Characterization of Cholinergic Receptors in Madin-Darby Canine Kidney Cells[1,2]

Dagmara Mohuczy-Dominiak and Lal C. Garg[3]

D. Mohuczy-Dominiak, L.C. Garg, Department of Pharmacology and Therapeutics and Division of Nephrology, Hypertension and Transplantation, University of Florida College of Medicine, Gainesville, FL

(J. Am. Soc. Nephrol. 1992; 3:170-181)

ABSTRACT

Muscarinic-type cholinergic receptors coupled to the phosphoinositide (PI) second messenger system are reported to be present in the inner medullary collecting duct cells. Madin-Darby canine kidney (MDCK) cells have several characteristics of collecting duct cells and have been shown to respond to muscarinic agonists. To determine if MDCK cells have PI-coupled muscarinic receptors, the radioligand binding and the effects of cholinergic agonists and antagonists on PI hydrolysis in MDCK cells were studied. The specific binding of (^{3}H)1-quinuclidinyl benzilate ((^{3}H)QNB), a muscarinic antagonist, to MDCK cell membranes had a K_d = 88 ± 7 pM and a B_{max} = 1464 ± 88 fmol/mg of protein. The displacement of (^{3}H) QNB from MDCK cell membranes by various cholinergic antagonists and agonists showed the order of potency: atropine > 4-diphenylacetoxy *N*-methylpiperidine (4-DAMP) > *p*-fluorohexahydrosiladifenidol > pirenzepine > methoctramine > arecoline > carbachol. The cholinergic agonists carbachol and arecoline stimulated PI hydrolysis in a concentration-dependent manner with an EC$_{50}$ of 3.7 and 1.3 μM, respectively. Muscarinic antagonists abolished carbachol-stimulated PI hydrolysis in the following order of potency: atropine > 4-DAMP > pirenzepine ≫ methoctramine. The order of potency of muscarinic antagonists is consistent with the characteristics of the M$_3$ subtype of muscarinic receptors. It is concluded that: (*1*) muscarinic receptor density in MDCK cells is 50 times higher than that in inner medullary collecting duct cells; (*2*) muscarinic receptors in MDCK cells are putative M$_3$ subtype; and (*3*) muscarinic receptors in MDCK cells are functionally coupled to the PI second messenger system. This intracellular messenger system may, at least, be partially responsible for the action of cholinergic agonists in these cells and in the kidney.

Key Words: *Arecoline, atropine, carbachol, cholinergic receptor, muscarinic receptor, phosphoinositide hydrolysis, 4-diphenylacetoxy* N-*methypiperidine pirenzepine,* p-*fluorohexahydrosiladifenidol, methoctramine, cAMP, 1-quinuclidinyl benzilate*

Although renal actions of cholinergic drugs have been recognized for more than 25 yr (1,2), the specific cholinergic receptors in the inner medullary collecting duct (IMCD) cells have been reported only recently (3). However, the receptor density in these cells is small (27.5 fmol/mg of protein). Madin-Darby canine kidney (MDCK) cells have several characteristics of renal collecting duct cells (4). In addition, acetylcholine has been shown to hyperpolarize the plasma membrane of MDCK cells and the action is blocked by atropine (5), suggesting that the effect is mediated by muscarinic receptors. However, muscarinic receptors in MDCK cells have not been characterized.

Pharmacologically, muscarinic receptors have been classified into three subtypes (6). M$_1$ receptors have a high affinity for pirenzepine, whereas M$_2$ receptors have a low affinity for pirenzepine and a high affinity for methoctramine. M$_3$ receptors have a low affinity for pirenzepine but have a high affinity for 4-diphenylacetoxy *N*-methylpiperidine (4-DAMP). Up to five different molecular forms of muscarinic receptors (denoted by m$_1$ to m$_5$) have been cloned from rat and human tissues (6). The receptor subtypes m$_1$, m$_2$, and m$_3$ have characteristics similar to those of M$_1$, M$_2$, and M$_3$, respectively.

Depending on the receptor subtype, the activation of muscarinic receptors has been shown to produce

[1] Received January 10, 1992. Accepted April 10, 1992.

[2] A part of this work has been published in abstract form (J Am Soc Nephrol 1991;2:460).

[3] Correspondence to Dr. L.C. Garg, Department of Pharmacology and Therapeutics, University of Florida Health Science Center, P.O. Box 100267, Gainesville, FL 32610-0267.

1046-6673/0302-0170$03.00/0
Journal of the American Society of Nephrology
Copyright © 1992 by the American Society of Nephrology

an increase in phosphoinositide (PI) hydrolysis or a decrease in cAMP. In general, M_1 and M_3 receptors are coupled to PI hydrolysis, whereas M_2 receptors are coupled to inhibition of the adenylate cyclase system (6). Recently, we have demonstrated that carbachol, a cholinergic agonist, stimulates PI hydrolysis in IMCD cells via the activation of muscarinic receptors (7). However, it is not known which subtype of muscarinic receptor is involved in PI hydrolysis in IMCD cells.

The purpose of this study was to characterize muscarinic receptors in MDCK cells, including the determination of the subtype and coupling with second messenger system, namely PI hydrolysis. To perform these studies, we used [^{3}H]1-quinuclidinyl(phenyl-4-^{3}H)-benzilate ([^{3}H]QNB), a muscarinic antagonist, as a radioligand and atropine as a nonselective muscarinic antagonist. Carbachol and arecoline were used as cholinergic agonists and pirenzepine, methoctramine and 4-DAMP were used as M_1, M_2, and M_3 selective muscarinic antagonists, respectively.

METHODS

Culturing and Preparing MDCK Cells

MDCK cells (ATCC No. CCL 34) were grown in monolayers in 75-cm^2 culture flasks at 37°C in 95% air–5% CO_2 in Dulbecco's modified Eagle's medium (Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (Sigma), $NaHCO_3$ (44 mM), penicillin G (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (0.25 μg/mL). Cells were passaged by trypsinization, seeded into 60-mm culture dishes, and used for experiments between the seventh and ninth days of growth, while confluent.

For ligand binding experiments, cells were washed with phosphate-buffered saline (pH 7.4) and scraped from the plates with a rubber policeman. The cells were then washed with ice-cold 50 mM Tris buffer containing 10 mM $MgCl_2$ (pH 7.6) and homogenized three times for 10 s with a Polytron homogenizer (Brinkmann Instruments Inc., Westbury, NY) at setting 5. Cell homogenate was centrifuged at 4°C at 35,000 × *g* for 15 min, and the membrane pellet was resuspended in Tris buffer at a protein concentration of ~300 μg/mL. The protein concentration was determined by the method of Lowry *et al.* (8) with BSA as a standard.

For PI hydrolysis, cells were labeled with 2-[^{3}H] myo-inositol (2 μCi/mL) in MDCK medium 48 h before the experiment. In our pilot experiments, we had determined that the labeling of membrane PI with 2-[^{3}H]inositol reached a plateau in 48 h. Cell surfaces

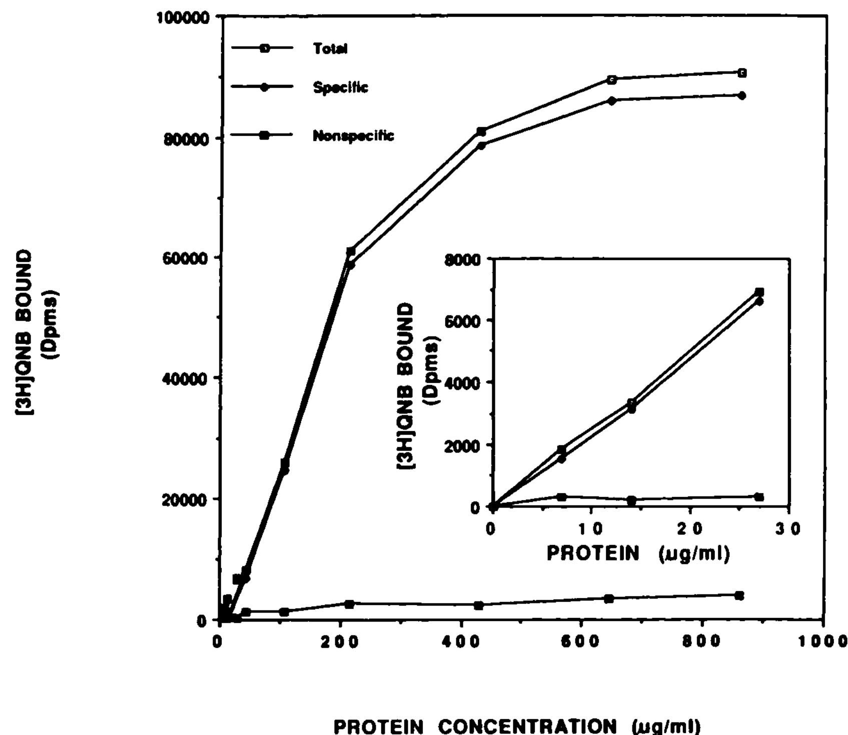

Figure 1. Protein dependency of (^{3}H)QNB binding to MDCK cell membranes. Different amounts of cell membranes were incubated with 0.6 nM (^{3}H)QNB for 1 h at 37°C. The total binding was determined in the absence of atropine, and the nonspecific binding was counted in the presence of 1 μM atropine at each protein concentration. The specific binding was calculated as a difference between the total and the nonspecific binding. Inset: Amplification of the protein concentration range from 0 to 30 μg/mL. Each datum point is an average of three experiments done in duplicate.

were washed with Krebs-Ringer bicarbonate (KRB) buffer, which contained (millimolar): NaCl, 118; KCl, 4.7; $CaCl_2$, 0.75; $MgSO_4$, 1.18; KH_2PO_4, 1.18; $NaHCO_3$, 24.8; glucose, 10; supplemented with 50 mM LiCl to inhibit the conversion of inositol-1-phosphate to inositol (9). MDCK cells require a relatively high concentration (50 mM) of LiCl. This was observed in our preliminary experiments and has also been reported by other investigators (10). Cells were scraped from the plates with rubber policeman. Cells were washed three times with KRB and centrifuged every time at low speed for 3 min. The cell pellet was dissolved in KRB in a protein concentration of about 10 mg/mL and preincubated in this buffer for 15 min in 4°C.

Radioligand Binding Assays

Cholinergic receptors were determined by measuring the binding of [^{3}H]QNB to MDCK cell membrane preparations. Membranes (~30 μg of protein) were incubated with the radioligand in Tris buffer in a final volume of 2 mL for 1 h at 37°C. Nonspecific binding was determined in the presence of 1 μM atropine. Duplicate samples were used for each treatment. The reaction was terminated by filtering the samples under vacuum over Whatman GF/B filters in a Brandel M24 Cell Harvester (Brandel Biomedical Research and Development Laboratories, Gaithersburg, MD), and the filters were washed three times with 5 mL of cold Tris buffer. Ten milliliters of Liquiscint (National Diagnostics, Manville, NJ) was added to the filters, and radioactivity was counted in a Beckman LS7000 scintillation counter (Beckman Instruments, Fullerton, CA) with an efficiency of approximately 60%.

For the determination of the time required to reach the binding equilibrium, membranes were incubated with 0.6 nM [^{3}H]QNB for various times before filtration. For receptor saturation studies, six increasing concentrations of radioligand were used. In competition studies, 0.6 nM [^{3}H]QNB was used and usually nine concentrations of each compound were examined. The radioligand binding data were analyzed by computerized program EBDA. Values of IC_{50} and slope factor (Hill coefficient) were estimated by using an iterative curve-fitting technique (Equilibrium Binding Data Analysis of a competition study). K_i values were calculated by using the formula of Cheng and Prusoff (11).

PI Hydrolysis

Incubation was performed in a total volume of 250 μL. To 130 to 140 μL of KRB (containing LiCl), 10 μL of agonist alone or 10 μL of agonist and 10 μL of

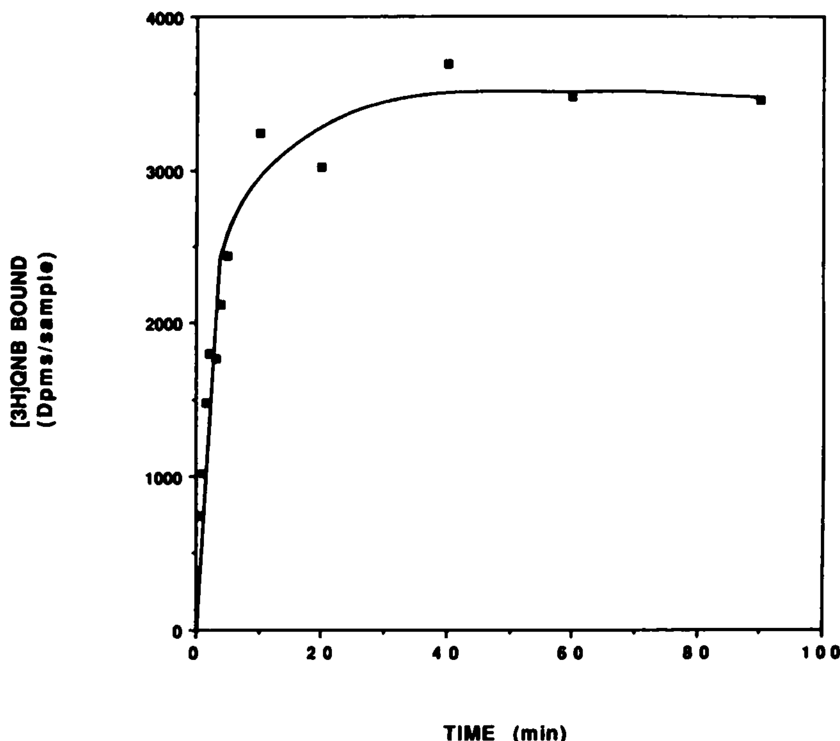

Figure 2. Time course of specific (^{3}H)QNB binding to MDCK cell membranes. Cell membranes (~15 μg/mL) were incubated with 0.6 nM (^{3}H)QNB at 37°C at the times indicated. The specific binding was calculated as a difference between the total (in the absence of atropine) and the nonspecific (in the presence 1 μM atropine) binding at each time point. Each datum point is an average of three experiments performed in duplicate.

antagonist were added. Reaction was started by the addition of 100 μL of cell suspension. The tubes were saturated with 95% O_2–5% CO_2 and capped. The tubes were incubated in a shaking water bath at 37°C for 60 min, unless otherwise indicated. The reaction was terminated by the addition of 1 mL of chloroform:methanol (1:2 vol/vol) to the tubes, followed by 0.35 mL of distilled water and 0.35 mL of chloroform. The tubes were capped and vortexed for 1 min before centrifugation at low speed for 5 min to separate aqueous and organic phases.

In studies with forskolin, there was a 10-min preincubation with this compound alone, followed by a 50-min incubation together with 1 mM carbachol. In experiments with 8-chloro-phenylthio–CAMP (8CPT–cAMP), there was a 15-min preincubation with this compound alone, followed by an incubation together with 1 mM carbachol for 5, 15, 30, and 50 min.

In studies with pertussis toxin, MDCK cells were incubated with pertussis toxin (1.5 to 150 ng/mL) for 4.5 h before the measurement of carbachol-stimulated PI hydrolysis. The incubation of cells with pertussis toxin for 4 h (or more) has been shown to block the agonist-stimulated PI hydrolysis and/or calcium release in cells where the effect is mediated via pertussis toxin–sensitive G proteins (12,13).

Determination of Inositol Phosphates

A 200-μL aliquot of the lower organic phase that contained unhydrolyzed [^{3}H]PI was placed in a glass scintillation vial. The chloroform was allowed to evaporate in a stream of air. Three milliliters of Organic Counting Scintillant (Amersham Corp., Arlington Heights, IL) was added, vials were vortexed, and radioactivity was determined in a Beckman LS7000 scintillation counter with an efficiency of about 60%.

A 750-μL aliquot was removed from the upper aqueous layer and diluted to 3 mL with water. One milliliter of 50% (vol/vol) Dowex AG 1-X8, 100 to 200 mesh, formate form (Bio-Rad Laboratories, Richmond, CA) was added. The slurry was poured over polypropylene columns (Bio-Rad Laboratories, Rockville Centre, NY). After the liquid was allowed to drain, the Dowex was washed three times with 5 mL of 5 mM inositol to remove all free [^{3}H]inositol. Glycerophosphoinositol was eluted with 5 mL of 5 mM sodium tetraborate–60 mM sodium formate. In our pilot experiments, we eluted inositol monophosphate ([^{3}H]IP$_1$), inositol bisphosphate ([^{3}H]IP$_2$), and inositol

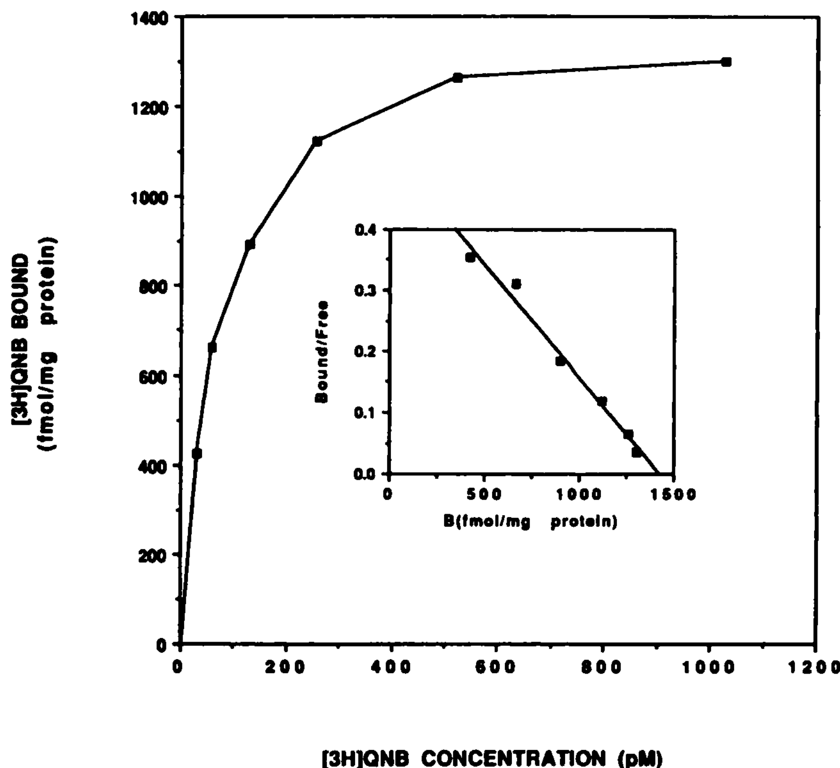

Figure 3. Saturation of (^{3}H)QNB binding to MDCK cell membranes. Cell membranes (~15 μg/mL) were incubated with increasing concentrations of (^{3}H)QNB for 60 min at 37°C. The specific binding was calculated as a difference between the total (in the absence of atropine) and the nonspecific (in the presence of 1 μM atropine) binding for each QNB concentration. Inset: Scatchard plot of the saturation curve. B, bound. Data are representative of results from six experiments performed in duplicate.

trisphosphate ([^{3}H]IP$_3$) from a Dowex-1-anion exchange column serially with 5 mL of 0.1 formic acid–0.2 M ammonium formate, 5 mL of 0.1 formic acid–0.4 M ammonium formate, and 5 mL of 0.1 M formic acid–1.0 M ammonium formate, respectively. The results showed that more than 90% of the radioactivity of inositol phosphates (IP) was in [^{3}H]IP$_1$ and that there was very little radioactivity in [^{3}H]IP$_2$ and [^{3}H]IP$_3$ because of the rapid hydrolysis of IP$_3$ to IP$_2$ and IP$_1$ (9,14). Therefore, after removing [^{3}H]inositol and [^{3}H]glycerophosphoinositol, we eluted total [^{3}H]IP ([^{3}H]IP$_1$ + [^{3}H]IP$_2$ + [^{3}H]IP$_3$) with 1.0 M ammonium formate–0.1 formic acid. Eluted radioactivity was determined after the addition of 10 mL of Liquiscint in a Beckman LS7000 scintillation counter with an efficiency of approximately 60%.

At the start of the assay (time zero), 1 mL of the chloroform-methanol mixture was added to the samples containing cells without any drug. These zero time samples were processed with other samples, and their radioactivity of the aqueous phase was subtracted from the radioactivity of all other samples after incubation.

Chemicals

The radiolabeled ligand [^{3}H]QNB (44 Ci/mmol) was obtained from DuPont, NEN Research Products (Boston, MA). Myo-2-[^{3}H]inositol (18 to 19 Ci/mmol) was supplied by Amersham Corp. Carbachol, atropine sulphate, pirenzepine dihydrochloride, myo-inositol, forskolin, and 8-(4-chlorophenylthio)–cAMP were obtained from Sigma Chemical Co. Lithium chloride was purchased from Matheson Coleman & Bell (Norwood, OH). Methoctramine tetrahydrochloride, arecoline hydrobromide, 4-DAMP methiodide, *p*-fluorohexahydrosiladifenidol hydrochloride (*p*-FHHSiD), and tetraethylammonium chloride (TEA) were obtained from Research Biochemicals Inc. (Natick, MA). Other reagents were purchased from Fisher Scientific (Orlando, FL).

Statistical Analysis

Unless stated otherwise, all experiments were performed in duplicate samples and the mean of this

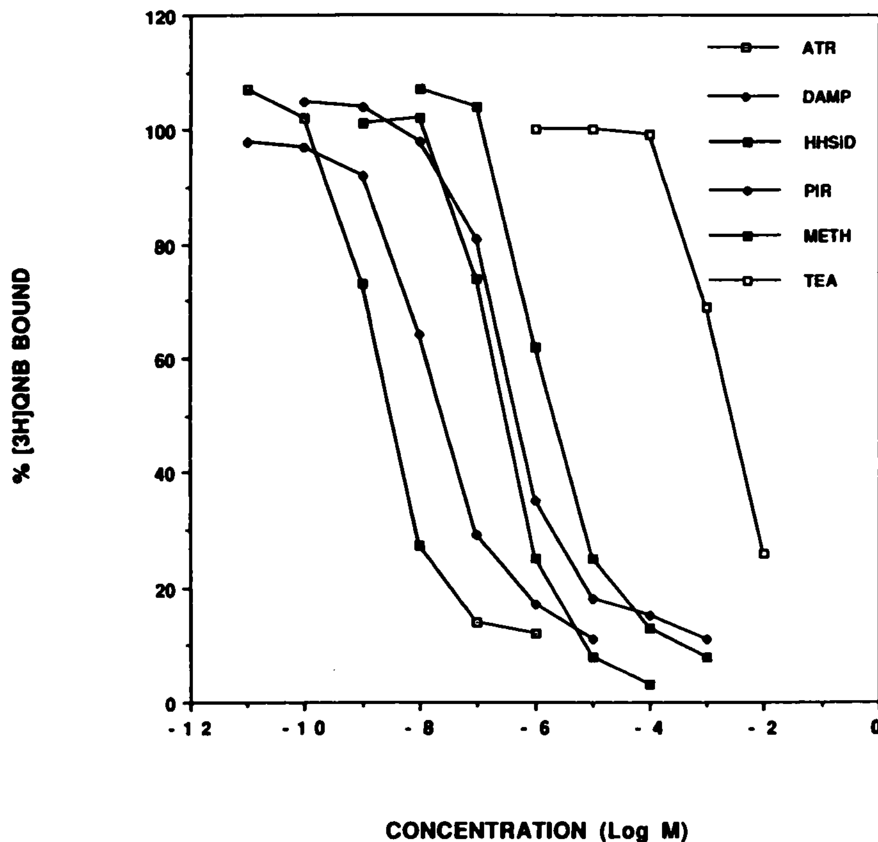

Figure 4. Displacement of the [^{3}H]QNB sites by muscarinic antagonists. MDCK cell membranes (~15 μg/mL) were incubated with 0.6 nM [^{3}H]QNB for 60 min at 37°C in the presence of different concentrations of each antagonist. ATR, atropine; DAMP, 4-DAMP; HHSID, *p*-FHHSiD; PIR, pirenzepine; METH, methoctramine. Each datum point is a mean of the results from three experiments performed in duplicate.

duplicate was taken as $N = 1$. The results were expressed as the means ± SE. Differences analyzed by t test were considered as significantly different from each other if $P < 0.05$.

RESULTS

Protein Dependency of (^{3}H)QNB Binding

The binding of [^{3}H]QNB (0.6 nM) at 37°C to MDCK cell membranes was dependent on the concentration of the protein (Figure 1). Binding increased with increasing protein concentration, and specific binding was saturated at a concentration of approximately 600 μg/mL. The nonspecific binding was very low—less than 10% of the total binding at all protein concentrations. For characterization of the cholinergic receptors in MDCK cells, we used a final concentration of protein ~15 μg/mL, because this concentration was in the linear range of [^{3}H]QNB binding (Figure 1, inset) and because not more than 10% of the radioligand added to the incubation mixture was bound.

Time Dependency of (^{3}H)QNB Binding

Figure 2 shows that the specific binding of [^{3}H]QNB (0.6 nM) to the membranes of MDCK cells reached at its maximum in approximately 20 min at 37°C. The specific binding remained the same at least up to 90 min. We used 60 min of incubation in all of our experiments.

Saturation of (^{3}H)QNB Binding Sites

The specific binding of [^{3}H]QNB to MDCK cell membranes increased with increasing concentrations of the radiolabeled ligand and reached a plateau at 0.6 nM (Figure 3). These results indicate that the specific binding sites for [^{3}H]QNB in MDCK are saturable. Scatchard analysis of the saturation data resulted in a straight line and indicated one type of binding site with a K_d of 88 ± 7 pM and a B_{max} of 1,464 ± 88 fmol/mg of protein (Figure 3, inset).

Displacement of (^{3}H)QNB by Cholinergic Agonists and Antagonists

The incubation of the membranes prepared from MDCK cells with [^{3}H]QNB in the presence of various

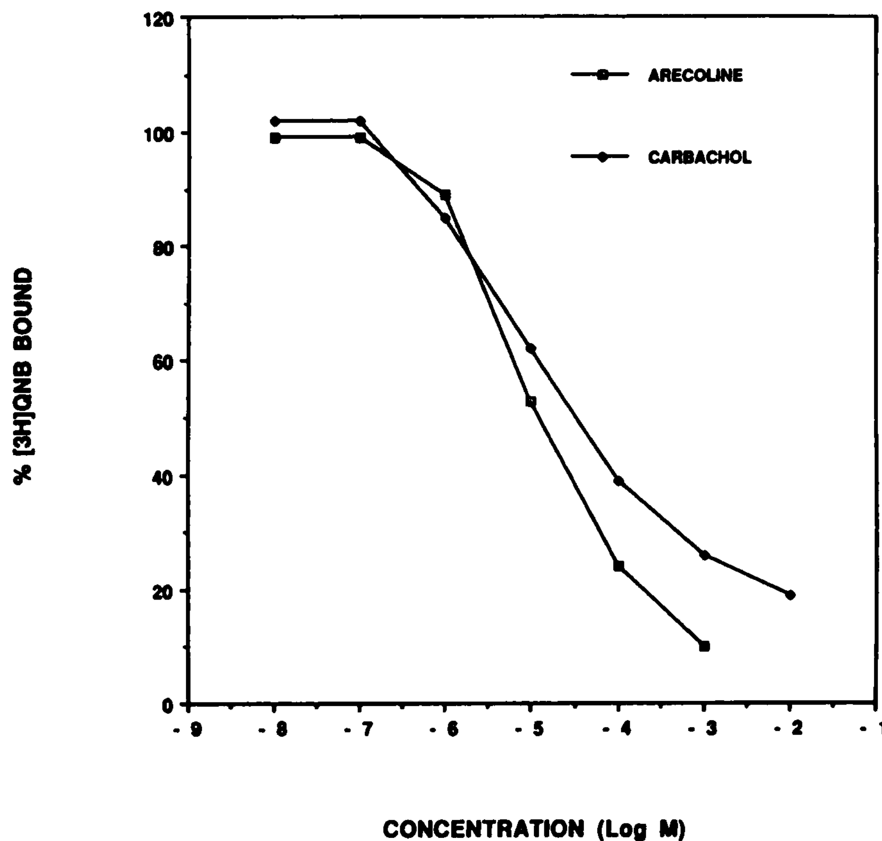

Figure 5. Displacement of the (^{3}H)QNB sites by cholinergic agonists. MDCK cell membranes (~15 μg/mL) were incubated with 0.6 nM (^{3}H)QNB for 60 min at 37°C in the presence of different concentrations of arecoline or carbachol. Each datum point is a mean of results from three experiments performed in duplicate.

cholinergic antagonists produced a concentration-dependent inhibition of the binding (Figure 4). Atropine was the most active and methoctramine was the least active in displacing [^{3}H]QNB from MDCK cell membranes (Figure 4). TEA, a nicotinic-type cholinergic antagonist, did not affect the binding of [^{3}H]QNB at a concentration as high as 1 mM (Figure 4). Of the cholinergic agonists, arecoline was more active than carbachol (Figure 5). The IC_{50} and K_i values of all cholinergic compounds used are given in Table 1. The displacement of [^{3}H]QNB binding to MDCK cells by various cholinergic antagonists and agonists expressed the following order of potency: atropine > 4-DAMP > *p*-FHHSiD > pirenzepine > methoctramine > arecoline > carbachol > TEA.

TABLE 1. Comparison of IC_{50}, K_i, and Hill coefficients of cholinergic agonists and antagonists for the displacement of (^{3}H)QNB binding to MDCK cell membranes[a]

Drug	IC_{50} (nM)	K_i (nM)	Hill Coefficient
Antagonists			
Atropine	2	0.3	0.98
4-DAMP	16	NC[b]	0.67
p-FHHSiD	280	39	0.98
Pirenzepine	310	36	0.91
Methoctramine	1,200	150	0.99
TEA	>1 mM	0.22 mM	1.07
Agonists			
Arecoline	11,000	NC[b]	0.78
Carbachol	20,000	NC[b]	0.54

[a] Values represent the means from three experiments done in duplicate.

[b] NC, K_i was not calculated for the ligands that have a Hill coefficient significantly less than 1.

Time Course of PI Hydrolysis

The effect of 1 mM carbachol on the accumulation of total IP was measured for 5 to 60 min (Figure 6). The maximal production of IP was reached at 30 min and remained at the same level at 60 min. An incubation time of 60 min was chosen for all further experiments.

Concentration Response Curves of Cholinergic Agonists for PI Hydrolysis

The formation of [^{3}H]IP was dependent upon the concentrations of the cholinergic agonists carbachol and arecoline (Figure 7). The maximal effective con-

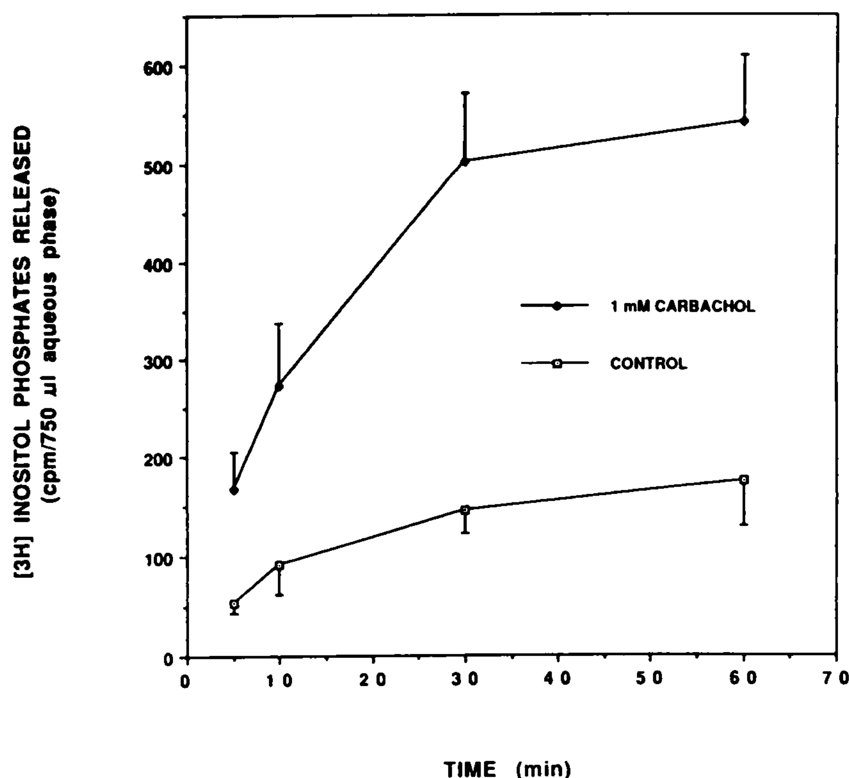

Figure 6. Time course of the formation of total IP in MDCK cells with and without carbachol (1 mM). Values are means ± SE of three experiments performed in duplicate.

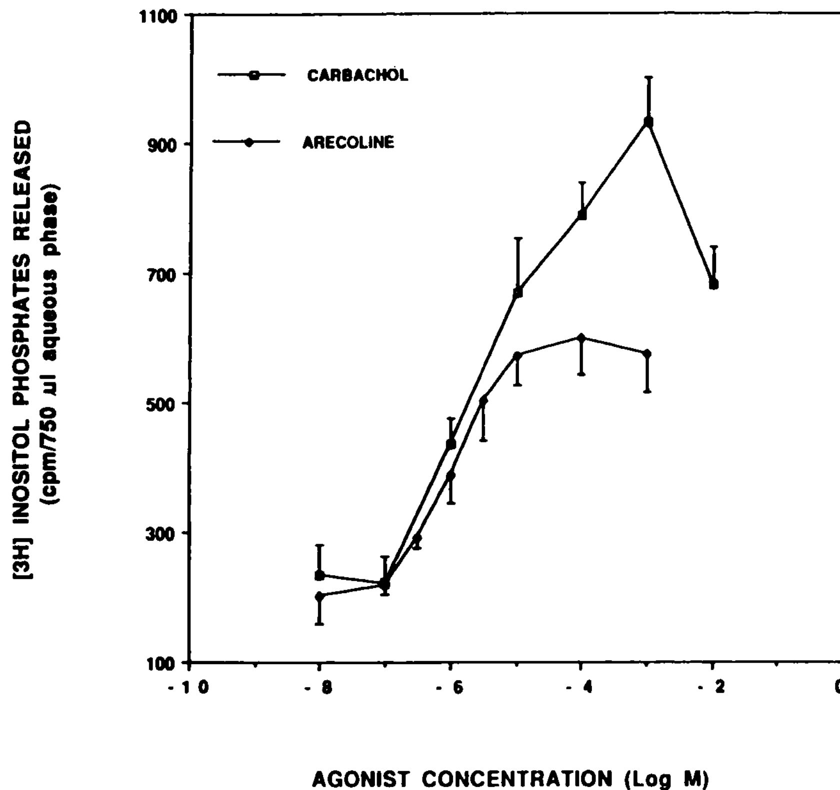

Figure 7. Concentration-response curve of carbachol and arecoline for the stimulation of PI hydrolysis in MDCK cells. Values are means ± SE of three to four experiments done in duplicate.

centration of carbachol was 1 mM, and the EC_{50} was 3.7 μM. The maximal stimulation with arecoline occurred at 0.1 mM, and its EC_{50} was 1.3 μM. However, the maximal [^{3}H]IP released by arecoline was only 65% of that obtained with 1 mM carbachol. Therefore, we chose 1 mM carbachol for further experiments.

Effect of Pertussis Toxin Treatment on Carbachol-Stimulated PI Hydrolysis

Pertussis toxin treatment at a concentration of 1.5 ng/mL decreased carbachol-stimulated PI hydrolysis in MDCK cells by 66% (Table 2). Carbachol response was completely blocked at 150 ng of pertussis toxin/mL. These data suggest that carbachol stimulates PI hydrolysis in MDCK cells via a pertussis toxin-sensitive G protein.

TABLE 2. Effect of pertussis toxin treatment on PI hydrolysis[a]

Treatment	[^{3}H]IP released (dpm/sample)		% Inhibition
	Control	Carbachol (1 mM)	
None	169 ± 48	2107 ± 261	0
Pertussis toxin (ng/mL)			
1.5	271 ± 90	936 ± 168	66
15	242 ± 56	595 ± 52	82
50	153 ± 47	309 ± 42	92
150	144 ± 50	191 ± 39	>97

[a] Values represent mean ± SE from three experiments.

Effects of Cholinergic Antagonists on Carbachol-Stimulated PI Hydrolysis

Three muscarinic antagonists decreased the carbachol-stimulated PI hydrolysis by 80% at 10^{-4} M (or lower) concentrations. The potency order of these compounds was: atropine (EC_{50} = 51 nM) > 4-DAMP (EC_{50} = 0.56 μM) > pirenzepine (EC_{50} = 10 μM) (Figure 8). The M_2-selective muscarinic antagonist methoctramine, at 10^{-4} M concentration, caused only a 30% decrease in carbachol-stimulated [^{3}H]IP formation. At 10^{-3} M concentration of methoctramine, there was a small but consistent stimulation of PI hydrolysis. The M_3 muscarinic antagonist *p*-FHHSiD up to 10^{-4} M did not antagonize the effect of carbachol on IP formation. *p*-FHHSiD (3×10^{-4} M) caused the stimulation

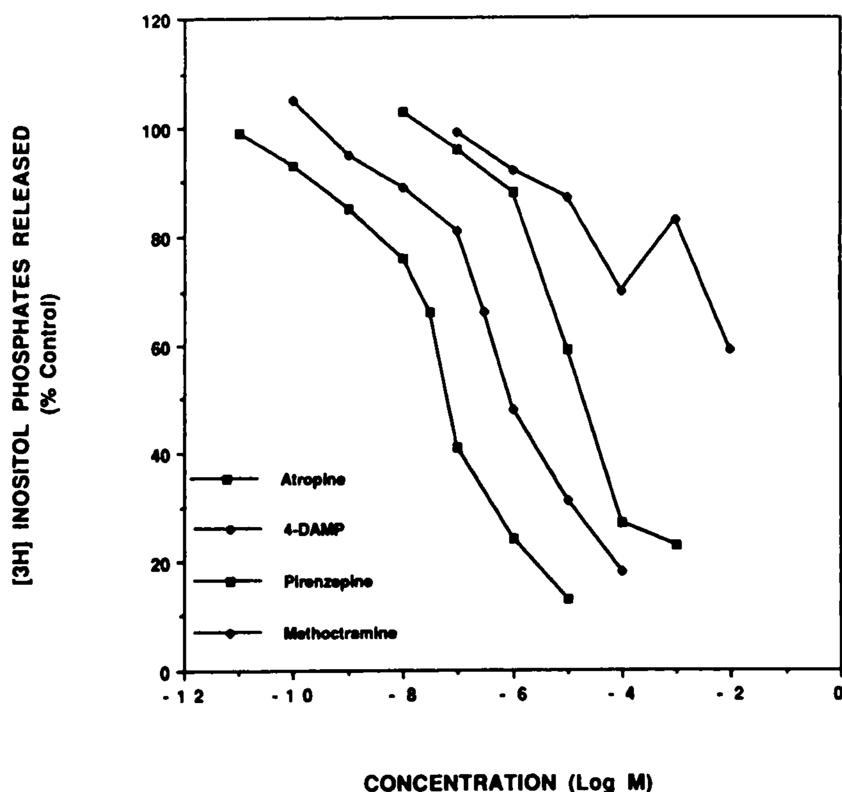

Figure 8. Effects of muscarinic antagonists on 1 mM carbachol-stimulated PI hydrolysis in MDCK cells. Values are means ± SE of three to four experiments performed in duplicate.

of IP formation similar to methoctramine. The stimulatory effects of methoctramine and *p*-FHHSiD on PI hydrolysis were even greater in the absence of carbachol and were not reversed by 1 μM atropine (data not shown); therefore, the stimulatory effect of these compounds was not mediated by muscarinic receptors. Methoctramine (>10^{-4} M) and *p*-FHHSiD (>10^{-4} M) caused increases in the radioactivity in the lipid fraction (data not shown), suggesting a stimulation of PI synthesis by these drugs.

Effects of cAMP on Carbachol-Stimulated PI Hydrolysis

When MDCK cells were exposed to 10^{-4} M 8-CPT-cAMP (an analog of cAMP resistant to phosphodiesterase) for 15 min before incubation in the absence (control) or presence of 1 mM carbachol for different times, a decrease in IP release was observed (Table 3). The maximal inhibition of IP formation was seen after 15 min of incubation with or without carbachol. Forskolin at 10^{-4} M caused a 20% decrease in [^{3}H]IP release in the presence of 1 mM carbachol (Figure 9). These results indicate that cAMP produces a small decrease in PI hydrolysis in MDCK cells.

TABLE 3. Effect of cAMP on PI hydrolysis in MDCK cells

Experiment	[^{3}H]IP released (% of carbachol stimulated)			
	5 min	15 min	30 min	50 min
Carbachol	100	100	100	100
Carbachol + cAMP[a]	75 ± 7.4	69 ± 6.5[b]	73 ± 7.9[b]	90 ± 5
Basal	52 ± 1.4	37 ± 3.5	36 ± 3.6	27 ± 0.3
Basal + cAMP[a]	45 ± 3.5	22 ± 3.4[b]	24 ± 1.7	29 ± 1.4

[a] Cells were exposed to 0.1 mM 8-CPT-cAMP for 15 min before incubation with or without 1 mM carbachol for 5, 15, 30, and 50 min. Values are means ± SE of three to five experiments done in duplicate.
[b] $P < 0.05$ versus same treatment but without cAMP.

DISCUSSION

Although a recent electrophysiologic study suggested that muscarinic-type cholinergic receptors are present in MDCK cells (5), this is the first study that characterizes these receptors. We have demonstrated that high-affinity specific binding sites for the muscarinic receptor antagonist [^{3}H]QNB are present in

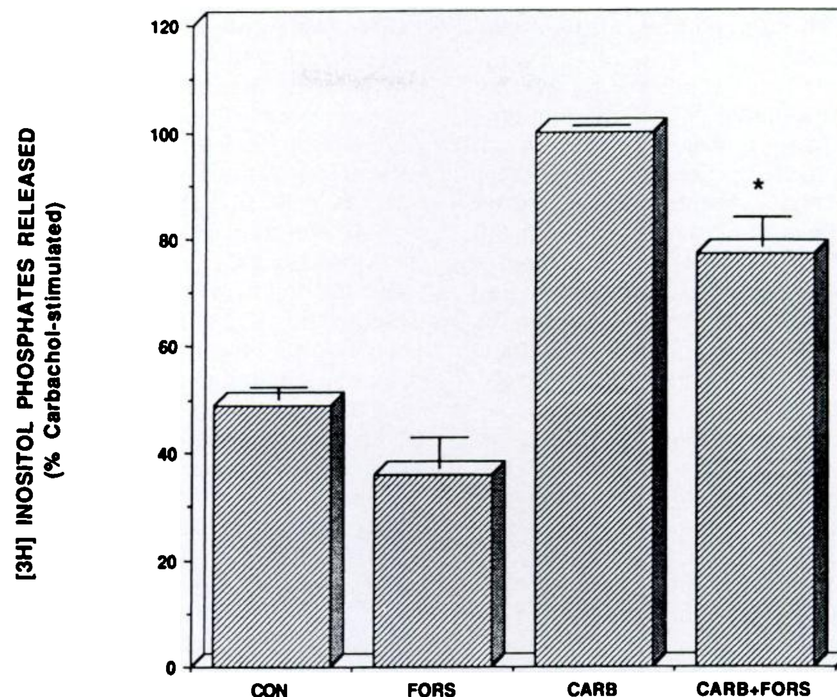

Figure 9. Effects of forskolin on carbachol-stimulated PI hydrolysis in MDCK cells. CON, control; FORS, forskolin; CARB, carbachol. Cells were exposed to 10^{-4} M forskolin for 10 min before 50 min of incubation with 1 mM carbachol. Values are means ± SE of four experiments done in duplicate.

MDCK cells, a renal epithelial cell line having characteristics of collecting ducts. Previously, we have shown that muscarinic-type cholinergic receptors are present in the IMCD cells of the rabbit kidney (3). Specific muscarinic receptors have also been demonstrated in the rat kidney (15). In one report, two types of binding sites for [^{3}H]QNB have been shown to be present in the dog kidney (16). The outer cortical sites have a high affinity, and the inner cortical sites have a low affinity, for [^{3}H]QNB. MDCK cells have only high-affinity binding sites for [^{3}H]QNB. The affinities of [^{3}H]QNB for high-affinity binding sites in all of the renal epithelial cells are comparable (Table 4). On the other hand, the density of muscarinic receptors in MDCK cells is more than 50 times greater than that in IMCD cells (Table 4) and is similar to the value obtained in a cerebral preparation (17). These results indicate that the MDCK cell line is a useful model for the elucidation of the function of muscarinic receptors in renal tubular cells.

In our radioligand binding studies, we have shown that the muscarinic receptors in MDCK cells have the highest affinity for the M_3-selective muscarinic antagonist 4-DAMP, intermediate affinity for M_1-selective pirenzepine, and the lowest affinity for M_2-selective methoctramine. These results suggest that the muscarinic receptors in MDCK cells are probably M_3 subtype.

We have also demonstrated that the muscarinic receptors in MDCK cells are coupled to the PI second messenger system. Two cholinergic agonists, carbachol and arecoline, stimulated PI hydrolysis in these cells in a concentration-dependent manner. The cholinergic-stimulated PI hydrolysis was blocked by several muscarinic antagonists. The order of potency of muscarinic antagonists was: atropine > 4-DAMP > pirenzepine ≫ methoctramine. This order of potency is similar to that obtained in our radioligand studies for muscarinic receptors in MDCK cells. Taken together, these results indicate that the cholinergic-stimulated PI hydrolysis in MDCK cells is

TABLE 4. [^{3}H]QNB binding data in renal cells

Tissue	K_d (nM)	B_{max} (fmol/mg of protein)	Reference No.
Kidney (rat)	0.14	3.5[a]	15
Kidney (dog)			
Outer cortex	0.11	4.1	16
Inner cortex	3.70	9.0	
IMCD cells (rabbit)	0.27	27.5	3
MDCK cells	0.09	1464	This study
Cerebral cortex (rat)[b]	0.05	1394	17

[a] Femtomoles per milligram of tissue.
[b] Given here for comparison.

mediated by putative M_3 subtype muscarinic receptors.

Experiments with other cell types have shown that muscarinic receptor–stimulated PI hydrolysis may or may not be sensitive to pertussis toxin (18). When muscarinic receptors were expressed in Chinese hamster ovary cells, carbachol-stimulated PI hydrolysis was completely blocked by pertussin toxin (30 ng/mL) (12). On the other hand, muscarinic receptor–stimulated PI hydrolysis in astrocytoma cells and cardiac myocytes is not sensitive to inhibition by pertussis toxin (18). Our results indicate that the G protein involved in carbachol-stimulated PI hydrolysis in MDCK cells is sensitive to pertussis toxin.

In some systems, cAMP has been shown to attenuate agonist-stimulated PI hydrolysis. In cortical slices, cAMP has been reported to slightly decrease α_1-adrenergic–stimulated phospholipase C activity (19). Also, the stimulation of cAMP formation by vasopressin or forskolin or the addition of exogenous cAMP (10^{-4} M) inhibits epidermal growth factor–stimulated phospholipase C in cultured rat IMCD cells (20). Furthermore, in our preliminary study, we found that cholinergic agonists inhibit vasopressin- and forskolin-stimulated cAMP formation in MDCK cells (21). In this study, we found that forskolin, which stimulates cAMP formation, and exogenous 8-CPT–cAMP inhibited PI hydrolysis in MDCK cells. The effect was produced at a concentration of 10^{-4} M, and the maximal reduction in PI hydrolysis was approximately 30%. In addition, the effect of 8-CPT–cAMP was time dependent and transient. Therefore, the effect of muscarinic agonists on PI hydrolysis in MDCK cells may be modulated to some extent by cAMP stimulated by hormones.

Although EC_{50} values of muscarinic antagonists and agonists for PI hydrolysis in MDCK cells were higher than IC_{50} values obtained for the displacement of [^{3}H]QNB in cell membranes, there was a linear relationship between the functional (EC_{50}) and binding (IC_{50}) data (Figure 10). These results indicate that both PI hydrolysis and radioligand binding were mediated by the same subtype of muscarinic receptor.

The physiologic effects of cholinergic-stimulated PI hydrolysis in renal tubules are not well established at this time. The hydrolysis of PI releases IP_3 and diacylgycerol; the former increases intracellular Ca^{2+}, and the latter activates protein kinase C (PKC) (9). Carbachol has been shown to increase cell Ca^{2+} in the rat outer medullary collecting duct (22) and in the rabbit cortical collecting duct (CCD) (23). The activation of PKC has been shown to inhibit Na^+ reabsorption and K^+ secretion in the rabbit CCD (24). The activation of PKC also inhibits Na^+ transport in

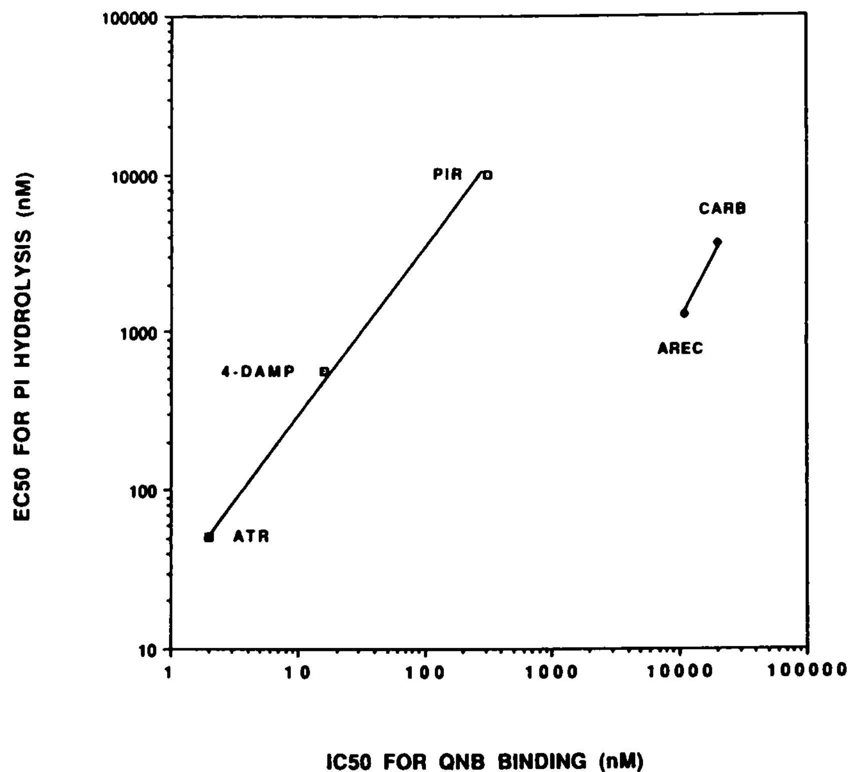

Figure 10. Relationship of muscarinic antagonists and agonists between their EC_{50} for PI hydrolysis and their IC_{50} for the displacement of [^{3}H]QNB in MDCK cells. Each datum point is mean of three to four experiments. ATR, atropine; PIR, pirenzepine; CARB, carbachol; AREC, arecoline.

A6 cells (25) and in frog urinary bladder (26), two experimental models used for the mammalian collecting duct. In addition, the inhibition of PKC has been shown to reverse the effect of carbachol on water permeability in the CCD (23). Therefore, it is possible that cholinergic-stimulated PI hydrolysis may affect electrolyte and water transport in MDCK cells as well as in the renal collecting ducts. The latter effect may be in part responsible for the diuretic and natriuretic actions of cholinergic drugs.

In summary, we have demonstrated that: (*1*) high-affinity muscarinic receptors are present in MDCK cells; (*2*) the receptor density in MDCK cells is 50 times greater than that in IMCD cells; (*3*) the muscarinic receptors in MDCK cells are coupled to the PI hydrolysis second messenger system via a pertussis toxin-sensitive G protein; (*4*) PI hydrolysis in MDCK cells can be modulated by cAMP; and (*5*) the muscarinic receptors in MDCK cells are putative M_3 subtype. It is concluded that the MDCK cell line is a useful cellular model for studying the physiologic role of the cholinergic-stimulated PI messenger system in the kidney.

ACKNOWLEDGMENTS

This work was supported in part by a NSF grant. We thank Dr. Elzbieta McMurtrie for her help in some of the experiments and Ms. Judy Adams for secretarial assistance.

REFERENCES

1. **Vander AJ:** Effects of acetylcholine, atropine, and physostigmine on renal function in the dog. Am J Physiol 1964;206:492–498.
2. **Williams RL, Pearson JE, Carter MK:** The saluretic effects of arecoline hydrochloride infused into left renal artery of dogs. J Pharmacol Exp Ther 1965;147:32–39.
3. **McArdle S, Garg LC, Crews FT:** Cholinergic receptors in renal medullary collecting duct cells. J Pharmacol Exp Ther 1989;248:12–16.
4. **Gstraunthaler GJA:** Epithelial cells in culture. Renal Physiol Biochem 1988;11:1–42.
5. **Lang F, Klotz L, Paulmichl M:** Effect of acetylcholine on electrical properties of subconfluent Madin-Darby canine kidney cells. Biochim Biophys Acta 1988;941:217–224.
6. **Hulme EC, Birdsall NJM, Buckley NJ:** Muscarinic receptor subtypes. Annu Rev Pharmacol Toxicol 1990;30:633–673.
7. **McArdle S, Garg LC:** Stimulation of phosphoinositide hydrolysis by carbachol in renal medullary collecting duct cells. J Pharmacol Exp Ther 1989;248:682–686.
8. **Lowry OH, Rosebrough NJ, Farr AL, Randall RJ:** Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–275.
9. **Berridge MJ:** Rapid accumulation of inositol trisphosphate reveals that agonists hydrolyse polyphosphoinositides instead of phosphatidylinositol. Biochem J 1983;212:849–858.
10. **Slivka SR, Insel PA:** Adrenergic receptor-mediated phosphoinositide hydrolysis and prostaglandin E_2 formation in Madin-Darby canine kidney cells. J Biol Chem 1987;262:4200–4207.
11. **Cheng Y, Prusoff WH:** Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (IC_{50}) of an enzymatic reaction. Biochem Pharmacol 1973;22:3099–3108.
12. **Ashkenazi A, Winslow JW, Peralta EG, *et al*:** An M_2 muscarinic receptor subtype coupled to both adenyl cyclase and phosphoinositide turnover. Science 1987;238:672–675.
13. **Burnatowska-Hledin MA, Spielman WS:** Vasopressin V_1 receptors on the principal cells of the rabbit cortical collecting tubule: Stimulation of cytosolic free calcium and inositol phosphate production via coupling to pertussis toxin substrate. J Clin Invest 1989;83:84–89.
14. **Garg LC, Kapturczak E, McArdle S:** Effects of osmolality on carbachol-stimulated phosphoinositide hydrolysis in renal medulla. J Pharmacol Exp Ther 1988;247:495–501.
15. **Yamada S, Yamamura HI, Roeske WR:** Effects of chemical sympathectomy with 6-hydroxydopamine on α- and β-adrenoceptors and muscarinic cholinoceptors in rat kidney. Eur J Pharmacol 1986;121:345–353.
16. **Pirola CJ, Alvarez AL, Balda MS, Finkielman S, Nahmod VE:** Evidence for cholinergic innervation in dog renal tissue. Am J Physiol 1989;257:F746–F754.
17. **Luthin GR, Wolfe BB:** Comparison of [^{3}H]pirenzepine and [^{3}H]quinuclidinyl benzilate binding to muscarinic receptors in rat brain. J Pharmacol Exp Ther 1984;228:648–655.
18. **Fain JN, Wallace MA, Wojcikiewicz JH:** Evidence for involvement of guanine nucleotide-binding regulatory proteins in the activation of phospholipases by hormones. FASEB J 1988;2: 2569–2574.
19. **Neylon CB, Summers RJ:** Inhibition by cAMP of the phosphoinositide response to α_1-adrenergic stimulation in rat kidney. Eur J Pharmacol 1988;148:441–444.
20. **Teitelbaum I:** Cyclic adenosine monophosphate and diacylgycerol: Mutually inhibitory second messengers in cultured rat inner medullary collecting duct cells. J Clin Invest 1990;86:46–51.
21. **Garg LC, Mohuczy-Dominiak D:** Inhibition of vasopressin- and forskolin-stimulated cyclic AMP formation by cholinergic agonists in MDCK cells [Abstract]. J Am Soc Nephrol 1991;2:453.
22. **Marchetti J, Taniguchi S, Lebrun F, Morel F:** Cholinergic agonists increase cell calcium in rat medullary collecting tubules: A fura-2 study. Pflügers Arch 1990;416:561–567.
23. **Snyder HM, Fredin DM, Breyer MD:** Muscarinic receptor activation inhibits AVP-induced water flow in rabbit cortical collecting ducts. Am J Physiol 1991;260:F929–F936.
24. **Hays SR, Baum M, Kokko JP:** Effects of protein kinase C activation on sodium, potassium, chloride and total CO_2 transport in the rabbit cortical collecting tubule. J Clin Invest 1987;80:1561–1570.
25. **Yanase M, Handler JS:** Activators of protein kinase C inhibit sodium transport in A6 epithelia. Am J Physiol 1986;250:C517–C522.
26. **Satoh T, Endou H:** Inhibitory effect of phorbol ester on sodium transport in frog urinary bladder. Am J Physiol 1990;259:F425–F431.