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COMMENTARY

SOLUBILIZATION OF THE DOPAMINE RECEPTOR: A STATUS REPORT

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Over the last decade, scientific research has identified a number of physiological, biochemical and pharmacological properties of the CNS dopamine receptor. This is a pre- or post-synaptic protein that specifically binds dopamine and is thought to affect the transmission of impulses between certain neurons. Unfortunately, adequate biochemical characterization of the molecular properties of the dopamine receptor has been hindered by the difficulties encountered in effecting the removal of the receptor from the membrane. However, the recent successful solubilization of the dopamine receptor with chaotropic agents and with selected detergents represents an important technical advance in the solution of this problem and facilitates the elucidation of the physical properties of this receptor. The purpose of this commentary is to review some of the recent developments in the solubilization of the dopamine receptor and to critically evaluate these findings.

Clearly, the process of solubilization and purification of any receptor protein is a formidable task. This is certainly the case for the dopamine receptor which constitutes less than 0.1% of the dry weight of the brain. The other brain material is composed of numerous proteins, many of which exhibit properties closely resembling those of the dopamine receptor. Despite the obstacles that are likely to be encountered by the researcher in the solubilization and the purification of the dopamine receptor, it is a worthwhile endeavor. If successful, the studies will provide useful information on the physical characteristics of the receptor, which is desirable for the design of novel therapeutic agents. Moreover, in the process of purifying the receptor, new knowledge may be gained about components and chemically reactive groups that are important for the regulation of the receptor.

Selection of tissue source

The first consideration in the solubilization and ultimate purification of the dopamine receptor is the selection of an appropriate tissue source. The ideal material would be a tissue that contains dopamine receptors almost exclusively, as is true for the acetylcholine receptor in *Electrophorus* or *Torpedo* [1–6]. Such a source has not been identified for the dopamine receptor, but alternative possibilities exist, which include discrete brain regions with substantial innervation from dopaminergic neurons. To date, only the corpus striatum (caudate nucleus and putamen) has been used for the solubilization and purification of the dopamine receptor. The striatum, however, contains many other neurotransmitter receptors which are likely to complicate the solubilization procedure. It is desirable, therefore, to subfractionate the tissue prior to solubilization in order to increase the number of dopamine receptors. The 100,000 *g* pellet (P_3), which is enriched with microsomes, has been accepted generally as the preferred source for solubilization of the dopamine receptor. Other subcellular fractions containing binding activity can be used, but these produce lower yields of soluble receptor protein.

Solubilization of the dopamine receptor with chaotropic agents

A variety of methods have been used to disrupt lipophilic membranes and release their constituents for further study. Treatments include solubilization with chaotropic agents, such as detergents and organic solvents. The term "chaotropic" was first used by Hamaguchi and Geiduscheck [7] to apply to anions that tend to disorder the structure of DNA, but it has since been applied to those inorganic anions that favor the transfer of apolar groups to water [8]. They cause water to become disordered and lipophilic and, thus, weaken hydrophobic bonds and increase the water solubility of particulate proteins and nonelectrolytes. Detergents have a mechanism of action different than that of chaotropic substances. They solubilize membranes through disruption of the lipid bilayer and formation of mixed micelles with membrane components, leading to an extract that is different both quantitatively and qualitatively from extracts of chaotropic agents. Collins and Salton [9] found that chaotropic agents, in general, were less effective than detergents in extraction of bulk protein but often produced extracts enriched with some membrane components. Additionally, upon rocket immunoelectrophoresis, salt extracts of *Micrococcus lysodeikticus* produced solubilized antigens that migrated toward both the cathode and the anode, whereas detergent extracts produced only anode-migrating proteins.

Neutral salts markedly perturb the secondary–tertiary structure of macromolecules [10]. This accounts, in part, for their suitability in solubilization. They tend to neither inactivate enzymes by denaturation, as organic solvents can [11], nor alter the specificity of binding [12, 13]. The chaotropic

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salt, potassium chloride, has been used in a variety of protocols, from solubilizing membrane-associated histocompatibility (HL-A) antigens that retain marked immunologic specificity [14, 15] to releasing membrane-bound ribosomes that remain active in peptide synthesis [16, 17]. Use of this salt has also proven successful in solubilizing striatal dopamine receptor binding proteins that exhibit the same stereoselectivity, saturability and pharmacological activity [13] as the membrane bound receptor.

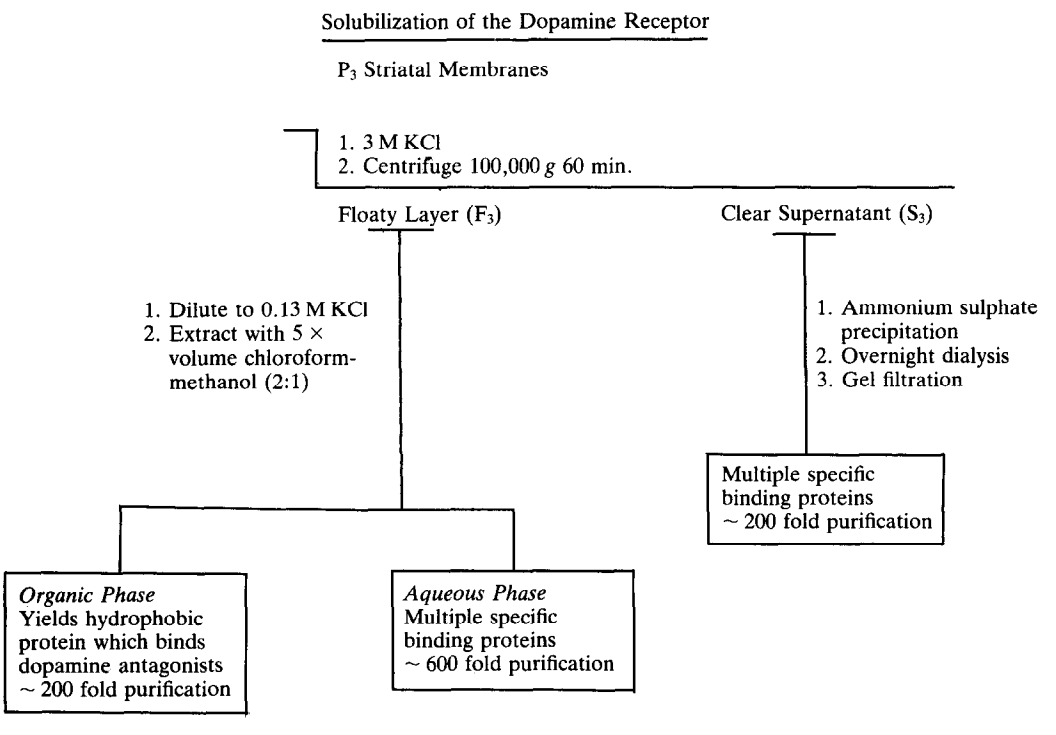
To solubilize the dopamine receptor with salt, potassium chloride is added to resuspended P_3 membranes, which are homogenized and then immediately centrifuged at 100,000 *g* for 60 min. Similar to the post-centrifugation finding of a fluffy layer with detergent treatment of membranes [18], a floaty layer (F_3) is produced following treatment of striatal membranes with potassium chloride, in addition to the supernatant fraction (S_3). Both the lipid-enriched F_3 and the clear S_3, when assayed separately, exhibit stereospecific, saturable binding for [^{3}H]-spiroperidol, a dopamine antagonist. The salt-P_3 suspension can also be agitated in the cold for 16 hr prior to centrifugation at 100,000 *g*. This procedure increases the amount of floaty layer and the yield of receptors in the S_3.

The concentration of potassium chloride is based on per cent w/w (grams salt/grams original wet weight of tissue) and is presently employed at 50% w/w in our laboratory. However, both 100% w/w and 300% w/w were used in the past when the tissue was homogenized in a larger volume of buffer, which could dissolve this quantity of salt. A more accurate representation, therefore, would state that the P_3 membranes are exposed to a saturating concentration of potassium chloride. In good agreement with the work of Collins and Salton [9], potassium chloride solubilizes less of the total P_3 protein than any detergent tried, but a comparison of salt versus detergent extraction of receptor protein revealed that about 25 per cent of the dopamine receptor binding sites are solubilized with salt compared to 10 per cent with detergents. Salt extraction, however, like detergent solubilization [21], is not complete in one treatment. Repeated extraction not only solubilizes increasing numbers of receptors, but it apparently unmasks additional dopamine receptor binding proteins as well [13].

Solubilization of the dopamine receptor with detergents

Many attempts to isolate and purify membrane proteins in a native form and in aqueous buffers have employed detergents. These molecules, like phospholipids, are amphipathic structures with an apolar end of aliphatic, aromatic character and a polar end, which may or may not be charged. Detergents are incorporated into the lipid bilayer of membranes as monomers. As their concentrations in the membrane increases, they solubilize the membrane components by forming mixed micelles, some containing protein and detergent, some containing lipid and detergent, and others containing lipid, protein and detergent [22–24].

SUMMARY*



* Taken from Refs. 13, 19, and 20.

In all cases, a protein micelle results from detergent treatment of membranes. The micelle is an aggregate that is presumably organized with hydrophobic surfaces enclosed within the interior of the structure. It is possible in some cases to remove bound detergent from membrane proteins and obtain a water-soluble preparation, but this depends on the manner in which the detergent is removed [22]. If the detergent cannot be removed, the molecular weight measurements are inaccurate. These must be corrected by estimating the amount of lipid remaining bound to the protein.

The detergents digitonin, deoxycholate and octyl-β-glucosylpyranoside have been used to solubilize the dopamine receptor [25, 26]. [^{3}H]-Spiroperiodol binding in a digitonin extract of striatal membranes is saturable and stereospecific for butaclamol, and the K_d for [^{3}H]spiroperidol is 2×10^{-9} M. These results are similar to those reported previously by Gorissen and Laduron [25]. Deoxycholate and octyl-β-glucosylpyranoside solubilize sites specific for the agonist [^{3}H]*N*-propylnorapomorphine and for [^{3}H]spiroperidol that are saturable, stereospecific, and exhibit high affinity [26]. Apomorphine displaces 30 per cent of the total counts labeled with [^{3}H]*N*-propylnorapomorphine in the deoxycholate extract and 45 per cent of the total counts in the octyl-β-glucosylpyranoside extract. Both deoxycholate and octyl-β-glucosylpyranoside appear more useful than digitonin for the solubilization of the agonist conformation of the dopamine receptor. These ionic detergents may prove useful for the reconstitution of the receptor complex, as has been reported by Hoffman [27].

Solubilization of the dopamine receptor with organic solvents

Organic solvents have also proven efficacious for the solubilization and purification of the dopamine receptor. When the F_3 fraction is diluted and then extracted under non-denaturing conditions with chloroform–methanol (2:1, v/v) overnight at 4°, evaporated to dryness, and resuspended in buffer, a dopaminergic hydrophobic protein (60% lipid/40% protein) is recovered. This proteolipid binds dopamine antagonists with high affinity and stereoselectivity [19]. The presence of this protein, a part of the crude phospholipid component, indicates a probable role for a proteolipid (i.e. hydrophobic protein) in the receptor complex.

Methods for detecting soluble binding sites

The standard means of quantifying receptors is by measuring radioligand binding to a preparation. Such a method has been used not only for the dopamine receptor but for the soluble acetylcholine and β-adrenergic receptors as well. Nonetheless, the use of radioligand binding as a definite measure of biological activity is somewhat controversial. This is because the binding sites of most neurotransmitters can be physically separated from other constituents which are used to detect their biological activity [28–30]. For example, binding sites can be separated by gel filtration chromatography from an enzyme which, in the case of certain dopamine receptors, is associated with its biological activity, i.e. dopamine-sensitive adenylate cyclase [31]. To overcome this problem, a number of criteria have been suggested for receptor binding, which help to identify whether the binding site is associated with the neurotransmitter–receptor complex. These criteria include saturability, high affinity, stereospecificity, and perhaps the most critical, pharmacological activity. The importance of the criterion of pharmacological activity is supported by the observation that the order of clinical potency of neuroleptic drugs closely correlates with dopaminergic activity in the binding assay [32]. However, in spite of the general acceptability of this methodology as the only alternative for detecting soluble receptor binding sites, the results from these studies must still be cautiously interpreted.

Binding assays for soluble receptors have employed precipitation with polyethylene glycol for the β-receptor [33] and the insulin receptor [34], adsorption to DEAE filters for glucocorticoid receptors [35], charcoal–dextran adsorption for melatonin receptors [36, 37], and adsorption to hydroxyapatite for steroid receptors [38]. The salt-extracted dopamine receptor can be conveniently and reproducibly measured by the charcoal adsorption method or by filtration through glass fiber filters. Filtration on Sephadex G-50 columns can be used for both the salt and detergent solubilized receptor, but it is cumbersome, with a fair amount of variability in the results. This method has been used successfully by others [25] and is somewhat useful as an adjunct for verifying results. We routinely use precipitation with polyethylene glycol followed by filtration to measure receptor extracted with detergent. A brief description of the methods we routinely use to measure soluble dopamine receptors follows.

Binding assay. The assay mixture varies according to the experimental design, but the basic standard mixture is 15 mM Tris or phosphate buffer at pH 7.4, 0.02% ascorbate, an amount of tissue, ^{3}H-ligand, and test substances as called for, all in a 1.0 ml final volume. The reaction is initiated by the addition of the tissue homogenate or the radioligand, and it is usually carried out for 30 min at 25°. Termination of the reaction involves separation of the free ligand and the ligand–receptor complex, and varies depending on the method chosen for solubilization of the receptor.

Specific binding is defined as the difference between total binding and that measured in the presence of (+)-butaclamol (10^{-6} M) for [^{3}H]spiroperidol or (−)-apomorphine (10^{-6} M) for [^{3}H]*N*-propylnorapomorphine. These conditions must be carefully determined for each ligand used. In this way, nonspecific binding to a large number of proteins, lipids and the glass filter is accounted for and removed from consideration in data analysis.

Protein precipitation. For those samples where the reaction is terminated by the addition of polyethylene glycol, 1 mg of gamma globulin in 20 mM Na_2HPO_4 (pH 7.4) is added to each tube in 25 μl aliquots, followed by 75 μl of 30% polyethylene glycol–20 mM Na_2HPO_4, pH 7.4. For [^{3}H]spiroperidol binding the samples are kept on ice for 8 min and then filtered on Whatman GF/B. When

[^{3}H]*N*-propylnorapomorphine is the ligand used in the binding assay, the samples are incubated for 2 min on ice after the addition of the polyethylene glycol and gamma globulin. The filters are then washed three times with 5 ml of 1% polyethylene glycol–20 mM Na_2HPO_4 for [^{3}H]spiroperidol or with 3.5 ml of 2.5% polyethylene glycol–20 mM Na_2HPO_4 for [^{3}H]*N*-propylnorapomorphine. The amount of time that is allowed for adsorption of the free ligand varies with the labeled compounds because of the differences in their association constants.

Adsorption. Talcum powder (hydrated magnesium silicate) and precipitated silica ("Quso silica") have been used in the past [39] for adsorption of free ligand and, thus, separation of free from bound complex. Miller [40] proposed the use of another substance, activated charcoal, based on the observation that bound B_{12} resists adsorption by this substance. Both dextran and plasma coatings [41] on the charcoal have been shown to improve the efficiency of separation in different applications. The method presented here utilizes bovine serum albumin (BSA) for this purpose. The principle of the method is that free radioligand (peptides) will bind to the charcoal particles, whereas the receptor-bound radioligand will not. Aliquots for counting, taken from the supernatant fraction after centrifugation (pelleting of the charcoal-free ligand complex), allow measurement of the soluble-labeled receptor. Charcoal separation of free and bound is rapid, being complete within 5 min. Once equilibrium has been reached, the distribution of radioactivity remains constant for over 2 hr, allowing time for the handling of samples. The charcoal method we use for the separation of soluble dopamine receptor involves the addition of 100 μl of a charcoal slurry, containing 2% bovine serum albumin and 10% Norit SGX charcoal in a buffer of 50 mM Tris, 8 mM theophylline, and 6 mM mercaptoethanol (pH 7.4), to each tube. After 5 min these tubes are centrifuged for 4 min in a microfuge, after which 400 μl aliquots of the supernatant fraction are placed in scintillation vials with 1 ml 95% ethanol and 6.5 ml of scintillation mixture. This is a simple, reliable, and rapid method for assaying soluble dopamine receptors. The charcoal adsorption method has not been as reliable for the detergent preparation as we had anticipated, probably because the detergent interfered with the adsorption of free ligand to activated charcoal.

Filtration. Filtration of the detergent solubilized extract on a Sephadex G-50 medium (Pharmacia) column (13 × 0.5 cm) has been reported to separate bound from free ligand [25]. We have found that the bound ligand in salt-extracted samples can be separated from free by filtration through a Whatman GF/B glass fiber filter with reliable results. The unbound ligand passes through the filter while the bound complex is retained. The filters are washed with 3 × 5 ml of ice-cold buffer, placed in scintillation vials with 1 ml of 95% ethanol, and incubated for 30 min, after which 6.5 ml of scintillation mixture is added to each vial. This method gives poor results with detergent solubilized preparations. The mechanism by which the procedure works for the salt extract is not known. It may be due, however, to the ability of salt to produce differently sized or differently charged proteins which are retained on the filter by adsorption and/or filtration. The question of whether the ligand is bound to the protein and not to the filter is valid, and can be answered with the appropriate controls, i.e. measuring radioactive ligand binding in the presence and absence of unlabeled drugs.

Criteria for solubility. A criterion we use for solubilization is that the protein from a 100,000 g × 1 hr pellet should remain in the supernatant fraction after repeated centrifugation under the same conditions but after treatment with the solubilizing agent. Thus, the S_3 in our preparation is considered a soluble receptor, whereas the F_3 is regarded as an emulsion containing soluble receptor. To verify the soluble state of the potassium chloride extracted binding site, the solubilized preparation has been subjected to a number of tests. For example, when the potassium chloride extract was centrifuged at 100,000 g for 2 hr, no sedimented material was observed, and after passage of the supernatant fraction through a 0.45 μm or a 0.22 μm millipore filter, no decrease in binding was observed in the filtrate. Extensive dialysis of the potassium chloride extract, followed by centrifugation a 100,000 g for 2 hr, did not alter binding, nor did filtration through BioGel P_2. In addition, electron microscopy indicated the total absence of any recognizable membrane vesicles or fragments in the solubilized preparation.

Purification

A number of fractionation procedures have been employed successfully, in the purification of dopamine receptor binding proteins. Chromatography of the potassium chloride extract on Sephadex G-200 has yielded multiple peaks of binding activity for [^{3}H]spiroperidol, [^{3}H]apomorphine (Fig. 1) and [^{3}H]-2-amino-6,7-dihydroxy 1,2,3,4-tetrahydronaphthalene ([^{3}H]ADTN) [20]. Because the elution profile of pure proteins can sometimes appear as skewed, diffuse, or split peaks on a gel filtration column being run with salt [42], some precaution must be taken with the sample prior to column application. It appears that aromatic and heteroaromatic anions, in the presence of some salts, can exhibit enhanced adsorption to the column material, thus retarding their progress. As the salt continues to move through the column and these molecules find themselves in a relatively salt-free environment, electrostatic influences again dominate and the compounds speed up, eluting just after the salt peak where the two contrary effects balance one another. To avoid this type of effect, we dialyze the potassium chloride extract against buffer prior to applying the sample to a gel filtration column [43]. Under these conditions multiple binding sites are still present. This has increased the difficulty attendant in a purification procedure and has led to an attempt to purify one component at a time. We find that a combination of gel filtration and ion exchange chromatography is a useful procedure for selectively enriching two peaks of binding activity. Salt precipitation with ammonium sulfate yields a fraction which, when applied to CM cellulose for ion exchange chromatography and finally filtered on Sephadex G-200, is enriched for two receptor binding proteins: one that co-elutes with a 69,000

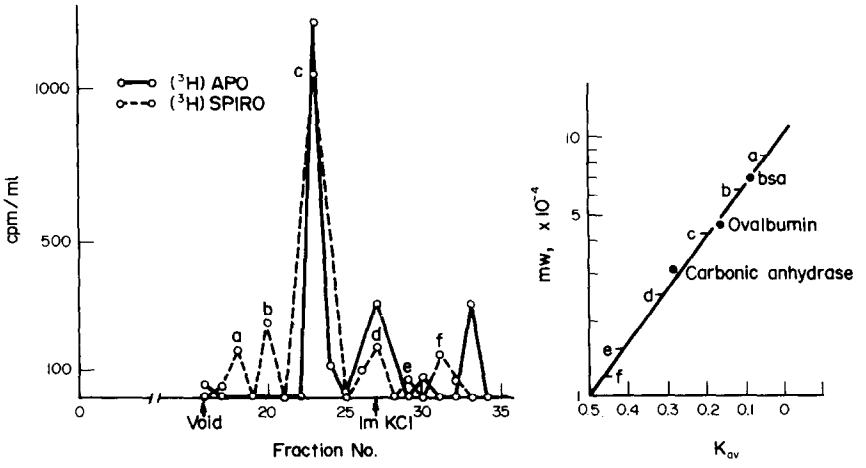


Fig. 1. Gel filtration chromatography of the striatal potassium chloride extract on sephacryl S-200. Taken from Ref. 20.

molecular weight standard and one that elutes with a 15,000 molecular weight standard. Both samples are purified about 200-fold at this step.

Affinity chromatography is a promising possibility for further, and dramatic, purification of the dopamine receptor. From previous work it was known that the dopamine receptor bound to gamma-labeled [^{32}P]ATP [37]. Thus, it was reasonable to expect the receptor to bind to ATP-sepharose. It appeared that the binding did occur, but was unstable. Fluphenazine-sepharose was also given a series of trial runs because fluphenazine is a competitive antagonist of dopamine-sensitive adenylate cyclase. This complex is presently being used to purify calmodulin, an activator of phosphodiesterase and brain adenylate cyclase, among other activities [44]. With the proper controls to avoid the binding of contaminating calmodulin, the potassium chloride extract was applied to the column in a variety of conditions, but the dopamine receptor appeared to have no affinity for the column under the conditions we tested. Our other attempts with chromatography included red and blue affinity columns, haloperidol-sepharose, octyl-sepharose and phenyl-sepharose. The dopamine receptor formed a tight complex with the hydrophobic columns, but numerous difficulties were encountered in desorbing the receptor, even under stringent conditions. Despite these negative findings, affinity chromatography appears to be the key to achieving the extent of purification necessary for the disappointingly small numbers of dopamine receptor proteins found in striatal membranes. Hopefully, the identification of the appropriate affinity column and conditions for the purification of the dopamine receptor will soon be forthcoming.

Conclusion

Solubilization and purification of membrane-bound receptors are complex and difficult procedures. There is a general consensus that the orientation of the receptor in the membrane is influenced by phospholipids and membrane water. Therefore, removal of these membrane lipids with detergents, etc., will undoubtedly influence the receptor topography. Whether this soluble receptor will resemble anything comparable to the functional receptor in terms of the affinity or response can be questioned. The recent solubilization of the dopamine receptor and the successful reconstitution of it with adenylate cyclase help to alleviate some of these concerns. It is unlikely that such solubilization and reconstruction experiments will precisely mimic all of the characteristics of the membrane bound complex, but the information to be gained about the physical nature of the components that constitute the receptor and their interaction with one another make this a worthwhile endeavor for future research.

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