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MUSCARINIC CHOLINERGIC RECEPTORS IN A RAT PHAEOCHROMOCYTOMA CELL LINE

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The high affinity binding of the muscarinic ligand ^{3}H-N-methylscopolamine (^{3}H-NMS) to intact cells of the rat pheochromocytoma cell line PC12 was studied. A single class of saturable binding sites was observed with a density of about 5000 sites per cell, and a pharmacological profile consistent with muscarinic receptors. The inhibition of ^{3}H-NMS binding by the antagonist pirenzepine was of low affinity suggesting PC12 muscarinic receptors may be similar to those found on rat sympathetic nerve terminals.

The rat phaechromocytoma cell line PC12 (1) displays many properties associated with differentiated sympathetic neurones, including storage and release of catecholamines (2) and responsiveness to nerve growth factor (3). In addition, PC12 cells exhibit the capacity to synthesise and release acetylcholine (4). Studies of acetylcholine receptors on PC12 cells have revealed that whilst sodium ion permeability responses to cholinergic agonists are present (5) these do not display a pharmacological profile similar to muscle nicotinic receptors, and do not involve the α-bungarotoxin binding component present in these cells. More recently it has been demonstrated that membrane preparations of PC12 cells contain a muscarinic ligand binding site which is elevated after nerve growth factor treatment (6).

It has been demonstrated that muscarinic binding sites in both brain and peripheral tissues display marked heterogeneity with respect to both agonist (7) and antagonist (8) displacement. Whilst complex agonist displacement may be explained by interconvertible states of a single receptor, this does not appear to be the case for the heterogeneity displayed by the antagonist pirenzepine (8). In tissues where binding is associated with sympathetic innervation, pirenzepine displacement is consistent with a single

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class of low affinity binding sites (8). As PC12 cells have several properties in common with sympathetic neurones it was of interest to determine the properties of PC12 muscarinic receptors. Thus in the present study we have characterised the binding of a muscarinic ligand to intact PC12 cells, with respect to agonist and particularly antagonist heterogeneity.

MATERIALS AND METHODS

^{3}H-N-methylscopolamine (^{3}H-NMS, 79 Ci/mmol) was obtained from Amersham International, U.K. Muscarinic drugs were either obtained commercially, or were generously supplied by Dr. Malcolm Caulfield, Glaxo Group Research, U.K.

Cells: PC12 cells were grown on plastic tissue culture vessels in MEM; supplemented with 10% foetal calf serum, non essential amino acids, penicillin and streptomycin. The medium was buffered with bicarbonate in a 5% CO_2 atmosphere. Once a week the cells were passaged at a split ratio of 1 : 3.

Binding assays: For binding assays growth medium was replaced with Dulbecco's modified Eagle's medium containing 5 mM HEPES pH 7.4 ^{3}H-NMS was added at the appropriate concentration (routinely 1 nM) and where appropriate drugs were added to give a final concentration of 1.0 ml in each tissue culture well. Tissue culture trays were incubated at 37 °C for 20 min, the medium removed by aspiration, and the cells were superficially washed twice with either phosphate-buffered saline or Hank's balanced salt solution. Cells were removed by treatment with trypsin EDTA, and bound ^{3}H-NMS determined by scintillation counting. Non-specific binding was determined from parallel incubations in the presence of 10 µM atropine. All binding assays were performed in quadruplicate.

Data Analysis: For saturation and drug displacement studies, parameters were calculated from Hill plots and Scatchard plots using linear regression analysis. Data from displacement studies were also analysed using derivative-free non-linear regression, based on models of one and two site interactions (9).

RESULTS AND DISCUSSION

Over the range of concentrations used (0.25 - 2.5 nM), ^{3}H-NMS bound to PC12 cells in a saturable manner. A linear Scatchard plot was obtained consistent with a single population of binding sites. The dissociation constant was 0.43 ± 0.11 nM (mean ± SD of four determinations), the maximum number of binding sites being 11.2 ± 1.1 fmol per tissue culture well. Each well contained ~ 10^6 cells, giving a value of approximately 5000 binding sites/cell. This is considerably greater than the value of 1400 sites/cell observed using PC12 membrane preparations (6). In preliminary studies we have observed an approximately 50% loss of binding sites after preparation of membranes, and this has been observed previously with muscarinic

Table 1
Interactions of muscarinic drugs with ^{3}H-NMS binding to PC12 cells

DRUG	K_i nM(a)	n_H(b)
ANTAGONISTS		
Scopolamine	1.4 ± 0.08	0.93 ± 0.07
Atropine	0.83 ± 0.05	0.91 ± 0.06
N-methyl atropine	0.17 ± 0.02	0.99 ± 0.12
Benztropine	3.0 ± 0.6	0.98 ± 0.12
AGONISTS		
Methacholine	1300 ± 1000	0.54 ± 0.08
Pilocarpine	10700 ± 4400	0.87 ± 0.14
Oxotremorine	140 ± 50	0.63 ± 0.05
Arecoline	5500 ± 2100	0.67 ± 0.09
Aceclidine	18000 ± 9000	0.93 ± 0.13

(a) K_i values were calculated from IC_{50} values using the equation $K_i = IC_{50}/_1 + L/_{KD}$ where L = concentration of ligand and KD = dissociation constant of ligand.
(b) Hill coefficients derived from Hill plots. Values are mean ± SD of 3 - 5 determinations using 5 - 6 drug concentrations.

receptors on neuroblastoma clones (10,11). The number of muscarinic binding sites approximately equals the number of α-bungarotoxin binding sites on these cells (5).

The binding of ^{3}H-NMS was inhibited by a range of muscarinic receptor antagonists and agonists (Table 1). The dissociation constants obtained from inhibition of ^{3}H-NMS binding are similar to those obtained in a variety of tissues and in a previous study of PC12 cells using ^{3}H-QNB as ligand (6). The Hill coefficients obtained for antagonist inhibition of ^{3}H-NMS binding were not significantly different from unity (Table 1), consistent with an interaction at a single class of binding sites. In general the inhibition of ^{3}H-NMS binding by agonists was of a complex nature with Hill slopes significantly less than unity.

The inhibition of ^{3}H-NMS binding in PC12 cells by the novel antagonist pirenzepine is shown in Fig 1. Similar data for ^{3}H-NMS binding to rat cortical membranes is included for comparison. The Hill coefficient for the inhibition of ^{3}H-NMS binding to rat cortical membranes was significantly less than unity. Using non-linear regression analysis the presence of two pirenzepine binding components could be detected, with affinities of 35 ± 6

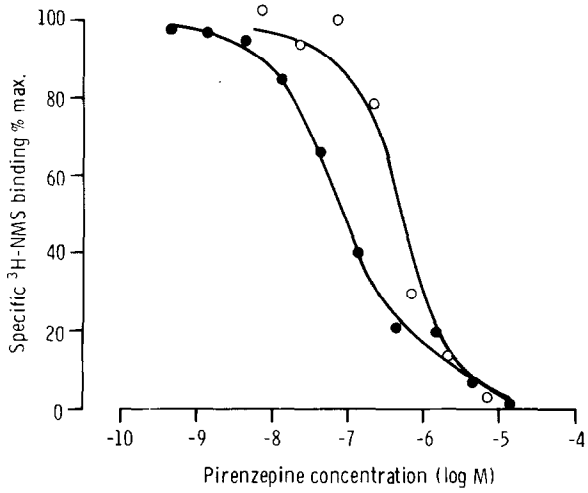

Fig 1. Inhibition of ^{3}H-NMS binding to rat cortex (•) and PC12 cells (o) by pirenzepine. Curves were derived using non-linear regression (9). Data is corrected for radioligand occupancy as described in the legend to table 1.

nM and 1780 ± 620 nM. In contrast, in PC12 cells the Hill coefficient for pirenzepine inhibition was not significantly different from unity, and only one low affinity (K_i = 870 ± 70 nM) pirenzepine binding component could be detected. The pirenzepine binding data for rat cortex gave a significantly better fit to a two site binding model than a one site model ($F_{8,7} = 35.1$ $p < 0.01$), in contrast binding to PC12 cells did not give a statistically better fit to a two site model ($F_{8,4} = 0.01$ NS). The dissociation constant for pirenzepine binding to PC12 cells was not significantly different to the low affinity site in rat cortex. This result suggests that muscarinic receptors present on PC12 cells are predominantly of low pirenzepine affinity, and in this respect may be similar to those present in heart and smooth muscle (8). Muscarinic receptors in heart are thought to be present on sympathetic nerve terminals (12) and to modulate the release of noradrenaline (13). Thus the similarity in pirenzepine affinity between heart and PC12 muscarinic receptors found in the present study adds to the analogous properties of these tissues. Moreover the presence of a single sub-class of muscarinic receptors suggests that PC12 cells may provide a useful system to study the properties and modulation of muscarinic receptors in intact cells.

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