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Solubilization and characterization of high and low affinity pirenzepine binding sites from rat cerebral cortex

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1 An apparently monomeric form of the digitonin-solubilized muscarinic acetylcholine receptor from the rat cerebral cortex retains a high affinity of 7×10^7 M^{-1} for pirenzepine.

2 Muscarinic receptor binding sites in the rat cerebral cortex with a low affinity for pirenzepine are solubilized with relatively little change in affinity.

3 The ability of pirenzepine to distinguish between subtypes of muscarinic binding site in the cerebral cortex is manifest in both the membrane-bound and soluble state.

Introduction

In certain tissues it is possible to demonstrate the presence of subclasses of muscarinic receptor binding sites which may be discriminated by the selective antagonist, pirenzepine (Hammer *et al.*, 1980; Hammer, 1982; Hammer & Giachetti, 1982; Watson *et al.*, 1982; 1983; 1984; Birdsall & Hulme, 1983; Birdsall *et al.*, 1983; 1984; Luthin & Wolfe, 1984). This selectivity is evident in both binding and functional studies (Hammer *et al.*, 1980; Brown *et al.*, 1980; Hammer & Giachetti, 1982; 1984; Caulfield & Straughan, 1983; Caulfield *et al.*, 1983) but its origin is unknown. One approach to this problem is to solubilize the receptor and to attempt to isolate, characterize and purify the subclasses of receptor binding site which have high and low affinities for pirenzepine. In this paper we demonstrate that both classes of binding site may be solubilized from rat cerebral cortex membranes, but that they exhibit different stabilities.

Methods

EDTA-washed membranes from rat cerebral cortex were prepared as described by Hulme *et al.* (1983). Solubilization, assay and analytical methods were identical to those described in the above paper by Hulme *et al.* (1983) and Berrie *et al.* (1984a), as was the sucrose gradient centrifugation technique. Solubilization was conducted at 4–5 mg ml^{-1} protein and 1% digitonin. Incubations with [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) and [^{3}H]-pirenzepine ([^{3}H]-Pir) were generally performed for at least 24 h at 4°C. As the rate of equilibration of pirenzepine binding was very slow, pirenzepine-[^{3}H]-NMS competition experiments were carried out by preincubating the soluble receptor with pirenzepine for 24 h before addition of [^{3}H]-NMS, at a concentration below its dissociation constant, and incubation for a further 24 h. All assays were conducted in a buffer containing 20 mM NaHEPES, 1 mM Mg^{2+}, 1% digitonin, pH 7.5 at 4°C unless otherwise specified. (−)-[^{3}H]-N-methylscopolamine, 53.5 Ci mmol^{-1} or 84.8 Ci mmol^{-1}), [^{3}H]-oxotremorine-M ([^{3}H]-oxoM, 82.5 Ci mmol^{-1}) and [^{3}H]-pirenzepine (75 Ci mmol^{-1}) were obtained from New England Nuclear. [^{3}H]-propylbenzilylcholine (40 Ci mmol^{-1}) was prepared by the method of Hulme *et al.* (1978). Digitonin was obtained from Wako Chemical Co., Osaka, Japan. Guanylylimidodiphosphate (GppNHp) was from Boehringer Mannheim. Binding data were analysed by non-linear least squares analysis (Birdsall *et al.*, 1978).

Results

Solubilizaton of [^{3}H]-pirenzepine binding sites from rat cerebral cortex

Several groups have shown that it is possible to define a component of [^{3}H]-pirenzepine binding to cerebral

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Table 1 Solubilization of specifically bound [^{3}H]-pirenzepine ([^{3}H]-Pir), [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) and [^{3}H]-oxotremorine-M ([^{3}H]-oxoM) from prelabelled rat cortex membranes at 0°C using digitonin*

Digitonin (%)	*% of binding recovered in supernatant*			*% of binding recovered in supernatant + membranes*		
	[^{3}H]-NMS	[^{3}H]-Pir	[^{3}H]-oxoM	[^{3}H]-NMS	[^{3}H]-Pir	[^{3}H]-oxoM
0.4	1.6	1.5	3.9	87	81	76
0.7	25	18	28	100	87	74
1.0	46	25	25	102	95	59
1.5	51	33	26	104	98	58
2.0	51	34	22	100	99	27

* EDTA-washed cortical membrane preparations were resuspended to ca. 5 mg ml^{-1} in 20 mM NaHEPES, pH 7.5, containing 1 mM Mg^{2+} and incubated with the radiolabelled ligands (10^{-8} M) for ca. 18 h at 0°C in the absence and presence of 10^{-6} M quinuclidinylbenzilate (QNB). The initial levels of QNB-displaceable binding were 4473 fmol ml^{-1} (NMS), 2464 fmol ml^{-1} (Pir) and 1058 fmol ml^{-1} (oxoM). Solubilization was accomplished by addition of 10% digitonin to the indicated concentrations, and stirring for 30 min at 0°C before centrifugation to remove insoluble material. Both supernatants and pellets were assayed for the presence of specifically bound radioligands.

cortex membranes which is inhibited by muscarinic drugs and displays a K_A of about 10^8 M^{-1} (Hammer *et al.*, 1980; Watson *et al.*, 1982; 1983; Birdsall *et al.*, 1983; 1984; Luthin & Wolfe, 1984). The concentration of these high affinity pirenzepine binding sites is ca. 50–80% of the total concentration of muscarinic acetylcholine receptor (MAChR) sites which may be labelled with a nonselective antagonist such as 3-quinuclidinylbenzilate (QNB) or N-methylscopolamine (NMS).

When cortical membranes were labelled with [^{3}H]-Pir (10^{-8} M, 24 h at 0°C) in the absence and presence of QNB (10^{-6} M), and the resultant labelled preparation solubilized by stirring with increasing concentrations of digitonin, a significant proportion of the labelled sites was recovered in the supernatant, as assessed by Sephadex gel-filtration (Table 1). The recovery of [^{3}H]-Pir binding sites rose to a maximum of ca. 30–34% at 1.5–2% digitonin. The unsolubilized binding activity was recovered in the pellet without denaturation. The recovery of [^{3}H]-NMS binding sites in the supernatant was ca. 50% under the same conditions, and once again the remaining sites were recovered undenatured in the pellet.

When the solubilization (30 min) was carried out at 30°C, up to 85% of the [^{3}H]-NMS sites but only 35% of the [^{3}H]-Pir sites were recovered in the supernatant using 1–2% digitonin. Furthermore, there was a 40–50% loss in overall [^{3}H]-Pir binding (supernatant + pellet) upon treatment with digitonin at 30°C (but not at 0°C).

Recovery of binding of the agonist, [^{3}H]-oxotremorine-M was optimum (28%) at 0°C at a slightly lower concentration of digitonin (0.7%, Table 1) and there was marked loss of overall binding at higher digitonin: protein ratios, probably reflecting disruption of the MAChR-nucleotide binding protein coupling which has been shown, in the myocardium, to be necessary for the expression of super-high affinity oxoM binding (Berrie *et al.*, 1984a).

Postlabelling of digitonin-solubilized cortical supernatants at 0°C yielded a similar solubilization profile (Table 2) although the yield of [^{3}H]-NMS binding sites was somewhat reduced. Again, there was no evidence

Table 2 Labelling of digitonin-solubilized muscarinic acetylcholine receptors (MAChR) from rat cerebral cortex*

Digitonin (%)	*% of initial membrane binding recovered in S/N*	
	[^{3}H]-NMS	*[^{3}H]- Pir*
0.4	0.9	2.2
0.8	20	19
1.0	30	37
1.5	38	36
2.0	37	32

* Cortical membranes (ca.4 mg ml^{-1}) were solubilized at 0°C in the indicated concentrations of digitonin and then labelled with [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) (10^{-8} M) or [^{3}H]-pirenzipine ([^{3}H]-Pir) (2.9×10^{-9} M) for 24 h at 0°C. Initial levels of membrane binding were 4450 fmol ml^{-1} ([^{3}H]-NMS, 10^{-8} M) and 543 fmol ml^{-1} ([^{3}H]-Pir, 2.9×10^{-9} M).

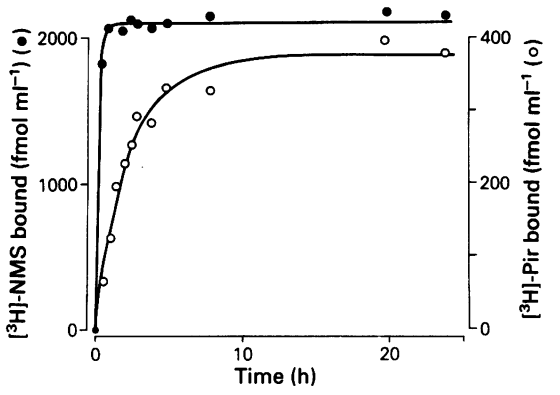

Figure 1 Time course of quinuclidinylbenzilate (QNB)-displaceable binding of [^{3}H]-pirenzepine ([^{3}H]-Pir, ○) and [^{3}H]-N-methylscopolamine ([^{3}H]-NMS, ●) to a digitonin supernatant of cerebral cortex membranes. Cortical membranes (5 mg protein ml^{-1}) were solubilized in 1% digitonin. The concentration of [^{3}H]-NMS and [^{3}H]-Pir was 10^{-8} M. The [^{3}H]-Pir time course was fitted by a single exponential of the form $[R.Pir] = [R.Pir](1 - e^{-kt})$ where $[R.Pir] = 371 \pm 16$ fmol ml^{-1} and $k = 0.453\ h^{-1}$. The comparable rate constant for [^{3}H]-NMS could not be determined accurately from the given data, but was ca. 10 fold greater ($\sim 3.8\ h^{-1}$).

to suggest that [^{3}H]-Pir binding was very sensitive to high digitonin: protein ratios at 0°C.

Binding properties of soluble [^{3}H]-pirenzepine binding sites

The time-course of [^{3}H]-Pir binding to digitonin-solubilized MAChR (1% digitonin) at 0°C or 4°C was very slow, much slower than that of NMS (Figure 1). It was necessary to continue incubations for 18–24 h at these low temperatures in order to allow an equilibrium state to be achieved.

The high affinity component of binding, inhibited by unlabelled Pir (and by nanomolar concentrations of atropine, NMS and QNB and higher concentrations of oxoM (Figure 6)), was identical to that inhibited by 10^{-6} M QNB (Figure 2). This demonstrates the muscarinic character of the [^{3}H]-Pir binding sites. The IC_{50} values for Pir were the same, ca. 2.0×10^{-8} M, in both membranes and soluble preparations. Pirenzepine inhibition of [^{3}H]-Pir binding to the solubilized preparation was well described by a simple mass-action curve with a log K_A of 7.81 ± 0.06 (mean ± s.d., $n = 6$), a figure almost exactly the same as that found for membranes (Figure 3; Watson *et al.*, 1982). Using this value, it was possible to calculate the total concentration of Pir high affinity sites present in different digitonin-solubilized cortical preparations. Values ranged from 42–80% of the total concentration of binding sites assayed using a saturating concentration of [^{3}H]-NMS, with a mean value of $59 \pm 13\%$ (mean ± s.d., $n = 6$), which is again very similar to the value found in cortical membranes (Birdsall *et al.*, 1984).

A high affinity component of [^{3}H]-Pir binding to the digitonin solubilized cortical preparation was also detectable in competition experiments in which unlabelled Pir was used to inhibit the binding of a low, fixed concentration of a second ^{3}H-antagonist, either [^{3}H]-NMS (Figure 4) or [^{3}H]-propylbenzilylcholine (data not shown). Because of the very slow binding of

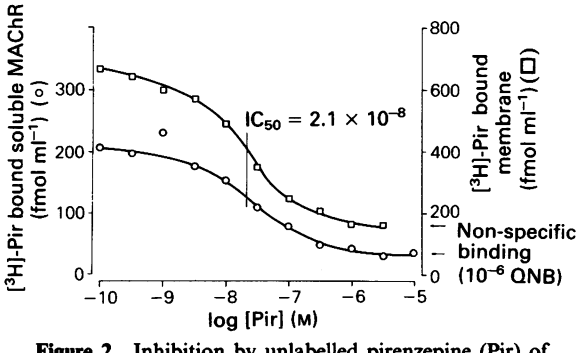

Figure 2 Inhibition by unlabelled pirenzepine (Pir) of binding of [^{3}H]-Pir (3×10^{-9} M) to a 1% digitonin supernatant (○) of cerebral cortex membranes (5 mg ml^{-1}) and to unsolubilized membranes (□). Binding to membranes was measured at 1 mg protein ml^{-1}, and has been scaled by a factor of 5 to allow a direct comparison with the supernatant measurements.

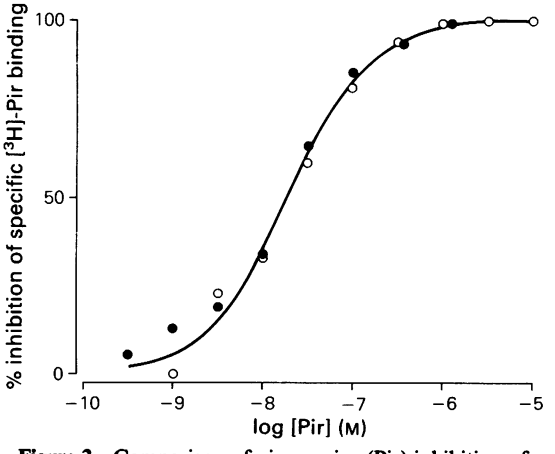

Figure 3 Comparison of pirenzepine (Pir) inhibition of the binding of [^{3}H]-Pir (3×10^{-9} M) to cortical supernatant (○) and membranes (●). The full curve is a simple Langmuir isotherm, yielding an apparent affinity constant of $5.5 \times 10^7\ M^{-1}$ from which a corrected affinity constant of $6.4 \times 10^7\ M^{-1}$ may be calculated.

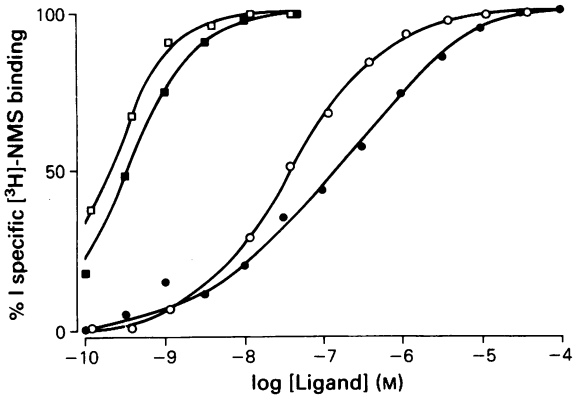

Figure 4 Inhibition of the binding of [^{3}H]-N-methylscopolamine ([^{3}H]-NMS, 2×10^{-10} M) to cortical membranes (closed symbols) and digitonin supernatant (open symbols) by unlabelled NMS (□, ■) and pirenzepine (Pir, ○, ●). The supernatant (derived from 5 mg ml^{-1} membrane protein) was diluted 1:5, and the concentration of membranes was 0.2 mg ml^{-1}, to avoid undue depletion of the [^{3}H]-NMS free concentration as a result of receptor-specific binding. The membrane and soluble preparations were preincubated with the unlabelled ligands for 24 h at 4°C, before addition of [^{3}H]-NMS, after which the incubation was continued for a further 20 h.

The NMS vs [^{3}H]-NMS competition curves were fitted to a one-site model of binding, yielding apparent affinity constants of 3×10^9 M^{-1} (membranes) and 5×10^9 M^{-1} (supernatant). The Pir vs [^{3}H]-NMS curves were significantly flatter than the simple Langmuir isotherm, with Hill coefficients of 0.55 (membranes) and 0.74 (supernatant). The curves were fitted to a 2-site model of binding yielding the following parameters:
Supernatant – log $K_H = 7.75 \pm 0.08$; log $K_L = 6.56 \pm 0.16$; % H sites = 67% ± 8; membranes – log $K_H = 7.98 \pm 0.12$; log $K_L = 6.08 \pm 0.09$; % H sites = 42 ± 4. The 95% confidence intervals of these parameters overlap.

pirenzepine, it was necessary to preincubate the soluble preparation with the drug for 24 h before addition of the [^{3}H]-NMS, to allow approach to equilibrium. The inhibition curve so obtained was flatter than the simple Langmuir isotherm. Analysis using a 2-site model of binding showed the presence of a high affinity subpopulation of sites with log $K_A = 7.75$ comprising 57% of the total sites, values in excellent agreement with those determined by inhibition of [^{3}H]-Pir binding. The remaining 43% of the total sites exhibited a log K_A of 6.56 for pirenzepine. Once more, these findings closely paralleled the results of membrane binding studies (cf. Figure 4), although there was evidence to suggest that the low affinity binding constant was somewhat enhanced after solubilization. In contrast, NMS inhibition of [^{3}H]-NMS binding to the solubilized preparation followed the simple Langmuir isotherm defining an apparent affinity constant of 5×10^9 M^{-1}, a value similar to and slightly higher than that found in membranes under the same conditions (3×10^9 M^{-1}).

Hydrodynamic properties of [^{3}H]-pirenzepine binding sites

Labelling of cortical supernatants with [^{3}H]-Pir followed by analysis by sucrose density gradient centrifugation yielded a sharp labelled peak with a sedimentation coefficient of 11.8 ± 0.14 S (mean ± s.e.mean, $n = 5$). The peak width was only 15% wider than the catalase standard. The [^{3}H]-NMS-labelled binding sites in the same preparation (Figure 5) gave a slightly assymetric and broader peak (40% wider than the catalase peak). However based on the fraction with maximum radioactivity, these sites had an identical S value (11.8 ± 0.14S, $n = 5$) to that of the [^{3}H]-Pir sites. Labelling of these peaks was suppressed by QNB (Figure 5). The recovery of [^{3}H]-Pir-labelled binding sites after centrifugation was 25–35%, rising to 50–70% when 10^{-8} M [^{3}H]-Pir was included in the gradient. Comparable recovery figures for [^{3}H]-NMS were ca. 50% when [^{3}H]-NMS was not present in the gradient and 80–90% when the gradient was supplemented with 10^{-8} M [^{3}H]-NMS. It is notable that the sedimentation coefficient of the cortical MAChR, whether labelled with [^{3}H]-Pir or [^{3}H]-NMS is identical to that of the (major) uncoupled component of the myocardial MAChR solubilized with digitonin and labelled under the same conditions (Berrie *et al.*, 1984a) and to the cortical muscarinic receptor labelled with [^{3}H]-QNB or [^{3}H]- propylbenzilylcholine mustard (Berrie *et al.*, 1984b).

We have previously shown that the sites labelled with [^{3}H]-Pir in cortical membranes can couple to a guanine nucleotide binding protein, which is indicated by a guanine-nucleotide-sensitive agonist inhibition of [^{3}H]-Pir binding (Birdsall *et al.*, 1984). However, the sedimentation coefficient of the MAChR-[^{3}H]-Pir complex is less than the value of 13.5 S characteristic of guanine nucleotide-sensitive superhigh affinity [^{3}H]-oxoM binding sites in the myocardium (Berrie *et al.*, 1984a), suggesting that the solubilized cortical [^{3}H]-Pir binding site may be monomeric. There is no evidence that under these conditions the [^{3}H]-Pir site is associated with a nucleotide binding protein since its affinity for oxoM is low ($K_A = 2 \times 10^5$ M^{-1}), and unaffected by the GTP analogue guanylylimidodiphosphate (Figure 6).

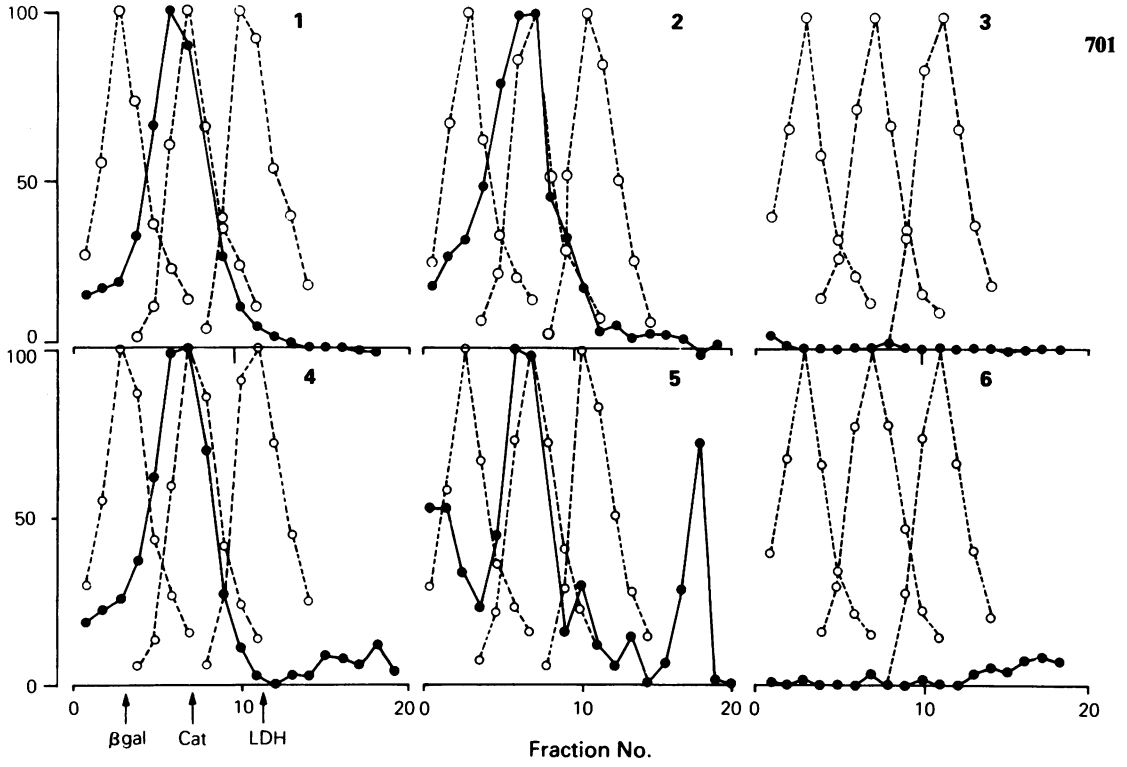

Figure 5 Sucrose density gradient analysis of [^{3}H]-pirenzepine ([^{3}H]-Pir) and [^{3}H]-N-methylscopolamine ([^{3}H]-NMS)-labelled muscarinic binding sites from rat cerebral cortex. A cortical supernatant was prepared from 4 mg ml^{-1} membranes and was labelled with [^{3}H]-NMS (10^{-8} M) and [^{3}H]-Pir (10^{-8} M) for 16 h at 0°C. The resultant levels of specific binding were 1394 fmol ml^{-1} ([^{3}H]-NMS) and 294 fmol ml^{-1} ([^{3}H]-Pir). The concentration of pirenzepine binding sites; calculated using affinity of 6.5×10^7 M^{-1}, is 54% of the NMS sites; 0.425 ml aliquots were analysed on 5–20% sucrose gradients containing 0.2% digitonin as previously described (Berrie *et al.*, 1984a); 10^{-8} M [^{3}H]-NMS and 10^{-8} M [^{3}H]-Pir were present in gradients 2 and 5 respectively, but not in gradients 1 and 4. Gradients 3 and 6 show suppression of the labelled peaks when labelling was conducted in the presence of 10^{-6} M quinuclidinylbenzilate. Recoveries of bound [^{3}H]-NMS were 57% (gradient 1) and 78% (gradient 2), and of bound [^{3}H]-Pir 34% (gradient 4) and 50% (gradient 5). β-Galactosidase (βGal) ($S_{20,w}$ = 15.93 S) catalase (Cat) ($S_{20,w}$ = 11.3 S) and lactate dehydrogenase (LDH) (7.3 S) were used as internal standards, and their activities are indicated by dotted lines.

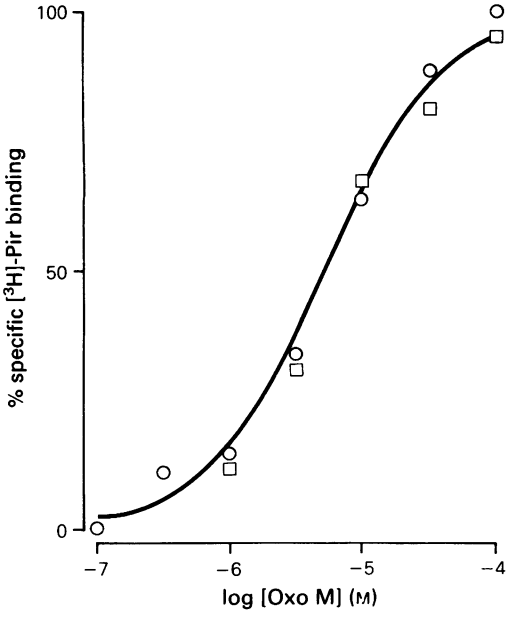

Figure 6 Oxotremorine-M (OxoM) inhibition of the binding of [^{3}H]-pirenzepine ([^{3}H]-Pir) (3×10^{-9} M) to cortical supernatant in the absence (○) and presence (□) of guanylylimidodiphosphate (GppNHP, 10^{-4} M). The full curve is a simple Langmuir isotherm with an affinity constant of 1.9×10^5 M^{-1}.

Stability of soluble [^{3}H]-pirenzepine binding sites

The soluble [^{3}H]-Pir and [^{3}H]-NMS sites are very stable at 4°C, less than 15% loss of binding being observed after 24 h. However, muscarinic sites solubilized at 4°C and assayed for [^{3}H]-Pir binding at 30°C exhibit lower levels of binding than are observed at 4°C. Self-competition experiments at 30°C (30 min incubation) gave an affinity constant for pirenzepine (log K_A = 7.69, data not shown) almost identical to that found at 4°C. However, the concentration of [^{3}H]-Pir binding sites measured at 30°C represented only 30% of the [^{3}H]-NMS sites compared to 70% at 4°C.

Table 3 Stability of soluble [^{3}H]-pirenzepine ([^{3}H]-Pir) and [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) binding sites at 30°C

Preincubation time (min)	*Loss in [^{3}H]-NMS binding sites (pmol ml^{-1})*	*Loss in [^{3}H]-Pir binding sites* (pmol ml^{-1})
15	0.71	0.61
30	0.91	0.82
60	1.11	1.19
120	1.43	1.48
180	1.59	1.64

Cortical membranes (5 mg ml^{-1}) were solubilized in 1% digitonin at 0°C. The supernatant was incubated at 30°C for the indicated times and then cooled to 0°C. [^{3}H]-NMS (30 nM) or [^{3}H]-Pir (3 nM) were added and the samples were incubated for 40 h at 4°C. At zero time the [^{3}H]-NMS binding capacity was 2.46 pmol ml^{-1}. The concentration of [^{3}H]-Pir binding sites was calculated from the amount of specific [^{3}H]-Pir binding and the affinity constant for the high affinity sites (K) 6.5×10^7 M^{-1} using the equation: [Binding sites] = [Bound pirenzepine] (1 + K[Pir])/K[Pir] where [Pir] is the concentration of unbound [^{3}H]-Pir. At zero time the [^{3}H]-Pir binding capacity was calculated to be 1.99 pmol ml^{-1}.

The reason for these differing results is that there is a relative instability of the [^{3}H]-Pir sites at 30°C. This is illustrated in Table 3 in which it is shown that the number of [^{3}H]-NMS binding sites decreased in parallel with the number of [^{3}H]-Pir sites if the incubations were carried out in the absence of ligands. If the stability study was carried out in the presence of a saturating concentration of [^{3}H]-NMS (10^{-8} M) no loss of binding was observed after 3 h. This reflects the ability of NMS to protect against receptor denaturation.

Discussion

The major finding of this study is that the high affinity Pir binding site from the cerebral cortex can be solubilized in good yield without loss of affinity using digitonin as the solubilizing agent.

High affinity [^{3}H]-Pir binding is stable at 4°C but unstable at 30°C. When the instability is seen it is accompanied by an equivalent loss of [^{3}H]-NMS binding. This suggests that the loss in [^{3}H]-Pir binding is due to a selective degradation of the subpopulation of muscarinic receptors with a high affinity for Pir. This conclusion contrasts with that of Roeske & Venter (1984) who have suggested that, under different experimental conditions and protocol, the high affinity [^{3}H]-Pir sites could be converted into low affinity sites.

After solubilization, muscarinic binding sites with a low affinity for Pir could also be detected in competition experiments. The presence of low affinity sites in addition to high affinity sites engenders a non-mass action inhibition curve which is also seen in the membranes. Analysis of these competition experiments suggests that the affinity of the population of sites which have a low affinity for pirenzepine may have increased slightly on solubilization. The sedimentation characteristics of the sites labelled by [^{3}H]-NMS suggest that they are somewhat more disperse than the subset of sites labelled by [^{3}H]-Pir.

Several pieces of evidence support the view that the high affinity Pir binding site is associated with a monomeric subtype of the MAChR:- (1) its sedimentation coefficient is 11.8 S, which is not only identical with that of the [^{3}H]-NMS binding site in the cortex, but also corresponds to the S-value of the decoupled form of the myocardial receptor (Berrie *et al.*, 1984a); the peak width is almost identical to those of the soluble standards (2) in contrast to the situation existing in cortical membranes (Birdsall *et al.*, 1984), agonist inhibition of [^{3}H]-Pir binding to the solubilized site is insensitive to guanine nucleotides, and (3) in contrast to the superhigh affinity [^{3}H]-oxoM binding site in the myocardium, whose expression depends on the maintenance of the integrity of MAChR-Ni coupling, [^{3}H]-Pir binding to solubilized cortical membranes is not adversely affected by high digitonin: protein ratios.

The molecular basis of Pir binding heterogeneity is the subject of intensive investigation. The present study shows that in digitonin supernatants of rat cerebral cortex membranes both high and low affinity Pir binding sites can be detected, the sites being solubilized with relatively little change in affinity. In the myocardium, however, we have recently shown that solubilization leads to a substantial (ca. 20 fold) increase in Pir affinity (Berrie *et al.*, 1985). In contrast, preliminary indications are that MAChRs from the lacrimal gland retain a lower affinity for Pir after solubilization (M. Keen, unpublished results). These findings support the suggestion (Birdsall & Hulme, 1983) that it is incorrect to regard the low affinity Pir

binding sites as a homogeneous population, and indicate that several factors contribute to the genesis of Pir selectivity.

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