[^{14}C]aminopyrine was linear during 6 hr of collection. The effects of phenobarbital induction and testosterone inhibition, as well as dose-dependent CCl_4 toxicity, were clearly demonstrable and the system was suited for enzyme kinetic studies of aminopyrine *N*-demethylation. It is suggested that this simple and noninvasive approach may prove useful for the study of drug metabolism and drug toxicity in hepatocyte monolayers, and for the rapid assessment of culture viability.

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Cholate-salt solubilisation of bovine brain muscarinic receptors

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Muscarinic cholinergic receptors have been extensively characterised in their membrane state (for recent review see [1]). The presence of receptors can be detected *in vitro* using radioligands (usually antagonists such as propylbenzilycholine [2], quinuclidinyl benzilate [3], *N*-methyl-4-piperidylbenzilate [4]) which label a saturable high affinity binding component able to be specifically displaced by muscarinic agonists and antagonists. These antagonists bind to a single high affinity receptor site while strong agonists bind with a lower potency and give binding curves with Hill coefficients less than 1 suggesting a possible heterogeneity of binding sites of high and low affinities [5]. In order to understand the precise functioning of the muscarinic receptor it is necessary to study the receptor in an isolated state; however, purification attempts have been hampered by the lack of an efficient solubilising agent. Earlier attempts to solubilise the muscarinic receptor made use of high salt [6, 7] or the detergent digitonin [8]. More recently several other laboratories have characterised the receptors using this detergent; however, the binding characteristics of the soluble material varied between reports. In two reports, strong agonists no longer bound to multiple sites but instead to a single binding site of low affinity in one case [9] and of high affinity in the other [10]. The ability of digitonin to solubilise muscarinic receptors also appeared to vary between batches [11]. A more useful solubilising agent would combine a reproducible and efficient solubilisation of membrane-bound receptors with retention of muscarinic binding characteristics. Reported here is the solubilisation of specific [^{3}H]quinuclidinyl benzilate binding sites from bovine brain caudate nucleus using a combination of 0.1% sodium cholate and 1 M NaCl.

* Abbreviations: QNB, quinuclidinyl benzilate; EGTA, ethylene-glycol-bis (β-amino-ethyl ether) *N*,*N*′-tetraacetic acid.

Materials and methods

Ox brains were obtained fresh from the slaughterhouse within 4 hr of death. The caudate nucleus was dissected out and a microsomal pellet (1500–2500 pmoles [^{3}H]QNB* binding sites/g protein) was prepared as described in [12]. Protein was assayed as in [13].

Microsomal-P_3 pellets (2 mg protein/ml) were homogenised in 0.1% Na cholate, 1 M NaCl, 1 mM EGTA, 25 mM Na-phosphate buffer (pH 7.4) for 10 strokes and shaken at room temperature for 1 hr. The mixture was then diluted 1:1 in phosphate–EGTA buffer (final concn now 0.05% Na cholate, 0.5 M NaCl) and centrifuged for 1 hr at 187,000 *g* in a Beckman 50.2 Ti rotor at 4°. The resulting supernatant was stored at 4° (stable up to 1 week) and is referred to as soluble material. The remaining pellet could be reextracted yielding a preparation of increased specific activity (3–5-fold).

Binding studies on membrane material were performed in duplicate at 0.015–0.03 mg protein/ml using (±)-[^{3}H]QNB as the ligand essentially as in [3] for 1 hr at 37°. When necessary, unlabeled ligands were preincubated with the membranes for 15 min at 37° before the addition of [^{3}H]QNB. Binding studies on soluble material (150–300 μg protein/ml) were performed by a protein precipitation method using saturated ammonium sulphate (pH 7) essentially as described in [14]: after incubation with the appropriate ligands as above, 1 ml of soluble material was precipitated with 1 ml of saturated ammonium sulphate (pH 7) and left on ice for 2 min. The mixture was then filtered through Whatman GF/B glass fiber filters and washed twice with 5 ml of 50% saturated ammonium sulphate. There was no specific [^{3}H]QNB binding to filters. The filters were dried and radioactivity determined by scintillation counting using Packard Toluene Scintillator and counting in a Packard Tri-carb 2660. (±)-[^{3}H]-QNB (32 Ci/mmole) was obtained from the Radiochemical Centre (Amersham, U.K.). Benzhexol, propylbenzilylcholine and benzilylcholine were gifts from Dr. J. M. Young. All other ligands and supplies were from Sigma Chemical Co. (London, U.K.).

Analysis of the binding data from the kinetic studies was based on the work of Maelicke *et al.* [15] and has been thoroughly described previously [16].

Results

Preliminary experiments established that a combination of 0.1% Na cholate and 1 M NaCl would solubilise specific [^{3}H]QNB binding sites from bovine brain as revealed by equilibrium dialysis [17]. Further work on the soluble material was done using a more rapid and efficient filtration assay as described in Materials and Methods. Cholate-salt routinely solubilised between 3 and 7% of total membrane receptor binding sites and between 15 and 30% of total specific activity in pmoles [^{3}H]QNB binding sites/g protein (results from 7 experiments, data not shown). Reextraction of the pellet remaining after the first solubilisation resulted in a 3–5-fold increase in specific activity of the soluble material and solubilised a further 2–4% of total membrane binding sites. 0.1% Na cholate or 1 M NaCl alone solubilised less than 1% of total specific activity. The soluble material was relatively stable at 4° and lost approx. 30% initial binding activity per week; all activity was, however, lost after 5 min at 100°. Efforts to reduce the salt concentration below 0.5 M NaCl resulted in a loss of binding sites, therefore all present studies were carried out at a final cholate–salt concn of 0.05% and 0.5 M respectively.

Figure 1 shows a typical saturation curve for the binding of the antagonist [^{3}H]QNB to a soluble preparation from bovine caudate nucleus. Non-specific binding (binding in the presence of 5 μM atropine) was 10–20%. Scatchard plot analysis of the data (inset) yields a B_{max} of 800 pmoles/g protein and an apparent dissociation constant (K_d) of 200 pM. The total number of binding sites varied from 150 to 900 pmoles/g protein and K_d values varied from 150 to 300 pM for soluble preparations.

The association and dissociation of specific [^{3}H]QNB binding is shown in Fig. 2. The average half times of association and dissociation were respectively 10 ± 2 min and 70 ± 9 min (n = 4). Analysis of the kinetic data gave

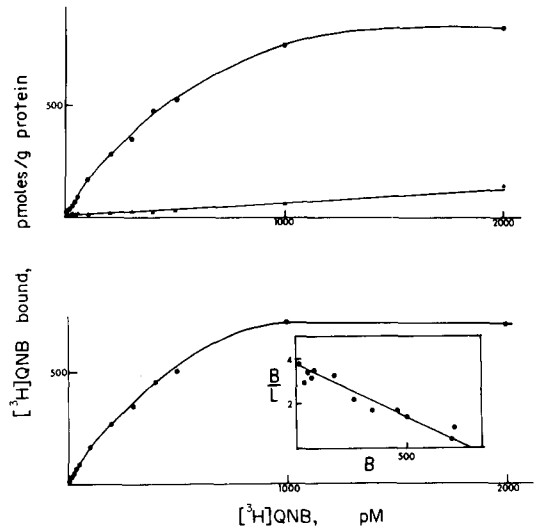

Fig. 1. Specific and non-specific binding of [^{3}H]QNB to a soluble preparation from bovine caudate nucleus. Binding was performed in duplicate at 37° as described in Materials and Methods. Upper figure: binding of [^{3}H]QNB in the absence (●) and presence (▲) of 5 μM atropine. Lower figure: subtraction of the two curves gives specific binding. The inset represents Scatchard plot analysis of specific [^{3}H]QNB binding data: B_{max} = 800 pmoles/g protein; K_d = 200 pM.

rate constants of $1.85 \pm 0.6 \times 10^8 M^{-1}min^{-1}$ for k_1 and $1.0 \pm 0.03 \times 10^{-2}min^{-1}$ for k_{-1}. Assuming a simple binding process, the K_d calculated from the ratio k_{-1}/k_1 was 54 pM as compared to 20 pM for the membrane value [17].

Inhibition of the binding of [^{3}H]QNB at 100 pM by representative muscarinic agonists and antagonists is shown in Fig. 3. Non-muscarinic and non-cholinergic ligands had no effect up to 10 μM. The results for both membrane and soluble receptors are summarised in Table 1. As can be seen the membrane values for ox brain are comparable to those of other workers on the rat brain. Inhibition constants

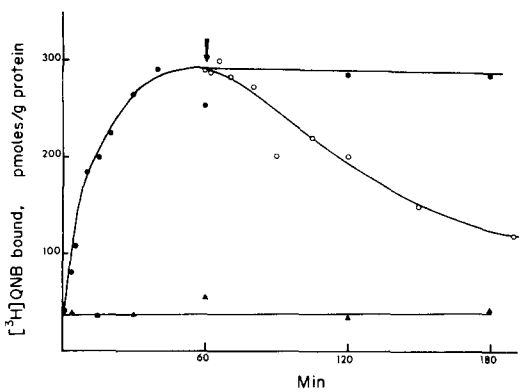

Fig. 2. Association and dissociation of QNB binding. Assays were performed in duplicate at 500 pM [^{3}H]QNB at 37°. Analysis of this particular experiment gave a half-time ($t_{1/2}$) for association and dissociation of 8 min and 84 min respectively; $k_{-1} = 8.25 \times 10^{-3}$ min^{-1} and $k_1 = 1.48 \times 10^8$ M^{-1} min^{-1} yielding a $K_d(k_{-1}/k_1)$ of 55 pM if one assumes a simple binding process. Total binding (●); non-specific binding (▲) in the presence of 5 μM atropine; total binding (○) after addition of 5 μM atropine at 60 min.

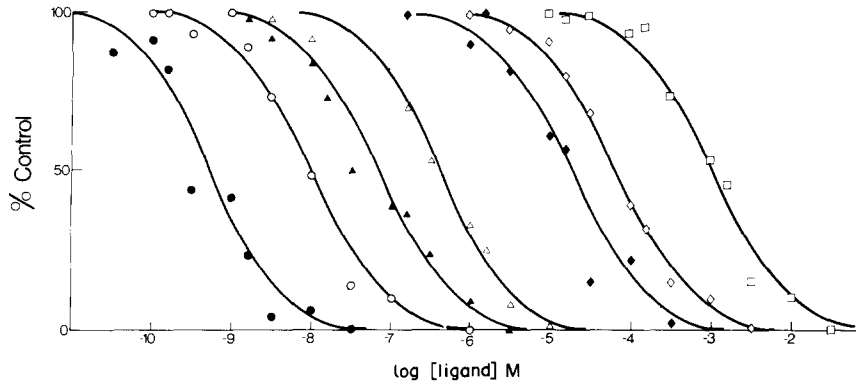

Fig. 3. Inhibition of [^{3}H]QNB binding to soluble material by muscarinic agonists and antagonists. Appropriate ligands were incubated with soluble material for 15 min at 37° before the addition of 100 pM [^{3}H]QNB as in Materials and Methods. Curves were drawn to fit a simple mass action equation. QNB (●); atropine (○); benzilylcholine (▲); chlorpromazine (△); oxotremorine (◆); methylfurmethide (◇); carbamylcholine (□).

for soluble material were 10 to 20-fold greater than in the membrane state. Hill coefficients remained the same after solubilisation except for the agonists methylfurmethide and carbamylcholine where the Hill coefficient was increased to 1.0 from 0.65 and 0.55 respectively.

Discussion

This paper has presented evidence that a combination of 0.1% Na cholate and 1 M NaCl will solubilise [^{3}H]QNB binding sites from bovine caudate nucleus. Approximately 15–30% of total sp. act. was solubilised following the procedure described in Materials and Methods. Unsolubilised receptor activity can be recovered in the pellet indicating that the combination of 0.1% Na cholate and 1 M NaCl by itself does not destroy binding sites. Using the muscarinic antagonist QNB a single saturable binding component was found with an equilibrium constant of 200 pM, approx. 10-fold higher than the membrane receptor and 4-fold higher than the ratio k_{-1}/k_1 determined kinetically in the soluble material. This latter difference may indicate that binding of QNB to the solubilised receptor is not a simple second-order process. Inhibition constants for a variety of muscarinic agonists and antagonists were 10 to 20-fold higher than in the membrane state and non-muscarinic ligands were without effect. The decrease in affinity of the

Table 1. Inhibition constants for muscarinic agonists and antagonists in membrane and soluble material*

Ligand	Rat brain	Ox brain			
		Membrane		Soluble	
	K_i[M]	K_i[M]	n_H	$ID_{50\,cor}$[M]	n_H
QNB	2×10^{-10} [3]	2.5×10^{-11}	1.0	4.6×10^{-10}	1.0
Atropine	7×10^{-10} [3]	3.6×10^{-10}	0.95	6.6×10^{-9}	1.0
Scopolamine	4×10^{-10}[3]	2.1×10^{-9}	1.0	6×10^{-9}	0.9
Benzhexol	7.6×10^{-9} [2]	1×10^{-9}	1.0	1.3×10^{-8}	0.9
Benzilylcholine	1.8×10^{-9}[19]	2.8×10^{-9}	0.9	4.6×10^{-8}	0.85
Propyl-benzilylcholine	1.3×10^{-8}[2]	2×10^{-8}	0.9	2×10^{-7}	0.85
Chlorpromazine	5×10^{-8}[20]	2.5×10^{-8}	0.9	4×10^{-7}	0.85
Oxotremorine	3×10^{-7}[3]	5×10^{-7}	0.85	8×10^{-6}	0.95
Pilocarpine	3×10^{-6}[3]	2.1×10^{-6}	1.0	1.3×10^{-5}	0.9
Methylfurmethide	4×10^{-6}[5]	8.5×10^{-6}	0.65	5.3×10^{-5}	1.0
Carbamylcholine	1×10^{-5}[3]	2×10^{-5}	0.55	4.6×10^{-4}	1.0

* Inhibition constants were calculated from the equation:

$$K_i = \frac{ID_{50}}{1 + C/K_d}$$

where C is the concentration of the radioactive ligand (50 pM for membranes and 100 pM for soluble material) and K_d its equilibrium constant for [^{3}H]QNB (20 pM and 200 pM for membrane and soluble material respectively). Due to the discrepancy between the equilibrium constant (200 pM) and the ratio k_{-1}/k_1 (54 pM) only the $ID_{50\,cor}$ can be calculated from the above equation for the soluble material. Binding assays were performed as in Materials and Methods. Each value represents the average of at least 2 experiments agreeing with 10%. ID_{50} values were determined graphically. Non-muscarinic drugs (hexamethonium, decamethonium, *d*-tubocurare, nicotine and neostigmine) were without effect up to 10 μM.

Values for rat brain were recalculated from the appropriate reference except for Ref. [19] which was determined in guinea pig ileum.

soluble receptor for muscarinic ligands is probably a result of the high salt concentration present in the preparation as indicated by the work of Birdsall *et al.* [18]. The Hill coefficients for the soluble material were all close to unity indicating the presence of a single binding site, as also described by Hurko using digitonin [9]. Whether the multiple binding sites of the potent agonists methylfurmethide and carbamylcholine were transformed to the low affinity site or whether the low affinity site was selectively solubilised cannot be decided at present. The fact that such mild conditions have solubilised receptor binding sites seems to indicate the importance of lipids in maintaining the solubilised receptor in an active state [21]. In conclusion, the results reported here indicate a novel means of receptor solubilisation which could be very useful in future purification work.

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