Molecular and Pharmacological Characterization of Muscarinic Receptor Subtypes in a Rat Parotid Gland Cell Line: Comparison with Native Parotid Gland

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ABSTRACT

The molecular and pharmacological characteristics of muscarinic receptor subtypes in the rat parotid acinar cell line, PAR-C5, were determined and compared with native rat parotid glands to evaluate the PAR-C5 cell line as a model to study receptor-mediated secretion. Reverse transcription-polymerase chain reaction (RT-PCR) identified mRNAs for M3, M4, and M5 receptor subtypes in both PAR-C5 cells and parotid glands. Specific [N-methyl-3H]scopolamine binding in PAR-C5 and parotid membranes was to a single class of sites with mean Kd values of 0.38 and 0.64 nM, respectively. Binding affinities (Ki values) of muscarinic receptor subtype-selective drugs were obtained in side-by-side experiments comparing PAR-C5 cells with parotid glands. Nonlinear regression analysis indicated that competition binding curves for drugs in PAR-C5 cells and parotid glands fit best to a one-site binding model. Ki values (nM) in PAR-C5 cells and parotid glands, respectively, for atropine (1.0, 2.1), darifenacin (1.2, 2.0), 4-diphenylacetoxy-N-methylpiperidine methiodide (4-DAMP) (2.9, 2.4), tripitramine (220, 180), pirenzepine (320, 720), and methoctramine (1400, 1700) were consistent with their known affinities at the M3 receptor subtype. Affinities (Kd values) of muscarinic receptor subtype-selective drugs for blocking methacholine-stimulated Ca2+ mobilization were determined to show which subtype mediates Ca2+-dependent secretion in Fura-2-loaded PAR-C5 cells. Kd values (nM) for atropine (0.44), 4-DAMP (0.38), pirenzepine (140), and methoctramine (320) for blocking Ca2+ responses correlated well with their known affinities at the M3 receptor (r2 = 0.99). These results show that at the level of mRNA, receptor protein and function, PAR-C5 cells and parotid glands are similar, establishing PAR-C5 cells as an important model for muscarinic receptor-mediated secretion.

One important obstacle to a better understanding of receptor regulation of salivary gland secretion has been the lack of an immortalized cell line maintaining the phenotypical characteristics of an epithelial cell of acinar origin. Acinar cells generate the secretory product of fluid and electrolytes; thus, a cell line that is similar in phenotype to native parotid acinar cells would be a useful model for the study of receptor-mediated secretion. Recently, Quissell et al. (1998) reported the immortalization of two clonal rat parotid gland acinar cell lines established from the same preparation (PAR-C5 and PAR-C10). They, as well as others (Turner et al., 1998), have shown that both cell lines exhibit similar morphological, biochemical, and functional characteristics as those in native parotid acinar cells.

In native parotid acinar cells, the neurotransmitter acetylcholine stimulates muscarinic receptors on the basolateral membrane, causing an elevation in intracellular free calcium, which then activates apical calcium-dependent chloride channels, resulting in fluid and electrolyte secretion. This muscarinic receptor pathway is thought to be an important mechanism for water and electrolyte secretion in salivary glands. PAR-C5 and PAR-C10 cells are polarized in culture and express basolateral muscarinic receptors and apical anion channels that mediate transepithelial anion secretion (Turner et al., 1998). In addition, PAR-C5 and PAR-C10 cells, like native acinar cells, express functional purinergic and adrenergic receptors that couple to an elevation in intracellular free calcium. The similarities between native parotid acinar cells and PAR-C5/C10 cells with respect to polarity, receptor distribution, signal transduction pathways, and other morphological characteristics suggest that these immortalized acinar cell lines are useful tools for studying salivary gland secretion.

It is now known that there are five subtypes of muscarinic receptors called M1, M2, M3, M4, and M5 (Caulfield and Birdsall, 1998). All five of the muscarinic receptor subtypes

ABBREVIATIONS: RT-PCR, reverse transcription-polymerase chain reaction; 4-DAMP, 4-diphenylacetoxy-N-methylpiperidine methiodide; S, sense; AS, antisense; bp, base pair; HBK, HEPES-buffered Krebs’ solution.
have been cloned and originate from individual genes. With the exception of the M₅ receptor subtype, the muscarinic receptor subtypes can be pharmacologically distinguished from one another by determining affinity constants for muscarinic receptor subtype-selective drugs. It is generally accepted that the M₅ receptor subtype is coupled to the elevation of intracellular free calcium, thus causing fluid secretion in native rat parotid acinar cells (Dai et al., 1991; Sawaki et al., 1993). However, the muscarinic receptor subtype mediating calcium mobilization in PAR-C5/C10 cells and native parotid glands is unknown. The possibility that immortalization, culture conditions, or both alter expression patterns and/or functionality of muscarinic receptor subtypes in PAR-C5/C10 cells should be considered if these cell lines are to be used as a model in secretion studies.

Thus, the overall aim of this study was to characterize the muscarinic receptor subtypes in side-by-side experiments comparing PAR-C5 cells and native rat parotid glands. We used reverse transcription-polymerase chain reaction (RT-PCR) to identify the mRNA for the muscarinic receptor subtypes present in PAR-C5 cells and native parotid glands. In addition, we characterized the muscarinic receptor subtypes expressed in PAR-C5 cells and native rat parotid glands by determining the affinities of several muscarinic receptor subtype-selective drugs for inhibiting specific [N-methyl-³H]scopolamine binding. Finally, we examined which muscarinic receptor subtype is coupled to an elevation in intracellular free calcium in PAR-C5 cells by determining affinity constants of several subtype-selective antagonists for blocking methacholine-stimulated fluorescence in PAR-C5 cells loaded with the calcium indicator Fura-2.

Materials and Methods

Drugs. The drugs used were obtained from the following sources: methacholine chloride, atropine sulfate, 4-diphenylacetoxyl-N-methylpiperidine methiodide (4-DAMP methiodide), pirenzepine dihydrochloride, methotrexate tetrahydrochloride, physostigmine sulfate, and triptilamine tetraoxalate (RBI/Sigma, Natick, MA); darifenacin hydrobromide (a generous gift from Pfizer Limited, Sandwich, Kent, England); and [N-methyl-³H]scopolamine (70–87 Ci/mmol; PerkinElmer Life Science Products, Boston, MA).

Cell Culture. PAR-C5 cells were seeded onto T-25 (2 × 10⁵ cells) or T-75 (8 × 10⁵ cells) Falcon Primaria tissue culture flasks (Becton Dickinson, Franklin Lakes, NJ) and grown in Dulbecco’s Modified Eagle’s Medium/F-12 (1:1) and 2.5% fetal bovine serum (Life Technologies, Grand Island, NY). Growth medium was supplemented with 2 mM glutamine and 4 μg/ml transferrin (Life Technologies); 0.1 μM retinoic acid, 2 mM triiodothyronine, 1 μM hydrocortisone, 5 μg/ml insulin, and 50 μg/ml gentamicin (Sigma, St. Louis, MO); 50 ng/ml epidermal growth factor (Collaborative Biomedical Products/Becton Dickinson); and trace element mix (Biofluids, Rockville, MD). Cells were grown to confluence at 37°C in a humidified 95% air, 5% CO₂ incubator and used for experiments between passages 53 and 59.

Total RNA Isolation. Total cellular RNA was prepared from 100 mg of pulverized frozen rat brains, rat parotid glands, and confluent PAR-C5 cultures grown in T-75 tissue culture flasks using TRIzol (Life Technologies) according to the manufacturer’s instructions. Contaminating genomic DNA was removed from total RNA by treatment with RNase-free DNase I, followed by RNA extraction with water-saturated phenol-chloroform and precipitation with ethanol. The integrity of the RNA was confirmed by agarose gel electrophoresis. Total RNA was determined by measuring the absorbance at 260 nm with a Beckman DU-650 spectrophotometer (Fullerton, CA). Preparations were stored at −70°C.

RT-PCR. Approximately 1 μg of total RNA from either rat brains, rat parotid glands, or PAR-C5 cells was reverse transcribed using 25 pmol of random hexamers and 25 pmol of oligo (dT) primers. First-strand cDNA was synthesized from RNA preparations using 50 units of murine leukemia virus reverse transcriptase (PerkinElmer, Foster City, CA) in a 10-μl reaction volume containing 20 units of RNasin (Promega, Madison, WI), 1 mM dNTP, and 2.5 mM MgCl₂ in PCR buffer (Life Technologies). The reaction was incubated at room temperature for 15 min, and then at 42°C for 50 min, followed by denaturation for 5 min at 95°C in a RoboCycler Gradient 96 thermal cycler (Stratagene, La Jolla, CA). PCR was performed using muscarinic receptor subtype-specific oligonucleotide primers synthesized on an Applied Biosystems synthesizer (PerkinElmer). GenBank accession numbers are indicated for their respective muscarinic receptor subtype-specific primers: M₁, M16406; M₂, M68296; M₅, M16409; and M₆, M22992. The sense (S) and antisense (AS) primer sequences and their locations were as follows: CTGTTT-TCCTCGTGCTCTG (M₁-S; 593); GTCGCTTTCTTCTCTGT (M₂-AS; 1233); GCACAGAAGATCTAATATCAAA (M₅-S; 1084); GCCAAGGATGCGCAAGAT (M₆-AS; 1635); GTGTGTAGTG-ATTGCTGCTG (M₅-S; 2682); TCTGCCGGAGGGTTGGTGC (M₆-AS; 3471); TGGAGACAGTGGAGATGGTG (M₄-S; 72); AGGTAAAG-GGACGACTG (M₅-AS; 609); CTTACTTATCGCATCTTCCT (M₆-S; 1199). RT-PCR followed by agarose gel electrophoresis was performed with 50 μl of PCR mixture for 40 cycles of denaturation at 95°C for 45 s and annealing for 45 s (55°C for M₃₅₆₇ primers; 57°C for M₄ primers), followed by extension at 72°C for 45 s. After amplification, products were extended further by incubation at 72°C for 7 min.

Membrane Preparation. PAR-C5 cells were washed twice with phosphate-buffered saline and removed from T-75 tissue culture flasks with a rubber policeman. Cells were homogenized twice in 10 volumes of ice-cold 20 mM Tris buffer, pH 7.4, containing 154 mM NaCl and 2 mM MgCl₂ using a Janke & Kunkel Ultra-Turrax T25 homogenizer (Staufen, Germany) at 22,000 rpm for 10 s. The homogenate was centrifuged at 30,000 g for 15 min and the supernatant discarded. The membrane pellet was resuspended in Tris buffer, washed twice more by centrifugation, and stored at −78°C.

Male Sprague-Dawley rats (140–190 g) anesthetized with pentobarbital (50 mg/kg i.p.) and exsanguinated by cutting the abdominal aorta were used to obtain parotid glands. Parotid glands were removed; placed in ice-cold Krebs’ solution at pH 7.4 containing 118 mM NaCl, 4.7 mM KCl, 1.3 mM CaCl₂, 1.2 mM MgSO₄, 25 mM NaHCO₃, 1.2 mM KH₂PO₄, 11.7 mM dextrose; trimmed of visible fat, fascia, and lymph nodes; and then minced with iris scissors. The mince was centrifuged at 1000 g for 5 min at 4°C and the supernatant discarded. The pellet was resuspended in 20 volumes of Tris buffer and membranes prepared as described for PAR-C5 cells.

Radioligand Binding Assays. Membrane pellets were resuspended and homogenized in Tris buffer. For saturation binding experiments, total [N-methyl-³H]scopolamine binding was determined using duplicate tubes containing 300 μl of membrane suspension (45 and 60 μg/0.5 ml of assay volume for PAR-C5 cells and parotid...
glands, respectively), 100 μL of Tris buffer, and 100 μL of [N-methyl-3H]scopolamine ranging in concentration from 0.02 to 6 nM. To a parallel set of duplicate tubes, 100 μL of 1 μM atropine in Tris buffer was added to determine nonspecific binding. After a 30-min incubation in a shaking water bath at 37°C, membrane suspensions were filtered through GF/F glass fiber filter strips (Whatman, Maidstone, England) using a 48-sample cell harvester (Brandel, Gaithersburg, MD). Tubes and filters were washed three times with 5 mL of ice-cold Tris buffer and radioactivity retained on the filters counted by liquid scintillation spectroscopy. Specific binding was calculated as the difference between total and nonspecific binding. For competition binding experiments, duplicate tubes containing 300 μL of membrane suspension, 100 μL of 0.2 nM [N-methyl-3H]scopolamine, and 100 μL of increasing concentrations of various unlabeled drugs were incubated and processed as for saturation experiments. The protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as the standard.

Binding data were analyzed using a nonlinear least-squares curve-fitting program to determine IC50 and IC50 values from saturation binding experiments and IC50 values from competition binding experiments. IC50 values were calculated from IC50 values by using the method of Cheng and Prusoff (1973). All values are given as means ± S.E. The F test was used to determine whether the binding data fit best to a one- or two-site binding model. A value of p < 0.05 was used to conclude that the two-site model fit the data best.

Measurement of Intracellular Free Ca2+

Confluent PAR-C5 cultures were washed free of growth medium three times with room-temperature phosphate-buffered saline containing EDTA (Versene; Life Technologies). Aggregates of cells were harvested by incubation in an enzyme-free dissociation solution (Cellstripper; Meditech, Herndon, VA) supplemented with 2 mM EDTA for 20 min at 37°C and gentle trituration with a serological pipette. Cell aggregates were washed twice by centrifugation (3 min at 200g) with room-temperature HEPES-buffered Krebs’ solution (HBK), pH 7.4, containing 125 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 5 mM NaHCO3, 1.25 mM NaH2PO4, 11.1 mM dextrose, and 15 mM HEPES. Cell aggregates from one T-25 culture flask were divided into four equal volumes of HBK, plated onto four 35-mm tissue culture dishes modified with glass coverslip bottoms, placed in a 37°C room air incubator, and allowed to attach to the glass bottom (20 min). Attached cell aggregates were incubated in HBK containing 2 μM Fura-2 AM (Molecular Probes, Eugene, OR), 0.1 mg/ml bovine serum albumin, 0.02% (v/v) Cremophor EL, and 10 nM neostigmine bromide (Sigma) for 30 min in a 37°C room-air incubator. Cells were washed free of unincorporated Fura-2 AM three times with HBK containing either 200 nM physostigmine or 200 nM physostigmine and an appropriate concentration of competitive receptor antagonist, and then incubated for 15 min at 37°C before measurement of intracellular free Ca2+

An inverted fluorescence microscope connected to a PTI spectrofluorometer (Lawrenceville, NJ) was used to measure intracellular free Ca2+ in Fura-2-loaded cells in glass-bottom modified 35-mm culture dishes mounted on a 37°C heated stage. Concentration-response curves for methacholine-induced elevation in intracellular free Ca2+ were obtained in aggregates of ~30 cells and measured as the fluorescence emission ratio of Fura-2 alternately excited at 340 and 380 nm. Concentration-response curves were generated by exchanging HBK with HBK containing an appropriate concentration of methacholine, allowing the response to plateau, and then washing with HBK for several minutes before adding the next methacholine concentration. Concentration-response curves obtained from peak and plateau Ca2+ responses were compared and found not to be different (e.g., EC50 values were not significantly different). Thus, peak emission ratio responses to methacholine minus the emission ratios in the absence of methacholine were used to construct agonist concentration-response curves. Fura-2 loading of cells was staggered over time such that each dish of cells was exposed similarly to Fura-2. Half-maximal effective concentrations (EC50 values) of methacholine were calculated from concentration-response curves by nonlinear regression analysis of individual saturation binding isotherms indicated that [N-methyl-3H]scopolamine bound with high affinity to a single class of binding sites. The mean Kp value for [N-methyl-3H]scopolamine in binding to muscarinic receptors on PAR-C5 membranes was 0.38 ± 0.03 nM and the Bmax was

Results

RT-PCR Analysis of Muscarinic Receptor Subtypes in PAR-C5 Cells and Parotid Glands. We used RT-PCR to determine whether the pattern of expression of mRNA for the five muscarinic receptor subtypes was similar between native rat parotid glands and PAR-C5 cells. Figure 1 shows PAR-C5, parotid gland, and brain RT-PCR products using gene-specific primers for the five muscarinic receptor subtypes. Effectiveness of the primers was shown in rat brain, which expresses all five muscarinic receptor subtypes. For example, RT-PCR with M1, M2, M3, M4, and M5 receptor mRNA-specific primers resulted in products of 641, 552, 790, 538, and 451 bp, respectively, that were the correct size and identity to their respective published sequences. However, in rat parotid gland and PAR-C5 cells, mRNA for only the M3, M4, and M5 receptor subtypes was expressed, illustrated by the RT-PCR products of 790, 538, and 451 bp, respectively. These data suggest that there is a similar pattern of expression of muscarinic receptor subtype mRNA in PAR-C5 cells and rat parotid glands.

Radioligand Binding Characterization of Muscarinic Receptor Subtypes on PAR-C5 Cells and Parotid Glands. Specific [N-methyl-3H]scopolamine binding in PAR-C5 membranes is shown in Fig. 2. Nonlinear regression analysis of individual saturation binding isotherms indicated that [N-methyl-3H]scopolamine bound with high affinity to a single class of binding sites. The mean Kd value for [N-methyl-3H]scopolamine in binding to muscarinic receptors on PAR-C5 membranes was 0.38 ± 0.03 nM and the Bmax was
Specific N-methyl-3H]scopolamine binding was 80% of total binding at the K_D concentration. A mean Rosenthal plot derived from individual saturation binding isotherms is also illustrated in Fig. 2, inset. Results from [N-methyl-3H]scopolamine binding in parotid gland membranes were similar to those obtained in PAR-C5 membranes. The mean K_D value for [N-methyl-3H]scopol-

**Fig. 1.** Horizontal gel electrophoresis of products from reverse transcription and PCR amplification of total RNA from PAR-C5 cells, rat parotid, and rat brain using rat M_1, M_2, M_3, M_4, and M_5 gene-specific primers. M_1 and M_2 products from rat brain RNA reactions were synthesized in separate reactions but the products (641 and 552 bp, respectively) were electrophoresed together in one lane for this figure. Amplification of cDNA from reverse transcription reactions are indicated as a “+”. Control reactions to check for DNA contamination were run in the absence of reverse transcriptase and are indicated with a “−”. The marker consists of a ladder of multimers of a 100-bp DNA fragment. Effectiveness of primers is shown in brain (+ Brain), which expresses all five of the muscarinic receptor subtypes. M_3, M_4, and M_5, but not M_1 and M_2, receptor subtype mRNAs were present in PAR-C5 cells and rat parotid glands.

152 ± 25 fmol/mg of protein. Specific [N-methyl-3H]scopolamine binding was 80% of total binding at the K_D concentration. A mean Rosenthal plot derived from individual saturation binding isotherms is also illustrated in Fig. 2, inset. Results from [N-methyl-3H]scopolamine binding in parotid gland membranes were similar to those obtained in PAR-C5 membranes. The mean K_D value for [N-methyl-3H]scopol-

amino in binding to muscarinic receptors on parotid gland membranes was 0.64 ± 0.07 nM and the B_max was 82 ± 10 fmol/mg of protein (n = 3; data not shown).

We determined the muscarinic receptor subtypes present in membranes from PAR-C5 cells and parotid glands from competition binding experiments using subtype-selective drugs (Fig. 3). Table 1 lists the binding affinity constants (K_i values) of these drugs in PAR-C5 and parotid gland membranes and compares them with their previously reported affinity values at the five muscarinic receptor subtypes. Although our RT-PCR results showed that there was no mRNA for the M_1 and M_2 receptor subtypes, we obtained the K_i values for pirenzepine and methoctramine to determine whether receptor protein for these two subtypes was expressed in PAR-C5 cell and parotid gland membranes. Affinity values reported for pirenzepine range from 3.2 to 16 nM at the M_1 receptor subtype. Our K_i values for pirenzepine were 320 and 720 nM in PAR-C5 and parotid gland membranes, respectively. Our K_i values for methoctramine were 1400 nM (PAR-C5 cells) and 1700 nM (parotid glands), which are at least 100-fold different from methoctramine’s affinity at the M_2 receptor subtype. These data confirm our results from RT-PCR experiments and illustrate that PAR-C5 cells, like native parotid glands, do not express the M_1 or the M_2 receptor subtypes.

The results of our RT-PCR experiments identified mRNA for the M_3, M_4, and M_5 receptor subtypes in PAR-C5 cells and parotid glands. These data suggest PAR-C5 cells and parotid glands might coexpress these muscarinic receptor subtypes. However, competition binding curves for atropine, darifenacin, 4-DAMP, tripitramine, pirenzepine, and methoctramine inhibition of specific [N-methyl-3H]scopolamine binding fit best to a one-site binding model, suggesting the expression of only one receptor subtype or multiple subtypes with the same pharmacological characteristics. Because methoctramine, pirenzepine, and tripitramine exhibit relative high affinities for the M_4 receptor ranging from 6 to 79 nM, we were able to eliminate the possibility of M_4 receptor expression in PAR-C5 cells and parotid glands (Table 1). For example, we found relative low-affinity values for these drugs in PAR-C5 and parotid gland membranes, respectively (methoctramine,
Methacholine-Stimulated Ca\(^{2+}\) Mobilization in PAR-C5 Cells. Figure 4 illustrates a representative recording of the concentration-dependent responses for methacholine-induced elevation in intracellular free Ca\(^{2+}\) in PAR-C5 cells. In all cases, methacholine induced a biphasic increase in cytosolic Ca\(^{2+}\) characterized by an initial rapid, transient peak followed by a lower, sustained plateau. The inset (Fig. 4) shows the concentration-response curve for methacholine-induced Ca\(^{2+}\) mobilization obtained from the peak responses. The mean EC\(_{50}\) value for methacholine-stimulated Ca\(^{2+}\) mobilization in control PAR-C5 cells was 0.83 ± 0.1 μM (n = 22), consistent with the activation of muscarinic receptors.

**Functional Characterization of the Muscarinic Receptor Subtype Mediating Ca\(^{2+}\) Mobilization in PAR-C5 Cells.** To determine the muscarinic receptor subtype that mediates Ca\(^{2+}\) mobilization in PAR-C5 cells, we generated methacholine concentration-response curves in the presence of a variety of muscarinic receptor subtype-selective competitive antagonists. For example, Fig. 5A shows mean concentration-response curves in the absence and presence of increasing concentrations of 4-DAMP. 4-DAMP-induced shifts in the methacholine concentration-response curves are plotted according to the method of Arunlakshana and Schild (1959) (Fig. 5B). Table 1 compares our mean affinity values (K\(_B\) values) for 4-DAMP, atropine, pirenzepine, and methoctramine for inhibiting muscarinic receptor mediated Ca\(^{2+}\) mobilization in PAR-C5 cells with the published affinities of these drugs at all five of the muscarinic receptor subtypes. In the present study, the rank order of affinity values for antagonists inhibiting specific [N-methyl-\(^{3}\)H]scopolamine binding in PAR-C5 and parotid gland membranes, respectively, was atropine (1.0 and 2.1 nM) ≳ darifenacin (1.2 and 2.0 nM) ≳ 4-DAMP (2.9 and 2.4 nM) ≳ tripitramine (220 and 180 nM) ≳ pirenzepine (320 and 720 nM) ≳ methoctramine (1400 and 1700 nM). The affinity values obtained for individual antagonists in PAR-C5 cells compared with parotid glands are all within 2-fold of one another, illustrating the similarity of the pharmacological characteristics of the muscarinic receptors between PAR-C5 cells and native parotid glands.

**Fig. 3.** Mean competition binding curves showing muscarinic receptor antagonists inhibition of [N-methyl-\(^{3}\)H]scopolamine (\(^{3}\)H[NMS]) binding in membranes from PAR-C5 cells (A) and rat parotid glands (B). For each concentration of antagonists, \(^{3}\)H[NMS] binding is expressed as a percentage of the specific binding in the absence of any drug. One- and two-site binding models were fit to individual and mean competition binding curves by using a nonlinear least-squares curve-fitting program to obtain K\(_I\) values. Competition binding curves for all drugs examined fit best to a one-site binding model. The rank order of K\(_I\) values was atropine = darifenacin ≳ 4-DAMP ≳ tripitramine ≳ pirenzepine ≳ methoctramine and was the same in PAR-C5 cells and parotid glands.

1400 and 1700 nM; pirenzepine, 320 and 720 nM; tripitramine, 220 and 180 nM). However, the rank order of affinities of these drugs is consistent with the expression of the M\(_3\), the M\(_5\), or both receptor subtypes in PAR-C5 cells and parotid glands. The rank order of affinity values for antagonists inhibiting specific [N-methyl-\(^{3}\)H]scopolamine binding in PAR-C5 and parotid gland membranes, respectively, was atropine (1.0 and 2.1 nM) ≳ darifenacin (1.2 and 2.0 nM) ≳ 4-DAMP (2.9 and 2.4 nM) ≳ tripitramine (220 and 180 nM) ≳ pirenzepine (320 and 720 nM) ≳ methoctramine (1400 and 1700 nM). The affinity values obtained for individual antagonists in PAR-C5 cells compared with parotid glands are all within 2-fold of one another, illustrating the similarity of the pharmacological characteristics of the muscarinic receptors between PAR-C5 cells and native parotid glands.
affinities of drugs for inhibiting specific \(^{3}H\)scopolamine binding.

\(K_i\) for inhibiting muscarinic receptor-mediated elevation of intracellular free Ca\(^{2+}\).

\(N.D.,\) not determined.

**TABLE 1**

Comparison of affinities in nanomolar of various drugs for muscarinic receptor subtypes

<table>
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<tr>
<th>Drug</th>
<th>(K_i^a) (n = 4)</th>
<th>(K_i^b) (n = 4)</th>
<th>(K_i^c) (n = 4)</th>
<th>(K_i^d) (n = 4)</th>
<th>(K_i^e) (n = 4)</th>
<th>(K_i^f) (n = 4)</th>
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<tr>
<td>Atropine</td>
<td>0.44</td>
<td>1.0</td>
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<td>2.0</td>
<td>16–32</td>
<td>40–100</td>
<td>1.3–4.0</td>
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<tr>
<td>4-DAMP</td>
<td>0.38</td>
<td>2.9</td>
<td>2.4</td>
<td>0.6–2.5</td>
<td>4.0–16</td>
<td>0.5–1.3</td>
</tr>
<tr>
<td>Tripitramine</td>
<td>N.D.</td>
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<td>180</td>
<td>1.6–4.0</td>
<td>0.2–0.4</td>
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<tr>
<td>Pirenzepine</td>
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<td>3.2–16</td>
<td>200–500</td>
<td>79–200</td>
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<tr>
<td>Methoctramine</td>
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<td>1700</td>
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**Discussion**

Cell lines maintaining a high degree of cellular differentiation and similar phenotype to their native counterparts are indispensable tools for examining cellular function. For example, in vitro studies using immortalized epithelial cells from a variety of sources, including the human colon (Dharmathahorn et al., 1984) and canine kidney (Simmons, 1981) have contributed significantly to our knowledge of ion transport mechanisms. Studies with immortalized cells can also add to our understanding of the physiology and pharmacology of the tissues from which these cell lines originated. For instance, recently a human corneal epithelial cell line (Bockman et al., 1998) was used in the construction of functional corneal equivalents (Griffith et al., 1999). Unfortunately in salivary gland research, well differentiated acinar cell lines have only recently become available. The immortalized PAR-C5 cell line was recently cloned from rat parotid acinar epithelial cells and exhibits many of the phenotypical characteristics of fully differentiated native acinar cells that make it a useful model of salivary gland secretion (Quissell et al., 1998; Turner et al., 1998). However, the pharmacological characteristics of PAR-C5 cells have not been fully examined and compared with native parotid acinar cells. For example, muscarinic receptors are thought to be a major mechanism for fluid and electrolyte secretion in salivary glands, yet the muscarinic receptor subtypes on PAR-C5 cells have not been characterized. Thus, in side-by-side experiments comparing PAR-C5 cells and native parotid glands, we characterized the expression of muscarinic receptor subtype mRNA and receptor protein using RT-PCR and radioligand binding, respectively. We also determined the muscarinic receptor subtype mediating intracellular free Ca\(^{2+}\) mobilization to ascertain which subtype mediates Ca\(^{2+}\)-dependent secretion in PAR-C5 cells.

Reports of a heterogeneous population of muscarinic receptors in parotid glands from mice (Watson et al., 1996) and rats (Dehay et al., 1988) suggested that multiple muscarinic receptor subtypes might be present in PAR-C5 cells. Unfortunately, the pharmacological characterization of muscarinic receptor subtypes is hindered because there are no selective agonists, and antagonists lack high selectivity for any single subtype (Caulfield and Birdsall, 1998). In particular, lack of high antagonist selectivity can add to the difficulty of characterization when muscarinic receptor subtypes are coexpressed. Thus, we used RT-PCR in experiments comparing the expression of mRNAs for muscarinic receptor subtypes in PAR-C5 cells with those in parotid glands. RT-PCR analysis for muscarinic receptor subtypes identified mRNA for the \(M_1\), \(M_4\), and \(M_5\) receptor subtypes in both PAR-C5 cells and rat parotid glands. These results are consistent with reports by others who showed that the \(M_4\) receptor subtype, along with another incompletely characterized muscarinic receptor subtype, is present in native parotid glands (Dehay et al., 1988; Dai et al., 1991; Watson et al., 1996). Unlike these previous studies, we used RT-PCR with gene-specific primers for all five muscarinic receptor subtypes, which allowed us to obtain a more complete characterization of the muscarinic receptor subtypes present not only in parotid glands but also in PAR-C5 cells. Importantly, PAR-C5 cells express identical
our RT-PCR analysis showed that at the level of mRNA for muscarinic receptor subtype mRNAs as do native parotid glands, suggesting the PAR-C5 cell line is a valid model for secretion studies.

Our RT-PCR analysis showed that at the level of mRNA for muscarinic receptor subtypes, PAR-C5 cells and rat parotid glands are similar. However, we considered the possibility that immortalization and/or culture conditions may affect the pattern of expression of muscarinic receptor subtype mRNAs into receptor proteins. Thus, we used several muscarinic receptor subtype-selective antagonists in radioligand binding experiments comparing the pharmacological profile of the muscarinic receptor population in PAR-C5 cells with that of native parotid glands. The rank order of $K_I$ values (Table 1) was atropine = darifenacin > 4-DAMP > tripitramine > pirenzepine > methoctramine and was the same in both PAR-C5 cells and parotid glands. Moreover, $K_I$ values for all the drugs examined were essentially the same in PAR-C5 cells compared with parotid glands. We compared the $K_I$ values for atropine, darifenacin, 4-DAMP, tripitramine, pirenzepine, and methoctramine in our radioligand binding studies with their published affinity constants in Table 1. This comparison indicated that the M$_3$ receptor subtype is expressed in both PAR-C5 cells and parotid glands, because the $K_I$ values for all drugs examined agree with their known affinity constants at the M$_3$ receptor subtype. Additionally, competition binding curves fit best to a one-site binding model for all drugs examined in both PAR-C5 cells and parotid glands. These results indicate that the M$_3$ receptor is the predominant subtype in PAR-C5 cells, consistent with most reports that the M$_3$ receptor is also the major muscarinic receptor subtype in parotid glands (Dai et al., 1991; Sawaki et al., 1993). Overall, our data suggest PAR-C5 cells exhibit a similar phenotype to native parotid glands with respect to their muscarinic receptor subtype expression.

RT-PCR analysis also identified mRNA for the M$_4$ receptor subtype in both PAR-C5 cells and parotid glands. These results raised the possibility that in addition to the M$_3$ receptor subtype, receptor protein for the M$_4$ subtype might also be expressed in PAR-C5 cells and parotid glands. We used methoctramine, tripitramine, and pirenzepine in radioligand binding studies to determine whether the M$_4$ receptor subtype was expressed in PAR-C5 cell and parotid gland membranes. These drugs can distinguish the M$_4$ receptor subtype from the M$_3$ and M$_5$ receptor subtypes because they possess at least a 10-fold higher affinity for the M$_4$ receptor compared with the M$_3$ and M$_5$ receptor subtypes. We did not find any compelling evidence to suggest the presence of the M$_4$ receptor subtype protein expressed in PAR-C5 cell or parotid gland membranes. For example, $K_I$ values for the drugs examined were between 20- and 50-fold different from their reported affinities at the M$_4$ receptor subtype. Competition binding curves fit best to a one-site binding model. Our result showing that mRNA for the M$_4$ receptor subtype was not expressed as receptor protein in PAR-C5 cells or parotid glands is not unique to muscarinic receptors in salivary glands. For example in rat parotid gland, Abel et al. (1995) identified mRNA for the $\alpha_1$-adrenergic receptor subtype but found no evidence for its expression as receptor protein.

Because we identified mRNA for the M$_4$ receptor subtype in both PAR-C5 cells and parotid glands, we performed radioligand binding in membranes to determine whether M$_4$ receptor protein was present. We used darifenacin to distinguish the M$_4$ receptor subtype because it is reported to be between 5- and 10-fold selective for the M$_4$ over the M$_5$ receptor subtype (Eglen and Nahorski, 2000). $K_I$ values for darifenacin in PAR-C5 and parotid gland membranes were
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Fig. 6. Correlation plots of mean $pK_B$ values of atropine, 4-DAMP, pirenzepine, and methoctramine for inhibiting Ca\(^{2+}\) mobilization in PAR-C5 cells (y-axis) and the published affinity constants ($K_B$ values; x-axis) of these drugs at muscarinic receptor subtypes (M\(_1\), M\(_2\), M\(_3\), M\(_4\), and M\(_5\)). Mean $pK_B$ values for drugs in PAR-C5 cells are taken from Table 1. The $pK_B$ values and $pK_B$ values of drugs at muscarinic receptor subtypes are expressed as the range of literature values summarized in Caulfield and Birdsell (1998). The dashed line represents the line of identity and the solid line, the regression line of the plotted points. The correlation coefficient ($r^2$) is greatest for the comparison of the pharmacological profile between the muscarinic receptor mediating Ca\(^{2+}\) mobilization in PAR-C5 cells and the M\(_3\) receptor subtype.

1.0 and 2.1 nM, respectively, consistent with this drug’s high affinity at the M\(_3\) receptor subtype. Additionally, competition binding curves for darifenacin fit best to a one-site binding model. Taken together, these data suggest that M\(_3\) receptor subtype protein is not expressed in PAR-C5 cells or parotid glands. However, the selectivity of darifenacin for the M\(_3\) receptor subtype is modest. Thus, darifenacin may not be able to distinguish the M\(_2\) from the M\(_3\) receptor subtype in cells or tissues where the two receptor subtypes are coexpressed, particularly if the proportion of the M\(_3\) receptor subtype is low compared with the total muscarinic receptor population. Indeed, most muscarinic receptor antagonists have similar affinities for both of these receptor subtypes. However, a recent report summarizes current research that shows several drugs, including darifenacin, are preferential for the M\(_3\) over the M\(_2\) receptor subtype (Eglen and Nahorski, 2000). Nevertheless, none of these drugs possesses significantly greater ability than darifenacin to distinguish between the M\(_2\) and M\(_3\) receptor subtypes. It is noteworthy that radioligand binding techniques have revealed the presence of the M\(_3\) receptor subtype in rat submandibular glands (Flynn et al., 1997), suggesting the possibility that M\(_3\) receptors may also be expressed in low levels in PAR-C5 cells and parotid glands.

It is generally accepted that intracellular free Ca\(^{2+}\) mobilization is the primary signal transduction pathway for muscarinic receptor-stimulated salivary gland secretion (Ambudkar, 2000). Thus, for PAR-C5 cells to be a useful model of salivary secretion, it is important to show that the muscarinic receptor mediating Ca\(^{2+}\) mobilization in PAR-C5 cells and parotid acinar cells is the same. We then correlated these functionally determined drug affinity constants ($K_B$ values) with their published affinity constants at the five muscarinic receptor subtypes to show which muscarinic receptor subtype mediates the elevation in intracellular Ca\(^{2+}\) in PAR-C5 cells. The comparison of our $K_B$ values for atropine, 4-DAMP, pirenzepine, and methoctramine with their known affinities at the five muscarinic receptor subtypes correlated best with the M\(_3\) receptor subtype ($r^2 = 0.99$), followed by the M\(_2\) ($r^2 = 0.92$), the M\(_4\) ($r^2 = 0.90$), the M\(_5\) ($r^2 = 0.88$), and the M\(_1\) ($r^2 = 0.42$) receptor subtypes. Although none of these drugs is highly selective for one muscarinic receptor subtype over all the others, when taken together in correlation plots a complete pharmacological profile of the muscarinic receptor subtype mediating Ca\(^{2+}\) mobilization is revealed. These results show that the M\(_3\) receptor subtype mediates Ca\(^{2+}\) responses in PAR-C5 cells. It is generally accepted that in native rat parotid acinar cells, the M\(_2\) receptor subtype is also coupled to the elevation of intracellular free calcium and mediates fluid secretion (Dai et al., 1991; Sawaki et al., 1993). Thus, our results support the use of the PAR-C5 cell line as a model in functional studies of secretion.

In conclusion, the PAR-C5 cell line is novel because it has maintained a high degree of cellular differentiation and acinar function compared with previous parotid cell lines (Quissell et al., 1998; Turner et al., 1998). The similarity between PAR-C5 cells and native parotid acinar cells suggests an important use for the PAR-C5 cell line in vitro studies of salivary gland function. In the present study, we showed that PAR-C5 cells express a functioning M\(_2\) receptor subtype that is coupled to Ca\(^{2+}\) mobilization. The M\(_2\) receptor subtype and its coupling to Ca\(^{2+}\)-dependent secretion in salivary glands is thought to be a major mechanism for saliva formation; thus, our results add to the growing body of evidence that the PAR-C5 cell line is a valuable tool in salivary gland research. In addition, we found that at both the level of mRNA and receptor protein the pattern of expression of muscarinic receptor subtypes was identical between PAR-C5 cells and parotid glands. An unexpected but potentially important observation was the identification of mRNA for the M\(_3\) receptor subtype in both PAR-C5 cells and parotid glands. These results not only highlight the similarity between the PAR-C5 cell line and the parotid gland, but also provide new information about the possibility of additional muscarinic receptor subtypes in the native gland.

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