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THE MUSCARINIC RECEPTOR OF RAT PITUITARY GH_3 CELLS IS COUPLED WITH ADENYLATE CYCLASE INHIBITION, BUT NOT WITH PHOSPHOINOSITIDE TURNOVER

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Abstract—The effects of muscarinic stimulation on cyclic AMP accumulation and on basal and thyrotropin-releasing hormone (TRH)-induced phosphoinositide turnover have been studied in rat pituitary GH_3 tumour cells. Carbachol produced a dose-dependent atropine-sensitive inhibition of basal cyclic AMP accumulation. It had no effect on basal or TRH-stimulated production of inositol phosphates. The majority of the muscarinic receptors had a low affinity (K_i 0.22 μM) for pirenzepine and can be classified as M_2 type, inhibiting adenylate cyclase but having no links with phosphoinositide metabolism.

Rat pituitary GH_3 tumour cells provide a convenient model for studies of the mechanisms of hormone-stimulated secretion. These cells contain receptors for thyrotropin releasing hormone (TRH), stimulation of which leads to prolactin (PRL) release. This response appears to be mediated via activation of phosphoinositide turnover and production of inositol phosphates, diacylglycerol and arachidonate. It has been suggested on the basis of many observations [1–4] that receptor-mediated increase in phosphoinositide metabolism is responsible for the biphasic elevation of intracellular Ca^{2+} ion concentration believed to trigger PRL secretion.

The rat pituitary gland has been shown also to possess muscarinic acetylcholine receptors [5–7] as do GH_3 cells [8]. In contrast to TRH receptors, muscarinic receptors mediate inhibition of PRL release [9] although the mechanism involved has been little studied. Recent reports suggest the inhibition to be due to attenuation of adenylate cyclase activity [10, 11] and to be accompanied by a reduction in intracellular Ca^{2+} ion concentration [12]. To date little attention has been drawn to the effects of muscarinic receptor activation on basal or TRH-induced phosphoinositide metabolism.

Over the last few years, receptor binding studies using the muscarinic antagonist, pirenzepine, have provided evidence for receptor heterogeneity [13–15]. Pharmacologically distinguishable subtypes of the muscarinic receptor exist in different tissues, and have been classified as M_1 or M_2 types. M_1 receptors show a higher affinity for pirenzepine than M_2 receptors.

In this study the effects of muscarinic receptor stimulation on cyclic AMP accumulation and on basal and TRH-induced phosphoinositide turnover have been investigated. The effect of pirenzepine on radioligand binding to GH_3 cell preparations have also been determined, allowing identification of the muscarinic receptor involved as a member of the M_2 subclass.

MATERIALS AND METHODS

Materials

GH_3 rat pituitary cells, Dulbecco's Modified Eagle Medium (DMEM) and antibiotics (penicillin and streptomycin) were obtained from Flow Laboratories (Irvine, Scotland). Foetal calf serum (FCS) was purchased from GIBCO (Paisley, U.K.), TRH from Cambridge Research Biochemicals (Cambs., U.K.), Dowex 1-X8 and Dowex AG50WX4 from Bio-Rad Laboratories (Watford, U.K.) and pirenzepine was a generous gift from Fisons (Loughborough, U.K.). [^{3}H]-Inositol (16.3 and 17 Ci/mmol), [^{3}H]-*N*-methylscopolamine ([^{3}H]-NMS, 72 Ci/mmol) and [8-^{3}H]-adenosine 3′,5′ monophosphate (26.1 Ci/mmol) were from Amersham (Bucks., U.K.), and [^{3}H]-pirenzepine (85 Ci/mmol) was from New England Nuclear (Southampton, U.K.). All other reagents were obtained from Sigma (Poole, U.K.) or BDH (Poole, U.K.).

Cell culture

GH_3 cells were grown in DMEM supplemented with 5% FCS and antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin) in a humidified atmosphere of 5% CO_2 at 37°. Cells in passages 37 to 55 were used.

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Measurement of cyclic AMP accumulation

Adenylate cyclase activity was measured by accumulation of cyclic AMP in cells pretreated with a phosphodiesterase inhibitor, using a modification of the procedure of Wojcikiewicz *et al.* [8]. Plates of cells were incubated with 0.5% (w/v) bovine serum albumin (BSA) in DMEM for 45 min and for a further 15 min in 0.2 mM isobutylmethylxanthine and 0.1 mM dithiothreitol in this buffer (IBMX/BSA/DMEM). Subsequently, drugs were introduced into this medium and incubation was continued for 15 min before termination by aspiration of medium and addition of ice-cold 5% (w/v) trichloroacetic acid (TCA). In the cases where antagonists were used, these were introduced 15 min prior to agonists. Cells were detached and the acid-soluble fraction separated by centrifugation at 2000 *g* for 10 min. Protein content of the pellet was determined using the method of Lowry *et al.* [16]. Cyclic AMP was extracted from the acid-soluble fraction on Dowex-H^+ columns and assayed by competition with [^{3}H]-cyclic AMP for bovine adrenal cyclic nucleotide binding protein according to the method of Brown *et al.* [17].

Measurement of phosphoinositide turnover

Cells were incubated with 2 μCi/ml [^{3}H]-inositol in DMEM/5% FCS for 24 hr, and then washed with BSS-Li^+ as described by MacPhee and Drummond [3] (125 mM NaCl, 4.5 mM KCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, 10 mM HEPES (4(2 - hydroxyethyl)1 - piperazine - ethanesulphonic acid), pH 7.4, 0.1% (w/v) BSA, 10 mM LiCl). Following 10 min incubation in this buffer, drugs were introduced at appropriate concentrations for a further 10 min incubation. Reactions were terminated by aspiration of buffer and addition of ice-cold 10% TCA. The TCA-soluble fractions were neutralised and inositol phosphates separated using Dowex-formate anion exchange chromatography as originally described by Ellis *et al.* [18]. A total inositol phosphates fraction was obtained by elution with 0.1 M formic acid/1.0 M ammonium formate, following washing with water, 5 mM disodium tetraborate and 5 mM disodium tetraborate/60 mM sodium formate.

GH_3 cell membrane preparation

GH_3 cells were detached from plates by flushing with medium, and pelleted by centrifugation at 200 *g* for 5 min. The cells were rinsed twice with ice-cold saline and the final cell pellet was frozen in liquid nitrogen and stored at −80°. A crude membrane fraction was prepared by thawing the pellet, followed by homogenisation in 10 mM EDTA, 10 mM HEPES, pH 7.4 (HEPES/EDTA) in a Teflon–glass homogeniser, 10 strokes. Following centrifugation at 500 *g* for 5 min to remove unbroken cells and nuclei, membranes were isolated by centrifugation of the supernatant at 40,000 *g* for 10 min at 4°. The resulting pellet was washed by resuspension in

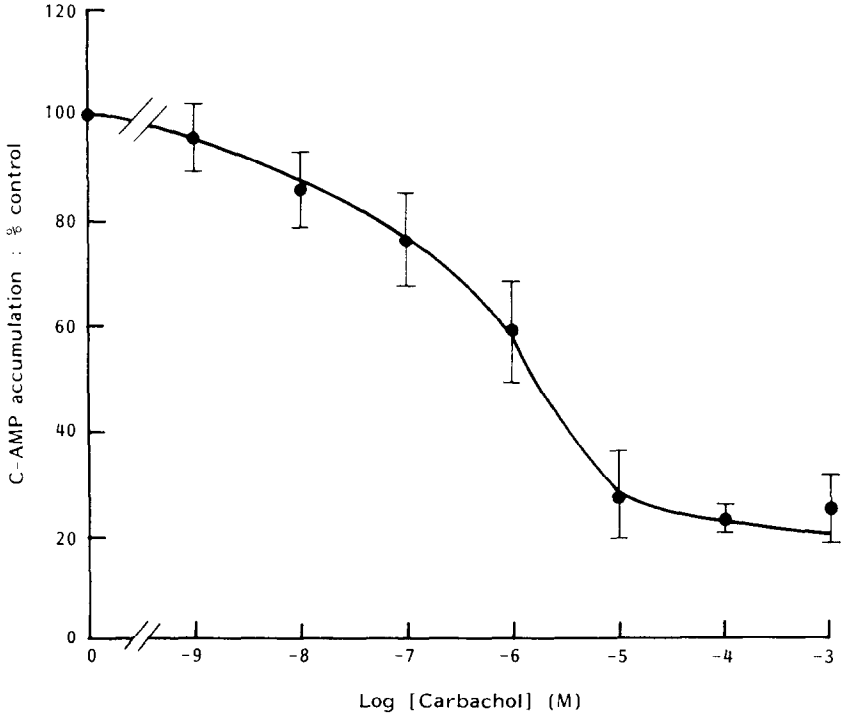

Fig. 1. Dose-dependent inhibition of basal cyclic AMP accumulation in IBMX-treated GH_3 cells by carbachol. Confluent 60 mm plates of GH_3 were incubated for 45 min in DMEM containing 0.5% BSA at 37° and then exposed to 0.2 mM IBMX in this medium for a further 15 min. Various concentrations of carbachol were then introduced into the dishes and incubation continued for 15 min longer. Cyclic AMP was extracted and assayed as described in Methods. Results are means ± SEM of 5 experiments carried out in duplicate.

HEPES/EDTA solution and centrifugation before final resuspension and storage at −80°.

[^{3}H]-*Radioligand binding assays*

Direct binding. Membranes or cells in suspension were incubated in binding buffer (110 mM NaCl, 5.3 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, 0.9 mM NaH_2PO_4, 25 mM glucose, 50 mM sucrose, 20 mM HEPES, pH 7.4), in a total volume of 1 ml, with varying concentrations of [^{3}H]-NMS or [^{3}H]-pirenzepine, in the presence and absence of 10 μM atropine for 2 hr at 25°. Reactions were terminated by addition of 4 ml ice-cold 10 mM Tris (pH 7.4), 0.9% (w/v) NaCl, 0.01% (w/v), BSA, 0.1% (v/v) ethanol, and filtration under reduced pressure over Whatman GF/C glass fibre filters. Filters were rinsed a further three times with 4 ml of this buffer and then dried. Radioactivity was determined by liquid scintillation counting. B_{max} and K_d values were determined using Scatchard analysis.

Competition binding. Competition experiments were performed by incubating varying concentrations of drugs with a fixed concentration of [^{3}H]-NMS (approximately 200 nM) in binding buffer for 2 hr at 25°. Reactions were terminated as above, and data were analysed using a nonlinear least squares curve-fitting computer program [19]. Calculated IC_{50} values were translated into K_i values using the method of Cheng and Prusoff [20].

RESULTS AND DISCUSSION

The muscarinic receptor employs various effector mechanisms resulting in increased phosphoinositide turnover, inhibition of adenylate cyclase, accumulation of cyclic GMP and modulation of K^+ channels. These functions appear to involve interactions of the receptor through guanine nucleotide binding proteins (G proteins, for review see Gilman [21]). It has been of great interest to consider whether different subtypes of receptor exist which are linked to individual effector mechanisms, possibly through distinct G proteins.

Figure 1 shows the dose-dependent inhibition of basal cyclic AMP accumulation by carbachol in GH_3 cells in the presence of a phosphodiesterase inhibitor. Inhibition is not complete, in agreement with others, occurs with an ED_{50} of approximately 1 μM, and is atropine-sensitive (not shown). Pertussis toxin has been reported to block the inhibitory effects of muscarinic agonists on cyclic AMP accumulation and PRL secretion [22] via ADP-ribosylation of a 41K protein [11], suggesting involvement of G_i in this response.

In Fig. 2 it can be seen that, in agreement with the results of others, TRH causes dose-dependent accumulation of [^{3}H]-inositol phosphates in Li^+-treated GH_3 cells. The ED_{50} was 2.8 (±1.4) nM (3 experiments). TRH-induced [^{3}H]-inositol phosphate accumulation was attenuated when 4 mM EGTA was included in experiments (not shown), suggesting a partial dependence of the receptor-linked phospholipase C on extracellular Ca^{2+}. However, results expressed in Fig. 3 indicate that carbachol at concentrations as high as 1 mM is unable to alter either basal or 10 nM TRH-stimulated production of inositol phosphates in these cells.

The fact that the muscarinic receptor of the GH_3 cell line is unable to couple to phospholipase C, under conditions where another receptor (the TRH receptor) can, led us to investigate the nature of this receptor with respect to pirenzepine sensitivity. In Fig. 4, direct binding of [^{3}H]-NMS to GH_3 membranes is demonstrated. Scatchard analysis indicates that this binding is to a single site showing a B_{max} of 27 ± 5 fmol/mg protein (mean ± SEM, 4 experiments) and a K_d of 76 ± 18 pM. Two independent experiments using intact cells gave a B_{max} of 7.9 ± 1.4 fmol/10^6 cell and a K_d of 76 ± 10 pM. Competition for [^{3}H]-NMS binding by pirenzepine (see Fig. 5) gives some evidence for the presence of two populations of muscarinic sites labelled by the radioligand, as determined by computer analysis. A minor subpopulation of sites (less than 10%) shows a high affinity for pirenzepine (K_i = 4 nM, 3 experiments), whilst the majority of the sites show a lower affinity for this antagonist (0.22 μM). In agreement with these observations, Fig. 6 shows that little, if any, specific binding of [^{3}H]-pirenzepine can be seen at concentrations in the range of the K_i value of the proposed high affinity site. These results are

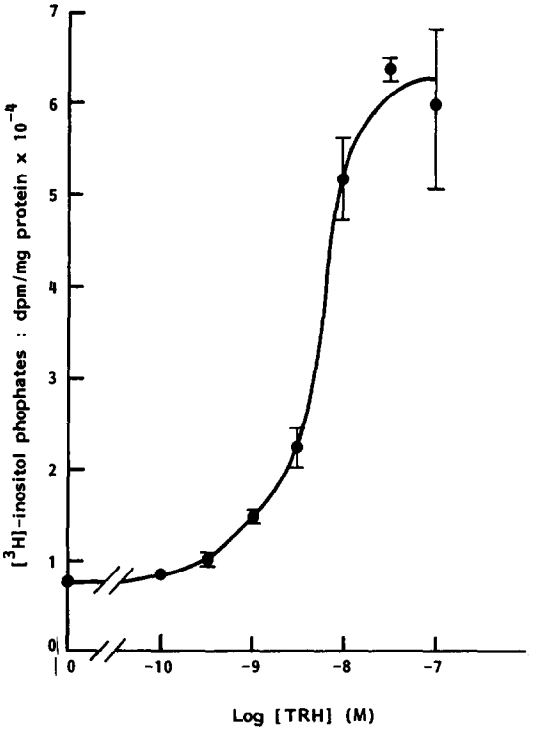

Fig. 2. Dose-dependent production of [^{3}H]inositol phosphates by TRH in Li^+-treated GH_3 cells. Cells grown to confluence on 60 mm dishes were incubated at 37° overnight with 2 μCi/ml [^{3}H]inositol. Following washing and 10 min preincubation with BSS-Li^+ (see Materials and Methods), various TRH concentrations were added and incubation continued for a further 10 min. [^{3}H]Inositol phosphates were extracted and quantified as described in Materials and Methods. Data shown are from one experiment in duplicate, which was repeated twice with similar results.

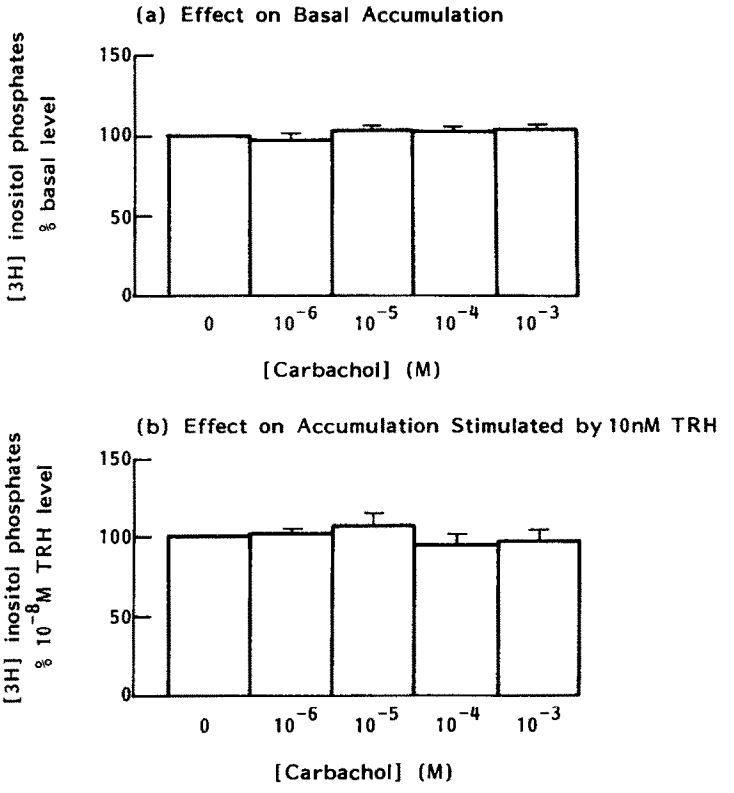

Fig. 3. Effects of carbachol concentration on [^{3}H]inositol phosphate accumulation in Li^+-treated GH_3 cells. Cells grown to confluence in 60 mm dishes were incubated for 24 hr at 37° with 2 μCi/ml [^{3}H]inositol. After washing and preincubation for 10 min in BSS-Li^+, cells were exposed for a further 10 min to various concentrations of carbachol with (a) or without (b) 10 nM TRH in BSS-Li^+. The [^{3}H]inositol phosphates were assayed as described in Methods. Results are means ± SEM of three experiments in duplicate.

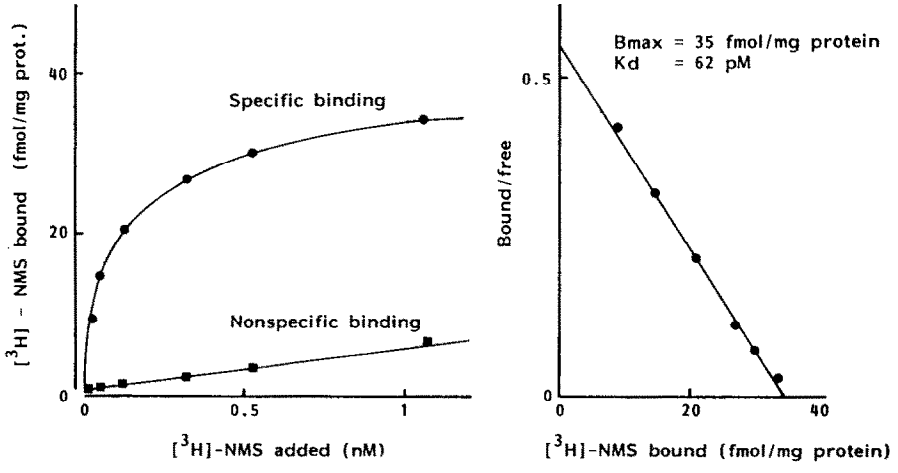

Fig. 4. Equilibrium binding of [^{3}H]-NMS to GH_3 cell membranes. Increasing concentrations of [^{3}H]-NMS were incubated with GH_3 cell membranes in the presence (non-specific binding) or absence (total binding) of 10 μM atropine. Specific binding was determined by subtracting non-specific from total binding. Saturation isotherm from a representative experiment (left) was converted to a Scatchard plot (right). Each point indicates the mean of two samples. Protein concentration was 0.4 mg/ml in this experiment. The experiment was repeated four times (protein concentrations, 0.3 to 1.2 mg/ml) and the mean values for B_{max} and K_d were calculated (shown in the text).

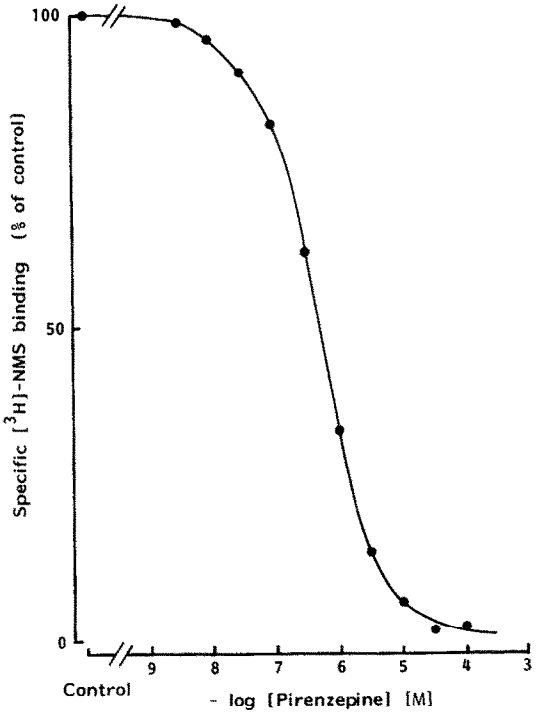

Fig. 5. Inhibition of [^{3}H]-NMS binding to GH_3 by pirenzepine. Experiments were carried out in triplicate and repeated three times with GH_3 membranes (0.4–0.6 mg protein/ml) and [^{3}H]-NMS (190–220 pM). Specific binding was defined using 10 μM atropine. A representative inhibition curve is shown. Standard deviations are less than 5% of specific [^{3}H]-NMS binding in control sample. Each inhibition curve was analysed using a two-site model by non-linear least squares analysis.

consistent with classification of the receptors as M_2 type. More detailed experiments (with more data points in the appropriate concentration range) will be required to determine the exact proportion and number of any high affinity pirenzepine binding sites which may be present. Table 1 indicates the affinities of the receptors for various ligands determined using binding studies.

The apparent K_i for carbachol of 7.54 μM belies the fact that binding of this ligand to the muscarinic sites labelled by [^{3}H]-NMS is complex. The pseudo-Hill coefficient obtained (0.56 ± 0.02) indicates that agonist binding is to more than one affinity state of the receptor, unlike binding of classical antagonists such as atropine (Hill slope = 1.03 ± 0.05). It is the case with many receptor types which induce second messenger systems via GTP-binding proteins [23] that agonists bind to receptor–G protein complexes with different affinities than to free receptor. As the muscarinic receptor under study here causes adenylate cyclase inhibition via interaction with G_i [11] heterogeneity of carbachol binding may be explained in terms of receptor–G_i interactions rather than the presence of multiple receptor subtypes. For a detailed review of receptor–G protein interactions see Dolphin [23]. Under normal cellular conditions, levels of GTP and Mg^{2+} are likely to be optimal for maintenance of receptors in stable low agonist affinity form. Indeed, in a single carbachol-[^{3}H]NMS displacement experiment carried out on intact GH_3 cells (not shown), a pseudo-Hill coefficient of 1.07 was obtained, with a K_i of 1.2×10^{-5} M. Thus *in vivo*, GH_3 muscarinic receptors are present in a single low affinity agonist state, rather than the mixed low-plus-high affinity states observed in washed membrane preparations.

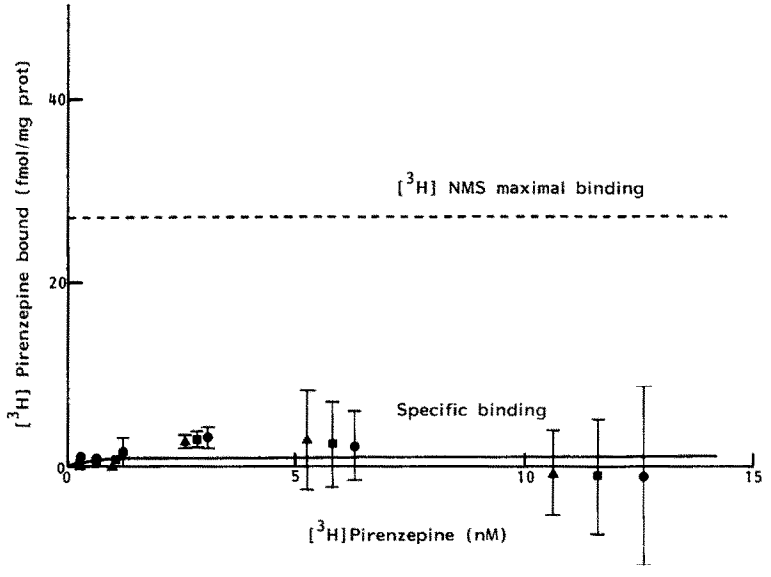

Fig. 6. Equilibrium binding of [^{3}H]pirenzepine to GH_3 cell membranes. The membranes were incubated with increasing concentrations of [^{3}H]pirenzepine in the presence (non-specific binding) or absence of 10 μM atropine. Specific binding was determined by subtracting non-specific from total. Saturation isotherms were constructed from three separate experiments (●, ▲, ■). Each point represents mean and range of two samples (protein concentration 0.4–0.6 mg/ml).

Table 1. Affinities of ligands for [^{3}H]NMS binding sites on GH_3 cell membranes

Competing drug	K_i/K_d*	Experiments
Atropine	0.22 nM	3
Carbachol	7.54 μM	3
Pirenzepine (high)	4 nM (8%)	3
Pirenzepine (low)	0.22 μM (92%)	3
NMS	76 pM	4

* Value for agonist is corrected IC_{50}. See legend to Fig. 5.

In conclusion, the muscarinic receptor effector mechanism involved in inhibition of PRL secretion from GH_3 cells appears to be attenuation of adenylate cyclase activity. No effect of carbachol on basal or TRH-stimulated phosphoinositide turnover occurs. As pharmacological analysis revealed that the receptors are primarily of the M_2 type, a correlation between a particular muscarinic receptor subtype and an individual effector system has been demonstrated. This is in accord with the results of Brown and Brown [24], who have indicated the presence of two muscarinic receptor types in embryonic chick heart, one linked to cyclase inhibition and one to phosphoinositide hydrolysis. More recently, direct evidence that M_1 and M_2 receptors are encoded by different genes and are expressed differently in various tissues has been provided by Kudo *et al.* [25]. Therefore it is very likely that different cell types within tissues such as the pituitary are regulated to express only one subtype of receptor to exert a particular function, and not to perturb other systems which are subserving different receptors in the same cell type. Indeed, there is some evidence that growth hormone release from somatotrophs is stimulated by carbachol via increased phosphoinositide turnover, in contrast to the inhibitory control this agonist exerts over PRL release from GH_3 cells via cyclase inhibition.

Classification of muscarinic receptors as either M_1 (phosphoinositide-linked, high affinity for pirenzepine) or M_2 (inhibiting adenylate cyclase, low affinity for pirenzepine) is not entirely satisfactory. There are phosphoinositide-linked muscarinic receptors in guinea-pig brain with a low affinity for pirenzepine [26]. Moreover, three muscarinic receptor types can be defined by their signalling systems, one linked to phosphoinositide hydrolysis, a second to adenylate cyclase inhibition and a third directly coupled to a K^+ channel [27].

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