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MOLECULAR PHARMACOLOGY, **52:**966–973 (1997).

Regulation of m2 Muscarinic Receptor Gene Expression by Platelet-Derived Growth Factor: Involvement of Extracellular Signal-Regulated Protein Kinases in the Down-Regulation Process

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Received March 26, 1997; Accepted August 21, 1997

SUMMARY

To study the role of mitogen-activated protein kinase in the regulation of M_2 receptors, we studied the effect of platelet-derived growth factor (PDGF) on M_2 receptor gene expression. PDGF (4 ng/ml) caused a time-dependent decrease in M_2 receptor number and in m2 receptor mRNA levels in HEL 299 cells. The PDGF-induced loss in m2 mRNA required *de novo* protein synthesis and occurred through a decrease in the rate of transcription of the m2 receptor gene. The down-regulation of M_2 receptors was not accompanied by an uncoupling of the remaining receptors, indicating a large receptor reserve in these cells. Preincubations with the phosphatidylinositol 3-kinase inhibitor wortmannin, the protein kinase C inhibitor GF 109203X and the cAMP-dependent protein kinase inhibitor H-8 did not attenuate PDGF-induced down-regulation, indicating a lack of involvement of these enzymes in the down-regulation process. Activation of the extracellular signal-regulated protein kinase (ERK) 1 and 2 proteins was measured by an "in gel" phosphorylation assay. Carbachol did not activate ERK1 or 2, whereas PDGF and 4β-phorbol 13,14-dibutyrate resulted in a large increase in ERK1 and 2 activity along with a decrease in m2 mRNA. Preincubation with PD 098059, an inhibitor of mitogen-activated protein kinase kinase, inhibited PDGF- and 4β-phorbol 13,14-dibutyrate-mediated activation of ERK 1 and 2 in a concentration-dependent manner. The inhibitory action of PD 098059 was reflected at the mRNA level attenuating both PDGF- and 4β-phorbol 13,14-dibutyrate-mediated decreases in m2 mRNA. These results suggest a role of ERK1 and 2 in the regulation of muscarinic m2 receptor gene expression.

Considerable interest surrounds the regulation of muscarinic acetylcholine and other G protein-coupled receptors. A number of studies have described the effects of agonist occupancy on muscarinic receptor regulation (1, 2) and have highlighted the role of a host of cellular kinases in this process (3). In addition, heterologous regulation has also been described for muscarinic receptors and kinases such as PKC and PKA have been shown to phosphorylate muscarinic receptors in an agonist-independent manner (4). Such cross-talk between the phospholipase C- and cAMP-linked second messenger pathways, which has been described in a variety of studies (5), enables cells greater plasticity in their response to external stimuli.

Recent advances have also been made in elucidating intracellular signaling through stimulation of receptor tyrosine kinases such as growth factor receptors (e.g., insulin and PDGF receptors). In this instance, ligand binding results in the autophosphorylation of key tyrosine residues within the receptor and subsequent receptor dimerization. This process promotes the interaction of the receptor with target proteins such as PI-3 kinase, phosphoinositide-specific phospholipase Cγ, and Ras-GTPase-activating protein, as well as others containing the SH2 binding domains for phosphotyrosine residues. In this pathway each receptor type interacts with its own characteristic battery of proteins, allowing the generation of a unique composite signaling cascade (for reviews, see Refs. 6–8).

One major consequence of stimulation of receptor tyrosine kinases is the activation of the MAPK cascades. MAPKs represent an expanding family of proteins located in one of

This work was funded by the Medical Research Council, UK, and by a European Union postdoctoral fellowship fund (E.-B.H.).

ABBREVIATIONS: PKC, protein kinase C; ERK extracellular-signal regulated protein kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GF 109203X, 2-[1-(3-dimethylaminopropyl)-inol-3-yl]-3-(indol-3-yl)maleimide; H-8, *N*-[2-(methylamino)ethyl]-5′-isoquinoline-sulfonamide; HBSS, Hanks' balanced salt solution; MAPK, mitogen-activated protein kinase; NMS, *N*-methyl-scopolamine; PD 098059, 2-(2-amino-3-methoxyphenyl)4*H*-1-benzopyran-4-one; PI, phosphatydylinositol; PDGF, platelet-derived growth factor BB chain; PDBu, 4β-phorbol 12,13-dibutyrate; PKA, cAMP-dependent protein kinase, TGF-β_1, transforming growth factor β_1; SDS, sodium dodecyl sulfate; EGF, epidermal growth factor; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

three fairly distinct protein phosphorylation cascades that are characterized by a unique dual phosphorylation motif. The three MAPK cascades elucidated so far are the ERKs, amino-terminal c-*jun* kinase/stress-activated protein kinase, and the p38 cascades. MAPKs have been shown to phosphorylate and activate a number of regulatory protein kinases (9) as well as a number of transcription factors such as *c-jun* (10) and ELK-1, a factor involved in *c-fos* expression (11). This places the MAPKs at a critical juncture within cells to mediate the expression of genes in response to various external stimuli.

In addition to receptor tyrosine kinases, stimulation of the MAPK cascade may also be achieved with a variety of cytokines and G protein-coupled receptors. In particular, $G_{q/11}$-coupled receptors such as muscarinic m3 receptors may activate MAPKs in a PKC-dependent and -independent manner (12), possibly through phosphorylation of Raf-1 (13). Significantly, G protein-coupled receptors not normally coupled to $G_{q/11}$ including the G_i-coupled α_2-adrenergic and M_2 muscarinic receptors may also activate MAPK via a pertussis toxin-sensitive activation of $p21^{ras}$ (14).

We have demonstrated previously that a number of stimuli, including phorbol ester and cytokines such as TGF-β_1 and tumor necrosis factor-α/interleukin-1β (15–17), result in a reduction in the transcription of m2 muscarinic receptors in HEL 299 cells. In this study, experiments were performed to determine whether stimulation of the PDGF-linked tyrosine kinase pathway leads to regulation of m2 receptor gene expression. Furthermore, with the key position of MAPK in cell signaling in mind, we wished to investigate whether activation of the 44-kDa ERK1 and the 42-kDa ERK2 MAPKs with PDGF and phorbol ester can be related to the regulation of M_2 receptor gene expression.

Experimental Procedures

Cell Culture

HEL 299 cells (American Type Culture Collection, Rockville, MD) were maintained in culture as described previously (15). Cells were maintained in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/liter amphotericin B in 95% air and 5% CO_2 at 37° in a humidifier incubator. Before treatment, cells were exposed to 1% fetal calf serum containing the aforementioned supplements for 24 hr and were harvested simultaneously at preconfluence. Cells were exposed to one or more of the following: PDGF peptide BB (4 ng/ml), GF 109203X (1 μM), wortmannin (10 nM), H-8 (30 μM), PD 098059 (0.1–100 μM), cycloheximide (10 μg/ml), actinomycin D (5 μg/ml), Org 20241 (30 μM), forskolin (50 μM), and carbachol (100 μM). All reagents, with the exception of wortmannin, GF 109203X, H-8 (CalBiochem-NovaBiochem, Nottingham, UK), and PD 098059 (Research Biochemicals, Natick, MA) were obtained from Sigma (Poole, UK).

Binding Studies

Radioligand binding experiments were performed on crude cell homogenates prepared at 4°. Cells (approximately 5–10 $\times$ 10^6 for each binding reaction) were washed twice with ice-cold Tris·HCl buffer (25 mM, pH 7.4), harvested by cell scraping, and homogenized with an Ultra-Turax homogenizer (one 30-sec burst). The crude membrane homogenates were isolated by centrifugation at 40,000 $\times$ *g* for 20 min and resuspended in an appropriate volume of Tris buffer. The protein concentration was measured according to the method of Lowry *et al.* (18).

[^{3}H]NMS (specific activity 80.4 Ci/mmol; New England Nuclear, Stevenage, UK) saturation curves were elucidated using concentrations varying from 0.06 to 2 nM, and nonspecific binding was measured in the presence of 1 μM atropine. Incubations were performed for 2 hr at 30° in 25 mM Tris·HCl buffer in a final volume of 1 ml and were terminated by rapid vacuum filtration over 0.2% polyethyleneimine-pretreated Whatman GF/C glass fiber filters using a Brandel cell harvester. Filters were washed three times with 4 ml of ice-cold Tris buffer and placed in vials with 4 ml of scintillation cocktail (Filtron X; National Diagnostics, Manville NJ) and counted on a Packard β counter (Packard model 2200 CA; Meriden, CT). Binding data were analyzed with the computerized iterative nonlinear regression program LIGAND (19).

Northern Analysis

Cells were washed twice with HBSS before total cellular RNA was prepared according to the method of Chomczynski and Sacchi (20). Isolation of poly(A)$^+$ RNA was achieved using a mRNA extraction kit (Promega, UK) according to the manufacturer's instructions. Northern blots to nylon N$^+$ membranes (Amersham, Buckinghamshire, UK) were prepared after size fractionation by gel electrophoresis of the denatured mRNA on 1% agarose/formaldehyde gels containing 20 mM morpholinosulfonic acid, 5 mM sodium acetate, and 1 mM EDTA, pH 7.0. A cloned Hm2 *Eco*RI/*Pst*I fragment of human muscarinic Hm2 cDNA and a 1272-base pair *Pst*I fragment specific to rat GAPDH mRNA were used as probes for the Northern analyses.

Prehybridizations and hybridizations were carried out at 42° with the probes labeled to approximately 1.5 $\times$ 10^6 cpm/ml in a buffer containing 50% formamide, 50 mM Tris·HCl, pH 7.5, 5 $\times$ Denhardt's solution, 0.1% SDS, 5 mM EDTA, and 250 μg/ml denatured salmon sperm DNA. After hybridization, the blots were washed to a stringency of 0.1 $\times$ standard saline citrate (1$\times$ = 15 mM sodium citrate, 0.15 M NaCl, pH 7.0), 0.1% SDS at 65° before exposure to Kodak X-Omat film. After suitable exposure times, autoradiographs were analyzed by laser densitometry (PDI Imageware System, Huntington Station, NY).

Cyclic AMP Measurements

After stimulation, cells were washed with HBSS, and the phosphodiesterase inhibitor Org 20241 (30 μM) was added to fresh medium for 15 min. Basal levels of cyclic AMP were measured as well as forskolin-stimulated (50 μM, 15 min) accumulation in the presence or absence of carbachol (10 nM to 1 mM; 15 min). Cells were harvested by addition of 1 ml of boiling water directly to each well followed by cell scraping before boiling for a further 2 min. After centrifugation at full speed in a microcentrifuge at 4° for 10 min, the supernatant was collected and stored at −20° before being assayed by radioimmunoassay as described by Brooker *et al.* (21). Protein concentrations were estimated using a Bio-Rad protein assay (Bio-Rad, Hemel Hempstead, UK), according to the manufacturer's instructions.

Measurement of Run-on Gene Transcription in Isolated Nuclei

Nuclei were prepared as described by Greenberg and Ziff (22) and nuclear run-on experiments were performed as described previously (15). Briefly, nuclei were incubated for 30 min at 27° in the presence of [^{32}P]UTP. Radiolabeled RNA was extracted using standard protocols and was hybridized to 10 μg of the immobilized plasmid pGEM3Z (control) or to pGEM3Z containing cDNA inserts of rat GAPDH and human m2 receptor in a buffer containing 50% formamide, 5 $\times$ standard saline citrate, 0.1% SDS, 1 mM EDTA, 10 mM Tris·Cl, pH 7.5, 5 $\times$ Denhardt's solution, 50 μg/ml yeast tRNA, 100 μg/ml salmon sperm DNA, and 0.02 μg of poly(A) and poly(G) RNA. The filters were washed in a buffer containing 300 mM NaCl, 10 mM Tris·HCl, pH 7.4, 2 mM EDTA, 0.1% SDS, 1 μg/ml RNase A, and 10 units/ml RNase T1 at 37° for 30 min and then in a buffer containing 10 mM NaCl, 10 mM Tris·HCl, pH 7.4, 2 mM EDTA, and 0.4% SDS to

a stringency of 55° for 30 min before exposure to Kodak X-Omat film for an appropriate time.

MAPK Studies

Extraction of cytosolic protein. After treatment, cells (approximately 1×10^6) were washed with HBSS supplemented with β-glycerophosphate, sodium orthovanadate, and NaF. Cells were lysed by addition of 100 μl of lysis buffer (1% Triton X-100, 0.5% SDS, 0.75% deoxycholate, 10 mM Tris, pH 7.4, 75 mM NaCl, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 100 μg/ml aprotinin, 5 mM NaF, and 10 mM sodium pyrophosphate) subsequent to centrifugation for 15 min at full speed in a microcentrifuge at 4°. Cell supernatants were boiled for 5 min in sample buffer (62.5 mM Tris·HCl, 20% glycerol 2% SDS, 10 mM 2-mercaptoethanol) and stored at −70° until use.

In gel phosphorylation assay. Assays were performed essentially as described by Kameshita and Fujisawa (23). Cytosolic protein (10 μg) was size fractionated by SDS-polyacrylamide gel electrophoresis on a 10% polyacrylamide gel containing 0.5 mg/ml myelin basic protein. After electrophoresis SDS was removed by three 20-min washes with 20% propan-2-ol in 50 mM Tris·HCl, pH 8.0, before a further 1-hr wash with 50 mM Tris·HCl, pH 8.0, 5 mM 2-mercaptoethanol (buffer A). Protein denaturation followed with two washes for 30 min in buffer A containing 6 M guanidine HCl before protein renaturation by several washes in buffer A containing 0.04% Tween 40 at 4° over 18 hr. The gel was then incubated in kinase assay buffer (40 mM HEPES/HCl, pH 8.0, 5 mM 2-mercaptoethanol, 100 nM EGTA, 5 mM MgAc, 25 μM ATP) containing 25 μCi of [γ-^{32}P]ATP for 1 hr. Nonspecific radioactivity was removed by five washes with 5% trichloroacetic acid/1% sodium pyrophosphate before drying under vacuum and subsequent exposure to Kodak X-Omat film for an appropriate time.

Results

Effect of PDGF on muscarinic M_2/m2 receptor expression. Receptor binding studies and Northern blot analyses were performed to measure changes in muscarinic receptor density and steady state mRNA levels after PDGF treatment (Fig. 1). Saturation studies performed with the non-subtype-selective muscarinic receptor antagonist [^{3}H]NMS revealed a single class of binding site (B_{max} 452 ± 23.4 fmol/mg of protein) with an equilibrium dissociation constant (K_D) of 0.20 ± 0.03 nmol. PDGF caused a time-dependent decrease in M_2 muscarinic receptor density. No significant change in M_2 muscarinic receptor density was observed after 4 hr, but fell to 60% of control levels after 14 hr of PDGF treatment (Fig. 1A) and 24 hr of treatment. A further reduction to approximately 40% of control was reported after 36-hr PDGF treatments. PDGF did not alter the affinity of [^{3}H]NMS for the remaining receptors (control, 0.2 ± 0.03 nmol; PDGF, 24 hr, 0.24 ± 0.03 nmol).

Northern blot analysis of isolated mRNA revealed the presence of a 6.1-kb transcript corresponding to the m2 receptor. PDGF treatments caused a time-dependent decrease in m2 muscarinic receptor mRNA (Fig. 1B) up to 14 hr (50% of control) that preceded the fall in receptor density. Longer incubations (24–36 hr) with PDGF resulted in m2 mRNA levels that remained significantly below control levels and indeed continued to fall up to 36 hr.

Receptor coupling studies. cAMP levels were measured in HEL 299 cells after short (up to 1 hr) PDGF treatments to determine whether stimulation of PDGF receptors resulted in the accumulation of cAMP. Longer incubations with PDGF were also performed to determine whether a functional desensitization of M_2 muscarinic receptor function accompanied the down-regulation in receptor number. PDGF induced a significant increase (150% control) in cAMP accumulation after incubations for up to 1 hr (Fig. 2A; 2.0 pmol/mg of protein in untreated cells up to 3.0 pmol/mg of protein after 30 min). In a second series of experiments (Fig. 2B), forskolin-stimulated cAMP accumulation was measured in control cells and in cells treated with PDGF for 14 hr. Concentration-response curves to carbachol-mediated inhibition of forskolin-stimulated cAMP accumulation were elucidated from cells treated with vehicle or PDGF for 14 hr. Although PDGF

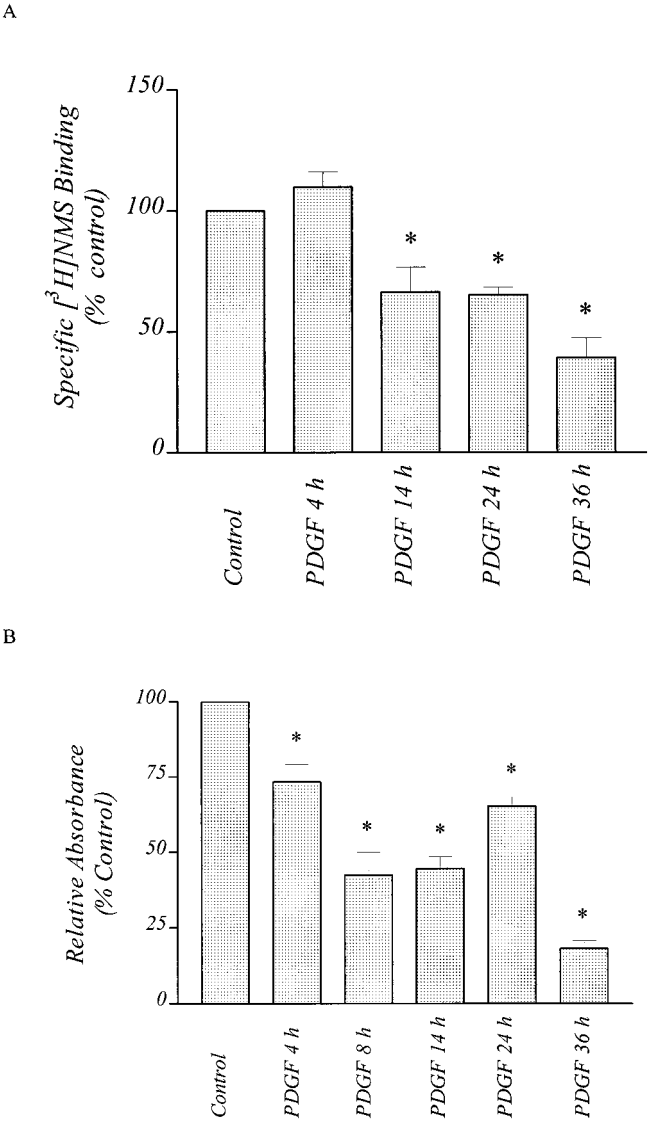

Fig. 1. Effects of PDGF on M_2 muscarinic receptor density and m2 mRNA levels. Cells were treated with PDGF (4 ng/ml) for the times shown before isolation of crude cell homogenates for radioligand binding studies or mRNA for Northern blot analyses. A, B_{max} values were obtained from saturation binding experiments as described in Experimental Procedures and are expressed as a percentage of control values. B, Northern blots were performed with ^{32}P-labeled cDNA probes specific to the m2 receptor and GAPDH mRNA. m2 receptor mRNA levels are expressed relative to GAPDH after assessment by laser densitometry. *Points,* mean (± standard error) of four or more independent experiments. ∗, Significant at $p < 0.05$ using Mann Whitney nonparametric test.

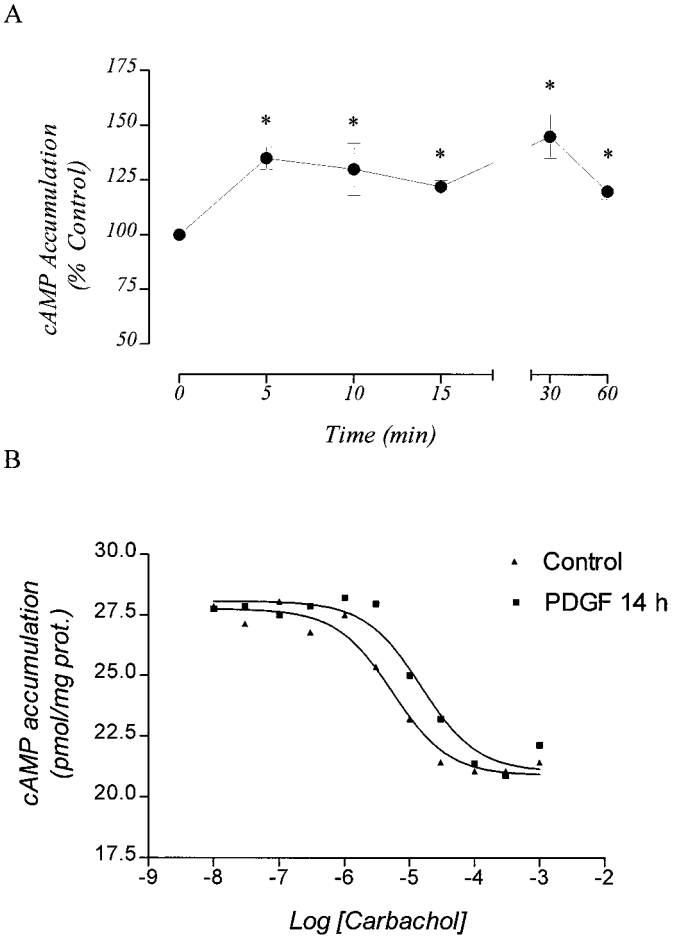

Fig. 2. cAMP accumulation studies. A, The effect of PDGF on cAMP accumulation. Cells were treated with PDGF for the times shown before the addition of the phosphodiesterase inhibitor Org 20241 for 15 min and extraction of cAMP. Data are mean ± standard error of four separate experiments performed in triplicate. B, A concentration-response curve to carbachol representative of three independent experiments. Cells were treated with drug vehicle or PDGF for 14 hr before the addition of forskolin (50 μM) for 15 min in the presence or absence of carbachol at the concentrations shown. *, Significant at $p < 0.05$ using Mann Whitney nonparametric test.

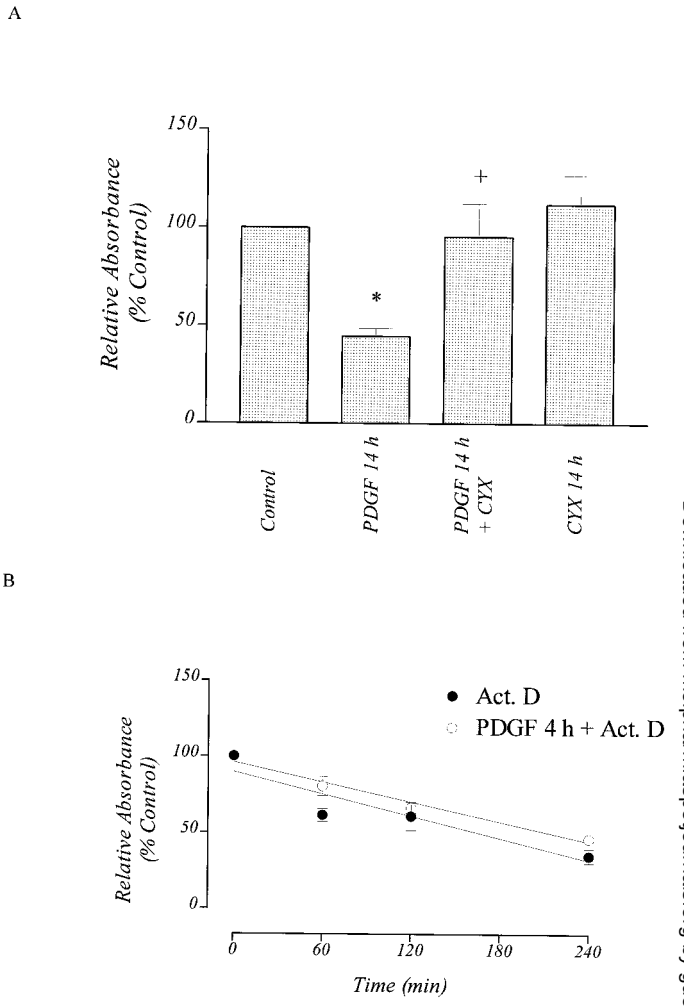

Fig. 3. Mechanisms involved in m2 mRNA down-regulation. A, Northern blots were performed on mRNA isolated from HEL 299 cells after treatments with drug vehicle (*control*) PDGF (4 ng/ml), cycloheximide (*CYX*, 10 μg/ml), and cycloheximide + PDGF. B, Cells were treated with vehicle (○) or PDGF for 4 hr (●) before the addition of actinomycin D (5 μg/ml) for the times indicated. Results are presented relative to the levels of GAPDH and are the mean (± standard error) of four independent experiments. *, Significant at $p < 0.05$ using Student's *t* test. +, Significantly different from PDGF 14 hr at $p < 0.05$ using Mann Whitney nonparametric test.

treatments shifted the concentration-response curve to the right, the IC_{50} values were not significantly different from vehicle-treated cells (control, 5.4 ± 0.8 μM; and PDGF, 14 hr, 11.8 ± 1.3 μM; $n = 3$). This modest shift in the potency of carbachol is, however, consistent with the fairly modest decrease in M_2 receptor density after 14 hr of PDGF incubation (60% of control).

The mechanisms involved in the down-regulation of m2 muscarinic receptor mRNA. Experiments were performed to investigate the mechanisms involved in the decrease of the steady state levels of mRNA. Preincubations with the protein synthesis inhibitor cycloheximide (10 μg/ml) inhibited the PDGF-induced reduction in steady state levels of m2 receptor mRNA, whereas cycloheximide alone had no effect (Fig. 3A). These data indicate *de novo* protein synthesis occurs after PDGF treatment that is required for the reduction in m2 mRNA. Half-life studies were also performed to determine whether the decrease in m2 mRNA was the result of any changes in the stability of the mRNA. The data obtained from treatments with the inhibitor of transcription, actinomycin D (5 μg/ml), indicated there was no change in the degradation rate of m2 mRNA after PDGF (4 hr) treatment (half-lives of 3 and 3.5 hr, respectively; Fig. 3B), indicating the decrease in m2 mRNA occurred through changes in the rate of transcription of the m2 receptor gene. This was confirmed by nuclear run-on transcription experiments where production of new m2-receptor mRNA was measured in isolated cell nuclei from control and PDGF-treated cells. The rate of transcription of new m2-receptor mRNA, compared with GAPDH (that remained unchanged), was reduced by 50% after 8 hr of PDGF treatment (Fig. 4).

Role of cellular kinases in m2 receptor mRNA down-regulation. To address the potential involvement of PKC, PI-3 kinase, and PKA in the m2 receptor down-regulation, experiments were performed with the PI-3 kinase inhibitor wortmannin, the PKC inhibitor GF 109203X, and the PKA inhibitor H-8. None of the above inhibitors attenuated the down-regulation observed with PDGF (PDGF, 14 hr; 44.6 ±

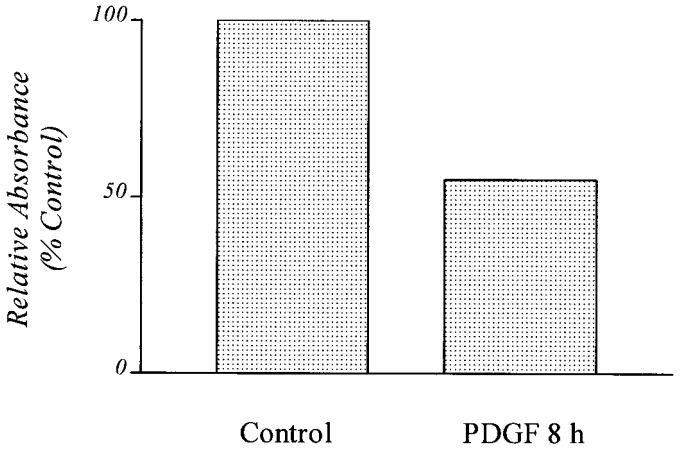

Fig. 4. Nuclear run-on transcription. [^{32}P]-labeled mRNA was transcribed *in vitro* from isolated cell nuclei (5×10^7) from PDGF treated (8 hr) and untreated cells before hybridization to plasmid cDNAs immobilized on nylon membranes. The plasmids used were pGEM3Z (negative control) and pGEM3Z containing m2-receptor and GAPDH cDNA inserts. Data are the average of two separate experiments and represent the ratio of the optical density of m2 to GAPDH in control and PDGF-treated cells.

3.9% control; PDGF + GF 109203X, 40 ± 2.2%; PDGF + wortmannin, 55 ± 7.0% and PDGF + H-8 55 ± 10%, respectively; Fig. 5). These results indicate that it is unlikely that these kinases are involved in m2 receptor mRNA down-regulation.

The role of MAPK in the down-regulation process was also investigated. Western blot analyses were performed to determine whether the 42- and 44-kDa ERK isoenzymes were present in HEL 299 cells. Western blots revealed the presence of both ERK1 and 2 isoenzymes in HEL 299 cells (data not shown). After treatments with PDGF and PDBu, there was a large and sustained increase in the activity of both ERK1 and 2, whereas treatments with carbachol did not affect the activity of either ERK isoenzymes suggesting M_2 receptors are not positively coupled to MAPK in these cells (Fig. 6). PD 098059, an inhibitor of MAPK kinase (24, 25), inhibited PDGF- and PDBu-mediated activation of ERK1 and 2 in a concentration-dependent manner (Fig. 7A). PD 098059 seemed to be a fairly potent inhibitor of PDGF-mediated activation of ERK1 and 2 (Fig. 7A), inhibiting between 1 and 10 μM concentrations in agreement with data from Alessi *et al.* (25), who found that PD 098059 inhibited phosphorylation of MEK1 with an IC_{50} of 2 μM. PDBu-mediated activation of ERK1 and 2 was also attenuated by PD 098059, although less potently than the activation induced by PDGF (Fig. 7B). Activation of ERK1 and 2 by PDBu was inhibited, although not completely, by incubations with the PKC inhibitor GF 109203X (1 μM; data not shown). The reasons for the residual activity of ERK1 and 2 after GF 109203X are not clear, although GF 109203X is a competitive inhibitor of PKC.

The attenuation of PD 098059 on PDGF- and PDBu-mediated down-regulation of m2 receptor mRNA mirrored that seen at the level of activation of ERK1 and 2 (Fig. 7). PDGF-mediated down-regulation of m2 mRNA levels was also attenuated in a concentration-dependent manner, suggesting a good correlation between changes in the activity of ERK1 and 2 and down-regulation of m2 mRNA. At 30 and 50 μM PD 098059, respectively, PDGF-and PDBu-mediated down-regulation of m2 receptor mRNA levels were inhibited signifi-

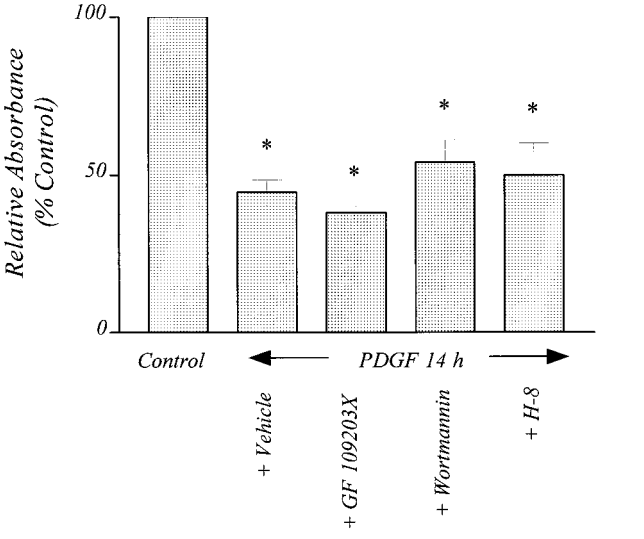

Fig. 5. Role of PKC, PI-3 kinase, and PKA in the down-regulation process. Northern blots were performed on isolated mRNA to determine whether PKC, PI-3 kinase, or PKA were involved in the PDGF-induced down-regulation of m2 receptor mRNA. Cells were treated with vehicle (*Control*) or with PDGF (4 ng/ml) for 14 hr in the absence (+ *vehicle*) or presence of a PKC inhibitor (+ *GF 109203X*; 1 μM), a PI-3 kinase inhibitor (+ *Wortmannin*; 10 nM) or a PKA inhibitor (+ *H-8*; 30 μM). Results are presented relative to the levels of GAPDH and are the mean ± standard error of four or more separate experiments. *, Significant at $p < 0.05$ using the Mann Whitney nonparametric test.

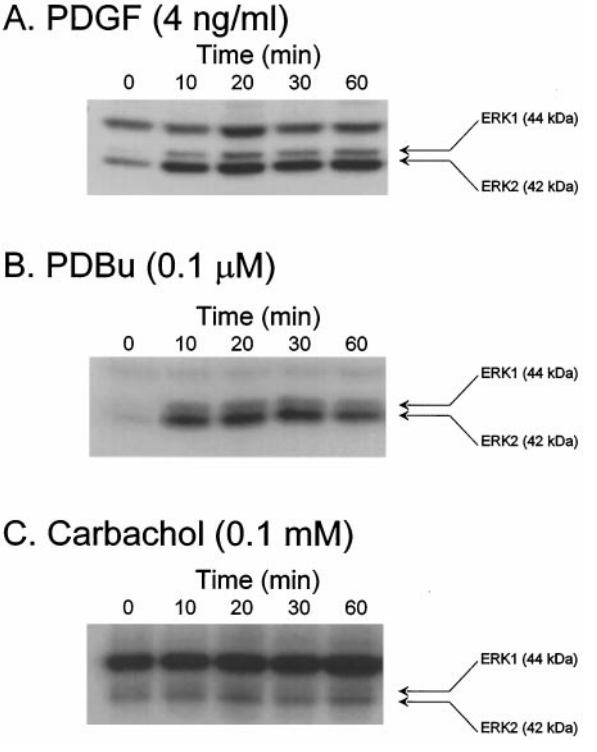

Fig. 6. Activation of ERK1 and 2 in HEL 299 cells. Experiments were performed to measure the activity of the 42- and 44-kDa ERK1 and 2 isoenzymes of MAPK. In gel phosphorylation assays were performed after isolation of cytosolic protein from cells treated with A, PDGF (4 ng/ml); B, PDBu (100 nM); or C, carbachol (0.1 mM) for the times indicated. Shown is a representative autoradiograph from at least four individual experiments.

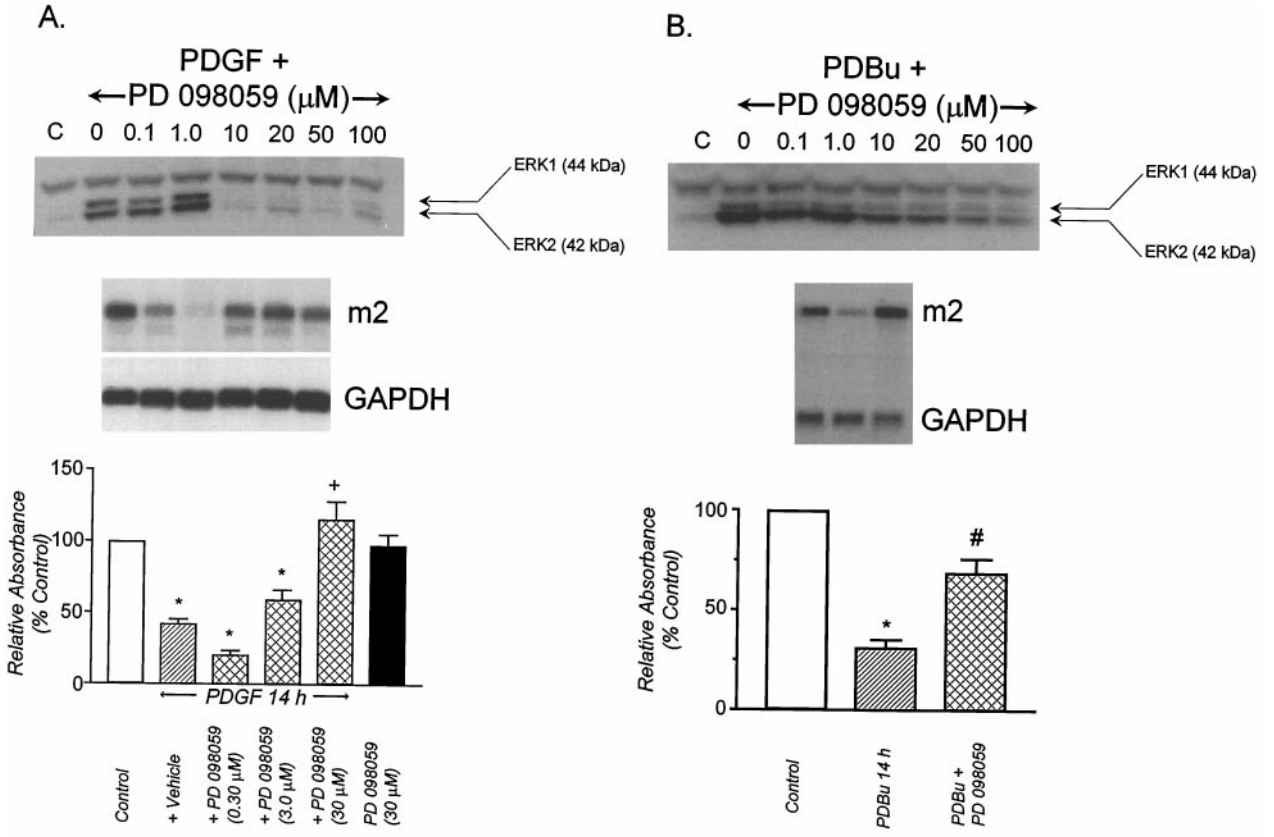

Fig. 7. Role of MAPK in the down-regulation process. *Top*, representative in gel phosphorylation assays after cells were pretreated with various concentrations of the MAPK kinase inhibitor PD 098059 before incubations with (A) PDGF (4 ng/ml) or (B) PDBu (0.1 μM) for 10 min. Northern blots were performed on isolated mRNA after cells were treated with dimethyl sulfoxide (*Control*), PDGF (4 ng/ml), or PDBu (100 nM) for 14 hr. Experiments were also performed in the presence of PD 098059 (0.3–50 μM) with PDGF (*PDGF + PD 098059*), PDBu (*PDBu + PD 098059*), or drug vehicle (*PD 098059*) for 14 hr. *Middle*, a representative Northern blot; *lower*, m2 mRNA levels are presented relative to the levels of GAPDH and are the mean ± standard error of at least four individual experiments. ∗, Significantly different from control at $p < 0.05$ using Mann Whitney nonparametric test; +, significantly different from PDGF at $p < 0.05$ using Mann Whitney nonparametric test; #, significantly different from PDBu at $p < 0.05$ using Mann Whitney nonparametric test.

cantly (PDGF 44.6% of control; PDGF + PD 098059 120 ± 12% of control; PDBu 31 ± 4%, PDBu + PD098059 70 ± 7% of control; Fig. 7). Conversely, at concentrations of 0.1 and 1 × the reported IC_{50} of PD 098059 (25), no significant inhibition was observed.

Discussion

We have conducted experiments to investigate whether stimulation of PDGF results in regulation of m2 receptor gene expression. We have also investigated whether the activation of the 42- and 44-kDa ERKs can be related to the down-regulation process.

PDGF treatments resulted in a time-dependent decrease in M_2 receptor density in HEL 299 cells that was preceded by a decrease in the steady state levels of m2 mRNA. The reduction in M_2 receptor density was accompanied by a modest decrease in the potency of carbachol to elicit inhibition of adenylyl cyclase. Although this 2-fold reduction in potency did not reach statistical significance, it is consistent with the fairly modest decrease in receptor density observed after 14-hr treatments with PDGF (60% of control). This is in contrast to previous studies on these cells. Long-term (24 hr) stimulations with the β_2-agonist procaterol (26), the muscarinic receptor agonist carbachol (27), PDBu (15), and TGF-β_1 (16) all resulted in a functional uncoupling of the remaining M_2 receptors. Phosphorylation is essential in the desensitization and internalization of G protein-coupled receptors and may be mediated by a number of cellular kinases in an agonist-dependent and -independent manner (3, 4). PDGF did not elicit any statistically significant change in the functional coupling of the remaining muscarinic receptors, suggesting phosphorylation of the majority of M_2 receptors does not occur. Furthermore, the reduction in M_2 receptor density was preceded by a reduction in the steady state levels of m2 mRNA suggesting the loss in receptor density was a consequence of the loss in m2 mRNA.

The mechanisms involved in the loss of the steady state levels of m2 mRNA were investigated. Preincubation with cycloheximide completely inhibited the down-regulation observed with PDGF indicating *de novo* protein synthesis is required in the down-regulation process. Actinomycin D treatments were also performed in untreated cells and in cells treated with PDGF for 4 hr. PDGF treatments did not result in any changes in the stability of the m2 mRNA, indicating changes must occur at the level of transcription. This was confirmed by nuclear run-on experiments that showed a large decrease in the rate of transcription after 8-hr PDGF treatments. The requirements for protein synthesis and reduced gene transcription are consistent with incuba-

tions of HEL 299 cells with other mitogens including phorbol ester (15) and TGF-β_1 (16).

Experiments were performed to determine whether protein kinase C or PI-3 kinase were involved in the down-regulation process. PI-3 kinase is known to be activated by PDGF, resulting in the generation of phosphatidyl-3,4,5-trisphosphate (28) and may also be important in the activation of atypical PKC isoenzymes (29). Preincubation with wortmannin, a selective and potent inhibitor of PI-3 kinase (30) did not significantly attenuate the PDGF-mediated reduction of m2 mRNA, indicating PI-3 kinase is not involved in the down-regulation process. Similarly, preincubation with the PKC inhibitor GF 109203X (31) did not inhibit the down-regulation induced by PDGF. This lack of involvement of PKC in the down-regulation process may be consistent with the lack of functional uncoupling of the remaining M_2 receptors after PDGF incubations. We have shown previously that stimulation of PKC results in the functional uncoupling of the M_2 receptors in these cells (15). In addition, we have shown that β-agonist-induced down-regulation and desensitization of M_2 receptors in these cells is partially reversed with GF 109203X (26).

As well as the possible roles for PKC and PI-3 kinase, experiments were performed to determine whether PKA was involved in the down-regulation process. cAMP accumulation was measured after PDGF treatment as it has been reported that PDGF stimulates cAMP formation in a number of cells including arterial smooth muscle (32). This large and rapid accumulation of cAMP (150-fold) was accompanied by activation of PKA and seems to occur through MAPK-mediated activation of phospholipase A_2 (32). In HEL 299 cells, treatments with PDGF caused a significant but small increase in cAMP levels (approximately 150% control). Preincubations with the PKA inhibitor H-8 did not, however, attenuate the down-regulation observed with PDGF, indicating that PKA does not play an important role in the down-regulation process.

The involvement of MAPK in m2 receptor down-regulation was investigated. Experiments were performed to measure the activity of the 44- and 42-kDa ERK1 and two isoenzymes of MAPK after PDGF, PDBu, and carbachol stimulations. All three stimuli have been reported to activate MAPK in a variety of systems (see Ref. 33 and references therein). PDGF and PDBu treatments resulted in a large and transient increase in the activity of ERK1 and 2 in agreement with a number of reports. In contrast to reports in other systems (14, 34), however, carbachol treatments did not activate MAPK. The "in gel" data are consistent with our findings at the mRNA level in HEL 299 cells where PDBu and PDGF, but not carbachol, treatments resulted in a down-regulation in the steady state levels of m2 mRNA through changes in gene transcription.

To further investigate the role of MAPK in the down-regulation process, experiments were performed with the MAPK kinase inhibitor PD 098059 (25). PD 098059 inhibited both PDGF- and PDBu-mediated activation of ERK1 and 2 in a concentration-dependent manner, although the inhibitor was more potent against PDGF-mediated than PDBu-mediated activation of ERK1 and 2. Neither PD 098059 nor the PKC inhibitor GF 109203X completely inhibited activation of MAPK by PDBu, suggesting some nonselective effect of PDBu. At the mRNA level, PD 098059 completely inhibited PDGF-induced, but only partially inhibited PDBu-mediated, down-regulation of m2 receptor mRNA, consistent with the ERK1 and 2 activation data. At concentrations below and around the reported IC_{50} of PD 098059 there was no significant inhibition of PDGF-mediated down-regulation in m2 mRNA. The reason for the differential inhibition of ERK activation by PD 098059 after PDGF or PDBu treatments is unclear, but even at the highest concentrations (30–50 μM) used PD 098059 does not inhibit a range of protein kinases including PKC (24). Furthermore, Alessi *et al.* (25) observed a similar resistance to PD 098059 in Swiss 3T3 cells where the inhibition of EGF-mediated activation of $p42^{MAPK}$ was dependent upon the initial concentration of EGF. Whether the initial concentration of PDBu in this system is important or not is debatable but may go some way to explain the residual activity. It should be noted that the exact mode of activation of MAPKs by PKC is not known. Although PKCα is known to phosphorylate Raf-1 *in vitro* (13), the effects of PKC phosphorylation on Raf-1 remain controversial (35).

In summary, we have shown that stimulation of the ERK1/2 pathway results in the down-regulation of m2 muscarinic receptor gene expression through the synthesis of unknown protein mediators. The ERK1 and 2 isoenzymes of MAPK enzymes seem to play an important role in down-regulation of m2 mRNA as inhibition of this pathway greatly attenuates down-regulation induced by PDGF and PDBu.

Acknowledgments

We wish to thank Marie Bogoyevitch and Monica Andersson for useful advice in establishing the "in gel" phosphorylation assay.

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