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The binding of pirenzepine to digitonin-solubilized muscarinic acetylcholine receptors from the rat myocardium

N.J.M. Birdsall, E.C. Hulme[1] & M. Keen

Division of Physical Biochemistry, National Institute for Medical Research, Mill Hill, London NW7 1AA

1 The binding of pirenzepine to digitonin-solubilized rat myocardial muscarinic acetylcholine receptors has been examined at 4°C.

2 Solubilization produced only small changes in the binding of N-methylscopolamine and atropine. In contrast to the low affinity binding of pirenzepine found to be present in the membranes, high affinity binding was detected in the soluble preparation. In both preparations, pirenzepine binding was complex.

3 High affinity pirenzepine binding ($K_D \sim 3 \times 10^{-8}$M) to the soluble myocardial receptors could be monitored directly using [^{3}H]-pirenzepine.

4 [^{3}H]-pirenzepine-labelled soluble myocardial receptors have a sedimentation coefficient of 11.1 *s*. This indicates that [^{3}H]-pirenzepine binds predominantly to the uncoupled form of the receptor. However, [^{3}H]- pirenzepine-agonist competition experiments indicated that the high affinity pirenzepine binding sites are capable of coupling with a guanosine 5'-triphosphate (GTP)-binding protein. Pirenzepine affinities for the soluble myocardial receptors were unaffected by their state of association with the GTP-binding proteins found in the heart.

5 The equilibrium binding properties of the soluble cortical and myocardial receptors were very similar. However, the binding kinetics of the myocardial receptor were much slower.

6 It appears that the membrane environment can affect the affinity of pirenzepine for the rat myocardial muscarinic receptor. Removal of the constraint by solubilization allows the expression of high affinity pirenzepine binding.

Introduction

There is both pharmacological and biochemical evidence to suggest that the antagonist pirenzepine (Pz) can discriminate between different populations of muscarinic acetylcholine receptors (mAChRs) (for reviews, see Birdsall & Hulme, 1983; Hammer & Giachetti, 1984). In particular, it has been shown that Pz blocks ganglionic mAChRs at lower concentrations than those at which it acts on receptors in ileum smooth muscle, or myocardium (Brown *et al.*, 1980; Barlow *et al.*, 1981). Furthermore, it preferentially antagonizes agonist-induced acetylcholine release from guinea-pig myenteric plexus neurones (Kilbinger, 1984). In biochemical assays, it has been shown that Pz is a more potent inhibitor of the breakdown of inositol phospholipids, or guanosine 3':5'-cyclic monophosphate (cyclic GMP) formation, resulting from stimulation of muscarinic receptors than of adenylate cyclase inhibition in clonal cell lines (Evans *et al.*, 1984; McKinney *et al.*, 1985) and rat brain, parotid and myocardium (Gil & Wolfe, 1985). These demonstrations of pharmacological and biochemical selectivity are supported by *in vitro* studies of the binding of Pz to membranes, which have demonstrated the presence of a population of binding sites with a dissociation constant (K_D) of ca.10–20 nM, which is abundant in mammalian forebrain and sympathetic ganglia but sparse or absent in hindbrain, exocrine glands, smooth muscle and myocardium, where Pz binds with K_Ds in the range of 10^{-6}–10^{-7}M (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; Berrie *et al.*, 1985b,c).

In contrast to these findings in mammalian tissues, Brown *et al.* (1985) have recently reported that mAChRs in the embryonic chick heart bind Pz with

[1]Author for correspondence.

high affinity, and that Pz is more active on adenosine 3':5'-cyclic monophosphate (cyclic AMP) than triphosphoinositide (TPI) metabolism in this tissue.

The origin of the selective pharmacological actions of Pz is still unknown. Suggested explanations fall into two categories, according to whether the differences in Pz affinity are attributed to an intrinsic property of the mAChR binding protein itself, (e.g. to differences in primary amino acid sequence (receptor isotypes) or tissue-specific post-translational modifications) or whether they reflect environmental influences, e.g. coupling to distinct effector mechanisms (McKinney *et al.*, 1985) or variations in membrane composition (Schreiber & Sokolovsky, 1985)). These two classes of possible explanation are not mutually exclusive; for instance, structural variations in receptor molecules may enable them to recognize selectively one effector species when several occur in the same membrane locale.

We have started to disentangle these possibilities by studying the binding of Pz to digitonin-solubilized mAChRs, where the influence of the original membrane environment is obliterated, and where the isolated binding protein can be distinguished from receptor-effector complexes by means of the difference in their sedimentation coefficients. The present paper is concerned with evaluation of the binding of Pz to digitonin-solubilized mAChRs from the rat myocardium.

Methods

KCl-pyrophosphate extracted, EDTA-washed membranes from rat myocardium were prepared and solubilized using digitonin as described previously (Berrie *et al.*, 1984b). Assay and analytical methods, and the sucrose density gradient centrifugation technique were as previously described (Hulme *et al.*, 1983a,b; Berrie *et al.*, 1984a,b; 1985c). 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 were generally performed for at least 24 h at 4°C. As the rate of equilibration of pirenzepine binding was slow relative to [^{3}H]-NMS, pirenzepine-[^{3}H]-NMS competition experiments were carried out by preincubating the soluble receptor with pirenzepine for 24 h before the 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. These conditions have previously been shown to allow the detection of stable mAChR-N protein complexes in addition to the apparently monomeric binding protein (Berrie *et al.*, 1984b). Furthermore, studies of Pz binding to solubilized cerebral cortex have shown that high affinity [^{3}H]-Pz and [^{3}H]-NMS binding sites are stable at 4°C, but not at 30°C (Berrie *et al.*, 1985c).

(−)-[^{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. In addition we used [^{3}H]-NMS (8 Ci mmol^{-1}) prepared by quaternization of (−)-scopolamine with [^{3}H]-methyl iodide, as previously described (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

N-methylscopolamine binding to myocardial membranes and supernatant

The potency of NMS for myocardial muscarinic binding sites was slightly increased after their solubilization in 1% digitonin. This increase (~1.5 fold) was evident whether the NMS binding curve was measured using a series of increasing concentrations of [^{3}H]-NMS (Figure 1) or by inhibition of the binding of a low, fixed concentration of [^{3}H]-NMS (data not shown). Examination of the curves in Figure 1 show that the binding of [^{3}H]-NMS to the solubilized mAChR followed a simple Langmuir isotherm with a K_D of 2.6×10^{-10}M. The concentration of [^{3}H]-NMS needed to occupy 50% of the solubilized receptors was in reasonable agreement with the concentration of unlabelled NMS needed to occlude 50% of the binding sites in a competition experiment (2.3×10^{-10}M, data not shown).

The [^{3}H]-NMS binding curve to membranes showed some signs of the deviation from simple mass-action behaviour, discerned in previous studies of the binding of this ligand to myocardial mAChRs under low ionic strength conditions (Hulme *et al.*, 1981; Burgisser *et al.*, 1982). The deviations from a simple binding curve were too small for the data to be analysed by a 2-site model to give precise estimates of the proportions and affinities of the two sites: 50% occupancy of the receptor occurred at 3.6×10^{-10}M. The binding of [^{3}H]-NMS was increased in the presence of 5'-guanylylimidodiphosphate (GppNHp, 10^{-4}M) to give a simple binding curve, [^{3}H]-NMS having a K_D of $1.5 \pm 0.1 \times 10^{-10}$M. A similar phenomenon is found with the same membrane preparation at 30°C where K_D values of 2.2×10^{-10}M and 1.8×10^{-10}M in the absence and presence of GppNHp (10^{-4}M) were found (Birdsall & Hulme, 1985). Comparison of the B_{max} values showed that 1% digitonin solubilized ca. 45%

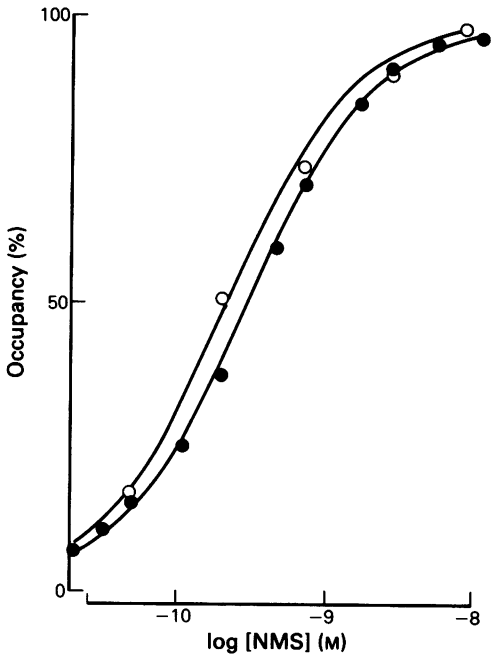

Figure 1 Binding of N-methylscopolamine (NMS) to membrane-bound and solubilized muscarinic acetylcholine receptors (mAChRs) from rat myocardium. Concentration-dependence of quinuclidinyl benzilate (QNB)-sensitive binding of [^{3}H]-NMS to membranes (●) and supernatant (○). Membrane protein concentration was 1 mg ml^{-1}, and the supernatant was diluted 1:5 in digitonin buffer to avoid undue depletion of the ^{3}H ligand as a result of receptor-specific binding. The binding curve for the supernatant is a simple Langmuir isotherm with $K_D = 2.6 \times 10^{-10}$M, and $B_{max} = 1.53 \times 10^{-10}$M. The binding curve for membranes is a simple mass action curve with a $K_D = 3.6 \times 10^{-10}$M.

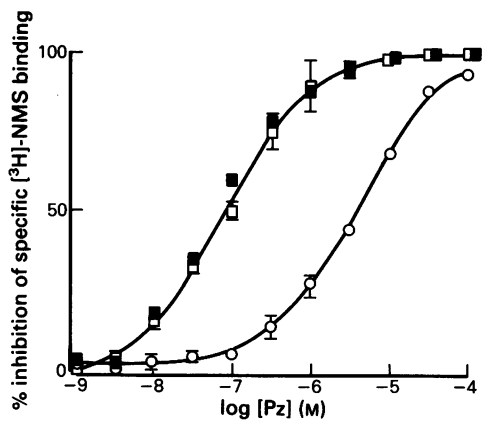

Figure 2 Pirenzepine (Pz) inhibition of the binding of [^{3}H]-N-methylscopolamine ([^{3}H]-NMS; $2-3 \times 10^{-10}$M) to myocardial membranes (○) (mean of 4 independent determinations), and digitonin supernatant (□,■) (mean of 3 and 2 independent determinations). The supernatant was diluted 1:2 (□) or 1:4 (■) with 1% digitonin buffer to avoid undue depletion of [^{3}H]-NMS. The preparations were preincubated with Pz for 24 h at 4°C before addition of [^{3}H]-NMS, and incubation for a further 18 h, in an effort to ensure that slowly-equilibrating high affinity Pz binding sites were detected.

The inhibition curves were fitted to a two-site model of binding as follows:

membranes: log $K_H = 6.45 \pm 0.32$ (22% ± 10% of total sites); $K_H = 3.5 \times 10^{-7}$M
log $K_2 = 5.18 \pm 0.10$ (78% ± 10% of total sites); $K_L = 6.6 \times 10^{-6}$M

supernatant: log $K_H = 7.66 \pm 0.34$ (39% ± 27% of total sites); $K_H = 2.2 \times 10^{-8}$M
log $K_L = 6.71 \pm 0.25$ (61% ± 17% of total sites); $K_L = 2.5 \times 10^{-7}$M

of the NMS binding sites, as found previously (Berrie *et al.*, 1984a,b). The NMS self-competition curve for membranes gave an IC_{50} of 5.5×10^{-10}M, using 2×10^{-10}M [^{3}H]-NMS to label the receptors. This value is in agreement with that predicted from the direct binding experiment.

Pirenzepine binding to myocardial membranes

The heterogeneity of the [^{3}H]-NMS binding site is a complicating factor in the quantitative assessment of Pz binding to myocardial membranes at 4°C. Under such conditions, the inhibition of the binding of [^{3}H]-NMS (2×10^{-10}M) by Pz took place with a very low apparent affinity, the IC_{50} being 4.6×10^{-6}M (Figure 2). Use of a higher concentration of [^{3}H]-NMS (10^{-9}M) to label a larger fraction of the total mAChR binding sites gave an IC_{50} of 6.3×10^{-6}M, while competition measurements at 30°C, where there was no evidence of deviation of the [^{3}H]-NMS binding curve from the simple Langmuir isotherm (Birdsall & Hulme, 1985), yielded an IC_{50} of 2×10^{-6}M for inhibition of the binding of 3.5×10^{-10}M [^{3}H]-NMS, giving a corrected K_D of 8.3×10^{-7}M.

The Pz binding curve at 4°C (Figure 2) appeared slightly flattened, and could be described by a two-site model of binding with a minor subpopulation of sites with a K_D of 3.5×10^{-7}M (22% of total sites) and a major subpopulation with a K_D of 6.6×10^{-6}M (78% of total sites). While a totally quantitative appraisal of these results is not possible, two points are clear (1) the major population of Pz binding sites assayed in the myocardial membrane preparation under the conditions used had a K_D of 10^{-6}M, (2) there was no evidence for the presence of a significant population of sites with a K_D of ca. 10^{-8}M.

In agreement with these findings, there was little or no quinuclidinyl benzilate (QNB)-sensitive [^{3}H]-Pz binding to the membrane preparation; the maximum estimate from such measurements indicated that high affinity Pz sites constituted less than 7% of the mAChRs present. [^{3}H]-Pz binding to membranes was not enhanced by GppNHp (10^{-4}M) under the present conditions (c.f. Hulme *et al.*, 1981).

Pirenzepine binding to digitonin-solubilized myocardial mAChRs

Measurement of Pz inhibition of the binding of [^{3}H]-NMS ($2-3 \times 10^{-10}$M) to the digitonin-solubilized preparation indicated a large shift of the binding curve towards a higher affinity, the EC_{50} of Pz being reduced ca. 20 fold to 1.0×10^{-7}M (Figure 2). In order to avoid artifacts caused by depletion of the tracer ligand the inhibition curve was determined at both 1:2 and 1:4 dilutions of the supernatant, with essentially identical results. Again, the binding curve appeared slightly flattened, and could be described by the summation of two populations of sites with K_Ds of 2.2×10^{-8}M (39% of total) and 2.5×10^{-7}M (61% of total). Because the deviation of the Pz binding curve from the simple Langmuir isotherm was not pronounced, the standard errors associated with these parameters were fairly high (see legend to Figure 2). However, a degree of flattening of the Pz binding curve was a reproducible feature of experiments in which supernatants were preincubated with Pz in an effort to ensure the detection of high-affinity, slowly equilibrating sites. As predicted from the competition experiments, it was possible to measure high affinity QNB-sensitive binding of [^{3}H]-Pz to myocardial supernatants, which was time-dependent (Figure 3), and unaffected by GppNHp (data not shown). The binding curve for [^{3}H]-Pz could be fit to a single exponential with a rate constant of $0.047\ h^{-1}$ ($t_{1/2} = 14$ h). This value is ten times slower than that found for the binding of a comparable concentration of [^{3}H]-Pz to soluble muscarinic receptors from rat cerebral cortex (Berrie *et al.*, 1985a). The binding of [^{3}H]-NMS (8.4 nM) to soluble heart receptors was much faster than that of pirenzepine (Figure 3, $t_{1/2} = 0.6$ h) but again was about four times slower than that found for [^{3}H]-NMS in the cortex. The occupancy of [^{3}H]-Pz (10^{-8}M) at very long incubation times (17%) agrees quantitatively with that estimated from the [^{3}H]-NMS/Pz competition experiments (Figure 2). The dissociation of bound [^{3}H]-Pz, induced by the addition of an excess of the unlabelled ligand, was extremely slow ($t_{1/2} \sim 24$ h), although it should be noted that the gel-filtration method used for assay would not have detected a very rapidly dissociating component of binding.

QNB-sensitive binding of [^{3}H]-Pz was inhibited by nanomolar concentrations of NMS and atropine,

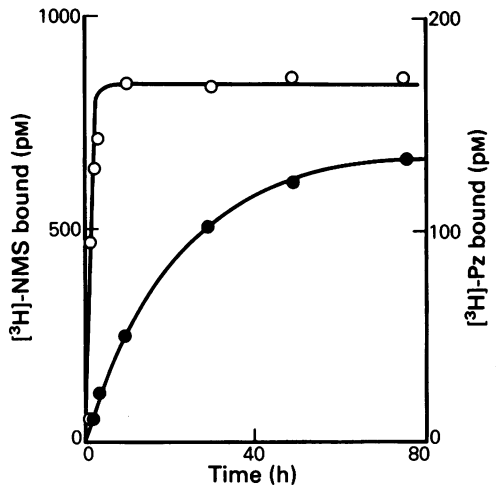

Figure 3 Time-dependence of specific [^{3}H]-pirenzepine ([^{3}H]-Pz; 10^{-8}M, ●) and [^{3}H]-N-methylscopolamine ([^{3}H]-NMS; 8.4×10^{-9}M, ○) binding to digitonin-solubilized myocardial muscarinic acetylcholine receptors. The curves are best fit curves to a simple exponential function $B_t = B_\infty (1 - e^{-kt})$. For [^{3}H]-Pz the values of B_∞ and K are 139 ± 15 fmol ml^{-1} and $0.047 \pm 0.005\ h^{-1}$ and for [^{3}H]-NMS, 840 ± 20 fmol ml^{-1} and $1.10 \pm 0.17\ h^{-1}$. It should be noted that the non-specific binding of the ^{3}H ligands increased by up to 150% during the first 8 h of the time course and thereafter remained constant. Hence estimates of non-specific binding were made at each time point.

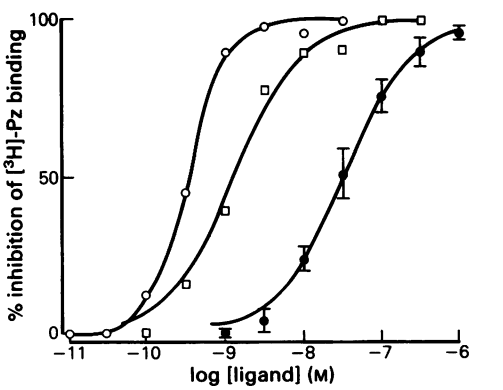

Figure 4 Inhibition of the quinuclidinyl benzilate (QBN)-sensitive binding of [^{3}H]-pirenzepine (Pz; 2.3×10^{-9}M) to digitonin-solubilized myocardial muscarinic acetylcholine receptors (NMS) (○), atropine (□) and unlabelled Pz (●) (mean of 4 independent determinations). To obtain reasonable levels of specific [^{3}H]-Pz binding (600 d.p.m.), the digitonin supernatant was not diluted in this experiment. The IC_{50} for NMS was 3.5×10^{-10}M, for atropine 1.3×10^{-9}M and for Pz 3.2×10^{-8}M.

as well as by unlabelled Pz (Figure 4). The curve for NMS-induced inhibition of [^{3}H]-Pz binding ($IC_{50} = 3.5 \times 10^{-10}$M) was perceptibly steep, again reflecting depletion of the unlabelled ligand by its binding to the receptor. It was necessary to carry out the assay at a higher receptor concentration in order to obtain readily measurable levels of [^{3}H]-Pz binding (see legends to Figures 1, 2 and 4). Evidence for significant deviation from the simple Langmuir isotherm was not obtained in the case of atropine ($IC_{50} = 1.3 \times 10^{-9}$M) or Pz ($IC_{50} = 3.3 \times 10^{-8}$M).

The properties of the high affinity Pz binding site were also defined by measurement of the saturation curve using the ^{3}H-ligand (Figure 5). Because of the modest affinity, and comparatively low ratio of specific: non-specific binding, measurements of [^{3}H]-Pz binding were not made at concentrations above 10^{-7}M. Nevertheless, the data given in Figure 5 yielded an estimate of the [^{3}H]-Pz dissociation constant, 3.9×10^{-8}M, entirely compatible with the value of 3.3×10^{-8}M obtained from the self-competition experiments. The ratio of the concentration of high affinity Pz binding sites to NMS binding sites was 0.72:1 in this experiment. Using a value of 3.3×10^{-8}M for the K_D of Pz, a number of independent estimates of this ratio were obtained. They fell in the range 0.52–0.87, with a mean of 0.64 ± 0.12 (mean ± s.d., $n = 6$). Both the K_D and relative proportion of the high affinity Pz sites obtained from studies of [^{3}H]-Pz binding are compatible with the estimates obtained from Pz/[^{3}H]-NMS competition data (Figure 2).

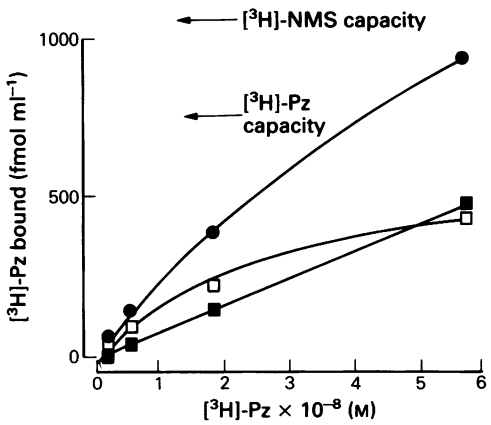

Figure 5 Concentration-dependence of [^{3}H]-pirenzepine ([^{3}H]-Pz) binding to digitonin-solubilized myocardial muscarinic acetylcholine receptors in the absence (●) and presence (■) of quinuclidinyl benzilate (QNB; 10^{-6}M). The QNB-sensitive binding component (□), is fitted to a simple Langmuir isotherm with a K_D of $3.9 \pm 0.2 \times 10^{-8}$M and a B_{max} of 0.764 ± 0.021 pmol ml^{-1}. The analysis was based on 7 data points, 3 of which ($< 10^{-9}$M [^{3}H]-Pz) are not shown in this figure. The B_{max} for [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) in this experiment was 1.06 pmol ml^{-1}, so the ratio of the concentrations of the Pz to NMS sites was 0.72.

Sucrose density gradient centrifugation of high affinity [^{3}H]-pirenzepine binding sites

We have studied previously the behaviour of the digitonin-solubilized myocardial mAChR labelled with [^{3}H]-NMS and [^{3}H]-oxoM on sucrose density gradient centrifugation (Berrie *et al.*, 1984b), showing that the [^{3}H]oxoM-mAChR complex has a slightly greater sedimentation coefficient ($s_{20,w} = 13.4$ *s*) than the major component of the [^{3}H]-NMS-mAChR complex ($s_{20,w} = 11.6$ *s*). Likewise, solubilized [^{3}H]-Pz and [^{3}H]-NMS sites from rat cerebral cortex display an $s_{20,w}$ of 11.85 (Berrie *et al.*, 1985b).

We have now studied the behaviour of the [^{3}H]-Pz-labelled myocardial mAChR by sucrose density gradient centrifugation, using the [^{3}H]-NMS-labelled receptor as a basis for comparison (Figure 6). The [^{3}H]-Pz-mAChR complex from the myocardium was typically recovered from a 5–20% sucrose gradient containing 1% digitonin as a somewhat asymmetrical peak whose sedimentation coefficient was 11.1 ± 0.12 *s* (mean ± s.e.mean, $n = 6$), a value essentially the same as the $s_{20,w}$ of the major component of the [^{3}H]NMS-mAChR complex (Figure 6). Recovery of ca. 75% of the bound [^{3}H]-Pz was obtained. The half-width of the [^{3}H]-Pz-labelled peak was slightly greater than that of the standard proteins (ratio of half-width to that of the catalase peak = 1.26:1) whilst, this ratio was significantly larger (1.64:1) for the [^{3}H]-NMS labelled species when labelling was conducted in the absence of guanine nucleotides and full saturation of the binding sites was maintained by inclusion of the ^{3}H-ligand in the gradient.

We have suggested previously that NMS binds to both the 13.4*s* and the 11.6*s* species which are presumed to represent the mAChR-N protein complex, and the uncomplexed form of the receptor respectively. The present results suggest that the major component of bound [^{3}H]-Pz recovered from the gradient was associated with the lower molecular weight species of the mAChR. However, minor labelling of higher molecular weight species is also in accord with the asymmetry of the [^{3}H]-Pz peak.

Interaction of oxotremorine-M and pirenzepine binding sites

In a previous study (Berrie *et al.*, 1984b) we have shown that a fraction of the digitonin-solubilized myocardial mAChRs retain high affinity ($K_D = 1.0 \times 10^{-9}$M) for the agonist oxoM, and that oxoM binding to these sites is inhibited by GTP analogues such as

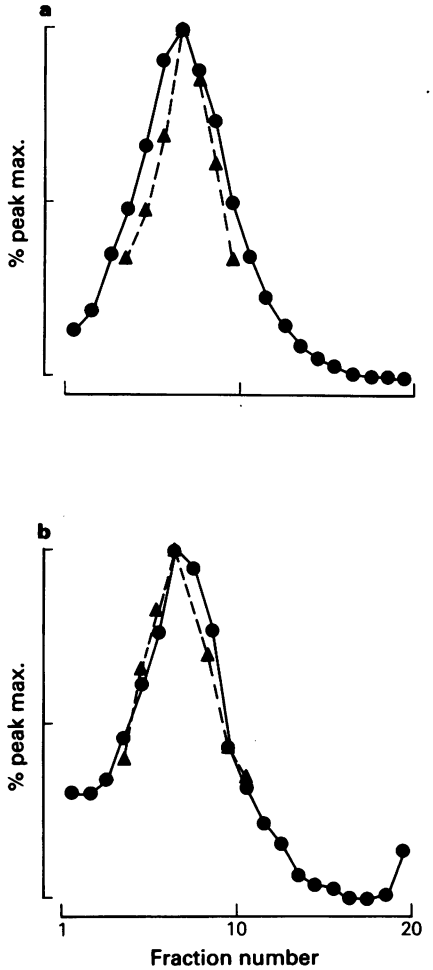

Figure 6 Sucrose density gradient centrifugation of (a) [^{3}H]-N-methylscopolamine ([^{3}H]-NMS) and (b) [^{3}H]-pirenzepine ([^{3}H]-Pz) binding sites in solubilized myocardium. A myocardial supernatant prepared by solubilizing membranes (4 mg ml^{-1}) in 1% digitonin was labelled with [^{3}H]-Pz (b), or [^{3}H]-NMS (a), both at 7×10^{-9}M for 24 h at 4°C. The levels of binding were 637 fmol ml^{-1} ([^{3}H]-NMS) and 68 fmol ml^{-1} ([^{3}H]-Pz); 0.4 ml aliquots were analysed on 5–20% gradients containing 0.2% digitonin as previously described. [^{3}H]-NMS 10^{-8}M and [^{3}H]-Pz 10^{-8}M were present in the gradients to attempt to maintain binding. Recovery of bound [^{3}H]-Pz from the peak was ca. 75% of specific binding applied, after subtracting a sloping baseline. Similar figures were obtained for the recovery of [^{3}H]-NMS. β-Galactosidase, $s_{20,w} = 15.93\,s$, catalase, $s_{20,w} = 11.3\,s$ and lactate dehydrogenase, $s_{20,w} = 7.3\,s$, were used as internal standards. The activity of catalase is indicated by dotted lines, to show the peak width of the standard proteins.

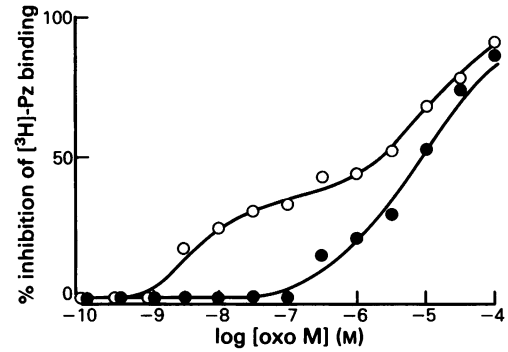

Figure 7 Inhibition of the binding of [^{3}H]-pirenzepine ([^{3}H]-Pz) to digitonin-solubilized myocardial muscarinic acetylcholine receptors by oxotremorine-M (oxo M) in the absence (○) and presence (●) of guanylylimidodiphosphate (GppNHp; 10^{-4}M). The inhibition curve in the absence of GppNHp was analysed by a two-site model of binding with $K_H = 6.3 \times 10^{-9}$M (39% of total sites) and $K_L = 1.3 \times 10^{-5}$M (61% of total sites), while in the presence of GppNHp values were $K_H = 3.9 \times 10^{-7}$M (22% of total sites) and 1.6×10^{-5}M (78% of total sites).

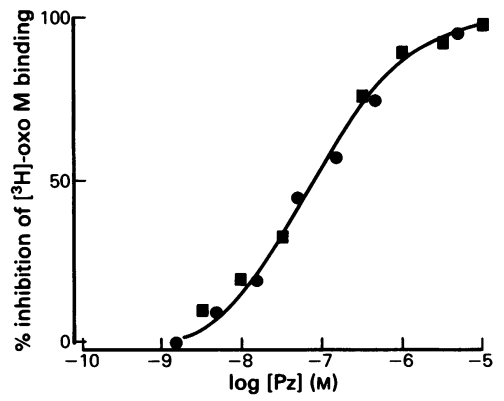

Figure 8 Pirenzepine (Pz)-induced inhibition of the binding of [^{3}H]-oxotremorine-M ([^{3}H]-oxoM; 1×10^{-9}M) to digitonin-solubilized muscarinic acetylcholine receptors from rat myocardium. The ligand-induced shift in this curve is anticipated to be similar to that in Figure 2, i.e. ca. 2 fold. Analysis using a 2-site model of binding yielded $K_H = 2.3 \times 10^{-8}$M (52% of total sites) and $K_L = 3.5 \times 10^{-7}$M (48% of total sites).

GppNHp. Such sites were either labelled directly with [^{3}H]-oxoM, or detected by oxoM inhibition of the binding of [^{3}H]-NMS.

Figure 7 shows that GppNHp-sensitive high-affinity oxoM binding sites were also detectable by oxoM inhibition of [^{3}H]-Pz binding to the digitonin-solubilized myocardial mAChR. In the absence of GppNHp, two populations of oxoM binding sites with K_Ds of 6.3×10^{-9}M (39% of total) and 1.3×10^{-5}M (61% of total) were clearly discernible. In the presence of GppNHp (10^{-4}M), the high affinity population of oxoM sites was no longer evident, although, a previously noted using [^{3}H]-NMS as the tracer ligand, conversion to the low affinity state was not complete, and a population of sites of K_D 3.9×10^{-7}M (20% of total) could still be detected by curve-fitting (Figure 7).

Pz inhibition of [^{3}H]-oxoM binding to the solubilized myocardium (Figure 8) was not distinguishable from the corresponding curve for inhibition of [^{3}H]-NMS binding (Figure 2). Once again, this curve showed evidence for slight but significant deviation from the simple Langmuir isotherm and required description by a two-site model of binding, yielding parameters which did not differ significantly from the corresponding values calculated from the [^{3}H]-NMS competition curve (see legend to Figure 2).

Discussion

The origin of the selective actions of Pz is controversial (Hammer & Giachetti, 1984; Roeske & Venter, 1984; Luthin & Wolfe, 1984; Schreiber & Sokolovsky, 1985). Solubilization of the membrane which, by liberating the receptor from its microenvironment, permits the study of the isolated receptors, specific receptor-effector complexes, and their interactions with selective ligands, might clarify this problem.

Before solubilization, there is evidence that the binding of both NMS and Pz is complex, under the conditions used. Analysis suggests that the major fraction of myocardial mAChRs bind Pz with very low affinity ($K_D \sim 10^{-6}$M) and that only a small percentage of the binding sites (less than 7%) have a high affinity for the ligand (c.f. Watson *et al.*, 1983). Digitonin (1%) solubilizes ca. 45% of the myocardial mAChRs. After solubilization, the affinity for NMS is slightly increased, and the complex binding properties are no longer evident. The binding affinity of Pz is substantially increased, but the binding remains complex. Approximately 64% of the sites now bind Pz with a K_D of 3×10^{-8}M. It is not possible for this enhancement to be caused by selective solubilization of the minor high-affinity population of Pz binding sites detectable in the membranes as over 45% of the total binding sites were solubilized: it must reflect an increase in Pz affinity consequent upon release of the receptor from a membrane constraint.

The properties of the [^{3}H]-Pz binding sites in the solubilized myocardium resemble those of the [^{3}H]-NMS binding sites in the following respects: (1) they have a similar sedimentation coefficient – 11.1 *s* for the [^{3}H]-Pz-mAChR-digitonin complex and 11.6 *s* for the [^{3}H]-NMS-mAChR-digitonin complex. (2) OxoM inhibition of both [^{3}H]-Pz and [^{3}H]-NMS binding reveals the presence of high and low affinity agonist binding sites and binding of oxoM to the high affinity population is inhibited by GppNHp in both cases. (3) Pz inhibits the binding of [^{3}H]-oxoM to the mAChR-N protein complex in a manner indistinguishable from its inhibition of the binding of [^{3}H]-NMS. This evidence indicates that sites which have a high affinity for Pz are not debarred from interacting with an N-protein.

Pz binding to the solubilized myocardial mAChR differs from that of NMS in that, as in solubilized cerebral cortex preparations, slight but detectable heterogeneity of the Pz binding sites persists.

Is this heterogeneity due to high affinity Pz binding to the free mAChR and low affinity binding to the mAChR-N protein complex? If so, can the increase in Pz affinity which takes place on solubilization be attributed to partial disruption of the mAChR-N protein interaction (Burgisser *et al.*, 1982)? This is an attractive hypothesis, but several lines of evidence militate against it:- (1) as noted above, there is no suggestion that Pz can differentiate between the mAChR-N protein complex, labelled by [^{3}H]-oxoM, and the totality of mAChR binding sites, labelled by [^{3}H]-NMS. (2) The kinetics and the equilibrium binding of [^{3}H]-Pz to the solubilized mAChR are both insensitive to GppNHp. (3) The mAChR purified from the cerebral cortex exhibits a low ($K_D = 2 \times 10^{-7}$M) affinity for Pz, yet it is clearly not complexed to a guanine nucleotide binding protein (Berrie *et al.*, 1985a).

If the affinity of Pz binding is not determined by the state of the mAChR-N protein interaction, it follows that the membrane constraint released on dissolution with digitonin is a more general microenvironmental one, as might be provided, for instance, by the presence of a high concentration of charged groups in the vicinity of the receptor which could affect the ionization state of Pz and/or the receptor (Barlow & Chan, 1982). In this regard, it is interesting that the quaternary methiodide of Pz has a K_D of ca. 10^{-7}M for both the myocardial and the cerebral cortical mAChRs, and thus is not selective (Stockton, personal communication).

It is evident that the increase in affinity produced by solubilization greatly reduces the ability of Pz to differentiate between myocardial and cerebral cortical receptors (Figure 9). It is arguable that a small difference between the binding properties of the

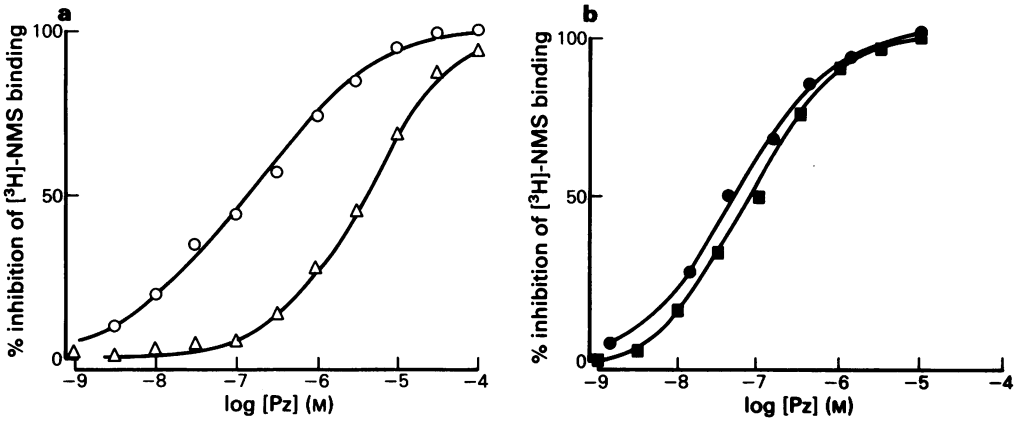

Figure 9 (a) Pirenzepine (Pz)-induced inhibition of [^{3}H]-N-methylscopolamine ([^{3}H]-NMS; 2×10^{-10}M) binding to cerebral cortex (○) and myocardial (△) membranes. The cortical curve is taken from Berrie *et al.* (1985c) and the myocardial curve from Figure 2. The curves were fitted to a 2-site model of binding as follows: cortex: $K_H = 1.04 \times 10^{-8}$M (42% of total sites), $K_L = 8.3 \times 10^{-7}$M (58% of total sites). Myocardium: $K_H = 3.5 \times 10^{-7}$M (22% of total sites), $K_L = 6.6 \times 10^{-6}$M (78% of total sites).

(b) Pirenzepine inhibition of the binding of [^{3}H]- NMS (2×10^{-10}M) to a digitonin supernatant of cerebral cortex (●) and myocardium (■). The cortical curve is taken from Berrie *et al.* (1985c) and the myocardial curve from Figure 2. The curves were fitted to a 2-site model of binding as follows: cortex: $K_H = 1.8 \times 10^{-8}$M (67% of total sites), $K_L = 2.8 \times 10^{-7}$M (33% of total sites). Myocardium: $K_H = 2.2 \times 10^{-8}$M (39% of total sites) $K_L = 2.5 \times 10^{-7}$M (61% of total sites).

solubilized mAChRs from those two tissues persists (Berrie *et al.*, 1985b), but in general the findings support the idea that there is little to distinguish the binding domains of the isolated receptors from cortex and myocardium (Laduron *et al.*, 1981; Venter *et al.*, 1984; Schreiber & Sokolovsky, 1985). Nonetheless, it would probably be premature to dismiss the notion of muscarinic receptor isotypes on this basis since (1) Pz high and low affinity binding sites survive solubilization from the cortex (Berrie *et al.*, 1985c), whereas high affinity sites actually appear after solubilization of the heart, and (2) the affinity of Pz for mAChRs from the rat lacrimal gland is lowered after solubilization (M. Keen *et al.*, unpublished observations), thereby enhancing the selectivity of the drug in this case.

One notable feature of the [^{3}H]-Pz/myocardial mAChR interaction at 4°C is the very slow kinetics which necessitate very long incubation times to achieve equilibrium. It is not known whether the slow kinetics are an intrinsic feature of the soluble receptor molecule or whether a form of the soluble receptor which binds pirenzepine with high affinity is slowly generated in an irreversible manner. The latter possibility is unlikely as the off-rate kinetics of [^{3}H]-Pz are so slow ($t_{1/2} \sim 24$ h) despite its affinity being relatively low. In fact the rate of approach to equilibrium of [^{3}H]-Pz, shown in Figure 3, is broadly compatible with its being dominated by the off-rate constant. The fact that the Pz binding kinetics are ca. 10 times slower for soluble myocardial receptors, as compared to the cortical receptor, and that this difference does not appear to be explicable by differences in the state of receptor-N protein coupling would argue for the existence of receptor isotypes.

The results of the present investigation have established some new points regarding the origin of Pz binding heterogeneity. (1) There is an effect of the membrane microenvironment on the affinity of Pz for the rat myocardial mAChR. (2) Removal of this constraint by solubilization with digitonin allows the expression of both high and low affinity Pz binding sites with equilibrium binding properties very similar to those noted in solubilized rat forebrain preparations (Berrie *et al.*, 1985c). However, considerable differences in the binding kinetics of Pz are present. (3) The affinities of solubilized myocardial mAChRs for Pz seem unaffected by their state of association with the GTP-binding proteins found in heart.

To some extent, these findings parallel the results of recent functional studies on the mechanism of action of Pz. Thus, Brown *et al.* (1985) have shown that Pz has high affinity for the mAChRs of chick embryo heart membranes, confirming that Pz high affinity sites are not confined to forebrain and ganglia. Secondly, Gil & Wolfe (1985) have found that Pz inhibits TPI metabolism stimulated by muscarinic agonists in rat brain slices with high affinity ($K_D = 21$ nM), whilst inhibition of cyclic AMP forma-

tion occurs with lower affinity (K_D = 210 nM). In contrast, Brown *et al.* (1985) have found precisely the converse situation in embryonic chick heart cells, namely high affinity inhibition of the cyclic AMP effect (K_D = 48 nM) and low affinity inhibition of the TPI response (K_D = 255 nM).

These observations show that different Pz affinities can be distinguished biochemically, but suggest that these affinities are not determined by the response to which the receptor is coupled. The findings of the present study are consistent with such properties. However, the precise determinants of differences in the Pz affinity of the solubilized mAChR binding protein, and the biological function (if any) of such differences remain to be determined.

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