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EXTERNALLY DISPOSED POLYPEPTIDES OF CHICK SYNAPTOSOMAL PLASMA MEMBRANE

IDENTIFICATION WITH PYRIDOXAL PHOSPHATE AND SODIUM BOROTRITIDE

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Summary

The topographical distribution of polypeptides in chick brain synaptic plasma membrane was studied using pyridoxal phosphate-sodium borotritide labeling. Labeling of intact synaptosomes was restricted to the external surface only by very careful adjustment of the reaction conditions. Fourteen major external polypeptides were labeled. These had apparent molecular weights of 210 000, 160 000, 130 000, 100 000, 92 000, 82 000, 60 000, 52 000, 42 000, 34 000, 29 000, 26 000, 24 000, and 19 000. Most of the label was incorporated into the 42 000, 29 000 and 26 000 dalton polypeptides.

Introduction

Many recent studies have examined the topographical distribution of membrane proteins [1—10]. It was found that membranes have an asymmetrical distribution of both lipids and proteins, which subserves the vectorial functions of the membrane. Some labeling studies used enzymes like lactoperoxidase [11—19] and galactose oxidase [2,20—27] to identify externally exposed proteins and glycoproteins. Various chemical reagents were also used to identify external membrane components [3,28—34].

Pyridoxal phosphate is one chemical reagent which has been used as an impermeant probe. It was considered impermeable to membranes because of

its hydrophilic nature and its negative charge. It was used first as an impermeant probe by Rifkin et al. [35] to locate proteins on the exterior of the influenza virus membrane. Since then pyridoxal phosphate has been used as a surface probe of several membrane systems [34,36,37], though not always successfully [38]. The labeling reaction involves pyridoxal phosphate forming a Schiff base with free amino groups of proteins [39—42]. The Schiff base can be reduced with sodium boro[^{3}H]hydride which introduces tritium label into the protein. We used pyridoxal phosphate to study the topographical arrangement of synaptic plasma membrane proteins under conditions in which it does not cross the synaptic membrane. By carefully controlling reaction conditions of medium, pH, temperature, and length of incubation, it has been possible to restrict labeling to the synaptosomal external surface.

Experimental procedures

Preparation of synaptosomes and synaptic plasma membranes. Synaptosomes and synaptic plasma membranes were prepared by the method of Babitch et al. [43] using 6—8-day-old chickens. Synaptosomes were isolated from a Ficoll 400 (Pharmacia) gradient at the 7%-14% interface. Synpatic plasma membranes were isolated from the sucrose gradient at the 0.8 M-0.95 M interface.

Labeling procedure. Pyridoxylation and tritiation was by the method of Rifkin et al. [35] with several modifications. In a typical experiment, synaptosomes were washed in medium P (0.32 M sucrose, 1 mM potassium phosphate, 0.1 mM EDTA, pH 7.6). They were resuspended in either medium P or Krebs-Ringer bicarbonate buffer, pH 7.6. 20 mM pyridoxal phosphate (Sigma) in the appropriate buffer was added to produce a final concentration of 2 mM (unless otherwise stated). The reaction with pyridoxal phosphate was allowed to proceed for 5 min or as indicated. Then ice-cold 1 M sodium boro[^{3}H]hydride (12.2 Ci/mol or 6.9 Ci/mmol, New England Nuclear or Amersham, freshly prepared in 20 mM sodium hydroxide or stored at −70° C in 20 mM sodium hydroxide) was added to the reaction mixture. The sodium boro[^{3}H]hydride added was in two-fold molar excess of the pyridoxal phosphate. Reduction was allowed to proceed for 5 min at 0° C. The reduction of Schiff bases with sodium borohydride is essentially complete within 3 min under these conditions [34]. The reaction mixture was then brought to the pyridoxal phosphate incubation temperature and unreacted sodium borotritide was removed by adding excess pyridoxal phosphate. The synaptosomes were diluted eight fold with medium P or Krebs-Ringer buffer, centrifuged at 17 000 × g_{av} for 20 min at 2—4° C, and washed twice with the same buffer to remove unreacted pyridoxal phosphate. With each experiment a control was performed simultaneously, in which only pyridoxal phosphate was omitted from the reaction mixture. When synaptosomes were prereduced, the washed synaptosomes were first treated with ice-cold 4 mM sodium borohydride, washed twice with buffer, and then subjected to pyridoxylation and titration by the above procedure. The labeled synaptosomes were lysed for 1 h in 1 mM potassium phosphate and 0.1 mM EDTA, pH 8.0 (lysis medium). Then synaptic plasma membranes and the synaptoplasmic polypeptides were prepared as described previously [43].

Protein purification. Samples not to be electrophoresed were subjected to purification as described by Chiu and Babitch [44]. The pellet was dissolved in 0.5 N sodium hydroxide. Aliquots of this solution were used for protein determination and counting of radioactivity. Protein was assayed by the Lowry method as described by Bruening et al. [45].

Sodium dodecyl sulfate polyacrylamide gel electrophoresis. One-dimensional electrophoresis was carried out in 5.6% polyacrylamide gels, according to the method of Fairbanks et al. [46]. Synaptic plasma membrane pellets were directly dissolved in the sample buffer containing 1.0% SDS, 10% glycerol, 10 mM Tris, 1 mM EDTA and 1% β-mercaptoethanol by incubating for 16 h at 37° C. Synaptoplasmic polypeptides were prepared as described by Chiu and Babitch [44], and fractions were dissolved as above. These solutions were used for electrophoresis after adding 0.001% bromophenol blue tracking dye. Protein bands on the gel were visualized by staining with Coomassie blue. Molecular weight markers for molecular weight determination were cytochrome *c* (12 400), ribonuclease (13 700), lysozyme (14 400), myoglobin (17 600), trypsin (23 800), pepsin (35 500), ovalbumin (46 000), catalase (60 000), phosphorylase *a* (92 000), and β-galactosidase (135 000) (all from Sigma). The gels were sliced into 1 mm slices with a gelslicer (Bio-Rad). Slices were incubated in 0.3 ml of TS-1 (Research Products International Corp.) and 0.03 ml of water at 60° C for 1 h until solubilized. The slices were then counted in a Packard Liquid Scintillation counter after adding 5 ml of Handi-fluor (Mallinckrodt).

All chemicals were reagent grade and water was twice deionized and glass distilled.

Results

Reactions of synaptosomal polypeptides as a function of pyridoxal phosphate concentration

Pyridoxal phosphate incubations were carried out at pH 7.6 after we observed little difference between the incubations at pH 7.6 and pH 8.0 (data

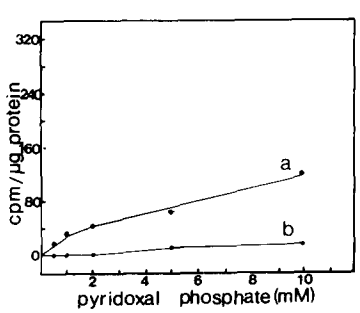

Fig. 1. Reaction of pyridoxal phosphate with intact synaptosomes as a function of pyridoxal phosphate concentration. Synaptosomes were prepared and incubated for 5 min at 0°C in medium P with various concentrations of pyridoxal phosphate and NaB^3H_4 as described in Experimental procedures. (a) Incorporation of pyridoxal phosphate into synaptic plasma membranes. (b) Incorporation of pyridoxal phosphate into synaptoplasmic polypeptides.

not shown). In these initial experiments the molar ratio of NaB^3H_4:pyridoxal phosphate was maintained at 2 : 1. Controls without pyridoxal phosphate were run in every case, and results shown are minus the control incorporation. It is evident from Fig. 1 that there was an increase in incorporation of label with increasing pyridoxal phosphate concentration. This indicates that the increased bound radioactivity was due to increased pyridoxal phosphate reaction and was not due to increased nonspecific labeling by NaB^3H_4. It is also evident that with 2 mM and lower concentrations of pyridoxal phosphate, synaptoplasmic polypeptides were not substantially labeled. At higher concentrations of pyridoxal phosphate, synaptoplasmic polypeptides incorporated label representing pyridoxal phosphate penetration into the synaptoplasm. With preparations of virus [35] or red blood cells [36], higher concentrations of pyridoxal phosphate were used without labeling the internal components.

Reaction of synaptosomal polypeptides with pyridoxal phosphate as a function of time

Fig. 2 shows that there is little incorporation of label into synaptoplasmic polypeptides for up to 5 min of incubation at 2 mM pyridoxal phosphate. However, with longer incubation periods the synaptoplasmic polypeptides began to incorporate label indicating that pyridoxal phosphate was forming Schiff bases with internal polypeptides. In red blood cells [36] it has been shown that the binding of pyridoxal phosphate to hemoglobin (representing pyridoxal phosphate penetration into the cell), begins only after an hour of incubation.

Effect of incubation temperature on synaptosomal labeling

Synaptosomes were incubated with 2 mM pyridoxal phosphate and 4 mM NaB^3H_4 in medium P for 5 min (each) at 0, 22, or 37° C. Results (not shown) indicated a good correlation between incubation temperature and tritium incorporation into both synaptoplasmic and synaptic plasma membrane poly-

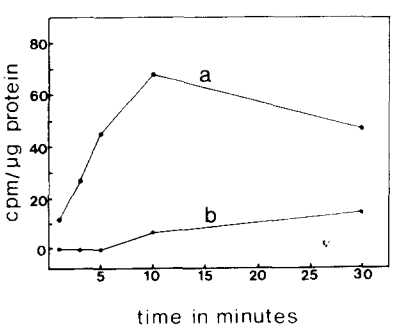

Fig. 2. Pyridoxal phosphate reaction with intact synaptosomes as a function of time. Synaptosomes were prepared and incubated at 0°C in medium P with 2 mM pyridoxal phosphate for various times as indicated before labeling with NaB^3H_4 as described under Experimental procedures. (a) Incorporation of pyridoxal phosphate into synaptic plasma membranes. (b) Incorporation of pyridoxal phosphate into synaptoplasmic polypeptides.

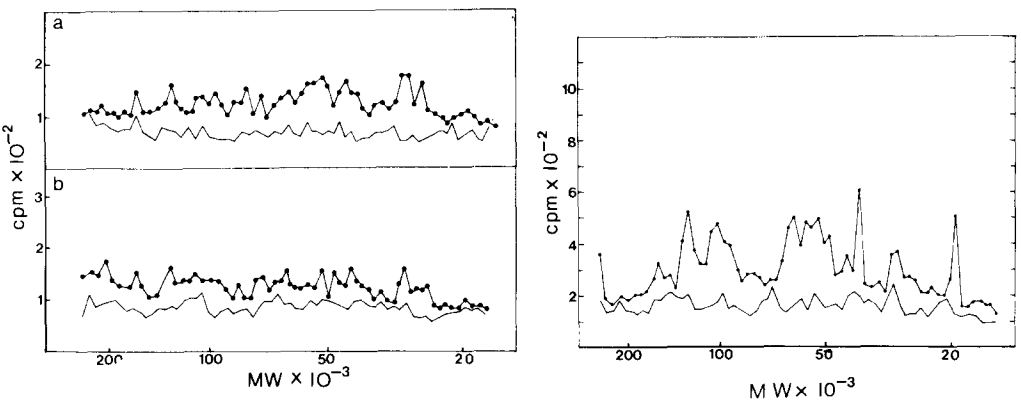

Fig. 3. Radioactivity profiles of synaptic plasma membrane polypeptides labeled with pyridoxal phosphate and NaB^3H_4 without (a) or with (b) prereduction by 4 mM NaB^3H_4. Synaptosomes were prepared and labeled as described in Experimental procedures and the text. •———•, incubation including pyridoxal phosphate; ———, control incubation without pyridoxal phosphate.

Fig. 4. Radioactivity profile of synaptic plasma membrane polypeptides after synaptosomal labeling in Krebs-Ringer buffer. Synaptosomes were prepared as described in Experimental procedures and were incubated with 2 mM pyridoxal phosphate in Krebs-Ringer buffer for 5 min at 0°C. Reduction was with 4 mM NaB^3H_4. •———•, pyridoxal phosphate treated; ———, control.

peptides. Only at 0°C were the synaptoplasmic polypeptides not labeled. This suggests that increasing membrane fluidity substantially contributes to passage of pyridoxal phosphate across the synaptosomal membrane. Similar effects were observed when incubations were conducted in Krebs-Ringer buffer (see below).

Effect of prereduction on synaptosomal labeling

Synaptosomes were prereduced with 4 mM sodium borohydride before pyridoxal phosphate and NaB^3H_4 labeling under conditions of minimal penetration, i.e., 2 mM pyridoxal phosphate at 0°C for 5 min followed by 4 mM NaB^3H_4 (specific activity 12.2 Ci/mol). Membranes from these synaptosomes were compared to synaptosomal membranes labeled with no prereduction. Radioactivity profiles (Fig. 3) show very little difference between the prereduced and non-prereduced polypeptides. Only low levels of incorporation were observed. The synaptoplasmic polypeptides in both cases do not incorporate specific label (not shown). No differences between the Coomassie blue-staining patterns of labeled, control or untreated polypeptides were observed (data not shown).

Effect of changing the incubation medium on synaptosomal incorporation

Synaptosomes were labeled with pyridoxal phosphate and NaB^3H_4 in a medium different from medium P to see if a different incubation medium might change the labeling pattern. Using Krebs-Ringer buffer, synaptosomes were reacted with 2 mM pyridoxal phosphate for 5 min at 0°C and were then reduced with 4 mM NaB^3H_4. The radioactivity profile of the synaptic plasma membrane polypeptides (Fig. 4) shows a pattern different from that of synap-

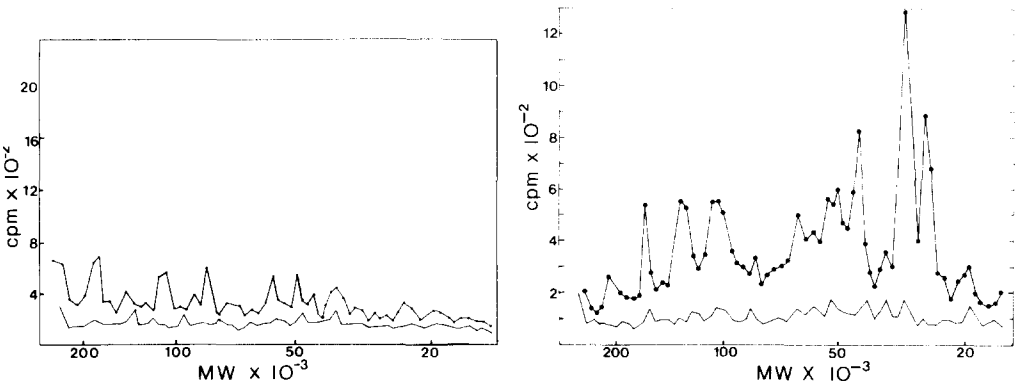

Fig. 5. Radioactivity profile of synaptoplasmic polypeptides after labeling intact synaptosomes in Krebs-Ringer buffer. For experimental details see legend to Fig. 4.

Fig. 6. Radioactivity profiles of synaptic plasma membrane polypeptides after synaptosomes were treated with 2 mM pyridoxal phosphate in medium P for 5 min at 0°C. Reduction was with 4 mM NaB^3H_4 (spec. act. 6.9 Ci/mmol). ●———●, pyridoxal phosphate treated; ———, control.

tosomes labeled in medium P. The radioactivity profile of the synaptoplasmic polypeptides (Fig. 5) shows considerable label incorporated, indicating that pyridoxal phosphate readily crossed the membrane under these conditions. Decreasing the pyridoxal phosphate concentration to as low as 0.1 mM still did not prevent labeling of the synaptoplasmic polypeptides. At 37°C incubations resulted in the apparent labeling of all synaptosomal polypeptides (not shown).

Identification of externally exposed synaptosomal plasma membrane polypeptides

We reacted synaptosomes in medium P with 2 mM pyridoxal phosphate at 0°C for 5 min, and then reduced with 4 mM NaB^3H_4 (spec. act. 6.9 Ci/mmol).

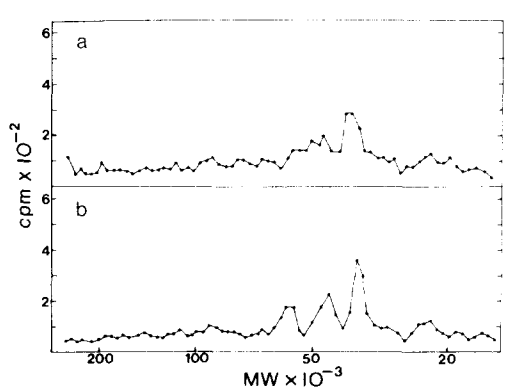

Fig. 7. Radioactivity profiles of synaptoplasmic polypeptides after labeling intact, prereduced synaptosomes with 2 mM pyridoxal phosphate in medium P at 0°C. Reduction with 4 mM NaB^3H_4 (spec. act. 6.9 Ci/mmol). (a) Pyridoxal phosphate treated. (b) Control.

The radioactivity profile of the synaptic plasma membrane polypeptides (Fig. 6) shows a high level of specific incorporation of label. The synaptoplasmic polypeptides did not incorporate any specific radioactivity (Fig. 7), indicating that pyridoxal phosphate did not cross the synaptic plasma membrane, and that labeling was restricted to the external surface. The major labeled peaks had apparent molecular weights of 210 000, 160 000, 130 000, 100 000, 92 000, 82 000, 60 000, 52 000, 42 000, 34 000, 29 000, 26 000, 24 000, and 19 000. Lactoperoxidase-catalysed iodination of the synaptosomal external surface does not label the 210 000, 160 000 and 82 000 dalton proteins [44]. All 14 peaks are labeled after neuraminidase pretreatment and galactose oxidase-NaB^3H_4 labeling of synaptosomes [47].

Discussion

Synaptosomes provide a good system for studying the mechanism of neurotransmission and the transport processes which occur across the neuronal membrane [48]. However, synaptosomes are nerve terminals that are torn away from the axon and from the postsynaptic site, so their intactness as a fully enclosed particle can be questioned. We have shown previously that our preparation of synaptosomes is quite intact by the use of trypsin which is not accessible to the synaptoplasmic polypeptides [44]. Still, extremely careful labeling conditions must be used to insure that the probe does not cross the membrane. This is particularly important when labeling with small molecules.

Another important consideration is the purity of the final synaptic plasma membrane preparation. The original description of purity [43] included electron microscopic and enzyme marker data for six enzymes. These data showed that maximal subcellular contamination was: mitochondrial (inner and outer membrane), 4% and 8%, respectively; microsomal and endoplasmic reticulum, 10%, and lysosomal, 1% or less. $(Na^+ + K^+)$-activated ATPase was enriched 13.3 fold above homogenate levels. Multilamellar myelin was absent.

We subsequently showed that the synaptic plasma membranes have a polypeptide pattern which is different from the polypeptide patterns of synaptic vesicles, mitochondria, microsomes, or synaptoplasm [49]. The surface labeling patterns of synaptic plasma membranes are very different from the labeling patterns of brain mitochondria, too [50]. We also showed that certain similarities between the synaptic vesicle and synaptic plasma membrane polypeptide patterns were not due to contamination of one fraction by the other. These data from two-dimensional gels confirm that our synaptic plasma membrane fraction is not contaminated by mitochondria, microsomes, endoplasmic reticulum, or synaptic vesicles.

In addition, we have results that demonstrate that our synaptic plasma membrane preparations have only low levels of carbonic anhydrase (unpublished data). Because carbonic anhydrase occurs in glial cytoplasm, glial membranes and in myelin [51], this suggests that the synaptic plasma membrane fraction only contains low levels of glial material. Further, we have removed the traces of glial contamination from the synaptic plasma membrane fraction by affinity chromatography and find that all of the peptides we describe in this report remain in the synaptic plasma membrane fraction

(Babitch, J.A., unpublished results). We believe that these data, when combined with our previous studies, demonstrate that this material is synaptic plasma membranes. Also, labeling patterns we observe are similar to those found by many others workers (reviewed by Smith and Loh [52]). It is unlikely that a mixed population of membranes would give such consistent results in the laboratories of several different groups.

Pyridoxal phosphate has been used as a membrane probe by different groups of researchers on various membranes [2,35,38,41,53]. Pyridoxal phosphate offers certain advantages as a membrane probe. It reacts specifically with amino groups, so it may label proteins which are not labeled by a probe such as lactoperoxidase which labels only tyrosine and histidine groups [54–57]. Second, pyridoxal phosphate may be able to reach and identify sites on proteins not accessible to a larger reagent like an enzyme. However, its small size apparently allows it to cross the synaptic membrane readily despite its net negative charge. We have achieved conditions for pyridoxal phosphate interaction with the synaptosomal plasma membrane where there is little or no penetration of the probe into the synaptoplasm. The conditions we had to employ to prevent pyridoxal phosphate from crossing the synaptosomal membrane are extremely mild compared to labeling conditions for red blood cells [36] or viruses [35]. This may be due to the many transport mechanisms for ions and small molecules in the synaptic plasma membrane.

After labeling with pyridoxal phosphate and NaB^3H_4 fourteen major labeled peaks were observed. These had apparent, molecular weights of 210 000, 160 000, 130 000, 100 000, 92 000, 82 000, 60 000, 52 000, 42 000 34 000, 29 000, 26 000, 24 000, and 19 000. After lactoperoxidase-catalysed iodination of the synaptosomes [44], 11 major labeled peaks were observed. These had the same molecular weights as above, except the 210 000, 160 000 and 82 000 dalton polypeptides which were not labeled. Thus, pyridoxal phosphate labels three more proteins which were not labeled by lactoperoxidase-catalysed iodination, either due to steric hinderance or lack of reactive groups. Neuraminidase treatment followed by galactose oxidase and NaB^3H_4 labeling of synaptosomes [47] labeled all 14 of the polypeptides labeled by pyridoxal phosphate and NaB^3H_4. Thus, pyridoxal phosphate-NaB^3H_4 labeling reinforces previous data that these 14 polypeptides are exposed at the outside of the membrane, since they are labeled by two probes which are not accessible to the synaptoplasm. Because the two methods label the same polypeptides both carbohydrate moieties and lysine residues must be externally disposed in all fourteen. That lactoperoxidase-catalysed iodination labels almost as many polypeptides as does pyridoxal phosphate and NaB^3H_4 suggests that most external proteins have substantial peptide areas exposed at the external surface.

Pyridoxal phosphate labeling gives some extra information about the polypeptides. There are three major peaks which incorporate much of the total radioactivity. These are all relatively low molecular weight polypeptides which may have a large number of exposed lysines or may participate in the transport of anions across the membrane. The 34 000 dalton protein (which is strongly labeled by galactose oxidase and NaB^3H_4) is a major Coomassie blue-stained peak. But it is only slightly labeled by pyridoxal phosphate suggesting that its orientation may not expose a large number of lysine residues or that the

negatively charged sialic acid groups of this glycoprotein may repel negatively charged molecules like pyridoxal phosphate [58].

In Krebs-Ringer buffer synaptoplasmic polypeptides seem to be accessible even at very low concentrations of pyridoxal phosphate. Sperk and Baldessarini [59] have shown that synaptosomes incubated in isotonic Krebs-Ringer buffer are susceptible to leakage of their cytoplasmic contents, and that addition of sucrose to the medium has a stabilizing effect on the membrane. This is in agreement with our observations that the synaptosomes are more impermeable to pyridoxal phosphate in an isotonic sucrose medium than in a medium where tonicity is maintained with ions. The 185 000 dalton protein labeled in Krebs-Ringer buffer by pyridoxal phosphate-NaB^3H_4 could be present in the inside surface of the membrane because it is not labeled by pyridoxal phosphate and NaB^3H_4 in medium P.

Acknowledgements

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