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Effect of purification followed by solubilization of receptor material on quantitative receptor assays for anticholinergic drugs

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Abstract

In order to optimize quantitative receptor assays for anticholinergics, the different receptor preparations resulting from the purification and the solubilization of the P2 pellet from the calf striatum were evaluated. The dissociation constants for two chemically different anticholinergics, the tertiary amine scopolamine and the quaternary amine oxyphenonium, were calculated from inhibition studies of ^{3}H-NMS binding in buffer and plasma.

The K_d values for both anticholinergics were similar for all the membrane-bound receptor preparations (unpurified and the purified P2 pellet) either in buffer or in plasma. More pronounced differences were observed between the membrane-bound and solubilized receptors. By introducing the solubilized receptor as well, differences between the individual anticholinergics appeared. On the one hand, for scopolamine, a gain in sensitivity of 1.5–2.8 in plasma was observed for the solubilized receptor. On the other hand, in the case of oxyphenonium, a dramatic loss in sensitivity (by a factor of about 24) was observed with the solubilized receptor, as compared to the membrane-bound receptor, in buffer. Very interestingly, however, when the solubilized receptor was used in plasma, a lowering of the K_d value was found for both antichloinergics, i.e. the assays became more sensitive. Such an effect (not observed for the membrane-bound receptor) could be obtained only when the percentage of digitonin present in the assay was at least 0.12% (w/v) or higher.

Keywords: Anticholinergics, Digitonin; Plasma assays; Receptor assay; Solubilized muscarinic receptor

1. Introduction

Receptor assays (RAs) together with immunoassays (IAs) belong to the group of competitive binding assays. These assays have a special place among the methods used in bioanalysis as they do not utilize the physicochemical properties of the analyte (drug , hormone, neurotransmitter, etc.). Instead, they are based on the principle that the analyte can bind to a specific protein and, in

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doing so, competitively displace a labelled ligand from the binding site.

In the case of RAs, the binding site of the analyte is represented by the agonist recognition site of the receptor molecule, or by the recognition site of an intracellular messenger, such as c-AMP, calmodulin or phosphoinositide. Thus, an analyte that exerts its pharmacological action through interaction with a given specific binding site can be analyzed, qualitatively as well as quantitatively, when an aliquot of the sample is added to a solution that contains a fixed amount of binding sites and a fixed amount of labelled ligand. The unknown quantity of the analyte can be calculated by determining the percentage inhibition of labelled ligand and comparing this to the inhibition produced by known quantities of analyte in calibration samples.

RAs, however, possess an advantage over IAs and also over chemical detection in that they offer additional information about the biological activity of the analytes in a specimen by distinguishing compounds on the basis of their receptor-binding affinity. Only those molecules whose structures possess affinity to the agonist recognition site are detected. This property of RAs makes these assays useful in therapeutic drug monitoring. Results of RAs (depending on the class of drug) can better reflect the overall effect of a drug. Also, RAs may be used for drug abuse testing. The application of RAs for systematic toxicological analysis was recently evaluated by Ensing et al. [1,2].

RA is a relatively simple, specific and sensitive method. The sensitivity of RAs may be very high (picograms to nanograms of analyte can be measured) if the analyte has a high affinity to the receptor. However, RA also have some limitations due to the biological (receptor) material involved in the assay. The crude membrane homogenate, which in most cases represents the receptor preparation of RAs, may cause non-specific binding and batch-to-batch variation. Furthermore, aggregation and sedimentation of such receptor material can cause an uneven distribution of receptor in the assay vials, which may result in poor precision [3].

In order to improve RAs of anticholinergics, the receptor material used was optimized. The crude membrane homogenate from calf striatum was purified and solubilized. Fractionation of the crude membrane homogenate may increase the number of specific binding sites per milligram of protein (a higher specific/non-specific binding ratio). However, solubilization without further purification may result in a more homogenous preparation, which provides, better precision.

In addition to the above-mentioned factors, the presence of biofluid (e.g. plasma) may hamper a direct assay. Also, from this point of view, the use of solubilized receptors may bring a substantial improvement. The non-specific binding of labelled ligand to plasma could be reduced when the membrane-bound receptor was replaced by the solubilized receptor [4]. Also, variations in the plasma interference with ligand binding caused by batch-to-batch variations in the crude membrane homogenate could be overcome by using a solubilized preparation [5–8].

In this paper, purified receptors (the pellets after extraction with hexane and 2 M sodium chloride) and solubilized receptor material were applied to the RA of two anticholinergics, namely the tertiary amine scopolamine and the quarternary amine oxyphenonium. The results were compared with those obtained with the non-purified receptor material (the P2 pellet).

2. Materials and methods

^{3}H-dexetimide (^{3}H – DEX, 12.6 Ci mmol^{-1}, radiochemical purity 97.8%, checked by TLC) was a gift from Janssen Pharmaceutica (Beerse, Belgium). ^{3}H-N-methylscopolamine (^{3}H-NMS, 81.5 Ci mmol^{-1}, radiochemical purity 98.3%, checked by TLC) was supplied by Du Pont (Wilmington, DE).

Unlabelled dexetimide was kindly donated by Janssen Pharmaceutica. Unlabelled scopolamine hydrobromide and oxyphenonium bromide were of pharmacopoeial quality. Protease inhibitors and bovine serum albumin (fraction V, powder) were supplied by Sigma Chemical Co. (St. Louis, MO). Digitonin was obtained from Fluka Chemie

AG (Buchs, Switzerland). The activated charcoal (Carbo activus, Ph.Eur.) was obtained from OPG Farm (Utrecht, The Netherlands). All other chemicals and solvents were of analytical grade and obtained from Merck (Amsterdam, The Netherlands). The digitonin was purified as follows. Digitonin was dissolved in distilled water (2% w/v) by boiling for 10 min. After cooling, the solution was stored for 1 week in the refrigerator. After centrifugation, at 40 000*g* for 20 min, the supernatant was transferred to a glass flask and lyophilized for 48 h.

Rialuma was used as the scintillation cocktail and obtained from Lumac (Olen, Belgium).

In the plasma experiments, heparinized drug-free human plasma stored at −20°C was used.

2.1. Preparation of buffers

The PB7 buffer, used in the experiments with the P2 pellet and the hexane pellet, and the PBI buffer for the experiments with the solubilized receptor, were prepared as described before [9]. PBNa buffer used in the experiments with the NaCl pellet was prepared as follows: PB7 buffer was supplemented with phenylmethylsulphonylfluoride (PMSF, 0.1 mM) and trypsin inhibitor (1 mg ml^{-1}).

2.2. Preparation of solutions

Stock solutions of scopolamine hydrobromide (2.3×10^{-3} M) and oxyphenonium bromide (2.3×10^{-3} M) were prepared in ethanol. Working solutions were prepared by diluting with PB7 buffer. After dilution in assay less than 1% of organic solvent was present.

2.3. Preparation of receptor materials

2.3.1. Membrane-bound receptors

Calf striata were obtained fresh from the local slaughterhouse and stored at −80°C. The crude membrane homogenate (the P2 pellet) and the hexane pellet were prepared as described before [10].

In order to prepare the NaCl pellet, the hexane pellet was further extracted with 2 M sodium chloride, as also described in Ref. [10].

2.3.2. Solubilized receptors

The P2 pellet, the hexane pellet and the NaCl pellet were each suspended in an equal volume of PBI buffer, supplemented with 2% digitonin, to give a protein concentration of about 9 mg ml^{-1} and a final digitonin concentration of 1% (w/v). After solubiliztion at 0°C for 60 min and centrifugation at 13 000*g* for 15 min, the supernatant obtained represented the solubilized receptors (SR-P2, SR-hex and SR-NaCl respectively). Solubilized receptors were stored as aliquots at −20°C.

2.4. Protein determination

The amounts of protein in the membrane-bound and solubilized receptor preparations were determined according to the modification of the Lowry method by Clark, using human serum albumin as the protein reference standard [11].

2.5. Procedure for radioreceptor assays of anticholinergics

Inhibition experiments. To duplicate the polyethylene tubes 250 μl aliquots of solutions of either scopolamine hydrobromide or oxyphenonium bromide in buffer or plasma were added, giving final concentrations ranging from 3×10^{-11}–1×10^{-5} M (for scopolamine) and 1×10^{-12}–1×10^{-5} M (for oxyphenonium). Then an aliquot of the membrane-bound or solubilized receptor, giving 200 μg proteins per assay, was added to each tube, mixed for 5 s and the tubes were preincubated for 60 min at 0°C. After addition of 50 μl of ^{3}H-NMS (5.6×10^{-10} M) and mixing for 5 s, the incubation continued for another 60 min at 0°C. The total incubation volume was 500 μl.

When binding to the membrane-bound receptor was being determined, the incubation was terminated by the addition of 4 ml of ice-cold phosphate buffer, followed by filtration through Whatman GF/B filters. The tubes were rinsed twice with 4 ml of ice-cold PB7 buffer which was also filtered and the filters were dispersed in 3.5 ml of Rialuma by shaking for 2 h. The vials were counted for 4 min in a liquid scintillation counter (Packard Tri-Carb 4000, Downers Grove, IL).

In the case of the solubilized receptor, the incubation was terminated by the addition of 0.5 ml of an ice-cold charcoal solution (10% charcoal, 2% BSA) in PB7 buffer. After mixing and centrifugation at 13 000*g* for 5 min, an aliquot of 0.6 ml of the supernatant, representing the receptor-bound fraction, was transferred into a polyethylene counting vial and mixed with 3.5 ml of Rialuma. The bound fraction was mixed with 3.5 ml of Rialuma. The bound fraction was measured in a scintillation counter and corrected for the original volume of the sample.

2.6. Data analysis

Dissociation constants for the anticholinergics were calculated with the Ligand curve fitting program [12]. All the inhibition curves fitted the one-site model equation.

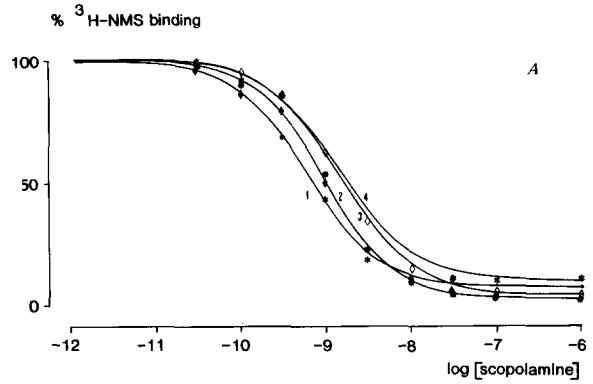
3. Results

3.1. Inhibition experiments with scopolamine on the membrane-bound and solubilized receptor

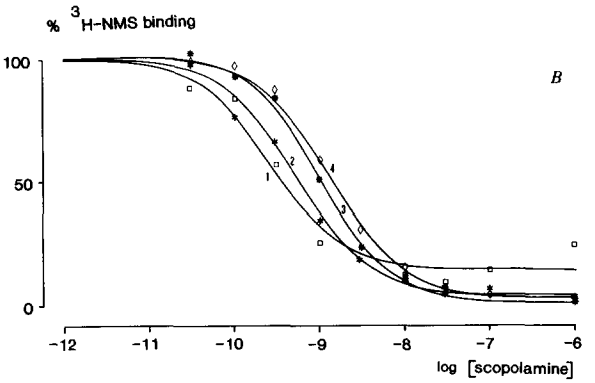
The inhibition curves of scopolamine in buffer and plasma, determined using ^{3}H-NMS as the labelled ligand, are shown in Fig. 1A and 1B respectively. The obtained dissociation constants for scopolamine are presented in Table 1.

In buffer, the dissociation constants for scopolamine remained much the same for the different membrane-bound receptors (see Fig. 1A and Table 1). The curves for the P2 pellet and the hexane pellet were virtually identical. When membrane-bound receptors were replaced by the solubilized NaCl pellet (SR-NaCl), a slight increase in the dissociation constant by a factor of about 2.0 was observed as compared to the P2 pellet.

In view of the consequences of the solubilization for applications in practice, the results obtained in plasma appear to be important. Here, by using purification followed by solubilization, a gain in sensitivity of 1.5 and 2.8 for SR-hex and SR-NaCl respectively, might be achieved (see Fig. 1B and Table 1). Furthermore, in plasma, there was no difference in sensitivity for the membrane-bound receptor preparations as compared to assays in buffer. The same was true for SR-hex. However, for SR-NaCl, the sensitivity of the assay in plasma improved when compared to buffer by a factor of about six. As will be shown later, the differences in K_d values between plasma and buffer for SR-NaCl can be explained by the different dilutions of the 1% digitonin-solubilized receptor in the assay. The dilution of SR-NaCl resulted in a digitonin assay concentration of



(A)



(B)

Fig. 1. Inhibition of ^{3}H-NMS binding to the different membrane-bound and solubilized receptors by scopolamine in buffer (A) and plasma (B). The membrane-bound receptor (200 μg proteins per assay) and the solubilized receptor (200 μg proteins per assay, 0.06–0.12% digitonin) were incubated with scopolamine for 1 h at 0°C in buffer or in the presence of 250 μl plasma. After the addition of ^{3}H-NMS (0.5 nM), the incubation continued for another 1 h at 0°C. (A): 1, SR-hex; 2, the P2 pellet = the hexane pellet; 3, NaCl-pellet; 4, SR-NaCl. (B): 1, SR-NaCl; 2, SR-hex; 3, the P2 pellet = the hexane pellet; 4, NaCl pellet.

Table 1
Dissociation constants (K_d; nM) of scopolamine in buffer and plasma for different receptor materials, using ^{3}H-NMS as ligand ($n = 1$ in duplicate; ± standard deviation of fitted curve)

Substance	Membrane-bound receptor			Solubilized receptor	
	P2 pellet	Hexane pellet	NaCl pellet	SR-hex[a]	SR-NaCl[b]
Buffer	0.35 ± 0.04	0.31 ± 0.04	0.68 ± 0.05	0.24 ± 0.06	0.72 ± 0.03
Plasma	0.33 ± 0.04	0.33 ± 0.02	0.58 ± 0.06	0.24 ± 0.06	0.12 ± 0.04

[a,b] The 1% digitonin-solubilized preparation was diluted in the assay volume of 0.5 ml to give digitonin percentages of 0.06% and 0.12% (w/v) respectively.

0.12% (w/v). SR-hex, for which the above-mentioned difference in K_d values between plasma and buffer was not observed, was diluted to 0.06% digitonin in the assay.

3.2. Inhibition experiments with oxyphenonium bromide on the membrane-bound and solubilized receptors

Fig. 2 shows the inhibition curves for oxyphenonium obtained with the purified and non-purified membrane-bound receptors and with the solubilized receptors. The calculated dissociation constants for oxyphenonium in the absence and presence of plasma for the different receptor materials are presented in Table 2.

In buffer (see Fig. 2A), the K_d values for the binding of oxyphenonium were similar for the purified and non-purified membrane-bound receptors with the K_d value for the NaCl pellet being slightly lower than those for the P2 pellet and the hexane pellet. However, the K_d value for solubilized receptor is some 10–20 times higher than those observed for the membrane-bound receptor preparation. No results for SR-NaCl could be obtained due to the limited amount of solubilized receptors available for this set of experiments.

Similar results were obtained in plasma, as shown in Fig. 2B and Table 2. The dissociation constants for oxyphenonium remained of the same order for the membrane-bound receptor preparations. Using the solubilized receptor resulted in a higher K_d value for oxyphenonium as compared to the membrane-bound receptor, but not as dramatic as seen in buffer.

Unlike for scopolamine, for oxyphenonium a decrease in the K_d value by a factor of about 1.9–3.2 was observed when the assay was performed with membrane-bound receptors in plasma as compared to buffer. This is due to non-specific binding of oxyphenonium to plasma proteins. However, interestingly, the use of plasma could partially offset the loss in sensitivity observed for the solubilized receptor in buffer (see Table 2). The dilution of the SR-hex in the assay resulted in the digitonin concentration of 0.24% (w/v). As will be shown later, the percentage of digitonin present in the assay has an impact on the sensitivity of the assay performed with the solubilized receptor in plasma.

3.3. Optimization of the RA of anticholinergics performed with the solubilized receptor

Due to the fact that by solubilization (even after the optimization) the receptor density per milligram proteins decreased [9], the absolute value of ^{3}H-NMS binding to the solubilized receptor was lower, as compared to the membrane-bound receptor. Fig. 3 depicts the inhibition of ^{3}H-NMS by scopolamine with the hexane pellet (200 μg proteins per assay) and the solubilized hexane pellet (200 μg proteins per assay) in buffer (the data were taken from Fig. 1A and expressed as the amount of total binding in Becquerels instead of percentage). A similar effect was observed for the solubilized NaCl pellet.

When the assay with the solubilized receptor was carried out in the presence of plasma a further reduction of ^{3}H-NMS binding took place. As can be seen in Fig. 4, plasma caused a reduction

in ^{3}H-NMS binding of about 60%, as compared to that in buffer.

In buffer, the lower binding to the solubilized receptor could be overcome by using a higher amount of solubilized proteins per assay. The limitation in this, however, is the capacity of the charcoal/BSA method. When using 0.53 nM ^{3}H-NMS, the binding was found to be linear for protein amounts up to at least 1 mg per assay volume of 0.67 ml. However, with plasma a curvilinear increase in binding was observed up to 800 μg proteins per assay. The increase in protein concentration was accompanied by an increase in the digitonin concentration in the assay. The latter was recognized as the crucial factor in the assay.

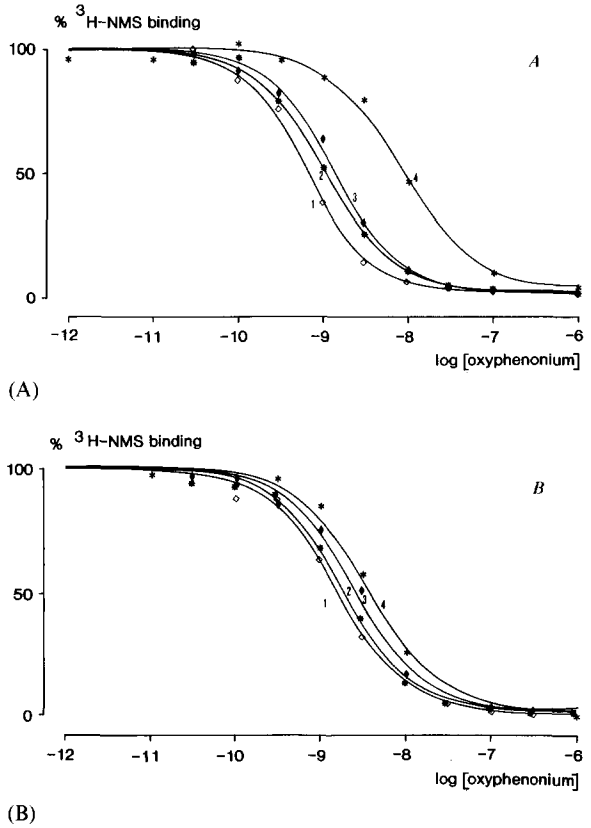


Fig. 2. Inhibition of ^{3}H-NMS binding to the different membrane-bound and solubilized receptors by oxyphenonium in buffer (A) and plasma (B). The membrane-bound receptor (200 μg proteins per assay) or solubilized receptor (600 μg proteins per assay, 0.24% digitonin) were pre-incubated with ^{3}H-NMS (0.5 nM) as described in Secion 2: 1, NaCl pellet; 2, P2 pellet; 3, hexane pellet; 4, solubilized hexane pellet.

It has already been observed [13] that in order to prevent the decline in binding of ^{3}H-NMS caused by the addition of 250 μl plasma, the solubilized receptor has to be diluted in the assay, so that the digitonin percentage reaches a value of at least 0.2%. In this paper, when the RA with the solubilized receptor was performed under these conditions, the plasma interference with the ^{3}H-NMS binding could not only be overcome, but the maximum binding could even be increased, as shown in Fig. 5. This resulted also in a higher sensitivity of the RA of oxyphenonium when performed with the solubilized receptor in plasma, as compared to the buffer (see Table 2). The inhibition curve for oxyphenonium in Fig. 5 is expressed as the total amount of ^{3}H-NMS binding (in Becquerels) in order to demonstrate the fact that more active binding sites were available in the solubilized receptor preparation in the presence of plasma.

4. Discussion

Quantitative RAs for anticholinergics have been applied in biofluids in this laboratory for the determination of the quaternary drugs oxyphenonium, ipratropium and oxitropium [14–16], as well as for the tertiary drugs scopolamine and atropine [17,18].

A substantial improvement in the sensitivity of these assays has been achieved by optimizing the incubation conditions (non-equilibrium conditions [17]) and the concentration of the labelled ligand [14]. The stability of the crude membrane homogenate (the P2 pellet) and the convenience of the assay were improved by using lyophilized receptor material [19]. However, interference of biofluids, e.g. plasma and urine, with the binding of the labelled ligand and/or analyte prevented a direct assay in these fluids, thus necessitating extensive sample work-up procedures. For example, because of the non-specific binding of ^{3}H-DEX an extraction of scopolamine from plasma with dichloroethane had to be included [19]. Also, when the lipophilic ligand ^{3}H-DEX was replaced

Table 2
Dissociation constants (K_d; nM) of oxyphenonium in buffer and plasma for different receptor materials, using ^{3}H-NMS as ligand ($n = 1$ in duplicate; ± standard deviation of fitted curve)

Substance	Membrane-bound receptor			Solubilized receptor
	P2 pellet	Hexane pellet	NaCl pellet	SR-hex[a]
Buffer	0.37 ± 0.05	0.43 ± 0.04	0.17 ± 0.02	4.1 ± 0.5
Plasma	0.69 ± 0.05	0.93 ± 0.09	0.55 ± 0.05	1.7 ± 0.03

[a] 1% digitonin-solubilized preparation was diluted in the assay volume of 0.5 ml to give a percentage of digitonin equal to 0.24% (w/v).

by ^{3}H-NMS, which has a lower non-specific binding to plasma proteins, extraction of scopolamine from plasma could not be avoided [20].

In the present study different receptor preparations were evaluated for use in QRA of anticholinergics, in order to see to what extent the character of the receptor material (membrane-bound vs. solubilized receptor, purified vs. non-purified receptor) influences the sensitivity of the assay, as well as to study the inhibitory effect of plasma on ligand binding. The preferable use of the solubilized receptor over the membrane-bound receptor in plasma assays of beta-blockers has already been reported [21].

The purified membrane-bound receptor materials, the hexane pellet and the NaCl pellet did not show a considerable improvement in sensitivities for both scopolamine and oxyphenonium, as can be seen in Tables 1 and 2. The non-specific binding of oxyphenonium to plasma proteins resulted in a small shift of the curves to the right and a slightly higher dissociation constant for oxyphenonium for all membrane-bound preparations in the presence of plasma. The addition of plasma had no effect on the sensitivity of the RA for scopolamine.

Similar to purified membrane-bound receptors, it was also found for the solubilized receptor that the improvement in sensitivity achieved did not seem to be sufficient motivation for solubilizing the muscarinic receptor for the purpose of quantitative receptor assays in buffer. Yet, the use of solubilized receptors in direct plasma assays appears to be very promising, provided that the optimum amount of proteins/digitonin is used in the assay. Using solubilized receptors, plasma no

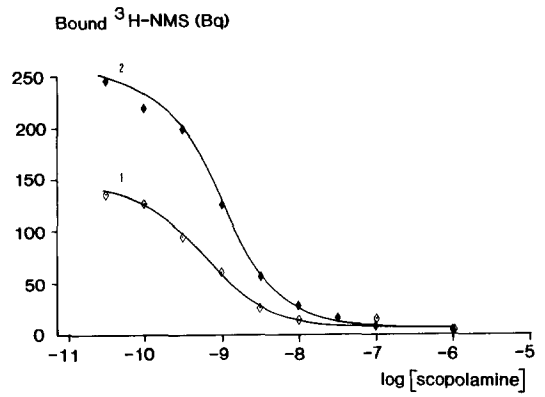


Fig. 3. Inhibition of ^{3}H-NMS binding to the solubilized (1) and the membrane-bound receptor (2) by scopolamine in buffer. The hexane pellet (200 μg proteins per assay) or solubilized hexane pellet (200 μg proteins per assay, 0.06% digitonin) were incubated with scopolamine under the conditions described in the legend to Fig. 1.

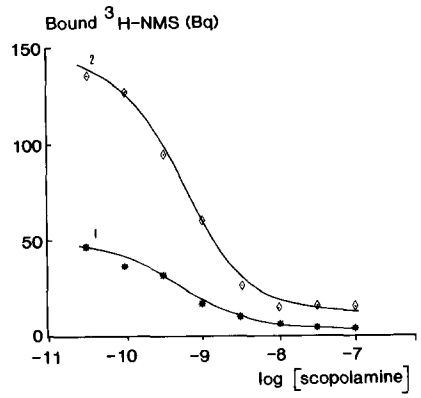


Fig. 4. Inhibition of ^{3}H-NMS binding to the solubilized receptor by scopolamine in plasma (1) and buffer (2). SR-hex (200 μg proteins per assay, 0.06% digitonin) was incubated with scopolamine under the same conditions as in Fig. 1.

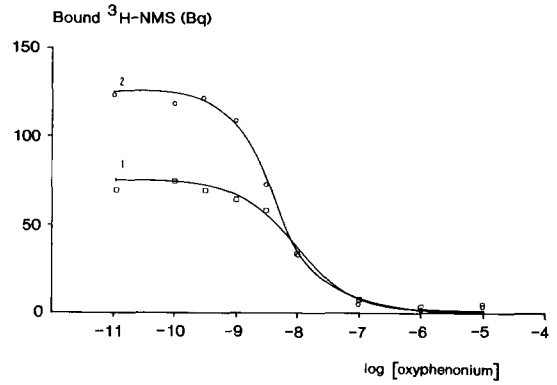


Fig. 5. Inhibition of ^{3}H-NMS binding to the solubilized hexane pellet by oxyphenonium bromide in buffer (1) and plasma (2). The results for SR-hex expressed in Fig. 2A,B as percentage of ^{3}H-NMS binding are here expressed in total binding in Becquerels. For details see legend of Fig. 2.

longer interfered in the analysis of oxyphenonium. When the precentage of digitonin in the assay reached 0.24%, the binding to the solubilized receptor was even higher in the presence of plasma, by a factor of 1.7. The current results suggest a special role of solubilized receptor-bound digitonin in this effect, since it could not be mimicked simply by the addition of digitonin to the assay with the membrane-bound receptor [13]. The assay of scopolamine with solubilized receptors was more sensitive in plasma than in buffer by a factor of 2.8.

It is conceivable that the lipids and lipoproteins present in plasma have a restoring effect on the solubilized receptor, after losing some essential lipid-like components during its exposure to digitonin. This could explain the fact that the ligand binding to the solubilized receptor increased in the presence of plasma. However, for an optimal effect, a certain ratio of added plasma to digitonin is required.

The role of physicochemical properties of the analyte in this effect remains to be established by extending this study to a larger group of anticholinergics, especially those for which the presence of plasma causes a serious loss in sensitivity.

Thus, under optimized conditions, the use of solubilized receptors may offer distinct advantages over membrane-bound receptors in the case where the interference of plasma with either the labelled ligand or the analyte causes a considerable loss in sensitivity. However, the exact mechanisms involved in the favourable effects of plasma on the ligand binding activity of digitonin-solubilized muscarinic receptors still remain unclear. Further explorations in this are are warranted, also with regard to other detergents and to other biofluids.

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