves as a substrate for both kinases provide another example to this rule. It should, however, be noted that we cannot conclude from our experiments which of the two serine residues present in peptide 346–359 is phosphorylated by either kinase.

The functional roles of the individual AChR subunits in ion transport and gating of the channel have been investigated (Sakmann et al., 1985; Mishina et al., 1986). Taken together, these studies indicate that both the δ and the γ (and/or ϵ) subunits may be functionally important in determining channel properties both at the mature state as well as during muscle development. While previous studies have implicated phosphorylation of the δ subunit in playing a role in the regulation of ion channeling (Miles et al., 1987), recent studies have suggested that PKA phosphorylation of the γ subunit may be responsible for enhanced AChR desensitization (Wagoner & Pallota, 1988). The present study indicates that, during activation of PKC, the γ and δ subunits are phosphorylated to different degrees. In contrast, under conditions that activate PKA, both the γ and δ subunits are similarly phosphorylated. This diversity may provide an additional mode of regulation of receptor function by phosphorylation.

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Purification and Reconstitution of the Sodium- and Potassium-Coupled Glutamate Transport Glycoprotein from Rat Brain†

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ABSTRACT: The sodium- and potassium-coupled L-glutamate transporter from rat brain has been purified to near homogeneity by reconstitution of transport as an assay, assuming that inactivated and active transporters cochromatograph. The purification steps involve lectin chromatography of the membrane proteins solubilized with 3-[(3-chloramidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), fractionation on hydroxylapatite, and ion-exchange chromatography. The specific activity is increased 30-fold. The actual purification is higher since 3-5-fold inactivation occurs during the purification. The efficiency of reconstitution was about 20%. The properties of the pure transporter are fully preserved. They include ion dependence, electrogenicity, affinity, substrate specificity, and stereospecificity. Sodium dodecyl sulfate-polyacrylamide electrophoresis revealed one main band with an apparent molecular mass of around 80 kDa and a few minor bands. Comparison of polypeptide composition with L-glutamate transport activity throughout the fractionation procedure reveals that only the 80-kDa band can be correlated with activity. The GABA transporter, which has the same apparent molecular mass (Radian et al., 1986), is separated from it during the last two purification steps. Immunoblot experiments reveal that the antibodies against the GABA transporter only reacted with fractions exhibiting GABA transport activity and not with those containing the glutamate transporter. We conclude that the 80-kDa band represents the functional sodium- and potassium-coupled L-glutamate transporter.

High-affinity neurotransmitter transport (reuptake) plays an important role in the process of synaptic transmission. After release and interaction with receptors, the transmitters have to be removed from the extracellular space either by degradation (acetylcholine and peptides) or by reuptake (amino acids and catecholamines). The latter process is catalyzed by sodium-coupled neurotransmitter transport systems [reviewed in Kanner (1983) and Kanner and Schuldiner (1987)], located in the plasma membranes of nerve endings and of glial cells. In recent years these uptake processes have been studied in detail with synaptic plasma membrane vesicles. It appears that these transport systems are coupled not only to sodium but also to additional ions like potassium or chloride.

In this paper we describe the purification and reconstitution of one of these neurotransmitter transporters, namely, the L-glutamate transporter from rat synaptic plasma membranes. A partial purification has previously been reported (Gordon & Kanner, 1988). This transporter catalyzes electrogenic cotransport of sodium, potassium, and L-glutamate (or aspartate).

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The reasons for undertaking research on the sodium- and potassium-coupled L-glutamate transport system are 4-fold. (1) L-Glutamate is considered to be the major excitatory transmitter in brain, and aspartate may have a similar action (Fonnum, 1984; Ottersen & Storm-Mathisen, 1984; Roberts et al., 1986). (2) This uptake system is held to be the mechanism by which synaptically released excitatory amino acids are inactivated (Johnston, 1981) and kept below toxic levels in the extracellular space (McBean & Roberts, 1985). (3) The uptake system is still the best marker for tracing of neurons which synaptically release glutamate and aspartate (Ottersen & Storm-Mathisen, 1984). Direct measurement of glutamatergic neurons in human brain has been hampered by lack of a suitable marker (Simpson et al., 1988). These methodological problems would be solved if a specific antibody could be raised against the transporter. (4) The L-glutamate transporter is, as indicated above, biochemically very interesting, since the molecular basis of sodium-coupled transport, and of cotransport in general, is not known.

Experimental Procedures

Materials. Soybean phospholipids (Sigma P-5638, type II-s, commercial grade) were partially purified by acetone precipitation. Crude bovine brain lipids were extracted with chloroform-methanol (Folch et al., 1957). Cholic acid (Sigma) was recrystallized from 70% ethanol (Kagawa & Racker, 1971) and neutralized with NaOH to pH 7.4. Sephadex G-50 fine and wheat germ (lectin) agglutinin-Sepharose 6MB were

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