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# Effects of detergent on the binding of solubilized sodium channels to immobilized wheat germ agglutinin: structural implications

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**The binding of the solubilized voltage-dependent sodium channel from rat brain to immobilized wheat germ agglutinin (WGA) is detergent-dependent. When solubilized in sodium cholate, only 11% of total recovered channels bound to a WGA-Sepharose column. When solubilized in Triton X-100 or CHAPS, however, 80% and 60% could bind, respectively. The effect of cholate on sodium channel binding is relatively specific: the major rat brain glycoproteins which bind to immobilized WGA are roughly the same in either Triton or cholate, as analyzed by SDS gel electrophoresis. The structural implications for the channel are discussed.**

The voltage-dependent sodium channel is the protein responsible for the depolarizing phase of the action potential in many excitable cells. It has been purified from several tissue sources [1–6], and cDNA's for its major polypeptide have been cloned and sequenced, thus elucidating primary structure [7,8]. Many protocols for channel purification utilized the observation that it could be purified 10–15-fold from rat sarcolemma and rat brain with wheat germ agglutinin (WGA) sepharose chromatography [9–11]. The results below demonstrate that efficacy in fractionating sodium channels from rat brain with WGA chromatography depends upon the solubilizing agent. As further discussed, this may indicate that channel carbohydrate and micelle surface significantly interact. The potential implications, with regard to channel function, are considered.

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Bovine serum albumin (globulin free), asolectin (crude soybean lecithin), tetrodotoxin, phenylmethanesulfonyl fluoride (PMSF), pepstatin A, iodoacetamide, Sephadex G-50–150, Triton X-100, cholic acid, sodium dodecyl sulfate, *N,N'*-methylene-bis-acrylamide, glycine, Trizma base, Hepes, and acrylamide were purchased from Sigma. TEMED and 2-mercaptoethanol were obtained from Bio-Rad. Wheat germ agglutinin coupled to Sepharose (6–10 mg WGA/mg resin) was obtained from E.Y. Laboratories. CHAPS was purchased from Calbiochem. All conventional materials were of analytical grade. Cholic acid obtained from Sigma was recrystallized according to the procedure of Kagawa and Racker [12]. Detergents were kept refrigerated, and lipids were stored at −70 °C in chloroform.

Lysates of synaptosomes can be fractionated to produce presynaptic plasma membranes, according to the procedures of Cotman and Matthews [13]. We used a variation of these procedures. The membrane preparation was kept on ice until use. All solutions in contact with the channel contained 0.1 mM PMSF, 1 μM pepstatin A, and 1

mM iodoacetamide unless otherwise stated, and all manipulations of the channel were done at 0 °C.

Membranes, prepared as described above, were solubilized by manual homogenization with ice-cold 100 mM KCl, 20 mM Hepes-Tris (pH 7.4, 0 °C), 0.1 mM PMSF, 1 μM pepstatin A, and 1 mM iodoacetamide containing the optimal detergent and lipid concentrations for channel stability [1]. These concentrations are presented below for each detergent. The homogenate was incubated on ice for 20 min, then the unsolubilized material was separated by centrifugation for 30 min at 100000 × g. The top 75% of the supernatant was collected and used.

The sodium channel was followed during its fractionation by its ability to bind saxitoxin (STX) with high affinity [14]. STX binding assays in detergent solution were performed essentially as first described by Levinson [15], incorporating some modifications [16]. Briefly, membranes solubilized as described above were incubated with 10 nM $^3$H-STX with and without 1 μM tetrodotoxin (TTX). After 20 min at 0 °C, the incubation mixtures were rapidly centrifuged through syringes packed with Sephadex G-50–150 (Sigma). The eluate was collected and counted for radioactivity. Total cpm were subtracted from nonspecific cpm to calculate the number of STX binding sites, correcting for the specific activity of $^3$H-STX [14]. The number of STX binding sites was normalized to the amount of total protein to calculate a specific activity of the STX receptor. Protein was determined either by the method of Lowry et al. [17] or the variation introduced by Peterson [18], using bovine serum albumin as a standard.

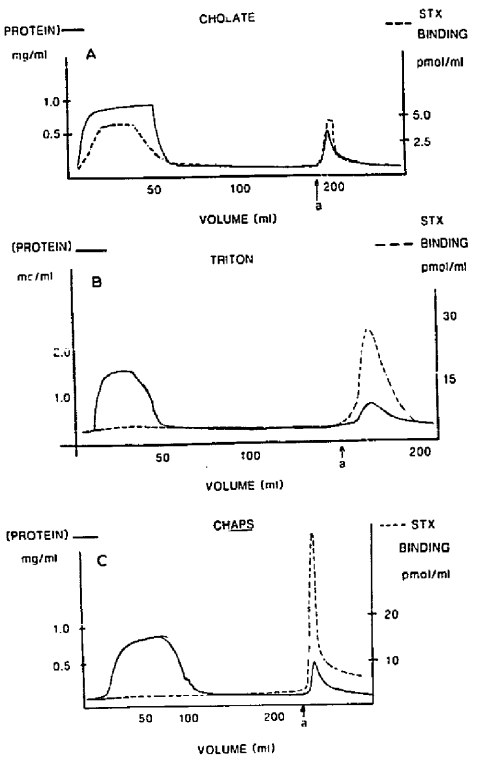


Fig. 1. Fractionation of STX receptors with WGA chromatography. (A) Fractionation of cholate-solubilized membranes. WGA-Sepharose, packed in a 2.5×4 cm column, was equilibrated with a solution containing 100 mM KCl, 20 mM Hepes-Tris (pH 7.4, 0 °C), 0.75% cholate, 0.4% asolectin, 0.1 mM PMSF, 1 μM pepstatin A, and 1 mM iodoacetamide (solution D). Membranes were solubilized in 2% (w/v) cholate and 0.4% (w/v) asolectin at a concentration of about 4 mg/ml of protein. After application of approx. 50 mg of solubilized protein with a specific activity of 8 pmol/mg, the column was washed with 4–5 column volumes of solution D. The abscissa indicates the total eluate volume at that time. At point "a" on the abscissa, the protein bound to the column was eluted with 75 mM *N*-acetyl-D-glucosamine in solution D. The eluate was collected in 2-ml fractions, and assayed for protein and STX binding. The ordinate depicts protein concentration (solid line) and STX binding (dashed line). (B) Fractionation of Triton X-100-solubilized membranes. The identical WGA column used in the fractionation of cholate-solubilized membranes was used with membranes solubilized in Triton X-100. The column was washed with a solution containing 0.1% Triton X-100, 0.02% egg phosphatidylcholine, 100 mM KCl, 20 mM Hepes-Tris (pH 7.4, 0 °C), and 10 mM $CaCl_2$ with the proteinase inhibitors mentioned above (solution T). Approx. 75 mg of Triton-solubilized protein was applied with an initial specific activity of 5 pmol/mg. The elution of bound protein was performed with 50 mM *N*-acetyl-D-glucosamine in solution T. (C) Fractionation of CHAPS-solubilized membranes. The protocol was identical to that for cholate-solubilized membranes except: (1) membranes were solubilized in 2% (w/v) CHAPS, (2) the WGA-Sepharose column was equilibrated with solution D, substituting CHAPS for cholate (solution E), and (3) elution of bound protein was performed with 25 mM *N*-acetyl-D-glucosamine in solution E. Elution with higher concentrations of glucosamine did not significantly improve recovery of either total bound protein or sodium channels. Approx. 70 mg of CHAPS-solubilized protein with an initial specific activity of 8 pmol/mg was applied to the column.

Fig. 1A illustrates the elution pattern from WGA chromatography of cholate-solubilized total protein and STX receptors in a typical experiment. Membranes were solubilized as described above, with 2% (w/v) sodium cholate and 0.4% (w/v) asolectin, the optimal concentrations of detergent and lipid for STX receptor stability [28,29]. The details of the chromatography are described in the figure legend. It is apparent that most of the applied protein passes through the column, unbound. Specifically bound protein was eluted with a step gradient of 75 mM *N*-acetyl-D-glucosamine, and, as seen in the figure, about 5% of the initially applied protein is eluted here. Applying higher concentrations of the saccharide increased neither the amount of eluted total protein or STX receptors.

STX binding profiles in Fig. 1A demonstrate that the overwhelming majority of sodium channels also pass through the column, unbound. Many experiments performed under various conditions verified this: an average of 5% of the total applied membrane protein bound to the column, as did only 11% of the total recovered STX receptors. Additionally, 75% of applied STX receptors passed through the column, unbound, accounting for the vast majority of applied sodium channels. The binding characteristics of STX receptors to the column did not change whether: (1) the cholate concentration was diluted to 0.75% before applying the protein to the column, (2) the protein was solubilized without supplemental asolectin, or (3) the solubilized protein was applied to a column equilibrated without asolectin.

As mentioned above, mammalian muscle and brain sodium channels, solubilized in neutral detergents, have been fractionated by other investigators with WGA chromatography. It is possible, therefore, that these results reflect unusual interactions of cholate with the column. To test this, we fractionated Triton X-100-solubilized rat brain membranes, closely following the procedures of Hartshorne and Catterall.

Membranes prepared as described above were solubilized in Triton X-100 as previously reported [4,16]. Briefly, the final pellet from the membrane preparation was homogenized with ice-cold 2.5% (w/v) Triton X-100, 0.25% (w/v) egg phosphatidylcholine (Sigma), 100 mM KCl, 20 mM Hepes-Tris (pH 7.4, 0°C), 0.1 mM PMSF, 1 μM pepstatin A, and 1 mM iodoacetamide. These were optimal detergent and lipid concentrations for STX receptor stability [16]. After incubating for 20 min at 0°C, the unsolubilized material was separated by centrifugation as described above. The lipid used to stabilize the sodium channel in Triton X-100 and cholate micelles differs as is required for better stability [28]. The supernatant from this centrifugation was brought upto 10 mM $CaCl_2$, then fractionated as detailed in Fig. 1B. Calcium chloride was used here to improve the stability of the Triton X-100-solubilized STX receptor [16].

As with cholate, most of the Triton-solubilized protein did not bind to the column (Fig. 1B). A noticeable difference, however, is seen with the fractionation of the STX receptor. In this experiment 76% of the total recovered STX receptors bound to the column. Because only about 4% of the total protein bound, up to 15-fold enrichments of the STX receptor were attained. Furthermore, this utilized a WGA column exposed first to cholate. In another experiment, using WGA-Sepharose not previously exposed to cholate, over 80% of the total recovered STX receptors bound to the column. This is comparable with previously reported results [11]. It is clear that the Triton-solubilized channel binds to WGA-Sepharose more effectively than the channel solubilized in cholate.

The total protein applied to WGA columns equilibrated with either cholate or Triton gave similar elution profiles, indicating that the weak binding observed with cholate-solubilized STX receptors might be specific. This was explored by running parallel WGA columns with both Triton and cholate-solubilized membranes. The specifically bound protein was eluted from each column with 75 mM glucosamine, then pooled. The gross protein composition from each column was then analyzed by SDS gel electrophoresis as described in Fig. 2.

Densitometer tracings of Comassie blue-stained protein from each gel demonstrate that the major glycoprotein species which bound to both columns are roughly the same (Fig. 2). Coupled with the observation that the total amount of protein bound to both columns is the same, one may conclude that the exclusion of cholate-solubilized sodium channels from WGA-Sepharose columns is not a

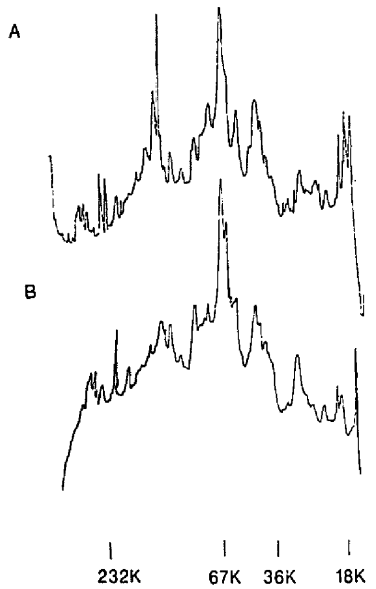


Fig. 2. SDS gel electrophoresis of WGA-fractionated protein–Triton vs. cholate. Samples for Coomassie blue-stained gels were treated as described by Hartshorne and Catterall [11]. Briefly, solubilized protein, specifically bound by WGA and eluted with *N*-acetyl-D-glucosamine, was pooled. The pooled eluate was precipitated with 10% (w/v) trichloroacetic acid and centrifuged for 30 min at 1500 × *g*. The pellet was washed with 95% acetone and 95% ethanol. This served to concentrate the protein and to remove substances which might otherwise interfere with the solubilization by the SDS solution. The pellet from the last centrifugation was solubilized by boiling for 5 min in 80 μl of 3% SDS, 50 mM Tris (pH 9.5), 1 mM EDTA, 10% sucrose, 10 mM dithiothreitol, and 0.01% phenol red. These samples were incubated at room temperature for 30 min then, after adding iodoacetamide to a final concentration of 40 mM, the samples were incubated for 1 h in the dark. Before applying the samples to the gel, 2-mercaptoethanol was added to 5%, the pH was adjusted to 6.8 with NaOH, and samples were boiled for an additional 5 min. A discontinuous gel system was used with a 5–15% acrylamide gradient in the separating gel along with a 3% stacking gel [27]. The tank buffer contained 0.025 M Tris (pH 8.3, 22°C), 0.192 M glycine, and 0.1% SDS. Gels were run for about 2.5 h at a constant current of 30 mA. Protein was visualized by staining with 0.125 % Coomassie blue, 50% methanol, and 10% acetic acid for 4–8 h. The gels were destained with progressively decreasing concentrations of methanol and acetic acid. The figure illustrates densitometer tracings of Coomassie blue-stained gels. (A) Pooled eluate from fractionation in cholate. (B) Pooled eluate from fractionation in Triton X-100.

general phenomenon for cholate-solubilized glycoprotein from rat brain.

We also fractionated sodium channels solubilized in CHAPS, a zwitterionic derivative of cholate, with WGA chromatography. This was part of an effort to purify the channel in an easily dialyzable detergent. In contrast to cholate-solubilized channels, those solubilized by CHAPS bound well to immobilized WGA. WGA chromatography, therefore, proved to be a useful fractionation step for CHAPS-solubilized channels from rat brain. Fractionation in CHAPS solution was performed as detailed for cholate, except for modifications described in Fig. 1C. In particular, membranes were solubilized with 2%(w/v) CHAPS and 0.4%(w/v) asolectin-optimal concentrations for STX receptor stability [29].

Fig. 1C illustrates a typical result from these fractionation studies. As with Triton or cholate-solubilized channels, most of the applied protein passed unbound through the column. However, the saxitoxin binding profiles show that most of the CHAPS-solubilized sodium channels were retained by the column, unlike those solubilized by cholate. Only 12% of the applied STX receptors passed through the column, unbound. In this experiment, 59.7% of the applied sodium channels both bound to, and were recovered from the column. Because 6% of the total applied protein was also recovered after binding, a peak purification of 10-fold resulted. In three such experiments, an average of 60% of the applied STX receptors were retained by the column, as was only 8% of total solubilized protein, attaining an average peak purification of 10-fold with a specific activity of 80 pmol/mg.

These observations may be explained by specific interactions between cholate and the sodium channel which decrease the accessibility of some of its sugars to the lectin. This would require, in turn, an immediate physical interaction between these sugars and the micelle. Such an interaction is unexpected: one commonly thinks of a glycoprotein's saccharides as floating away, like the bait of an angler fish.

However, it is quite possible that glycosylation of the channel occurs well into the mouth of its pore, as previously suggested [2]. If this were the case, both the surface of the micelle and the

relevant sugars could juxtapose, interfering with lectin binding. The speculation that glycosylation occurs close to the conducting pore is important: sialic acid, which comprises a large percent of the channel's sugars, would create a marked negative surface potential. This would increase the effective sodium concentration at the pore, which would, in turn, result in an increased single-channel conductance. A proposed model of sodium channel secondary structure, based on the primary amino acid sequence of the eel electroplax protein, contains a potential N-glycosylation site near the putative pore of the channel [19].

Models of sodium channel secondary structure [19,20], have proposed very little primary amino acid sequence existing outside the membrane on the channel's extracellular face. Therefore, even if glycosylation does not occur deep into the mouth of the pore, it could still be close to the micelle surface where it could interact with cholate. Related to this issue, site directed mutagenesis studies have been performed on the alpha subunit of nicotinic *Torpedo californa* acetylcholine receptors [20]. When N-glycosylation of this subunit is prevented, the other subunits do not properly assemble to form a functional molecule. This indicates N-glycosylation of the alpha subunit is required for necessary subunit interactions to occur. These carbohydrates might, therefore, be intimately related to the protein tertiary structure, as speculated above for the sodium channel.

CHAPS proved to be a good detergent for fractionating solubilized STX receptors with WGA chromatography. Interestingly, marked differences in rat brain channels binding WGA depended on solubilization in cholate, CHAPS, or Triton. The first two detergents are steroid derivatives, their major difference being one of charge: cholate is negatively charged; CHAPS is zwitterionic. Because CHAPS-solubilized channels bind well to WGA, there may be an electrostatic interaction which prevents cholate-solubilized channels from binding. Triton, like CHAPS, has no net charge. The channel, furthermore, is heavily glycosylated with sialic acid creating fixed negative charge (see above). Electrostatic repulsion between cholate and the sialic acid may alter the carbohydrate 'secondary structure' preventing binding of the relevant sugars to the WGA. Since both with CHAPS and with cholate we used asolectin to stabilize the protein in detergent this indicates it is not the phospholipid that interferes with the binding, as previous controls also suggested.

An alternative explanation for the channel's decreased binding to WGA is an interaction between cholate and the immobilized lectin. Such interactions, resulting in diminished capacities of immobilized lectins to fractionate glycoprotein, have been reported for several lectins and detergents [21–23]. The effect of a particular detergent, however, is empirical, dependent upon both the lectin and the protein being fractionated. In this case, the exclusion of cholate-solubilized sodium channels from WGA is a relatively specific phenomenon. Cholate, therefore, would have to interact with WGA so that the binding of only a minority of proteins, undetected in our gels, is reduced – not the most likely possibility.

The Lubrol-solubilized TTX receptor from eel electroplax has also been shown to have a decreased affinity for WGA [24]. The authors attributed this to differences between eel and mammalian channels. Based on the above findings with cholate, however, one may also speculate that this was a result of solubilization in Lubrol. The gross carbohydrate compositions of both eel electroplax and rat brain sodium channels were subsequently shown to be much the same [2,25,26]. As with cholate-solubilized sodium channels from rat brain, one cannot pinpoint the reason for this reduced affinity. Nevertheless, the same possibilities exist.

The importance of the sodium channel has generated much interest in its molecular properties. These results and their possible structural implications suggest further experiments to study the channel's tertiary structure.

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