ELSEVIER

Biochemical Pharmacology 61 (2001) 337–342

Biochemical Pharmacology

Muscarinic receptor subtypes and calcium signaling in Fischer rat thyroid cells

Eugenio Jiménez[a,*], José Pavía[b], Valentina Morell[a], Elisa Martín[b], Mercedes Montiel[a]

[a]*Departamento de Bioquimica y Biologia Molecular, Facultad de Medicina, Universidad de Malaga, 29080 Malaga, Spain*
[b]*Departamento de Farmacologia, Facultad de Medicina, Universidad de Malaga, 29080 Malaga, Spain*

Received 18 April 2000; accepted 19 July 2000

Abstract

A specific and saturable binding site for [^{3}H]N-methyl-scopolamine ([^{3}H]NMS) was observed in plasma membrane of Fischer rat thyroid (FRT) cells with an equilibrium dissociation constant (K_d) of 0.11 ± 0.02 nM and a concentration of receptor sites (B_{max}) of 14.1 ± 3.9 fmol/mg protein. Pharmacological characterization of this binding site using pirenzepine, himbacine, (11(2-diethyl-amino)methyl)-1-piperidinylacetyl-5-11-dihydro-6*H*-pyrido(14) benzodiazepine (AF-DX 116), dicyclomine, 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP), and hexahydro-sila-difenidol (HHSD) showed clear differences, in terms of affinities, between these muscarinic receptor antagonists. The order of potency for inhibiting [^{3}H]NMS binding was HHSD = dicyclomine > 4-DAMP > pirenzepine = himbacine > AF-DX 116. These findings suggest that the muscarinic receptors found in FRT cells belong to the M_3 subtype. Stimulation of FRT cells with carbachol produced a biphasic and dose-dependent increase in the intracellular calcium concentration ($[Ca^{2+}]_i$), which was blocked in pretreated cells with atropine and almost abolished by a low concentration of 4-DAMP and HHSD. Removal of extracellular Ca^{2+} from the incubation medium reduced the initial transient peak and completely abolished the plateau phase, while the transient phase was markedly reduced by the phospholipase C inhibitor U73122. These data indicate that $[Ca^{2+}]_i$ results from both Ca^{2+} influx across Ca^{2+} channels and mobilization of Ca^{2+} from intracellular Ca^{2+} stores. The present data showed the presence of the M_3 muscarinic acetylcholine receptor subtype in plasma membrane of FRT cells, which may influence cellular function via modulation of $[Ca^{2+}]_i$.

Key words: Muscarinic receptor subtypes; FRT cells; Intracellular calcium concentration; Carbachol

1. Introduction

It is known that the stimulation of muscarinic acetylcholine receptors produces important metabolic and functional changes in the thyroid gland which affect thyroid hormone formation [1,2]. At present, at least three different pharmacologically muscarinic acetylcholine receptor subtypes have been defined: the M_1 receptor subtype, which has a high affinity for pirenzepine, is preferentially localized in nervous tissue; the M_2 receptor subtype, which has a high affinity for AF-DX 116, is preferentially localized in cardiac tissue; and the M_3 receptor subtype, which has a high affinity for both HHSD and 4-DAMP antagonists, is localized preferentially in glandular tissue [3]. A fourth subtype, termed M_4, has been reported to be localized in rabbit lung, chicken heart, and NG108-15 cells [4]. Moreover, five different molecular subtypes have been delineated: m_1, m_2, m_3, m_4, and m_5. There seems to be general agreement that these molecular subtypes represent the pharmacological subtypes M_1, M_2, M_3, M_4, and M_5, respectively [5].

Radioligand binding studies in fully differentiated thyroid (FRTL-5) cell membranes have reported the existence of two populations of muscarinic receptors with low and high affinity for the [^{3}H]NMS antagonist [6]. In addition, two different muscarinic receptor subtypes in FRTL-5 cells, which are related to at least two separate intracellular pathway mechanisms, have also been identified: a pirenzepine-sensitive muscarinic receptor subtype implicated in the activation of phospholipase A_2 [6] and another, pirenzepine-

* Corresponding author. Tel.: +34-952-131-536; fax: +34-952-131-534.

E-mail address: ejg@uma.es (E. Jiménez).

Abbreviations: FRT, Fischer rat thyroid; FRTL-5, Fischer rat thyroid low serum; [^{3}H]NMS, [^{3}H]*N*-methyl-scopolamine; 4-DAMP, 4-diphenylacetoxy-*N*-methylpiperidine methiodide; HHSD, hexahydro-sila-difenidol, AF-DX 116, (11(2-diethyl-amino)methyl)-1-piperidinylacetyl-5-11-dihydro-6*H*-pyrido(14) benzodiazepine; $[Ca^{2+}]_i$, intracellular calcium concentration; and PLC, phospholipase C.

0006-2952/01/$ – see front matter
PII: S0006-2952(00)00565-7

insentitive, involved in the inhibition of PLC [7]. In accordance with these observations, further studies performed in primary cultures of dog thyroid cells have suggested that M_2 muscarinic receptor subtypes are not implicated in the desensitization of adenylyl cyclase activity via muscarinic receptors [8].

Controversy exists as to the effect of muscarinic receptor activation with carbachol in thyroid cells and its relationship to increased $[Ca^{2+}]_i$. In FRTL-5 cells, it has been described that carbachol produces either inhibition of PLC and inositol 1,4,5-trisphosphate (IP_3) formation and decreased $[Ca^{2+}]_i$ [7] or has no effect on Ca^{2+} mobilization [9]. However, in dog thyroid cells, carbachol significantly enhanced $[Ca^{2+}]_i$ [9].

Although there are reports confirming the presence of muscarinic receptors in dog thyroid and FRTL-5 cells [6–8], an exact pharmacological characterization of these binding sites has not yet been clearly performed in thyroid cells. The aim of the present study was to pharmacologically characterize the muscarinic receptor subtypes present in fully neoplastic FRT cells. In addition, on the basis of the above-described differential coupling of muscarinic receptors to Ca^{2+} mobilization in thyroid cells and other cell lines such as neuroblastoma cells [10], the effect of carbachol on intracellular Ca^{2+} mobilization was also analyzed.

2. Material and methods

2.1. Materials

[^{3}H]NMS (specific activity 80–84 Ci/mmol) was purchased from Amersham International plc. Carbachol was from Sigma Chemical Co. Dicyclomine, 4-DAMP, HHSD, and himbacine were from RBI. Atropine sulfate was a gift from B. Braun Medical S.A. Pirenzepine dihydrochloride and AF-DX 116 were gifts from Dr. Karl Thomae GmbH. Bovine serum was from GIBCO. Carbachol, Coon's modified Ham's F12 medium, antibiotics, proteinase inhibitors, fura 2-acetoxymethyl ester, pluronic F-127, and other drugs and reagents were from Sigma Chemical Co.

2.2. Cell culture

FRT cells were grown in Coon's modified Ham's F12 medium, supplemented with 5% bovine serum, containing gentamicin (50 μg/mL) and amphotericin B (0.25 μg/mL). The cells were maintained in a water-saturated atmosphere of 5% CO_2 and 95% air at 37°. Before the experiments, cells from one donor culture dish were harvested with a 0.05% trypsin–EDTA solution and plated onto plastic 150-mm culture dishes. The cells were grown for 7–8 days, with two to three changes of the culture medium. Fresh medium was always added 24 hr prior to an experiment.

2.3. Plasma membrane preparations

FRT cells were harvested from the plates by scraping the surface and then suspended in cold 20 mM HEPES buffer containing 10 mM EDTA, pH 7.4, homogenized, and centrifuged at 45,000 *g* for 30 min at 4° in a Beckman L80 centrifuge. The pellet was resuspended in cold 20 mM HEPES buffer containing 0.1 mM EDTA, pH 7.4, re-homogenized, re-centrifuged under the same conditions, and resuspended in 20 mM HEPES buffer, pH 7.4, containing 50 mM NaCl, 10 mM $MgCl_2$, and proteinase inhibitors (1 μg/mL of leupeptin and aprotinin and 0.1 μg/mL of bacitracin and phenylmethysulfonyl fluoride). The membranes were aliquoted and stored until use at −80°.

2.4. Binding assays

Binding of [^{3}H]NMS to plasma membrane fractions (300 μg protein) from FRT cells was performed in 1 mL incubation volume for 45 min at 30° in the absence or presence of 10 μM atropine to define the non-specific binding. Bound and free radioactivity was separated by adding 4 mL of ice-cold 20 mM Tris buffer, pH 7.4 to the assay tube, followed by vacuum filtration through Whatman GF/B glass fiber filters presoaked with 0.1% polyethylenimine. The assay tubes and filters were then rinsed with three additional washes of 4 mL cold distilled water. The filters were placed in vials containing scintillation liquid and then measured in a Wallac 1410 liquid scintillation counter (Pharmacia). Saturation experiments were carried out using eight [^{3}H]NMS concentrations, ranging from 0.05 to 2×10^{-9} M. Inhibition binding studies of 2×10^{-9} M [^{3}H]NMS with the muscarinic receptor antagonists pirenzepine, AF-DX 116, himbacine, 4-DAMP, dicyclomine, and HHSD were performed using ten concentrations of these drugs, ranging from 6×10^{-10} M to 8×10^{-5} M.

All binding data were analyzed by an iterative curve-fitting procedure using the LIGAND program modified by McPherson [11], which obtains K_i values by correction of 50% inhibitory concentration (IC_{50}) for receptor occupancy using the Cheng and Prusoff equation. Hill slopes were obtained by fitting the competition binding data to a four-parameter logistic equation using the GraphPad Prism (V 2.01) graphic program. Protein was measured by the method of Lowry [12] using BSA as standard.

2.5. Determination of $[Ca^{2+}]_i$

To measure the $[Ca^{2+}]_i$, the FRT cells were loaded with fura 2-acetoxymethyl ester (5 μM final concentration) at 37° with continuous shaking (100 cycles/min) for 30 min and in the presence of 20 μg/mL of pluronic F-127 to encourage dispersion of the probe. After the cells were washed and centrifuged (50 *g* for 2 min) twice in a modified Krebs–Ringer buffer in which bicarbonate was replaced by 20 mM HEPES, pH 7.4, they were incubated again at 37°

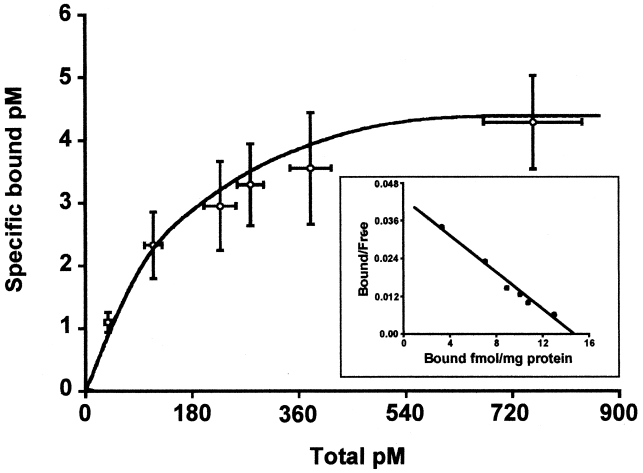

Fig. 1. Saturation plot of [^{3}H]NMS binding to FRT cell membranes. Inset: Scatchard representation from [^{3}H]NMS saturation experiments in plasma membrane from FRT cells. Means ± SEM from three individual experiments performed in triplicate are plotted.

for 10 min to facilitate hydrolysis of the esterified probe, and centrifuged once more. FRT cells were resuspended in the same buffer containing 0.1% BSA, and 2 mL of the cell suspension was placed in a fluorescence cuvette with continuous stirring. Cells preincubated at 37° with the different agents were stimulated with carbachol, and then the fluorescence intensity was recorded at 510 nm in an F-2000 Hitachi spectrofluorimeter (Hitachi Ltd.) using a dual-excitation source at 340 and 380 nm. The maximal fluorescence was determined at the end of the assay by adding 40 μL of 10% SDS and the minimal fluorescence by adding 30 μL of 0.5 M EGTA solution, pH 9.0. The cytoplasmic Ca^{2+} concentration was calculated from the fluorescence ratio [13].

3. Results

3.1. Binding assays

To perform a pharmacological characterization of the muscarinic cholinergic receptors in FRT cells, saturation and competition binding experiments were carried out. Specific binding of 2×10^{-9} M [^{3}H]NMS to plasma membrane from FRT cells reached equilibrium at 20 min of incubation and was stable for up to 90 min at 30° (data not shown). The results obtained in saturation experiments with [^{3}H]NMS revealed the existence of a homogeneous population of muscarinic receptors in plasma membrane preparation of FRT cells with a K_d of 0.11 ± 0.02 nM and a B_{max} of 14.1 ± 3.9 fmol/mg protein (Fig. 1).

Data from competition experiments with the muscarinic receptor antagonists pirenzepine, AF-DX 116, himbacine, 4-DAMP, dicyclomine, and HHSD are summarized in Table 1, and the competition curves for these antagonists are presented in Fig. 2. The results show clear differences, in terms of affinities, between these muscarinic receptor antagonists. The order of potency for the inhibition of [^{3}H]NMS binding was HHSD = dicyclomine > 4-DAMP > pirenzepine = himbacine > AF-DX 116. In all the competition experiments, the Hill coefficients (nH) were close to 1, and we were unable to fit our competition data to other models different from the one-site model.

Table 1
Results from competition experiments of the binding of 2×10^{-9} M [^{3}H]NMS versus HHSD, dicyclomide, 4-DAMP, pirenzepine, himbacine, and AF-DX 116 on plasma membranes from FRT cells

Drugs	K_i(nM)	nH
HHSD	3.62 ± 0.8	0.89 ± 0.11
Dicyclomine	3.93 ± 1.1	0.99 ± 0.12
4-DAMP	6.62 ± 1.0	0.89 ± 0.11
Pirenzepine	103 ± 20	0.91 ± 0.13
Himbacine	137 ± 22	0.95 ± 0.15
AF-DX 116	1920 ± 400	0.98 ± 0.12

Means ± SEM from three to five individual experiments performed in duplicate are shown.

3.2. Intracellular [Ca^{2+}]

Carbachol-induced Ca^{2+} mobilization in the FRT cells was examined by measuring the changes in the $[Ca^{2+}]_i$ using fura-2 as a Ca^{2+} indicator. Exposure of FRT cells to carbachol (10–100 μM) evoked a dose-dependent and rapid transient increase in $[Ca^{2+}]_i$, followed by a lower and sustained phase, which persisted for at least 5 min of the observation period (Fig. 3). Carbachol (100 μM) stimulated calcium mobilization to an average 6-fold increase above basal levels. In contrast, when extracellular Ca^{2+} was removed by adding 2 mM EGTA, the initial and transient

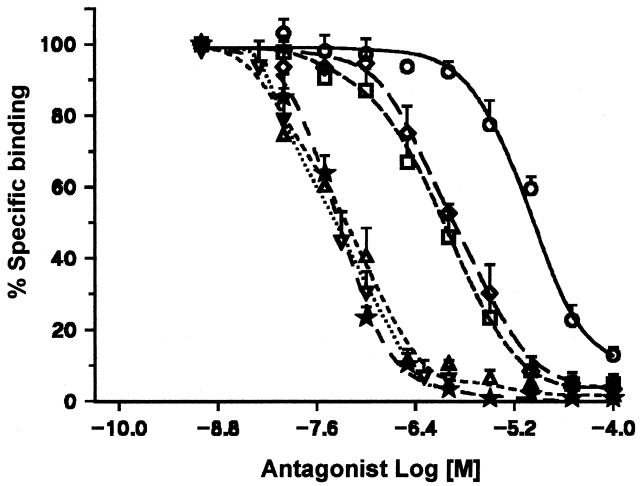

Fig. 2. Competition experiments of [^{3}H]NMS versus the antagonists pirenzepine (□), AF-DX 116 (○), himbacine (◇), 4-DAMP (△), dicyclomide (∗), and HHSD (▽) in plasma membrane from FRT cells. Means ± SEM from three to five individual experiments performed in duplicate are plotted.

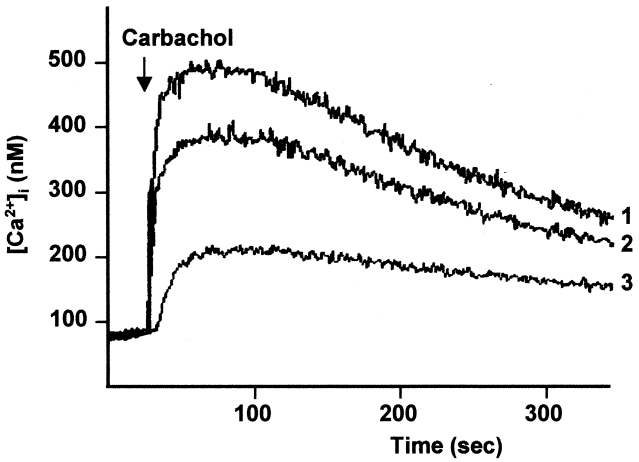

Fig. 3. Dose-dependent effect of carbachol on $[Ca^{2+}]_i$ in FRT cells. Cells were harvested and loaded with fura-2 as described in Materials and Methods. FRT cells were stimulated by 100 μM carbachol (1), 50 μM carbachol (2), and 10 μM carbachol (3).

$[Ca^{2+}]_i$ response was reduced and the sustained phase was completely abolished, while the transient phase was attenuated by U73122, a specific inhibitor of PLC (Fig. 4). The effects of several muscarinic receptor antagonists in FRT cells were also studied. The carbachol-induced Ca^{2+} response was completely blocked by atropine (10 μM) and almost abolished by 100 nM 4-DAMP and HHSD (Fig. 5), while AF-DX 116 and pirenzepine were ineffective at concentrations of up to 1 μM (data not shown).

6. Discussion

The results of the present study show the presence of specific and saturable binding sites for [^{3}H]NMS in FRT cells. The dissociation constant (K_d) for the tracer, as determined by saturation experiments, was in agreement with previously reported data obtained in several rat tissue preparations such as rat forebrain, submandibular gland, heart, pancreas and striatum, human placenta [14–16], and in transfected cell lines expressing different muscarinic receptor subtypes [17]. Although a previous report suggested the existence of two binding sites for [^{3}H]NMS in FRTL-5 cells [6], our findings unequivocally indicate the existence of a single population of muscarinic cholinergic receptors in FRT cell membranes.

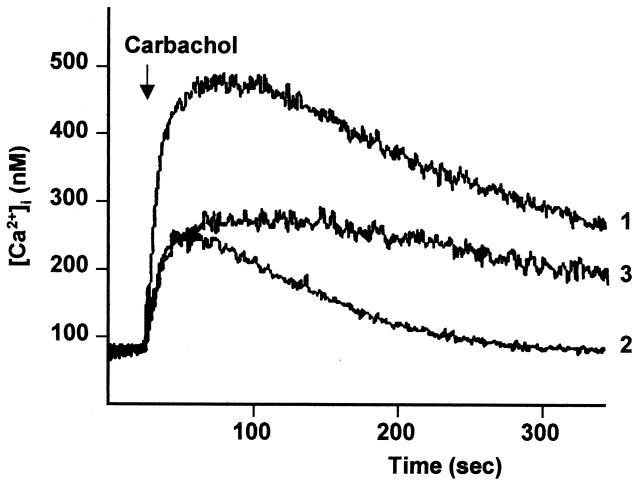

Fig. 4. Effect of carbachol on $[Ca^{2+}]_i$ in FRT cells pretreated with EGTA and U73122. Cells were harvested and loaded with fura-2 as described in Materials and Methods. FRT cells were stimulated by 100 μM carbachol (1), and FRT cells pretreated with 2 mM EGTA for 2 min (2) or 0.5 μM U73122 for 10 min (3) before stimulation with 100 μM carbachol.

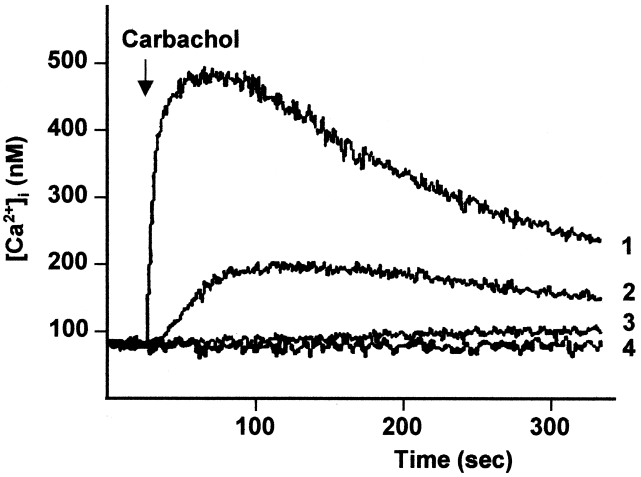

Fig. 5. Effects of atropine, HHSD, and 4-DAMP on carbachol-evoked $[Ca^{2+}]_i$ in FRT cells. Cells were harvested and loaded with fura-2 as described in Materials and Methods. FRT cells were stimulated by 100 μM carbachol (1), and FRT cells pretreated with 100 nM HHSD (2), 100 nM 4-DAMP (3), or 10 μM atropine (4) for 5 min before stimulation with 100 μM carbachol.

In order to pharmacologically characterize these binding sites, several antagonists competing with [^{3}H]NMS were used in this work. The inhibition constant (K_i) values obtained in plasma membrane from FRT cells with pirenzepine, a selective M_1 antagonist, were closer to the values found in mammalian tissues for the M_3 than for the M_1 receptor subtype [3,18] and were very similar to the values found in transfected cell lines expressing the M_3 receptor subtype [17,19]. The same applies to 4-DAMP, a selective M_3–M_1 antagonist, with K_i values similar to those found for the M_3 receptor in rat pancreas [14].

No clear data exist on the characteristics of muscarinic receptors in thyroid and FRTL-5 cells. Whereas some authors suggest the existence of an M_2 receptor based on the lack of inhibition of PLC activity by pirenzepine and the blocking effect produced by atropine [7], the same authors in a later report found the existence of two different receptors, a pirenzepine-sensitive receptor activating phospholipase A_2 and a pirenzepine-insensitive receptor inhibiting PLC [6].

In an attempt to clarify the muscarinic receptor subtypes present in FRT cells, further experiments were performed with the M_2 selective antagonist AF-DX 116, which showed

low affinity for the muscarinic receptors present in FRT cells. The K_i values for this antagonist were close to the value found in transfected cell lines expressing the M_3 receptor subtype [19] and very similar to the value found for the M_3 receptor in rat submaxillary gland and pancreas [14,18]. The results obtained with himbacine, an M_2–M_4 antagonist, were in agreement with those found with the above compounds, with K_i values very similar to those found for the M_3 receptor in rat submaxillary gland or in transfected cell lines; the same applies to HHSD, a selective M_3 antagonist, with K_i values closer to the M_3 receptor than to the remaining subtypes when expressed in transfected cell lines [19] and very similar values to those found in rat submandibular gland [4]. The data from experiments with dicyclomine also showed K_i values closer to the M_3 receptor subtype than to the other muscarinic receptor subtypes.

From the data obtained with pirenzepine, we can exclude the M_1 receptor subtype as a candidate for the receptor expressed in FRT cells. The results with AF-DX 116 also exclude the M_2 and M_4 receptor subtypes as candidates, with this also being supported by the results obtained with himbacine and dicyclomine. They usually show higher affinity for the M_4 receptor subtype than for the M_3 receptor subtype, but such was not the case here. The results obtained with 4-DAMP and HHSD point to the M_3 receptor subtype as the most important candidate present in FRT cells. All these findings taken together strongly suggest that the muscarinic receptor found in FRT cells belongs to the M_3 subtype.

Activation of muscarinic receptors has been shown to elicit diverse cellular effects including attenuation of cyclic adenosine 3′,5′-monophosphate (cAMP) accumulation [20], an increase in cellular cyclic guanosine 3′,5′-monophosphate (cGMP) levels [21], and activation of PLC [22]. Since intracellular Ca^{2+} plays a central role as a second messenger in a number of physiologically important processes determining thyroid cell function [23,24], the effect of carbachol in $[Ca^{2+}]_i$ was also analyzed in an attempt to determine the functional significance of these muscarinic receptors in FRT cells. Carbachol evoked an increase in $[Ca^{2+}]_i$ in these non-differentiated thyroid cells. This observation, which is consistent with earlier reports performed in dog thyroid cells [9], is especially relevant in FRT cells. First, it is important because it is the first report indicating a Ca^{2+} mobilization in response to stimulation of muscarinic receptor with carbachol in FRT cells. Second, the results show a functional interaction of the muscarinic receptors with guanine nucleotide binding proteins, indicating that the ability of this thyroid cell line to respond to muscarinic receptor agonist is not altered. Recently, it has been demonstrated that specific binding sites for somatostatin are expressed in both FRT and FRTL-5 thyroid cell lines; however, while FRTL-5 cells are sensitive to somatostatin, FRT cells are not sensitive to hormone, indicating that the expression of a specific receptor type does not directly correlate with the ability of a given cell line to respond to agonist [25].

Carbachol-induced Ca^{2+} mobilization has been extensively examined in several target cells. Carbachol evokes a rapid and transient increase in $[Ca^{2+}]_i$ resulting from both mobilization of intracellular Ca^{2+} stores, mediated by inositol 1,4,5-triphosphate (IP_3) and Ca^{2+} influx across Ca^{2+} channels, and a subsequent lower and sustained phase that is dependent on Ca^{2+} influx through the channels [26,27]. Our results confirm the existence of both Ca^{2+} phases of mobilization in FRT cells, indicating that the carbachol stimulation of muscarinic receptors in FRT cells causes not only extracellular Ca^{2+} entry, but also Ca^{2+} mobilization from IP_3-sensitive intracellular stores. This effect of carbachol on Ca^{2+} in FRT cells is a specifically muscarinic effect, as it was totally abolished by atropine.

In summary, the present data indicate the presence of the M_3 muscarinic acetylcholine receptor subtype in plasma membrane of FRT cells, which may influence cellular function via modulation of intracellular Ca^{2+}.

Acknowledgment

Fischer rat thyroid epithelial cells were a generous gift from Dr. Di Jeso (Centro di Endocrinologia ed Oncologia Sperimentale “G. Salvatore”, CNR, Italy). This work was supported by Grant PM98-0221 from the Dirección General de Enseñanza Superior e Investigación Científica, Madrid.

References

[1] Van Sande J, Dumont JE, Melander A, Sundler F. Presence and influence of cholinergic nerves in the human thyroid. J Clin Endocrinol Metab 1980;51:500–2.
[2] Rani CS, Field JB. Comparison of effects of thyrotropin, phorbol esters, norepinephrine, and carbachol on iodide organification in dog thyroid slices, follicles, and cultured cells. Endocrinology 1988;122:1915–22.
[3] Hulme EC, Birdsall NJ, Buckley NJ. Muscarinic receptor subtypes. Annu Rev Pharmacol Toxicol 1990;30:633–73.
[4] Lazareno S, Buckley NJ, Roberts FF. Characterization of muscarinic M_4 binding sites in rabbit lung, chicken heart, and NG108–15 cells. Mol Pharmacol 1990;38:805–15.
[5] Caulfield MP, Birdsall NJ. International Union of Pharmacology. XVII. Classification of muscarinic acetylcholine receptors. Pharmacol Rev 1998;50:279–90.
[6] Di Girolamo MD, D’Arcangelo D, Bizzarri C, Corda D. Muscarinic regulation of phospholipase A_2 and iode fluxes in FRTL-5 thyroid cells. Acta Endocrinol (Copenh) 1991;125:192–200.
[7] Bizzarri C, Di Girolamo MD, D’Orazio CD, Corda D. Evidence that a guanine nucleotide-binding protein linked to a muscarinic receptor inhibits directly phospholipase C. Proc Natl Acad Sci USA 1990;87:4889–93.
[8] Pasquali D, Rani CS, Deery WJ. Carbachol-induced decrease in thyroid cell adenylyl cyclase activity is dependent of calcium and phosphodiesterase. Mol Pharmacol 1992;41:163–7.
[9] Rani CS, Schilling WP, Field JB. Intracellular Ca^{2+} mobilization by thyrotropin, carbachol, and adenosine triphosphate in dog thyroid cells. Endocrinology 1989;125:1889–97.

[10] Heikkilä J, Jansson C, Akerman KE. Differential coupling of muscarinic receptors to Ca^{2+} mobilization and cyclic AMP in SH-SY5Y and IMR 32 neuroblastoma cells. Eur J Pharmacol 1991;208:9–15.
[11] McPherson GA. Analysis of radioligand binding experiments. A collection of computer programs for the IBM PC. J Pharmacol Methods 1985;14:213–28.
[12] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75.
[13] Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 1985;260:3440–50.
[14] Waelbroeck M, Tastenoy M, Camus J, Christophe J. Binding of selective antagonists to four muscarinic receptors (M_1 to M_4) in rat forebrain. Mol Pharmacol 1990;38:267–73.
[15] Pavía J, Marquez E, Laukkonen S, Martos F, Gomez A, Sánchez de la Cuesta F. M_1 and M_3 muscarinic receptor subtypes in rat forebrain. Methods Find Exp Clin Pharmacol 1991;13:653–60.
[16] Pavía J, Muñóz M, Jiménez E, Martos F, Gonzalez-Correa JA, De la Cruz JP, García V, Sánchez de la Cuesta F. Pharmacological characterization and distribution of muscarinic receptors in human syncytiotrophoblast brush-border and basal plasma membranes. Eur J Pharmacol 1997;320:209–14.
[17] Dörje F, Wess J, Lambrecht G, Tacke R, Mutschler E, Brann MR. Antagonist binding profiles to five cloned human muscarinic receptor subtypes. J Pharmacol Exp Ther 1991;256:727–33.
[18] Delmendo RE, Michel AD, Whiting RL. Affinity of muscarinic receptor antagonists for three putative muscarinic receptor binding sites. Br J Pharmacol 1989;96:457–64.
[19] Buckley NJ, Bonner TI, Buckley CM, Brann MR. Antagonist binding properties of five cloned muscarinic receptors expressed in CHO-K1 cells. Mol Pharmacol 1989;35:469–76.
[20] Nathanson NM, Klein WL, Nirenberg M. Regulation of adenylated cyclase activity mediated by muscarinic acetylcholine receptors. Proc Natl Acad Sci USA 1978;75:1788–91.
[21] El-Fakahany E, Richelson E. Regulation of muscarinic receptor-mediated cyclic GMP synthesis by cultured mouse neuroblastoma cells. J Neurochem 1980;35:941–8.
[22] Hepler JR, Harden TK. Guanine nucleotide-dependent pertussis-toxin-insensitive stimulation of inositol phosphate formation by carbachol in a membrane preparation from human astrocytoma cells. Biochem J 1986;239:141–6.
[23] Takada K, Amino N, Tada H, Miyai K. Relationship between proliferation and cell cycle-dependent Ca^{2+} influx induced by a combination of thyrotropin and insulin-like growth factor-1 in rat thyroid cells. J Clin Invest 1990;86:1548–55.
[24] Di Jeso B, Pereira R, Consiglio E, Formisano S, Satrustegui J, Sandoval IV. Demonstration of a Ca^{2+} requirement for thyroglobulin dimerization and export to the golgi complex. Eur J Biochem 1998; 252:583–90.
[25] Medina DL, Velasco JA, Santisteban P. Somatostatin is expressed in FRTL-5 thyroid cells and prevents thyrotropin-mediated down-regulation of the cyclin-dependent kinase inhibitor $p27^{kip1}$. Endocrinology 1999;140:87–95.
[26] Ohta T, Asano T, Ito S, Kitamura N, Nakazato Y. Characteristics of cytosolic Ca^{2+} elevation induced by muscarinic receptor activation in single adrenal chromaffin cells of the guinea-pig. Cell Calcium 1996; 20:303–14.
[27] Ding KH, Husai S, Akhtar RA, Isales CM, Abdel-Latif AA. Inhibition of muscarinic-stimulated polyphosphoinositide hydrolysis and Ca^{2+} mobilization in cat iris sphincter smooth muscle cells by cAMP-elevating agents. Cell Signal 1997;6:411–21.