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AN ULTRASTRUCTURAL AND CHEMICAL ANALYSIS OF THE EFFECT OF TRITON X-100 ON SYNAPTIC PLASMA MEMBRANES

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SUMMARY

Triton X-100 treatment of synaptic plasma membrane fractions selectively dissociates membrane constituents. At an ultrastructural level the synaptic complex resists Triton treatment when Ca^{2+} is present so that the synaptic complex is dissociated from the bulk of the adjoining plasma membrane. The synaptic complex retains its selective affinity for phosphotungstic acid and can therefore be unambiguously identified in the Triton-insoluble residue. At a chemical level Triton results in differential solubilization of membrane protein, phospholipid and sialic acid. Under conditions favorable for preserving synaptic complex, the insoluble residue retains about 60 % of its initial protein, 70 % of its initial phospholipid phosphorus, and 80 % of its initial sialic acid. Over 75 % of the acetylcholinesterase is solubilized, but essentially all of the 5'-nucleotidase and alkaline phosphatase of the synaptic plasma membrane fraction remains insoluble. The implications of these data for synaptic function and the structuring of synaptic plasma membranes are discussed.

INTRODUCTION

Synapses are the primary sites of interneuronal communication, "the key structures of the nervous system"[1]. Electron microscopic evidence indicates that synaptic transmission takes place in a specialized morphological framework, the "synaptic complex", characterized by apposed cell membranes separated by a synaptic cleft with adjacent pre- and post-synaptic cytoplasmic densities and a pre-synaptic accumulation of synaptic vesicles[2,3]. Complete understanding of synaptic function requires knowledge of the role of these structural specializations. This paper describes two steps in this direction based on partial solubilization of synaptic plasma membrane with Triton X-100 ("Triton"). We provide information on the structural organization of this membrane through biochemical analyses of synaptic plasma membrane solubilization. At the same time we examine the use of Triton treatment as a preliminary step toward isolation and purification of the synaptic complex.

Dissociation by solubilization can be used to study the structural and functional interactions in membranes at a variety of levels. Varying degrees of solubilization can be used (1) to reveal elemental membrane constituents by breaking all non-covalent

Abbreviation: SPM fraction, synaptic plasma membrane fraction.

bonds, (2) to isolate subunit complexes intrinsic or "native" to a membrane[4], (3) to preserve a membrane's ultrastructural integrity while eliminating non-essential components[5]. This latter approach can be extended to include preservation of additional membrane properties including selective permeability, molecular transport, surface-associated enzymatic activities, or antigenic properties, while dissecting free other non-involved components.

As a first step toward understanding the structure of synaptic plasma membrane, we investigate the solubilizing properties of Triton X-100. We measure the release of synaptic plasma membrane protein, phospholipid phosphate, and sialic acid as a function of amount of Triton used, ionic conditions and pH during solubilization. We also examine the solubilization of acetylcholinesterase, 5'-nucleotidase, and alkaline phosphatase. These experiments allow some tentative conclusions about forces important for protein and phospholipid binding in synaptic plasma membrane and about the possible structural role of membrane glycoproteins.

The second contribution of this paper involves the use of Triton X-100 as a preliminary step for purification of synaptic complexes. DE ROBERTIS *et al.*[6,7] have reported that treatment of synaptic plasma membrane-containing subcellular fractions with Triton leaves the synaptic complex intact while solubilizing or detaching the adjacent plasma membrane. Clear interpretation of these studies is hampered because identification of the synaptic complex was based primarily on the presence of electron-dense, osmiophilic patches after fractionation of the Triton-treated material. These densities could be artifactually produced by detergent action on non-junctional membrane. Furthermore, dense packing in the pelleted Triton residue could lead to the apposition of membrane fragments which could easily be mistaken for synaptic complexes.

To clarify this situation we describe the effect of Triton on a purified preparation of synaptic plasma membrane. We determine the presence of the synaptic complex after Triton treatment by electron microscopy, both by conventional procedures and with the use of ethanolic phosphotungstic acid. This latter procedure results in a relatively specific staining of the synaptic complex[8,9]. Based on these methods we conclude that the integrity of the synaptic complex is dependent not only on the amount of Triton used, but also on pH and the presence of Ca^{2+}. The details of the solubilization procedures should be of considerabe value for future progress in obtaining synaptic complex-rich fractions.

METHODS

Tissue preparation

Forebrains of 20–40-day-old, male, Sprague–Dawley rats were used in the preparation of tissue for this experiment. Tissue to be used for enzymatic or chemical analysis was maintained at 4° throughout the preparative procedures.

Synaptic plasma membrane was prepared essentially as described in the previous article[10]. Membrane from the 25–32.5 % sucrose band was used in these experiments. This membrane band was pelleted and resuspended in 3 mM Tris at the appropriate pH and at a protein concentration of 2 mg/ml. In the experiments in which membrane was to be assayed for sialic acid, the membrane was resuspended in twice distilled water and repelleted once to remove sucrose. Prior to addition of Triton the

pH was checked (at 4°) and adjusted, if necessary, with NaOH (a Corning combination triple-purpose electrode was used to monitor pH). Triton at concentrations ranging from 3.6 to 12.5 % (v/v) in 3 mM Tris at the appropriate pH was added to the desired final ratio of Triton to protein (Triton/protein, μl 100 % Triton/mg membrane protein). During addition of Triton, samples were rapidly mixed and then allowed to stand for 20 min at 4°. An aliquot was taken and the remaining material centrifuged in a Spinco Type 40 rotor for 40 min at 40000 rev./min to sediment the insoluble material. Supernatants and pellets were separated and frozen at —20° for subsequent analysis.

Analytical techniques

Protein was analyzed by the method of LOWRY *et al.*[11] with bovine serum albumin as standard. Total phosphate was determined as described by AMES[12] after washing in $Mg(NO_3)_2$. Sialic acid was determined by the method of WARREN[13] after hydrolysis in 0.05 M H_2SO_4 at 80° for 1 h. The absorbance was measured at 549 and 532 nm and the quantity of sialic acid (expressed in terms of *N*-acetylneuraminic acid) computed according to the formula of WARREN (Eqn. 2). Acetylcholinesterase (EC 3.1.1.7), alkaline phosphatase (EC 3.1.3.1), and 5'-nucleotidase (EC 3.1.3.5) were determined as described in the previous paper[10].

Electron microscopic techniques

Samples were prepared for analysis by electron microscopy in two ways: (1) by glutaraldehyde fixation followed by osmium fixation, or, (2) by glutaraldehyde followed by staining in ethanolic phosphotungstic acid according to the procedure of BLOOM AND AGHAJANIAN[8]. 4 % glutaraldehyde solutions in CAULFIELD[14] buffer were used. Tissue preparations were fixed with glutaraldehyde in suspension or thin pellets of the fraction were prepared by the Beem capsule technique previously described[15]. After 0.5 h of fixation, samples were either post-fixed in Caulfield osmium fixative or dehydrated in a graded ethanol to 100 % ethanol for phosphotungstic acid staining. Samples were stained with freshly prepared phosphotungstic acid (Fischer) for 2 h. Phosphotungstic acid treated samples were next embedded in Marglas. Phosphotungstic acid staining appeared to be stable if samples were stored in 70 % ethanol for 1 day. We found that in order to obtain reliable data it was essential to use Fischer phosphotungstic acid and to embed in Marglas resin. For most electron microscope experiments osmotic shock was carried out at room temperature for 1 h prior to separation on a discontinuous gradient.

RESULTS

After treatment of the synaptic plasma membrane fraction (SPM fraction) with relatively high concentrations of Triton X-100, the pellet, when examined by electron microscopy, consists of numerous membrane fragments with few, if any, structures resembling synaptic complexes. If the quantity of Triton added is decreased and $CaCl_2$ included (Triton/protein = 1.2, 3 mM $CaCl_2$), a number of structures resembling synaptic complex are seen (Fig. 1). Most synaptic complexes seen are detached from the non-junctional membrane. A higher magnification view of the more intact junctional structures reveals the presence of small pieces of both *pre-* and *post*-synaptic membranes (Fig. 2). However, the identity of many of these osmiophilic structures is not certain and artifacts are possible.

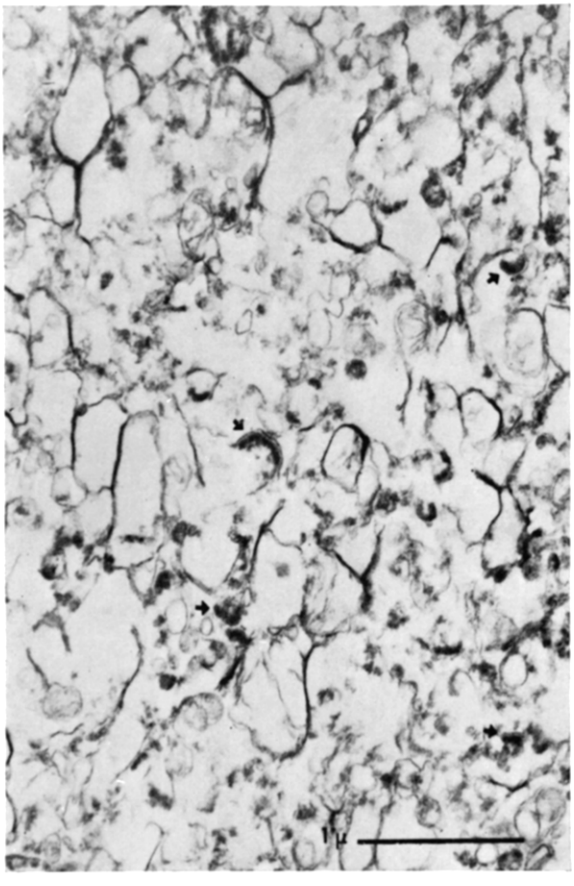


Fig. 1. Electron micrograph of insoluble residue obtained after solubilization of SPM fraction by Triton X-100 in the presence of 3 mM $CaCl_2$. The arrows point to structures which appear to be synaptic complexes. 1.2 μl of Triton was added per mg of membrane protein as described in methods section. Glutaraldehyde–osmium fixation.

In order to determine more precisely the presence of synaptic complex we used the ethanolic phosphotungstic acid procedure developed by BLOOM AND AGHAJANIAN[8]. These workers have shown that ethanolic phosphotungstic acid selectively stains the synaptic complex region in tissue sections prepared from brain. Fig. 3 shows an electron micrograph from a synaptosome preparation prepared by the ethanolic phosphotungstic acid technique. Synaptic complexes are clearly stained, as well as some undefined elements within the vesicular structures. Plasma membranes adjacent to synaptic complexes are not stained, nor are mitochondria. Thus ethanolic phosphotungstic acid can provide histochemical identification of synaptic complexes in these preparations.

After Triton treatment under moderately mild conditions (Triton/protein = 1.2, 3 mM $CaCl_2$) a substantial number of synaptic thickenings, identified by ethanolic phosphotungstic acid staining, are present (Fig. 4). As shown in Fig. 5b, at high magnification, both a pre- and post-synaptic thickening are present in some synaptic

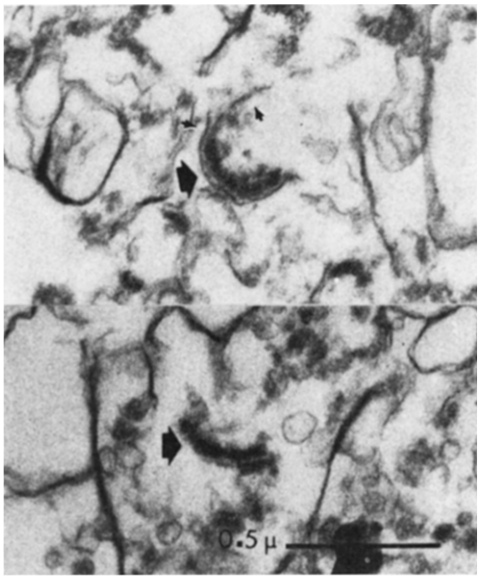


Fig. 2. High magnification field showing synaptic complex (large arrows). Small pieces of attached pre- and post-synaptic membrane are seen attached to one synaptic complex (small arrows), while in the other these membranes have been lost. Prepared as described in Fig. 1.

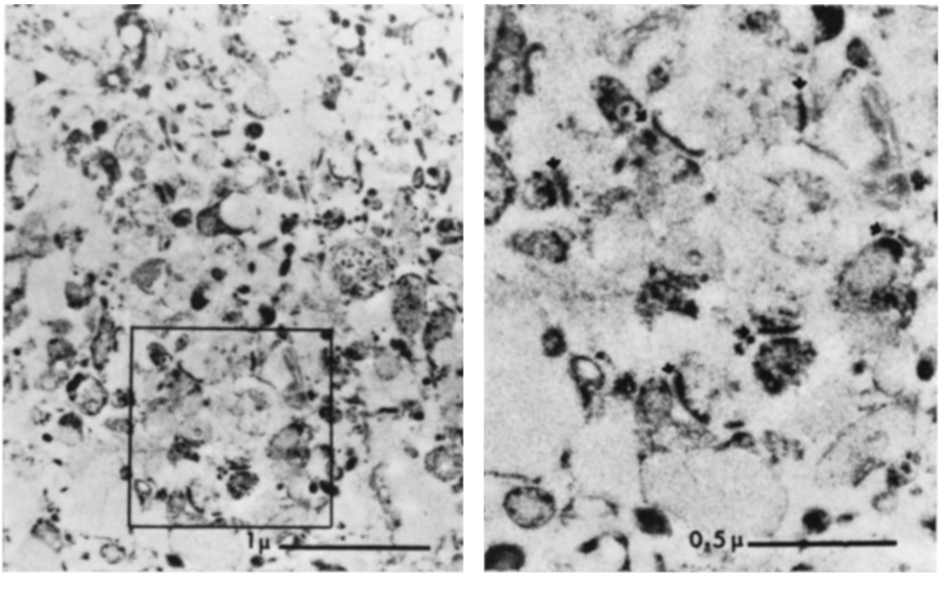


Fig. 3. An electron micrograph of a synaptosomal fraction stained by the ethanolic phosphotungstic acid technique of BLOOM AND AGHAJANIAN[8]. (a) is a low magnification field and (b) is a high magnification field taken from the enclosed area in (a). The fine intercleft band of synaptic complex is well preserved and stained. Synaptic complexes are heavily stained. In some structures there is also a diffuse staining of the cytoplasm. Neither mitochondria nor plasma membranes adjacent to synaptic complex are stained.

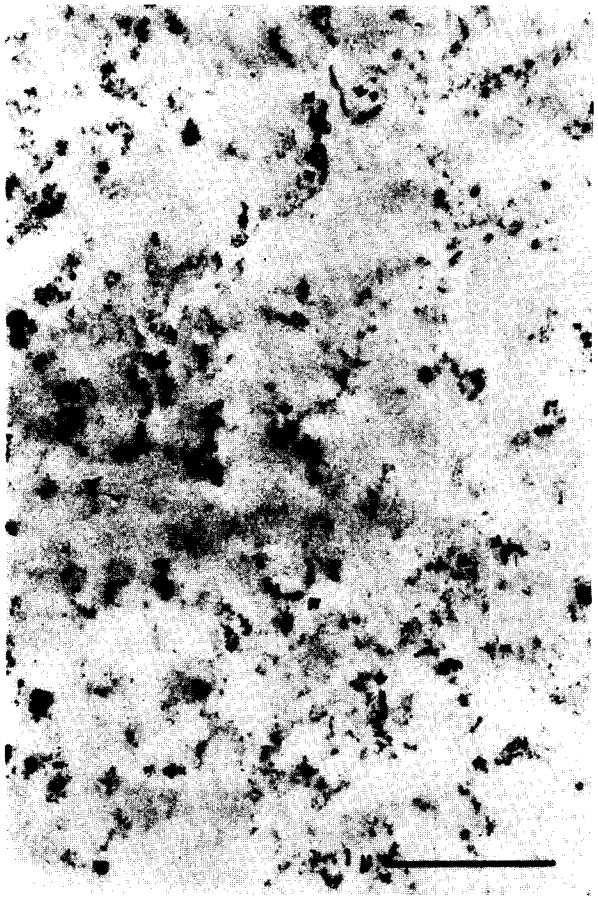


Fig. 4. Micrograph of insoluble residue, obtained after solubilization of SPM fraction by Triton and stained in ethanolic phosphotungstic acid. The arrows indicate structures which appear to be synaptic complexes. This micrograph was obtained from the same preparation illustrated in Fig. 1. An aliquot was pelleted in a Beem capsule and prepared with OsO_4 and another pelleted and prepared with ethanolic phosphotungstic acid.

complexes. This is in accord with the findings from osmium-treated material. Prior to Triton treatment, fewer synaptic complexes appear to be present in a representative field from an SPM fraction (Fig. 6). However, it is difficult to make precise comparisons since the packing of the two preparations may differ to some extent due to the presence of different sized particles.

The effects of Triton on synaptic plasma membrane can be more clearly defined by examination of the solubilization of representative membrane components under the conditions suggested by the morphological data. The nature and extent of solubilization is dependent on the quantity of detergent used, the pH during solubilization, and the presence of certain ions.

The effects of increasing Triton on solubilization of synaptic plasma membrane are shown in Fig. 7 (3 mM Tris, 3 mM $CaCl_2$, pH 8.0). Protein is preferentially released at low Triton concentrations. Solubilization of phosphate increases linearly with

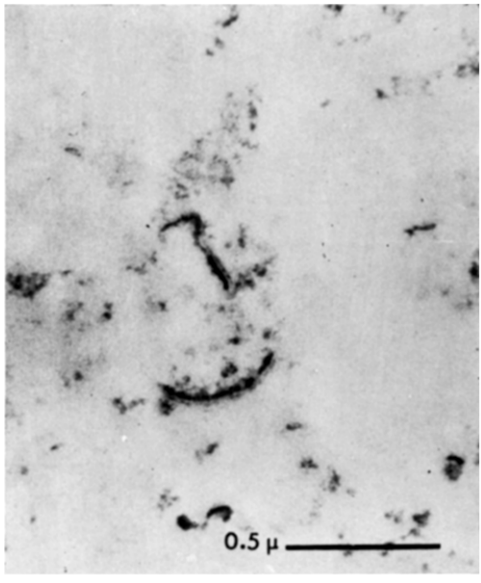


Fig. 5. A high magnification field of the Triton-insoluble residue stained by ethanolic phosphotungstic acid. Well preserved synaptic complexes include a thin band of attached material suggestive either of intracleft material or fragments of presynaptic membrane. Prepared as described in Fig. 4.

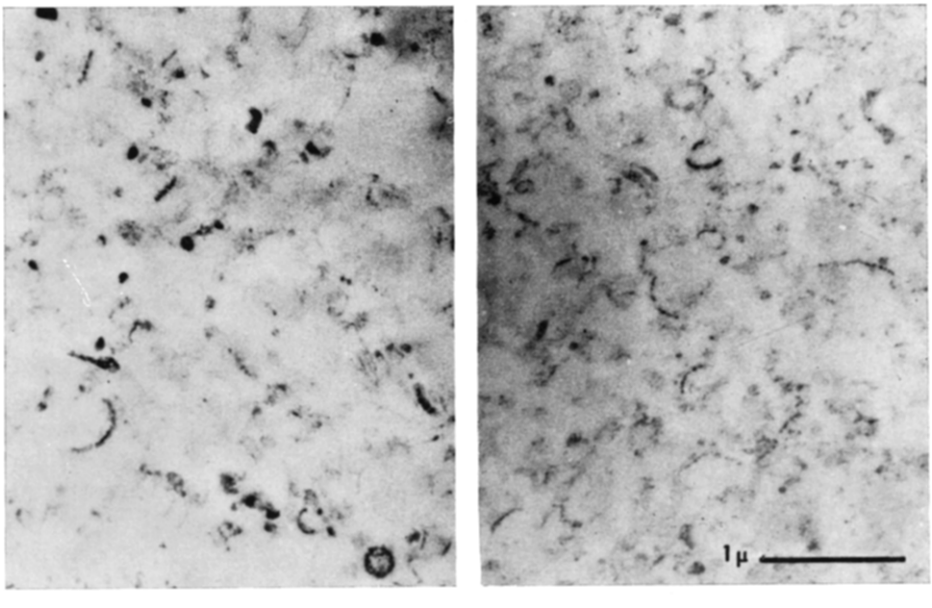
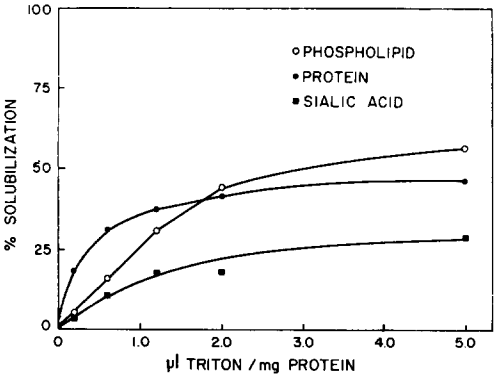


Fig. 6. Micrograph of untreated SPM fraction stained by ethanolic phosphotungstic acid. (a) and (b) are representative fields from 2 different preparations. Numerous bar-like structures which are synaptic complexes can be seen.

increasing Triton until approx. 40 % of the phosphate is released (Triton/protein = 2.0) and only slightly more thereafter. Relative to the other components, sialic acid is particularly resistant to the action of this detergent.

The pH dependence of Triton solubilization is quite striking (Fig. 8). The solubilization of membrane protein doubles between pH 6.0 and pH 10.0. In contrast membrane phosphate is little affected by changes in pH. The solubilization of sialic acid is also relatively unaffected by pH changes between 7.0 and 10.0. Less sialic acid is solubilized at pH 6.0.



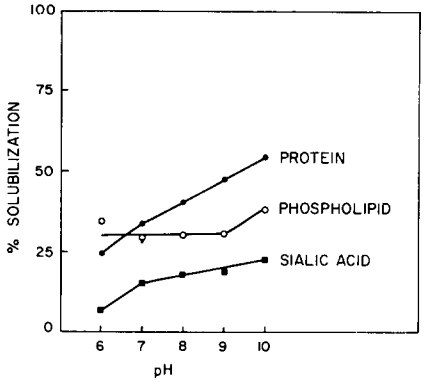


Fig. 7. Solubilization of synaptic plasma membrane protein, phospholipid phosphate, and sialic acid with increasing amounts of Triton X-100. Solubilization was carried out in 3 mM Tris, pH 8.0 with 3 mM $CaCl_2$; membrane protein concentration was 2 mg/ml. Triton was added at the Triton-to-protein ratios indicated (μl Triton/mg protein). Assays were performed on the Triton-soluble material, the Triton-insoluble residue (40000 rev./min pellet), and a sample of the total Triton-treated material before pelleting. Percent solubilization reported is the percent of the total Triton-treated material recovered in the soluble fraction. Recoveries were calculated for the studies reported and were 95.6 $\pm$ 4.8, 95.2 $\pm$ 5.8, and 90.7 $\pm$ 3.5, for protein, phosphate and sialic acid, respectively (mean $\pm$ S.D.). Recoveries did not vary systematically with any of the solubilization variables studied.

Fig. 8. Effect of pH on the Triton solubilization of synaptic plasma membrane protein, phospholipid phosphate, and sialic acid. Solubilization was carried out at a Triton-to-protein ratio of 1.2 in the presence of 3 mM $CaCl_2$ and 3 mM Tris at the appropriate pH. Protein concentration was 2 mg/ml. Additional details as described in Fig. 7.

The effects of Ca^{2+} on Triton solubilization of synaptic plasma membrane components is shown in Table I. These Triton conditions are those which give satisfactory preservation of synaptic complexes as determined by electron microscopy. Inclusion of $CaCl_2$ (3 mM) inhibited the solubilization of protein by 24 % compared to solubilization in the absence of added calcium. The inhibitory effect on solubilization of phosphate and sialic acid is even more marked, 34 and 40 % respectively. 50 μM $CaCl_2$ did not produce a detectable inhibition of the solubilization of any components examined.

The solubilization of several enzymes was also examined under the conditions of Triton concentration and pH previously described. Acetylcholinesterase is more readily solubilized than total membrane protein (over 75 % at Triton/protein = 1.2, pH 8.0) and its solubilization increases with increasing pH (49 % at pH 6.0 *vs.* 88 % at pH 10.0, Triton/protein = 1.2). By contrast, solubilization of alkaline phosphatase

TABLE I

EFFECT OF Ca^{2+} ON TRITON SOLUBILIZATION OF SYNAPTIC PLASMA MEMBRANE

Solubilization was carried out at Triton/protein ratio of 1.2 in 3 mM Tris, pH 8.0.

Ca^{2+} added (mM)	*% Solubilization*		
	Protein	*Phosphate*	*Sialic acid*
0.0	52	45	30
0.05	51	46	29
3.00	40	30	18
Ca^{2+} Inhibition (%) *	23	34	40

* Difference in presence and absence of Ca^{2+} (3 mM) as percent of solubilization in absence of Ca^{2+}.

and 5′-nucleotidase is less than 5 % under all of these conditions. It seems clear from these results that the solubilization of "total" membrane protein represents the cumulated effect on individual proteins with markedly different solubility properties.

In summary, the most favorable conditions for preservation of synaptic complexes on the basis of morphological evidence is at relatively low Triton concentrations in the presence of Ca^{2+}. Under these conditions the insoluble residue retains about 60 % of its initial protein, 70 % of its initial phosphate, and 80 % of its initial sialic acid. The membrane-associated enzymes 5′-nucleotidase and alkaline phosphatase are completely insoluble under these conditions.

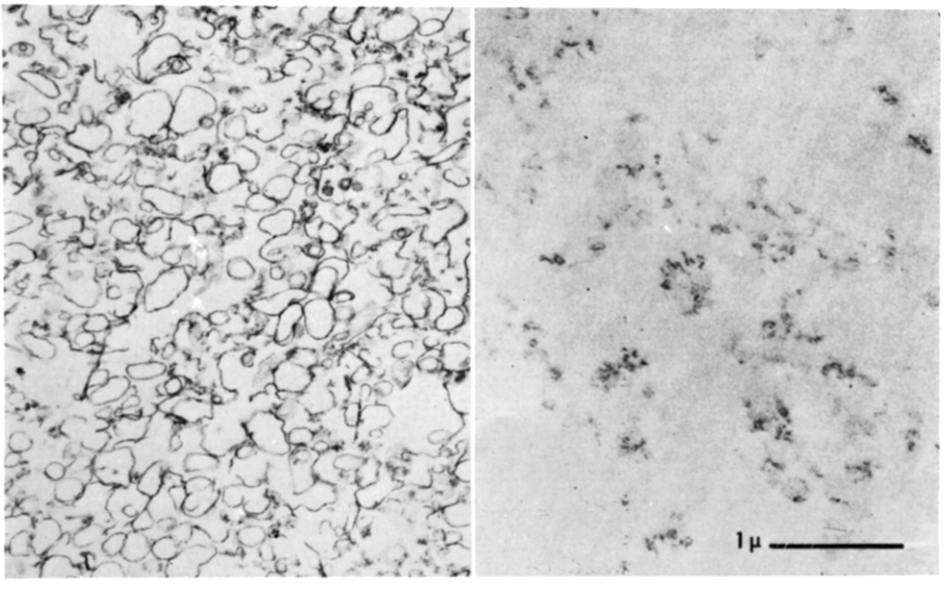


Fig. 9. Micrographs of synaptic plasma membrane fraction treated with Triton X-100 (Triton/protein = 0.55). (a) Prepared by glutaraldehyde–osmium fixation. (b) Prepared by ethanolic phosphotungstic acid procedure. Although some ethanolic phosphotungstic acid material is evident, synaptic complexes are not well preserved. At higher magnification in osmium material a double membrane structure can be seen.

The apparent Ca^{2+} protection of synaptic complex morphology can be further clarified experimentally. This effect could result in one of two ways. It could be due to the general inhibition of detergent effectiveness in the presence of Ca^{2+} (see Table I), or to a specific protection of the synaptic complex, presumably because of synaptic complex-specific Ca^{2+} binding. The quantitative data on Triton solubilization with and without Ca^{2+} allow the distinction of these possibilities. Triton solubilization of synaptic plasma membrane was carried out in the absence of Ca^{2+} but at a lowered Triton concentration (Triton/protein = 0.55). This was adjusted to give protein solubilization approximately equivalent to that seen with Ca^{2+} present when synaptic complex are preserved. Electron micrographs of the preparations are shown in Fig. 9. Tissue prepared by glutaraldehyde–osmium fixation shows numerous membrane fragments but no prominent synaptic complex (Fig. 9, a.). Ethanolic phosphotungstic acid stains only a few small regions in this material (Fig. 9, b.). Since synaptic complexes are present when a similar degree of solubilization occurs in the presence of Ca^{2+} (Figs. 1, 4) it appears that the action of Ca^{2+} is to specifically preserve the structural integrity of the junctional complex.

DISCUSSION

Triton treatment of synaptic plasma membrane allows a selective dissociation of membrane constituents. At a chemical level Triton results in differential solubilization of membrane protein, phospholipid phosphate and sialic acid. At the ultrastructural level the synaptic complex resists Triton treatment so that it is dissociated from the bulk of the adjoining plasma membrane.

The differential solubilization of proteins and phospholipids as a function of pH and Triton concentration suggests important differences in the forces stabilizing the organization of these components in synaptic plasma membrane. Solubilization of phospholipid increases linearly with increasing Triton up to a Triton-to-protein ratio of 2, indicating that the amount of phospholipid solubilized is proportional to the amount of Triton used. The solubilization of phospholipid from erythrocyte membrane by deoxycholate follows a linear relationship until 80 % of phospholipid is removed[16]. For synaptic plasma membrane, after about 45 % of phospholipid is released, the relationship is no longer linear, possibly because of differences in composition or structural relationship of the remaining components.

Phospholipid solubility is essentially pH independent over the range examined. We would interpret this to mean that bonds disrupted during phospholipid solubilization are non-ionic since all expected ion pairs would be affected by such pH changes.

Quite different results were obtained for protein solubilization. Initially solubilization of protein is more rapid than phospholipid and follows an exponential rather than linear relationship with amount of Triton. The initial release with low amounts of Triton may represent the removal of protein loosely bound to membranes, either adsorbed or native. At higher Triton concentrations, protein solubilization appears asymptotic. Protein and phospholipid solubilization also differ with respect to pH. With a constant amount of Triton, pH has a profound effect on protein solubilization; increases in pH from 6 to 10 result in a 2-fold increase in protein solubilized.

The solubilization data on proteins may be explained in a variety of ways.

The pH effect on protein solubilization suggests a relative importance of ionic interactions in protein bonding, since pH changes over this range will alter the ionization of several groups in proteins. However, alternative explanations are possible. Experiments with soluble proteins have shown that pH-induced denaturation can lead to increased detergent binding[17] which would promote solubilization. By analogy with colloidal floculation phenomena, pH changes can lead to increased surface potentials and decreased hydrophobicity[18], both of which would result in increased disaggregation. At constant pH, the asymptote reached in protein solubilization with larger amounts of Triton might suggest that a class of Triton-insusceptible interactions is important in protein binding in synaptic plasma membrane but is not involved in phospholipid binding. Alternatively, the asymptotic effect could result from the rearrangement and formation of numerous new interactions in the unsolubilized membrane residue. Colloidal aggregation is known to increase with increasing molecular size[18], and by analogy, the larger size of proteins could account for their incomplete solubilization by Triton. The variety of interactions and the possibility for rearrangements induced by solubilization make it difficult to assess the validity of analogies derived from model systems such as soluble proteins and colloids. Additional data are essential to determine the applicability of such arguments to biological membranes.

Sialic acid-containing compounds are markedly less soluble in Triton than total membrane proteins or phospholipids. Furthermore, preliminary data suggest that the majority of Triton-insoluble sialic acid is a constituent of glycoproteins. Thus synaptic plasma membrane glycoproteins probably are much more resistant to Triton solubilization than membrane protein as a whole.

The relative insolubility of the sialic acid-containing components of synaptic plasma membrane is unexpected. Electrophoretic[19,20], immunological[21], and histochemical[22,23] evidence suggests that these components are located at the outer surface of the plasma membrane of many cell types, including neurons[22,24,25]. Based on these data some membrane models now include carbohydrates superimposed on a lipid–protein core[26].

Our own solubilization data suggest that sialic acid-containing components may be more firmly enmeshed in the plasma membrane than these models indicate. With Triton solubilization of synaptic plasma membrane the release of sialic acid lags far behind the solubilization of total membrane protein and phospholipids. Analogous results have been seen in studies of erythrocyte plasma membrane[27]. Solubilization at low ionic strength in the presence of EDTA results in significant release of protein with no detectable removal of sialic acid. Reinforcing this suggestion that sialic acid-containing components are embedded within the membrane are three studies examining release of sialic acid following incubation with trypsin[28–30]. These studies show that in certain intact cells, trypsin incubation fails to release plasma membrane sialic acid. Taken together, these data suggest that sialic acid-containing components are much less susceptible to solubilization than their apparent surface location suggests. In synaptic plasma membrane, these components are more strongly bound to membrane than most phospholipid or protein, either by virtue of their own properties or their organization within the detergent-inaccessible core of the membrane. Recent studies on erythrocytes demonstrate that the major glycoproteins span the entire membrane[31,32].

Our ultrastructural data demonstrate that synaptic complexes are present after

Triton treatment of a purified SPM fraction and are separated from adjoining non-junctional membrane. This conclusion follows from the following observations in Triton-treated synaptic plasma membrane: First, the presence of osmiophilic densities of size and shape similar to the *post*-synaptic densities of intact synapses. These densities typically are attached to short segments of adjoining plasma membrane. Second, the presence of ethanolic phosphotungstic acid-staining densities of shape and size similar to ethanolic phosphotungstic acid-staining post-synaptic densities of intact synapses. The specificity of ethanolic phosphotungstic acid for the synaptic complex was verified in subcellular fractions. Third, well-preserved thickenings show the presence of an adjacent parallel band, which is seen with both glutaraldehyde–osmium and ethanolic phosphotungstic acid procedures. These structures unambiguously resemble synaptic complexes prior to Triton treatment, and it is exceedingly unlikely they are an artifact. These findings confirm and extend the results of DE ROBERTIS *et al.*[6,7] based on treatment of membrane fractions derived from the crude mitochondrial fraction. Our findings are in close agreement with the recent results of DAVIS AND BLOOM[33] obtained by a different preparative procedure; these workers also report the presence of ethanolic phosphotungstic acid staining synaptic complexes after Triton treatment.

Optimum preservation of the synaptic complex is dependent not only on the amount of Triton used, but also the presence of Ca^{2+} during solubilization. The best results were obtained at a Triton-to-protein ratio of 1.2 in the presence of 3 mM $CaCl_2$. Large increases in the amount of Triton used, or decreases in Ca^{2+}, result in the absence of clearly identifiable synaptic complexes in electron micrographs.

The specific protection of synaptic complexes by Ca^{2+} is not surprising in view of the physiological functions of Ca^{2+}. Ca^{2+} plays a critical role in synaptic transmitter release[34] and reception[35] and in the maintenance of certain classes of intercellular junctions[23,36,37]. These findings suggest that the molecules of the synaptic complex have a high affinity for Ca^{2+}, perhaps because of the presence of a high concentration of negative charges.

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