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Muscarinic Receptor-Mediated Prostacyclin and cGMP Synthesis in Cultured Vascular Cells

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SUMMARY

The purpose of the present study was to determine the subtype of muscarinic receptor involved in the action of cholinergic stimuli on synthesis of prostacyclin, measured as immunoreactive 6-keto-prostaglandin $F_{1\alpha}$ (6-keto-$PGF_{1\alpha}$), and cGMP in bovine aortic endothelial and rabbit vascular smooth muscle cells. Acetylcholine and arecaidine propargyl ester, a selective M_2 agonist, produced a dose-dependent increase in 6-keto-$PGF_{1\alpha}$ output and cGMP formation in confluent endothelial cells but not in confluent vascular smooth muscle cells. McN-A-343, a selective M_1 agonist, failed to alter basal 6-keto-$PGF_{1\alpha}$ or cGMP synthesis. Acetylcholine- and arecaidine propargyl ester-induced 6-keto-$PGF_{1\alpha}$ synthesis and cGMP formation in endothelial cells were attenuated by atropine, AF-DX 116 (M_2 antagonist), and hexahydrosiladifenidol (M_3 antagonist) but not by pirenzepine (M_1 antagonist). The cyclooxygenase inhibitor indomethacin abolished 6-keto-$PGF_{1\alpha}$ synthesis but not the increase in cGMP formation elicited by the cholinergic stimuli. Our data suggest that the effect of cholinergic stimuli to enhance prostacyclin and cGMP synthesis is mediated via activation of M_2 and M_3 receptors located on endothelial cells and that the increase in cGMP production is independent of prostaglandins.

A number of substances, including cholinergic stimuli, have been shown to stimulate prostacyclin (PGI_2) synthesis in various tissues (1–11). PGI_2, which is a potent vasodilator and antiaggregating substance, is believed to be important in the maintenance of vascular homeostasis (12). Because PGI_2 synthesis elicited by cholinergic stimuli in vascular tissues is abolished by atropine, it appears to be mediated via activation of muscarinic receptors. Many *in vitro* and *in vivo* studies support the hypothesis of muscarinic receptor heterogeneity. M_1 receptors show high affinity for the antagonist pirenzepine and are found mainly in neural tissues such as cerebral cortex, hippocampus, and autonomic ganglia. M_2 and M_3 receptors show low affinity for pirenzepine and are found mainly in peripheral effector organs and certain parts of central nervous system (13–15). Antagonists, such as AF-DX 116 and methoctramine, that show higher affinity for cardiac than for smooth muscle muscarinic receptors are classified as M_2 receptor antagonists, whereas HHSiD and *para*-fluoro-HHSiD, which show a higher affinity for smooth muscle than for cardiac muscarinic receptors, are classified as M_3 receptor antagonists (16, 17). It has been demonstrated that stimulation of endothelial cells with vasoactive substances such as ACh releases a short-lived EDRF, causing relaxation of underlying vascular smooth muscle (18) that can be correlated with an increase in cGMP concentration (19). ACh, histamine, and calcium ionophore A23187 have been shown to produce a large increase in cGMP levels in intact vascular strips but have no effect in endothelium-denuded aortic preparation (20).

In our previous studies of isolated perfused rabbit heart and rabbit aortic rings (10, 21), we have shown that PG synthesis induced by cholinergic agonists is due to activation of M_2 as well as M_3 muscarinic receptors. The purpose of this study was to determine the location of these muscarinic receptors that are linked to PGI_2 and cGMP synthesis in bovine aortic endothelial cells and rabbit vascular smooth muscle cells, by using selective muscarinic receptor agonists and antagonists.

Materials and Methods

Bovine Aortic Endothelial Cells

Bovine aortic endothelial cells were isolated and cultured as described by Luckhoff *et al.* (22). Cells were grown in Dulbecco's modified Eagle's medium supplemented with 20% FBS plus 100 mg/ml penicillin and 100 mg/ml streptomycin. Cells were allowed to grow in the incubator at 37°, under a controlled atmosphere (95% air and 5% CO_2), until confluent. Medium was replaced after 3 days. At confluency, cells were detached using trypsin (0.01% in Ca^{2+}- and Mg^{2+}-free HBBS) and plated at a density of 1×10^4 cells/cm^2. Cells in culture were characterized by the presence of factor VIII antigen (23) and the uptake of the fluorescent probe diacetylated low density lipoprotein (24).

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ABBREVIATIONS: PG, prostaglandin; HBSS, Hanks' balanced salt solution; FBS, fetal bovine serum; ACh, acetylcholine; APE, arecaidine propargyl ester; HHSiD, hexahydrosiladifenidol; RIA, radioimmunoassay; M199, medium 199; EDRF, endothelium-derived relaxing factor.

Isolation of Aortic Smooth Muscle Cells

Male New Zealand White rabbits were killed by pentobarbital anaesthesia. The thoracic aorta was removed, kept in HBSS, cleared of fat and connective tissues, and then cut lengthwise with scissors to expose the intima. Intima was washed free of blood clots with calcium-free HBSS, and the intima side was gently scraped with a scalpel blade. The adventitia and the outer third of media were removed from the inner media. The remaining inner media was cut into 2–3-mm slices and incubated in enzyme solution, containing 0.25 mg/ml elastase type III, 5 mg/ml collagenase, 4 mg/ml soybean trypsin inhibitor, and 2 mg/ml bovine serum albumin in M199 without serum, at 37° for 3 hr or until the medium became cloudy. Undigested tissue was allowed to settle, and the supernatant was removed and centrifuged at 500 rpm for 1 min. The pellet was suspended in M199 with 10% FBS and plated in tissue culture dishes. The cultures were washed at 24 hr to remove debris and refed at 1–3-day intervals. Cell number was determined using a hemocytometer. At confluency, cells were detached from the plates by trypsin (0.12% in Ca^{2+}- and Mg^{2+}-free HBSS) and passaged by plating at a density of 1×10^4 cells/cm^2.

Experimental Protocols

After reaching confluency, cells were washed two times with serum-free medium and incubated with different drugs for an appropriate period of time. At the end of the reaction, medium was removed for measurement of 6-keto-$PGF_{1\alpha}$. To extract cGMP, 1.0 ml of 0.1 N HCl was added to each well. For all the experiments, cells were used between passages 3 and 8. The following experimental protocols were used.

Protocol 1. This series of experiments were performed to investigate the time course of 6-keto-$PGF_{1\alpha}$ production caused by cholinergic stimuli. Cells were incubated for 5, 10, and 20 min with 1.0 ml of medium containing cholinergic agonists, at 37°. At the end of each time period, medium was removed for measurement of 6-keto-$PGF_{1\alpha}$ by RIA, whereas protein was measured in the cell pellet by the procedure described below.

Protocol 2. This series of experiments were performed to determine the effects of different concentrations (10^{-9} to 10^{-5} M) of the cholinergic agonists ACh, APE, and McN-A-343 on PG synthesis as well as on cGMP formation in vascular cells. For incubation, 1 ml of medium, with or without agonists, was added to each well, and cells were incubated for 10 min. Previous experiments have shown this time to be optimal for 6-keto-$PGF_{1\alpha}$ accumulation and cGMP formation during stimulation with cholinergic stimuli. The medium was aspirated for 6-keto-$PGF_{1\alpha}$ assay, and cells were extracted for cGMP and protein determination, as described below.

Protocol 3. This series of experiments were performed to investigate the effects of muscarinic receptor antagonists on PG and cGMP formation by vascular cells in response to cholinergic stimuli. Vascular cells were preincubated with the muscarinic receptor antagonists atropine (10^{-7} M), pirenzepine (10^{-7} or 10^{-6} M), AF-DX116 (10^{-8} to 10^{-6} M), and HHSiD (10^{-8} to 10^{-6} M), or their vehicle, at 37° for 15 min and then challenged with different cholinergic agonists, as described in protocol 2.

Protocol 4. This series of experiments were performed to determine the effects of the α-adrenergic receptor antagonist phentolamine (0.3 μM), the β-adrenergic receptor antagonist propranolol (4.0 μM), the nicotinic receptor antagonist hexamethonium (2.0 μM), and the cyclooxygenase inhibitor indomethacin (3.0 μM) on PG output and cGMP formation elicited by ACh and APE, as described in protocol 3. An additional series of experiments were performed to determine the effect of muscarinic receptor antagonists on 6-keto-$PGF_{1\alpha}$ and cGMP synthesis induced by bradykinin (10^{-6} M), norepinephrine (10^{-6} M), and calcium ionophore A23187 (10^{-6} M).

PG Assay

6-Keto-$PGF_{1\alpha}$ concentration was determined in the samples by RIA. In brief, 100 μl of sample were mixed with 2000–3000 cpm of tracer plus an appropriate concentration of antibody. The tracer and antibody were prepared in a buffer consisting of (in g/liter): NaN_3, 1.0; NaCl, 9.0; KH_2PO_4, 6.8; K_2HPO_4, 26.1; and gelatin, 2.0. The tubes were vortexed and incubated overnight at 4°. Bound and free tracer were separated by addition of 1.0 ml of dextran-coated charcoal to each tube, and radioactivity was determined by liquid scintillation counting. The antibody for 6-keto-$PGF_{1\alpha}$ was provided by Dr. C. Leffler (Department of Physiology, University of Tennessee, Memphis). Cross-reactivity of 6-keto-$PGF_{1\alpha}$ antibody was <0.1% with thromboxane B_2 and 13,14-dihydro-15-keto-PGE_2 and <0.5% with PGE_2 and $PGF_{2\alpha}$.

cGMP Assay

cGMP was extracted from the cells with 0.1 N HCl and then measured by RIA, as described previously (25). Briefly, duplicate 100-μl aliquots were acetylated with 5 μl of acetylation mixture (acetic anhydride and triethylamine, 1:2) and incubated with labeled antigen (succinylated cGMP ^{125}I-tyrosine methyl ester), normal rabbit serum, and antiserum for 16–18 hr at 4°. The reaction was terminated by addition of 1.0 ml of charcoal/bovine serum albumin solution to separate bound and unbound tracer. Recovery of cGMP was >90% and was not affected by different muscarinic agents.

Protein Assay

After removal of the acid extract, protein was solubilized by addition of 1.0 ml of 1 N NaOH to each well. Protein was measured by the method of Lowry *et al.* (26).

Drugs

ACh, atropine, hexamethonium, indomethacin, collagenase, soybean trypsin inhibitor, bovine serum albumin, FBS, penicillin, streptomycin, Dulbecco's modified Eagle's medium, and M199 were purchased from Sigma Chemical Co. (St. Louis, MO). The following drugs were donated: APE, HHSiD (Dr. G. Lambrecht, Pharmakologisches Institut, Frankfurt, FRG), McN-A-343 (Dr. R. Hammer, Institut de Angeli, Milan, Italy), pirenzepine, and AF-DX 116 (Boehringer Ingelheim Pharmaceuticals, Ridgefield, CT).

Statistical Analysis

Comparisons between two groups were made using Student's paired or unpaired *t* test, wherever appropriate. Comparisons involving more than two groups were made using one-way analysis of variance, followed by Dunnett's test when significant differences were present. A $p < 0.05$ was accepted as significant. The PGI_2 and cGMP syntheses elicited by various agents are expressed as percentage above basal, because of the variability in basal PG and cGMP formation among various cell preparations.

Results

Time course of 6-keto-$PGF_{1\alpha}$ synthesis in endothelial and smooth muscle cells. The time course of 6-keto-$PGF_{1\alpha}$ accumulation elicited by 10^{-6} M ACh in bovine aortic endothelial cells is shown in Fig. 1. In endothelial cells, 6-keto-$PGF_{1\alpha}$ levels were significantly increased (125% above basal level) at 5 min and reached a maximum at 10 min. Norepinephrine and calcium ionophore A23187, but not ACh or bradykinin, stimulated the synthesis of 6-keto-$PGF_{1\alpha}$ in smooth muscle cells (Table 1). The maximal increase in 6-keto-$PGF_{1\alpha}$ synthesis was observed at 10–20 min.

Effect of muscarinic agonists on 6-keto-$PGF_{1\alpha}$ accumulation in bovine aortic endothelial cells. Comparisons were made of the effects of the muscarinic receptor agonists ACh, McN-A-343, and APE on 6-keto-$PGF_{1\alpha}$ production in bovine aortic endothelial cells (Fig. 2). ACh (10^{-9} to 10^{-5} M) produced a dose-dependent increase in the release of 6-keto-$PGF_{1\alpha}$, eliciting a maximal response at 10^{-5} M (95 ± 2% above

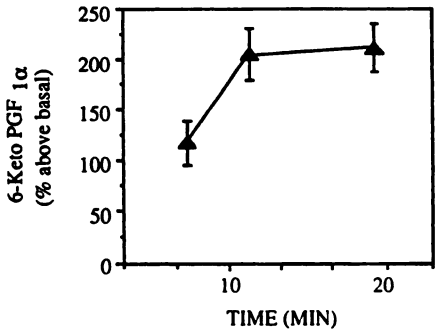

Fig. 1. Time course of 6-keto-$PGF_{1\alpha}$ synthesis in cultured bovine aortic endothelial cells. ACh (10^{-6} M) was added to confluent endothelial cells for the indicated periods of time. Each value is the mean ± standard error of four separate experiments. Basal 6-keto-$PGF_{1\alpha}$ was 24.90 ± 1.15 ng/mg of protein.

TABLE 1

Effect of ACh, bradykinin (BK), norepinephrine (NE), and A23187 on 6-keto-$PGF_{1\alpha}$ accumulation in rabbit vascular smooth muscle cells

Confluent cells were incubated with 1.0 ml of M199, with or without drugs for different periods of time, at 37°. Data are reported as mean ± standard error of quadruplicate samples.

Time	6-Keto-$PGF_{1\alpha}$ accumulation				
	Basal	ACh (10^{-6} M)	NE (10^{-6} M)	BK (10^{-6} M)	A23187 (1 μg)
min	*ng/mg of protein*				
5	2.23 ± 0.26	2.62 ± 1.15	2.58 ± 0.16	2.66 ± 0.67	11.45 ± 1.48[a]
10	2.69 ± 0.57	2.89 ± 0.89	7.44 ± 1.27[a]	2.80 ± 0.48	27.35 ± 7.55[a]
20	3.43 ± 0.31	3.62 ± 0.34	9.49 ± 0.90[c]	3.71 ± 0.69	29.20 ± 3.48[a]

[a] Significant difference from basal ($p < 0.05$).

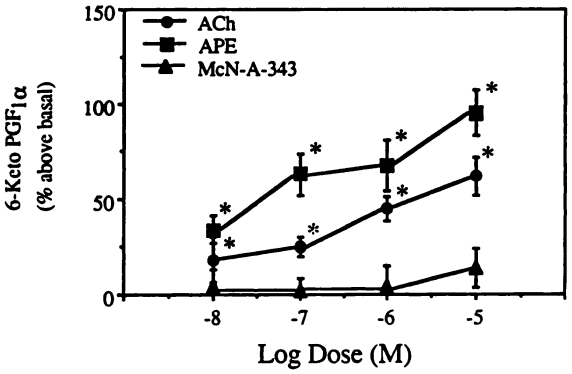

Fig. 2. Effect of the muscarinic agents ACh, McN-A-343, and APE on 6-keto-$PGF_{1\alpha}$ accumulation in bovine aortic endothelial cells. 6-Keto-$PGF_{1\alpha}$ was measured in the medium after 10 min of incubation of cells with muscarinic agents. Values are mean ± standard error from at least five experiments. *, Significant difference from basal ($p < 0.05$), using Dunnett's test for multiple comparisons. Basal 6-keto-$PGF_{1\alpha}$ was 34.17 ± 3.33 ng/mg of protein.

basal level; seven experiments; $p < 0.05$). McN-A-343, an M_1 receptor-selective agonist, did not alter the basal release of PG at a dose of 10^{-9} to 10^{-5} M, whereas APE, a selective M_2 agonist, enhanced 6-keto-$PGF_{1\alpha}$ output in a concentration-dependent manner (10^{-9} to 10^{-5} M). These agonists failed to alter the basal output of 6-keto-$PGF_{1\alpha}$ in rabbit aortic smooth muscle cells.

Effect of muscarinic receptor antagonists on 6-keto-$PGF_{1\alpha}$ release elicited by ACh in bovine aortic endothelial cells. As shown in Fig. 3a, atropine at 10^{-7} M inhibited the ACh-induced release of 6-keto-$PGF_{1\alpha}$. Release of 6-keto-$PGF_{1\alpha}$ induced by ACh was not significantly affected by the M_1 receptor antagonist pirenzepine (Fig. 3b). ACh-elicited 6-keto-$PGF_{1\alpha}$ accumulation was not altered by 10^{-8} M AF-DX 116 but was reduced significantly by this agent at 10^{-7} M and 10^{-6} M. The effect of ACh to increase the release of 6-keto-$PGF_{1\alpha}$ was not altered by HHSiD at 10^{-8} M, but it was reduced at 10^{-7} M and 5×10^{-7} M HHSiD; increasing the HHSiD concentration to 10^{-6} M did not further decrease the output of 6-keto-$PGF_{1\alpha}$ (Fig. 3d). None of the antagonists affected the basal output of 6-keto-$PGF_{1\alpha}$ from rabbit aortic endothelial cells.

Effect of muscarinic receptor antagonists on 6-keto-$PGF_{1\alpha}$ output elicited by APE in bovine aortic endothelial cells. Fig. 4 shows the effects of muscarinic receptor antagonists on 6-keto-$PGF_{1\alpha}$ release elicited by APE. Atropine (10^{-7} M) inhibited the action of APE on 6-keto-$PGF_{1\alpha}$ release in rabbit aortic endothelial cells. In the presence of 10^{-8} M AF-DX 116, the effect of APE to enhance 6-keto-$PGF_{1\alpha}$ release was unaltered. Higher concentrations of AF-DX 116 minimized APE-induced release of 6-keto-$PGF_{1\alpha}$ (Fig. 4c). The effect of APE (10^{-6} M) to increase the release of 6-keto-$PGF_{1\alpha}$ was not altered by 10^{-8} M HHSiD but was reduced by higher concentrations of HHSiD (Fig. 4d).

Effect of cholinergic agonists on cGMP content in vascular cells. In bovine aortic endothelial cells, ACh elicited a dose-dependent increase in cGMP accumulation (Fig. 5). A significant increase in cGMP levels was obtained at 10^{-7} M ACh (31 ± 3% above basal level; six experiments; $p < 0.05$). Peak levels of cGMP occurred at 10^{-6} M ACh. A further increase in ACh dose produced a decrease in cGMP accumulation. McN-A-343 had no effect on basal cGMP level. Incubation of intact

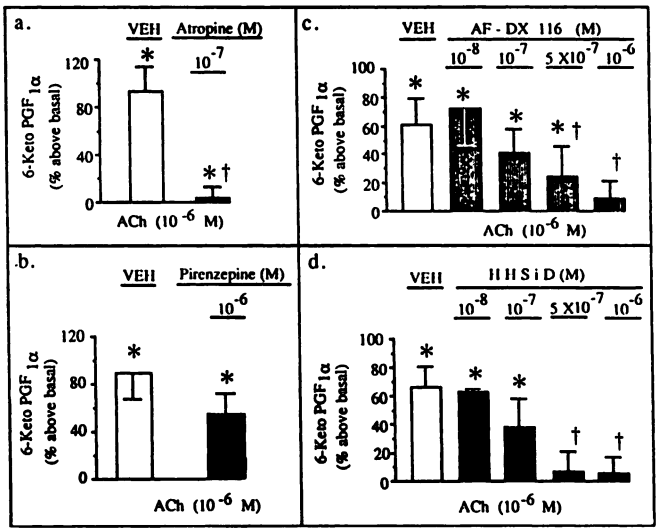

Fig. 3. Effect of the muscarinic receptor antagonists atropine (a), pirenzepine (b), AF-DX 116 (c), and HHSiD (d) on 6-keto-$PGF_{1\alpha}$ formation in bovine aortic endothelial cells stimulated by ACh (10^{-6} M). Data are expressed as mean ± standard error. *, Significant increase above basal; †, significant difference between values in the presence of an antagonist and its vehicle (*VEH*) ($p < 0.05$). Basal 6-keto-$PGF_{1\alpha}$ was 27.72 ± 3.43 ng/mg of protein.

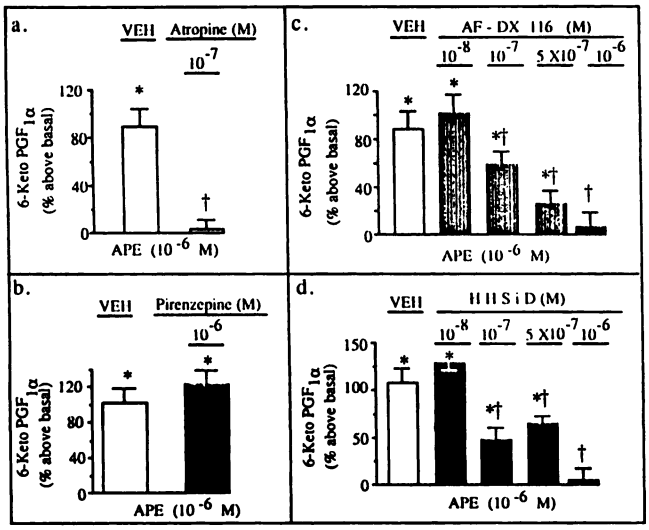

Fig. 4. Effect of the muscarinic receptor antagonists atropine (a), pirenzepine (b), AF-DX 116 (c), and HHSiD (d) on 6-keto-$PGF_{1\alpha}$ formation in bovine aortic endothelial cells stimulated by APE (10^{-6} M). Data are expressed as mean ± standard error. *, Significant increase above basal; †, significant difference between values in the presence of an antagonist and its vehicle (*VEH*) ($p < 0.05$). Basal 6-keto-$PGF_{1\alpha}$ was 28.7 ± 3.0 ng/mg of protein.

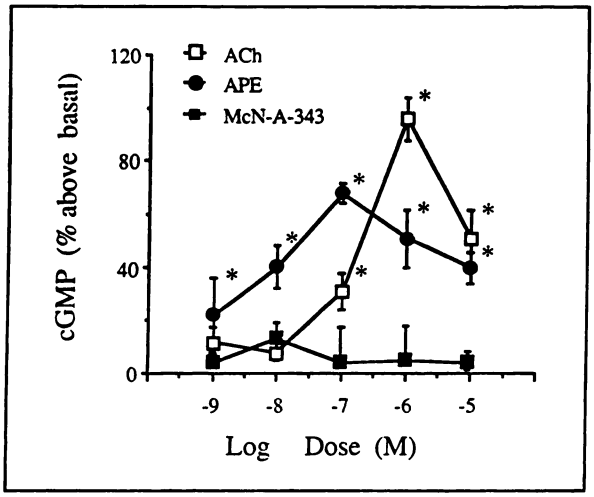

Fig. 5. Effect of the muscarinic agents ACh, McN-A-343, and APE on cGMP accumulation in bovine aortic endothelial cells. The reaction was stopped by aspiration of the medium, followed by rapid addition of 0.1 N HCl. cGMP was measured after 10 min of incubation of cells with muscarinic agents. Values are mean ± standard error from at least five experiments. *, Significant difference from basal ($p < 0.05$), using Dunnett's test for multiple comparisons. Basal cGMP was 22.96 ± 3.79 pmol/mg of protein.

endothelial monolayers with APE resulted in a marked increase in cGMP formation. The response was maximal at 10^{-7} M and then gradually decreased at 10^{-6} and 10^{-5} M. Dose-response analysis indicated that APE was a more potent stimulator of endothelial cell cGMP levels than was ACh, with EC_{50} values of ~10 nM and ~400 nM, respectively. An additional series of experiments were performed in vascular smooth muscle cells to determine the effects of ACh, McN-A-343, and APE on cGMP accumulation. All of these agents failed to alter the basal level of cGMP (data not shown).

Effect of muscarinic receptor antagonists on cGMP formation elicited by ACh in bovine aortic endothelial cells. The effect of ACh to enhance cGMP formation was abolished in the presence of 10^{-7} M atropine (Fig. 6a) but was not altered by the M_1 receptor antagonist pirenzepine (10^{-6} M) (Fig. 6b). Incubation of endothelial monolayers with the M_2 receptor antagonist AF-DX 116 (10^{-7} M) blunted the ACh-induced increase in cGMP levels by 50%. A 5-fold increase in AF-DX 116 concentration further attenuated the ACh-induced cGMP formation (Fig. 6c). As shown in Fig. 6d, 10^{-7} M HHSiD significantly inhibited cGMP accumulation elicited by ACh; increasing the HHSiD concentration to 10^{-6} M did not produce any further decrease in cGMP formation. Neither antagonist altered basal levels of cGMP.

Effect of phentolamine, propranolol, and hexamethonium on 6-keto-$PGF_{1\alpha}$ release and cGMP accumulation elicited by ACh and APE in bovine aortic endothelial cells. Incubation of endothelial monolayers with phentolamine (α-adrenergic receptor blocker) (0.3 μM), propranolol (β-adrenergic receptor blocker) (4.0 μM), or hexamethonium (nicotinic receptor blocker) (3.0 μM) had no effect on 6-keto-$PGF_{1\alpha}$ release or cGMP accumulation elicited by 10^{-6} M ACh or APE (data not shown). Indomethacin, a cyclooxygenase inhibitor (4.0 μM), inhibited basal as well as ACh- and APE-induced 6-keto-$PGF_{1\alpha}$ release, without altering basal or ACh- and APE-induced cGMP accumulation (data not shown).

Effect of muscarinic receptor antagonists on the action of norepinephrine, bradykinin, and A23187 in bovine aortic endothelial cells. Incubation of endothelial cells with norepinephrine (10^{-6} M), bradykinin (10^{-6} M), or A23187 (10^{-6} M) significantly enhanced 6-keto-$PGF_{1\alpha}$ release, by 84 ± 12%, 119 ± 5%, and 110 ± 6% above basal, respectively ($p < 0.05$; seven to nine experiments) (Table 1); basal as well as norepinephrine-, bradykinin-, and A23187-induced 6-keto-$PGF_{1\alpha}$ release was not altered by the muscarinic receptor antagonists atropine, pirenzepine, AF-DX 116, or HHSiD (data not shown).

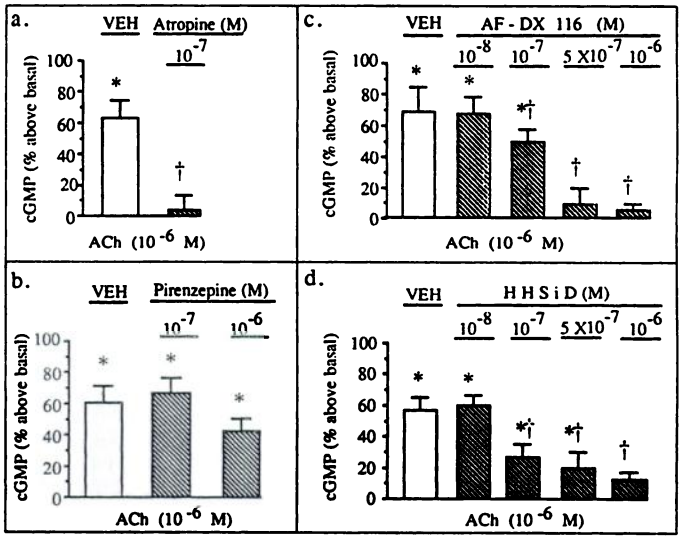

Fig. 6. Effect of the muscarinic receptor antagonists atropine (a), pirenzepine (b), AF-DX 116 (c), and HHSiD (d) on cGMP formation in bovine aortic endothelial cells stimulated by ACh (10^{-6} M). Data are expressed as mean ± standard error. *, Significant increase above basal; †, significant difference between values in the presence of an antagonist and its vehicle (*VEH*) ($p < 0.05$). Basal cGMP was 39.0 ± 8.19 pmol/mg of protein.

Discussion

Our previous study in isolated rabbit heart and rabbit aorta suggested that PG synthesis elicited by cholinergic stimuli is mediated via activation of M_2 ($M_{2\alpha}$) and M_3 ($M_{2\beta}$) receptors, because 1) ACh and APE, but not McN-A-343 (M_1-selective agonist), increased the output of 6-keto-$PGF_{1\alpha}$ and 2) ACh- and APE-induced 6-keto-$PGF_{1\alpha}$ release was attenuated by atropine, AF-DX 116 (M_2 receptor antagonist), and HHSiD (M_3 receptor antagonist), but not by the M_1 receptor antagonist pirenzepine (10, 11, 21). The present study, which was undertaken to determine the localization and the subtype of muscarinic receptors involved in PGI_2 synthesis in bovine endothelial and rabbit smooth muscle cells, indicates that PGI_2 (measured as 6-keto-$PGF_{1\alpha}$) synthesis elicited by muscarinic agents is due to activation of M_2 and M_3 receptors located on endothelial cells but not on smooth muscle cells.

The conclusion that 6-keto-$PGF_{1\alpha}$ synthesis elicited by cholinergic stimuli is due to activation of distinct subtypes of M_2 and M_3 receptors is based on our observation that ACh- and APE-induced PG synthesis in the bovine endothelial cells was attenuated by AF-DX 116 (an M_2 receptor antagonist having a greater affinity for cardiac muscarinic receptors than for smooth muscle or glandular muscarinic receptors) and HHSiD (an M_3 receptor antagonist having a greater affinity for glandular or smooth muscle receptor than for cardiac muscarinic receptors) (27) but not by pirenzepine. The conclusion that these receptors, which are linked to PG synthesis, are located on endothelial and not on smooth muscle cells is supported by our demonstration that cholinergic agonists failed to alter basal 6-keto-$PGF_{1\alpha}$ synthesis in rabbit smooth muscle cells. We performed our study in bovine endothelial cells because they grew at a much faster rate than rabbit endothelial cells. However, we did perform a few studies in rabbit endothelial cells, and qualitatively similar results as in bovine endothelial cells were obtained with the agents described above.[2] The conclusion that attenuation of ACh- and APE-induced PG synthesis by AF-DX 116 and HHSiD was due to selective blockade of muscarinic receptors in the endothelial cells was suggested by our observation that these antagonists did not alter norepinephrine-, bradykinin-, or A23817-induced 6-keto-$PGF_{1\alpha}$ synthesis. Furthermore, the nicotinic receptor blocker hexamethonium, the α-adrenergic receptor blocker phentolamine, and the β-adrenergic receptor blocker propranolol did not alter PG synthesis induced by ACh or APE in endothelial cells, suggesting the selectivity of muscarinic receptor antagonists to inhibit PG synthesis elicited by cholinergic stimuli.

Results obtained in the present study suggested that cholinergic agonists enhanced cGMP levels in endothelial cells, but not in smooth muscle cells in culture. The elevation in cGMP formation induced by cholinergic stimuli in endothelial cells was due to activation of M_2 and M_3 receptors because 1) ACh and APE, but not McN-A-343, increased cGMP formation and 2) ACh- and APE-induced cGMP formation was attenuated by atropine and by the M_2 receptor antagonist AF-DX 116 and the M_3 receptor antagonist HHSiD, but not by the M_1 receptor blocker pirenzepine. ACh has been reported to elevate cGMP levels in several tissues in a calcium-dependent manner (28–30). Moreover, the increase in cGMP in endothelial cells could result from activation of soluble and/or particulate guanylate cyclase, because glyceryl trinitrate and sodium azide, which directly stimulate soluble guanylate cyclase, and atriopeptin, which stimulates particulate guanylate cyclase, increased cGMP accumulation in endothelial cells (31, 32). EDRF, which is released by various vasoactive agents, including ACh, has been reported to increase guanylate cyclase activity and formation of cGMP (30, 33). However, in the pig aortic endothelial cells, ACh did not increase cGMP levels (31). In contrast, Kajikuri and Kuriyama (34) have shown that ACh markedly enhanced the synthesis of cGMP in endothelium-intact but not in endothelium-denuded rabbit aorta. Because activation of guanylate cyclase and the rise in cGMP level were blocked by methylene blue (an inhibitor of soluble guanylate cyclase), it appears that ACh stimulates cGMP formation in endothelial cells by activating soluble guanylate cyclase via release of EDRF. EDRF released from endothelium also stimulates soluble guanylate cyclase in smooth muscle cells and increases cGMP levels, which in turn promote vascular smooth muscle relaxation (19). Supporting this view was our finding that ACh and APE, which increased cGMP accumulation in endothelial cells, failed to alter cGMP levels in vascular smooth muscle cells, even though vascular smooth muscle contains M_2 muscarinic receptors (35–37), and that stimulation of these receptors by ACh and APE in the absence of endothelium caused vascular contraction (37).

In conclusion, the data of the present study suggest that the effect of cholinergic stimuli in enhancing PG synthesis and cGMP formation is mediated through activation of M_2 and M_3 receptors located on vascular endothelial cells and that cGMP production is independent of PGs.

Acknowledgments

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