Modulation of muscarinic-receptor expression in human embryonic lung fibroblasts by platelet-derived growth factor

Ahmet KOMAN,* Odile DURIEU-TRAUTMANN†, Pierre-Olivier COURAUD†, A. Donny STROSBERG† and Babette B. WEKSLER‡

Molecular Biology of Receptors Unit, Department of Biotechnology, Pasteur Institute, 28 rue du Dr. Roux, 75427 Paris, France

Platelet-derived growth factor (PDGF) is known to have regulatory control of a large number of cellular components, including various receptors. We show that muscarinic acetylcholine receptors of the m2 subtype on CCL 137 human fibroblasts in culture are affected by PDGF treatment. A time-dependent down-regulation is observed in steady-state RNA levels, followed by a decrease in ligand-binding capacity. Minimum RNA levels are attained at 11 h; minimum binding capacity is observed after 24 h of treatment. To our knowledge, this is the first example of negative gene control by PDGF.

INTRODUCTION

Muscarinic cholinergic receptors modulate physiological responses such as smooth-muscle contraction, vasodilatation, glandular secretion and neural transmission, and are widely distributed in tissues. Subtypes of muscarinic receptors, originally delineated by specific ligand-binding patterns, have more recently been further distinguished by their gene sequences (Kubo *et al.*, 1986; Braun *et al.*, 1987; Gocayne *et al.*, 1987; Peralta *et al.*, 1987a,b; Bonner *et al.*, 1988). The genetically defined subtypes m1–m5 have been localized to different areas of brain, and m3 is also expressed in exocrine secretory glands; subtype m2 corresponds to the pharmacologically defined M2 receptors in cardiac and vascular tissues (Bonner *et al.*, 1987; Peralta *et al.*, 1987a; Maeda *et al.*, 1988). Differences in inhibition of adenylate cyclase, activation of the phosphatidylinositol cycle and interactions with G-proteins also distinguish several of the muscarinic-receptor subtypes (Ashkenazi *et al.*, 1987; Kerlavage *et al.*, 1987; Peralta *et al.*, 1988; Stein *et al.*, 1988).

A human embryonic lung fibroblast line, CCL 137, displaying abundant muscarinic receptors, has been previously described by our laboratory, and the receptors have been pharmacologically characterized as belonging to the M2 subtype (André *et al.*, 1988). These receptors mediate inhibition of adenylate cyclase and, with less efficiency, stimulation of phosphatidylinositol hydrolysis.

Platelet-derived growth factor (PDGF) is a known growth factor for fibroblasts. Among its multiple effects are autoregulation of PDGF receptors (Heldin *et al.*, 1982) and transmodulation of a variety of membrane receptors such as those for 5-hydroxytryptamine (Coughlin *et al.*, 1981), low-density lipoprotein (Habenicht *et al.*, 1986), transferrin (Davis & Czech, 1986), interleukin 1 (Bonin & Singh, 1988) and epidermal growth factor (Wharton *et al.*, 1982; Olashaw *et al.*, 1986). The transcription of several genes is stimulated by PDGF (Cochran *et al.*, 1983).

In the present report, we have investigated whether m2 muscarinic receptors also come under the regulatory control of

PDGF. We evaluated binding of the cholinergic antagonist quinuclidinyl [*phenyl*-4-³H]benzylate (³H]QNB) to CCL 137 cells during the quiescent phase after confluence, and compared receptor binding at intervals after stimulation by PDGF with steady-state levels of m2-receptor mRNA.

We show that PDGF induces a time-dependent decrease in muscarinic-receptor number in confluent growth-arrested cells, without altering binding affinity of the receptors. Decreased muscarinic-ligand binding was preceded by a decrease in the steady-state level of RNA for the m2 subtype of muscarinic receptor. These results indicate that PDGF does participate in the regulation of a muscarinic receptor.

METHODS

Cell culture

The human lung fibroblast line CCL 137 (HEL 299) was obtained from the American Type Culture Collection. The cells were cultured as previously described (André *et al.*, 1988). At 1 day after confluence was reached, the medium was replaced with fresh medium supplemented with 1% (v/v) fetal-calf serum. Under these conditions, the cells became quiescent after 24 h, as measured by [³H]thymidine incorporation and by cell counts. We determined that the number of muscarinic binding sites was decreased by 40–50% in cells maintained for 48 h in medium containing 1% fetal-calf serum, compared with cells kept in medium with 10% fetal-calf serum. Experiments were all conducted with cell cultures maintained in medium containing 1% fetal-calf serum, designated 'serum-starved cultures'.

Stimulation

During the 48 h of serum starvation, CCL 137 fibroblasts were exposed to recombinant PDGF (B-chain; Amersham), at 4 ng/ml of culture medium, or to an equivalent volume of vehicle (0.1 M-acetic acid containing 0.5% BSA) for periods of 2–48 h before simultaneous harvest, after which binding of [³H]QNB (Amersham; 42 Ci/mmol) was assayed or total RNA was

Abbreviations used: PDGF, platelet-derived growth factor; [³H]QNB, quinuclidinyl [*phenyl*-4-³H]benzylate.

* To whom correspondence should be addressed. Present address: Laboratoire d'Immunologie, Faculté des Sciences Pharmaceutiques et Biologiques, Université René Descartes, Paris V, 4 Avenue de l'Observatoire, 75006 Paris, France.

† Present address: Laboratoire d'Immuno Pharmacologie Moléculaire, C.N.R.S. et Université Paris VII, Institut Cochin de Génétique Moléculaire, 22 rue Méchain, 75764 Paris Cedex 14, France.

‡ Present address: Division of Hematology–Oncology, Department of Medicine, The New York Hospital–Cornell Medical Center, 525 East 88th Street, New York, NY 10021, U.S.A.

prepared. In preliminary experiments we determined that a maximal effect on muscarinic-ligand binding was obtained at 4 ng of PDGF/ml and that DNA synthesis was initiated by PDGF within 18 h.

Muscarinic-ligand binding

Cells were detached from culture flasks with 0.025% trypsin/1 mM-EDTA, washed with Hepes-buffered (20 mM) Hanks salt solution, pH 7.4, supplemented with 0.1% BSA, and were suspended in this medium at $(2-4) \times 10^5$ cells/ml. Portions (0.8 ml) of this cell suspension were incubated at 37 °C with [3 H]QNB for 40 min in a final volume of 1 ml. The reaction was stopped by the addition of 4 ml of ice-cold phosphate-buffered saline [10 mM- $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ (pH 7.4)/145 mM- NaCl] and the cells were trapped on Whatman GF/C glass-fibre filters, washed, and bound radioactivity was measured in a liquid-scintillation counter. Non-specific binding was assessed in the presence of 100 μM -atropine and did not exceed 20% of total binding. In preliminary studies, it was established that binding experiments conducted with suspensions of enzyme-detached CCL 137 cells prepared as detailed above gave results equivalent to those of binding experiments carried out with cell monolayers treated *in situ* without detachment. Experimental variability was minimized in the assays with cell suspensions compared with the monolayers, so this method was employed for the results reported below.

RNA isolation and Northern blotting

Total cellular RNA was harvested from PDGF-treated and control cells by dissolving washed cell monolayers in 4 M-guanidinium isothiocyanate solution, followed by separation of RNA by centrifugation through CsCl by the method of Chirgwin *et al.* (1979) or by phenol extraction (Chomczynski & Sacchi, 1987). The two methods gave equivalent results. Northern blots on nitrocellulose (Schleicher and Schuell) were prepared from denatured RNA samples (15 μg /lane) after electrophoresis in 1.2%-agarose/formaldehyde gels. Slot blots of 10 μg of RNA were prepared on nitrocellulose. Blots were hybridized to a ^{32}P -labelled RNA probe specific for the m2 subtype of the human muscarinic receptor, kindly provided by Dr. Tom I. Bonner (National Institute of Mental Health, Bethesda, MD, U.S.A.). The probe consisted of the 510 bp (823-1333) *Ava*I fragment of the m2 cDNA sequence (corresponding to the third putative intracellular loop of the protein) inserted in the *Xma*I site of pSP64 and transcription-labelled to a specific radioactivity of $(0.5-1) \times 10^9$ c.p.m. Hybridization was carried out as specified by Promega (Madison, WI, U.S.A.) for Riboprobe systems at 55 °C for 18 h, and blots were washed for 3×20 min with $0.1 \times \text{SSC}/0.1\%$ SDS at 65 °C ($1 \times \text{SSC}$ is 150 mM- $\text{NaCl}/15$ mM-sodium citrate, pH 7.4). Under these conditions, a single hybridizing band of 6.5 kb was obtained on Northern blots. A probe for mouse α -actin was provided courtesy of Dr. Tomas Geijer (University of Uppsala, Sweden).

RESULTS

Binding of [3 H]QNB to confluent serum-starved CCL 137 cells was measured after PDGF or control treatment for 2-48 h. Binding declined in a time-dependent manner in PDGF-treated cells (Fig. 1), beginning at 11 h and reaching a minimum at 24 h. Scatchard analysis of the saturation-binding curve for [3 H]QNB revealed that a 24 h treatment with PDGF induced a decrease in m2 receptor density (54.6 ± 1.9 fmol/ 10^6 cells, versus 104.6 ± 4.0 fmol/ 10^6 cells in controls) (Fig. 2). The apparent dissociation constant remained unchanged by PDGF treatment of the cells ($K_D = 0.13 \pm 0.02$ nM after PDGF treatment versus 0.19 ± 0.02 nM in controls).

Fig. 1. Time-dependent effect of PDGF on [3 H]QNB binding in CCL 137 cells

Confluent cells were starved for 48 h. PDGF (4 ng/ml) was added during starvation at the indicated time before cell harvest. [3 H]QNB binding was measured as described in the Methods section. The [3 H]QNB binding results are expressed as percentages of the control level (measured in the presence of vehicle alone), 100% at zero time = 98.4 ± 13.6 fmol/ 10^6 cells. Means $\pm$ s.d. are given for three independent duplicate experiments.

Fig. 2. Inhibition of [3 H]QNB binding by PDGF in CCL 137 cells

At 24 h before harvest, serum-starved cultures were treated with 4 ng of PDGF/ml (■) or vehicle alone (□). Saturation experiments were done with [3 H]QNB concentrations ranging from 10 to 80 nM. Results are expressed as Scatchard plots, with B for bound radioactivity and F for free ligand concentration.

The steady-state level of RNA for the m2 muscarinic receptor was evaluated by Northern blotting of total cellular RNA hybridized with an RNA probe for the m2 subtype. In preliminary studies we established that the CCL 137 fibroblasts, as predicted from the characteristics of their binding of muscarinic ligands, expressed the m2-subtype muscarinic receptors (Fig. 3a) and did not express detectable m1 receptors (results not shown). After addition of PDGF to the cell cultures, a decrease in m2-receptor mRNA levels was observed after 5 and 9 h. In contrast, actin RNA levels did not change (Fig. 3b).

Autoradiographs of slot-blots from two independent experiments were quantified by densitometric scanning. The time course of the change in m2-receptor mRNA levels was compared with that of [3 H]QNB binding (Fig. 4). The decrease in the steady-state level of receptor mRNA begins about 6 h before the decrease in the binding capacity of the cells (5 versus 11 h). RNA levels return to control values after 17 h of treatment; the binding capacity does not significantly increase by 48 h.

Fig. 3. Northern-blot analysis of m2-receptor RNA levels

(a) CCL 137-cell total RNA (15 µg/lane) was run on a 1.2% agarose/formaldehyde gel; blotting and hybridization were performed as described in the Methods section, by using a ^{32}P -labelled m2-receptor RNA probe. (b) Slots were loaded with 10 µg of total RNA of CCL 137 cells treated for the indicated times with PDGF (4 ng/ml) or vehicle alone; blots were hybridized with either m2-receptor or actin (Act) RNA probe and autoradiographed for 3 days. Results are representative of three independent experiments.

Fig. 4. Comparison between the PDGF-induced changes in m2-receptor mRNA level and $[^3\text{H}]\text{QNB}$ -binding capacity of CCL 137 cells

Autoradiographs of slot-blot corresponding to m2-receptor-mRNA signals of cells treated with PDGF for the indicated times were quantified by densitometric scanning: results are expressed as percentages of the mean value of all time points with vehicle alone (■). Binding data (□) are from Fig. 1.

DISCUSSION

We have addressed the question whether PDGF affects the number of muscarinic receptors in normal human fibroblasts in culture. Although we have examined fetal lung fibroblasts, studies with adult lung fibroblasts have suggested that similar muscarinic receptors are also present (Van Riper *et al.*, 1985). Under the conditions used in the present experiments, control cells displayed approx. 6×10^4 muscarinic receptors/cell. We confirmed, by Northern-blot hybridization, previous pharmacological data that these cells express the m2 receptor subtype.

When CCL 137 fibroblasts were grown to confluence and synchronized by growth arrest in medium containing a low

concentration of serum, the addition of mitogenic concentrations of PDGF resulted in a consistent decrease in the binding of the muscarinic ligand $[^3\text{H}]\text{QNB}$. Analysis of the saturation-binding curves by the method of Scatchard revealed a 50% decrease in the numbers of a single population of receptors, without alteration in receptor affinity. The fall in receptor number was detected 11 h after exposure of the cells to PDGF, and this fall persisted with increasing exposure time up to 24 h. Receptor numbers remained decreased at 48 h, the latest time point examined. This time-dependent change in receptor number is unlikely to represent receptor internalization, since binding was assayed with a hydrophobic ligand that measures both membrane-exposed and internal binding sites. The change in receptor number is not related to cell division, since under the conditions employed the cell number does not change. The decrease in receptor number was preceded by a decrease in the steady-state RNA level for the m2 receptor, measured by Northern blotting of total RNA. This decrease in specific RNA was first detectable by 5 h after addition of PDGF to the cell culture and persisted up to 14 h after the addition of PDGF. The amount of RNA for the m2 muscarinic receptor returned to the baseline value by 17–24 h, so that the changes in steady-state RNA also preceded cell division. Whether the return of RNA levels to control values is followed by an increase in QNB-binding sites is not possible to elucidate under the conditions used, as cells could not be maintained under starving conditions for longer time periods than 48 h without loss of viability.

The order of events is consistent with a model where PDGF has an inhibitory effect on the transcription of the m2-receptor gene, followed by a decrease in receptor number. It will be of interest to determine if post-transcriptional control of the expression of muscarinic receptors, such as increased degradation of m2-receptor mRNA, contributes to the final effect on muscarinic ligand-binding sites after PDGF-mediated initiation of cell proliferation.

The observed changes in muscarinic-receptor RNA level and in receptor number occur later than PDGF-induced expression of the *c-myc* and *c-fos* genes, phosphorylation of the epidermal-growth-factor receptor, or the induction of RNA for interleukin-1 receptors, all 'early' effects observed within minutes of addition of PDGF. It is tempting to speculate that the PDGF effect on m2-receptor RNA levels might be mediated by an early-induced nuclear protein. PDGF elicits a vast number of biochemical responses, such as protein phosphorylation, phosphatidylinositol turnover, cyclic AMP synthesis, and changes in ionic flux. Several of these factors have been shown to affect gene regulation. Protein phosphorylation via protein kinase C may be implicated, since m2-receptor RNA levels are also found to decrease upon phorbol ester treatment of SHSY-5Y neuroblastoma cells with a similar time course (A. Koman & P.-O. Conrand, unpublished work), followed by a decrease in QNB binding (Adem *et al.*, 1987).

Some previous reports indicate that muscarinic agonists alter DNA synthesis either positively (Ashkenazi *et al.*, 1989a,b) or negatively (Conklin *et al.*, 1988). The physiological role of PDGF-induced down-regulation of m2 muscarinic receptors may be related to an involvement of these in cell proliferation. To our knowledge, this is the first example of negative control of RNA levels by PDGF.

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