Purification and solubilization of muscarinic receptors for the purpose of quantitative receptor assays of anticholinergics
Šmisterová, Jarmila

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SUMMARY

This thesis deals with the optimization of quantitative receptor assays (QRA) of anticholinergic drugs. QRA have recently emerged as a new tool in bioanalysis, offering challenging perspectives with regard to improving sensitivities and/or selectivities. Similar to immunoassays, QRA is a binding assay in which an analyte and a (radio)labelled ligand compete for the binding to a specific protein. In immunoassays the latter are antibodies raised against a given antigen. Yet, in QRA the specific protein is the isolated receptor with which the analyte interacts when exerting its pharmacological effect in vivo.

Chapter 1 in detail describes the state of the art of QRA. The factors influencing the analytical characteristics of QRA and the approach to their optimization are discussed. The review also points out the main directions in improving QRA by using the purified and solubilized receptor. In order to allow a wide-spread use of these assays in routine (clinical) practice, the development of solid-phase assays seems highly desirable.

Chapter 2 presents a two-step purification procedure for the muscarinic receptor from calf striatum for the purpose of QRA. This procedure includes the extraction of lipids with hexane in the first step and the removal of non-receptor proteins in the second step by means of 2 M sodium chloride. In order to check the influence of the purification on the ligand binding properties of the muscarinic receptor, two different types of labelled ligand, the tertiary amine $^3$H-dexetimide ($^3$H-DEX) and the quaternary amine $^3$H-N-methylscopolamine ($^3$H-NMS) were used.

Such a combination of the receptor material (purified or non-purified) and labelled ligand was sought, which gave the best results with regard to high affinity, the ratio specific/non-specific binding and lack of plasma interference. The high affinity of binding was preserved for both ligands for all the membrane-bound receptor preparations, which makes the purification procedure suitable for the purpose of solid-phase RA. No significant improvement was achieved in the ratio specific/non-specific binding, neither for $^3$H-DEX, nor for $^3$H-NMS by purifying muscarinic receptors with hexane. However, the use of the NaCl-pellet in combination with hydrophilic $^3$H-NMS appeared to be preferable over non-purified receptors, since receptor density per mg protein increased by a factor of about 1.7. This improves the counting precision in the assay. Also, the overall yield of receptors in the NaCl-pellet was higher when measured with $^3$H-NMS (80%) than that measured with $^3$H-DEX (40%). Thus, rather than in absolute terms, the improvement of the receptor material has to be considered by also taking into account the different physico-chemical properties of the labelled ligands. The use of the hydrophilic $^3$H-NMS over the lipophilic $^3$H-DEX in combination with the NaCl-pellet was considered preferable because of the higher ratio specific/non-specific binding and the negligible interference of plasma.

In Chapter 3, the possibilities to prepare solubilized muscarinic receptors with pre-
served binding properties for anticholinergics were studied. Also, the impact of optimizing the purification and solubilization procedure was evaluated. The factors addressed included the amounts of protein and digitonin introduced in the procedure and the time and temperature of the solubilization. The improvements achieved by first purifying and then solubilizing made this approach preferable over direct solubilization of non-purified receptor material. The treatment of purified receptors with digitonin preserved the high affinity for $^3$H-NMS ($K_d = 0.645$ nM), while the solubilization of non-purified receptor resulted in increased $K_d$-value ($K_d = 2.422$ nM). Also, the receptor density in the preparation solubilized after the purification was higher than that in non-purified solubilized material by a factor of about 1.7. The solubilized receptor can be used in the direct assay of plasma samples without any interfering effect on $^3$H-DEX binding to the muscarinic receptor. When purified membrane-bound receptors were used, plasma appeared to substantially interfere with $^3$H-DEX binding.

With regard to the choice of the appropriate labelled ligand, the results obtained in Chapter 2 with the membrane-bound receptor were similar for the solubilized receptor. Also for the solubilized receptor $^3$H-NMS was distinctly preferable over $^3$H-DEX. Using $^3$H-NMS, the percentage of non-specific binding dropped and the ratio specific/non-specific binding increased by a factor of about 20, when compared to the membrane-bound receptor.

The study presented in Chapter 4 was initiated by our observation that, by using the solubilized receptor, the detrimental effect of plasma on $^3$H-DEX binding can be avoided. In this chapter the factors involved in this effect were studied in more detail. Two of them, the amount of the digitonin-solubilized receptor in the assay and the temperature, appeared to be critical. Apparently, the presence of digitonin together with the solubilized receptor is of key importance, since avoidance of interference could not be obtained by the addition of digitonin to the assay with the membrane-bound receptor. Also, an interesting observation was made about the restoring effect of plasma on digitonin-inhibited ligand binding to the muscarinic receptor observed during the solubilization. The nature of the plasma components responsible for the latter effect is discussed. Our results suggest a synergistic effect of digitonin and plasma in preventing and overcoming the inhibitory effect that each of them have when present alone.

Chapter 5 deals with the impact of purification and solubilization of receptor material on the receptor assays of selected anticholinergic drugs. In Chapters 2 and 3, the improvements achieved by purification and solubilization were considered in terms of the labelled ligand binding only. Here, the different receptor preparations were evaluated in the assay system. Two different analytes, the tertiary amine scopolamine and the quaternary amine oryphenonium, were tested. $^3$H-NMS, which had turned out to be preferable over $^3$H-DEX for both the membrane-bound and the solubilized receptor, was used as the labelled ligand.

The $K_d$-values for both anticholinergics calculated from inhibition studies with $^3$H-NMS were similar for all the membrane-bound receptor preparations, either in absence or
presence of plasma. When the optimum amount of solubilized proteins/digitonin was used, a gain in sensitivity for scopolamine by a factor of 1.5 - 2.8 in plasma could be achieved. In case of oxyphenonium, a dramatic loss in sensitivity on the basis of $K_d$ was observed for the solubilized receptor. Interestingly, this loss could be offset partially when the assay was performed in the presence of plasma. Our results suggest that for every individual anticholinergic drug the conditions used have to be carefully chosen, especially when the RA is performed with solubilized receptors.

In Chapter 6 some final remarks about the importance of the results obtained are made. In view of the consequences of the purification and solubilization for applications in practice, we have clear indications that solubilized receptors are preferable over membrane-bound receptors in direct plasma assays.

The presented results are discussed here with regard to the application of different receptor materials for solid-phase RA with immobilized receptors. The possibilities of using the purified and the solubilized receptor in developing new RA, especially solid-phase RA and non-isotopic RA, are outlined.