Activation of platelet-derived growth factor (PDGF) receptor dephosphorylation in intact Swiss 3T3 cells by elevators of intracellular Ca^{2+} and cAMP

Frank-D. Böhmer[a,*], S.-Annette Böhmer[b]

[a] *Max-Planck Society, Research Unit 'Molecular Cell Biology', Medical Faculty, Friedrich-Schiller University, Drackendorfer Str. 1, D 07747 Jena, Germany*
[b] *Institute of Biochemistry II, Medical Faculty, Friedrich-Schiller University, Löbderstr. 3, D 07743 Jena, Germany*

Received 28 May 1996

Abstract We investigated the modulation of platelet-derived growth factor (PDGF) receptor dephosphorylation in Swiss 3T3 cells using a novel assay permitting monitoring of receptor dephosphorylation in intact cells. PDGF treatment of the cells reduced the receptor dephosphorylation rate to 41%, the elevators of intracellular Ca^{2+}, A23187 and thapsigargin increasing it to 227 and 138%, respectively. The cAMP elevators forskolin and isobutylmethylxanthine also accelerated PDGF receptor dephosphorylation. The involvement of Ca^{2+}- and cAMP-dependent protein kinases in the regulation of PDGF receptor dephosphorylation is suggested.

Key words: Platelet-derived growth factor receptor; Receptor dephosphorylation; Protein tyrosine phosphatase; Fibroblast (Swiss 3T3)

1. Introduction

Autophosphorylation of growth factor receptor tyrosine kinases is an important step for receptor signal transduction [1,2]. PTPases [3,4] dephosphorylate the autophosphorylated receptors [5,6] and thereby most likely antagonize the mitogenic signal. A number of transmembrane PTPases have been implicated in the attenuation of insulin receptor signaling [7–9]. The SH2-domain PTPase PTP1C (SHC, HCP, SH-PTP1; see [10] for review) has been shown to interact with multiple transmembrane receptors including Kit/SCF receptor [11], HER2 [12] and the EGF receptor [13] and is believed to contribute to negative regulation of signaling. Little is known about the identity and regulation of PTPases which attenuate PDGF receptor signaling [6,14]. In coexpression systems the capability of PTP1C [12], low-M_r PTPase [15] and the transmembrane PTPases CD45 and RPTPα ([16]; R. Lammers, personal communication) to dephosphorylate the PDGF receptor has been demonstrated. Distinct characteristics of EGF receptor and PDGF receptor dephosphorylation in the same cells suggest the involvement of different PTPases in the process [6].

Using a recently developed novel assay which permits for the first time the monitoring of growth factor receptor dephosphorylation in intact cells [17], we studied the modulation of PDGF receptor dephosphorylation in Swiss 3T3 cells. Agents known to elevate intracellular Ca^{2+} and cAMP accelerate receptor dephosphorylation, suggesting that the involved PTPases are positively regulated via Ca^{2+}- and cAMP-dependent pathways.

2. Materials and methods

2.1. Cells

Swiss 3T3 cells were obtained from the American Type Culture Collection (CCL 92) and grown in DMEM (Life Technologies) supplemented with 10% calf serum. Confluent, density inhibited cells (approx. $3–4\times10^5$ cells/cm^2) were used for all experiments except for the comparison between resting and growing cells, cultures at about 50% confluence (approx. $1–2\times10^5$cells per cm^2) also being included.

2.2. Assay of PDGF receptor dephosphorylation

The assay has been described and validated before in detail [17]. In brief, the medium of confluent cells in 24-well plates was changed to serum-free DMEM, buffered with 20 mM HEPES, pH 7.5 and the cells were treated for various lengths of time with the different agents, as outlined in the figure and table legends. Then, PDGF receptor autophosphorylation was initiated by the addition of PDGF-BB (100 ng/ml) for 5 min at room temperature. Thereafter, the PDGF receptor specific kinase inhibitor AG1296 [18] was added to a final concentration of 50 μM to quench the receptor kinase reaction. Cell extracts were prepared at different time points (from 0 to 10 min) thereafter as described [17] and the tyrosine phosphate content of the receptor was evaluated by immunoblotting of equal amounts of cell lysate protein. Under the used conditions, decay of receptor phosphotyrosine is completely caused by receptor-directed PTPase activity. To obtain semi-quantitative measurements of receptor dephosphorylation rates, the receptor signals on the immunoblots were quantified by densitometry. Usually, 50% dephosphorylation was observed 2–5 min after kinase inhibition. Relative dephosphorylation rates (% $PTPase_T$) in cells treated (T) under various conditions compared to control (C) dephosphorylation rates (100%) were calculated based on the formula: % $PTPase_T = (\Delta PY_T/\Delta PY_C)\times100\%$ with ΔPY being the difference in the signal intensity between time point 0 and a second measuring time point routinely chosen at 30–50% dephosphorylation in controls.

2.3. Raytide assays

These were performed as described [13]. Lysis buffer to obtain the cell extracts for Raytide dephosphorylation measurements was as described [17] but omitting PTPase inhibitors. Different protein concentrations were analyzed to ensure that the comparative assays were performed within the linear range of the method.

2.4. Reagents

Human recombinant PDGF-BB was obtained from Biomol (Hamburg). A23187, calpeptin and calphostin C were purchased from Calbiochem (Bad Soden). Thapsigargin, forskolin, 3-isobutyl-1-methylxanthine and phorbol-12-myristate-13-acetate were obtained from Sigma (Deisenhofen).

*Corresponding author. Fax: (49) (3641) 304 462

Abbreviations: DMEM, Dulbecco's modified Eagle medium; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; PKC, protein kinase C; PTPase, protein tyrosine phosphatase.

PII S0014-5793(96)00740-5

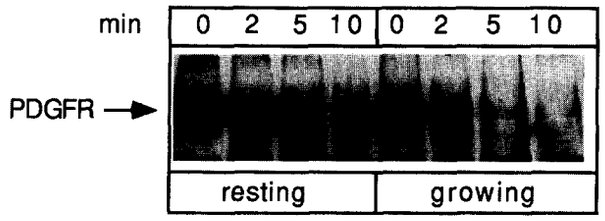

Fig. 1. Time course of PDGF receptor dephosphorylation in density-inhibited and growing Swiss 3T3 cells. Swiss 3T3 cells were seeded at different densities so that they reached confluency and growth arrest ('resting', $3–4\times10^5$ cells/cm^2) or grew actively ('growing', $1–2\times10^5$ cells/cm^2) at the time of experiment. Then, PDGF receptor dephosphorylation was measured as described under Section 2. The receptor kinase was stimulated briefly by PDGF and subsequently inhibited with the potent membrane permeable tyrosine kinase inhibitor AG1296. Then, the phosphotyrosine content of the receptor was evaluated by immunoblotting at different time points. A typical experiment is shown.

3. Results and discussion

A recently developed method to visualize growth factor receptor dephosphorylation in intact cells is based on rapid inhibition of the receptor kinase with cell-permeable tyrosine kinase inhibitors and subsequent monitoring of the time course of decay of receptor tyrosine phosphate content by immunoblotting [17]. Compared with previously described methods this approach offers at least two advantages: It allows unequivocal detection of receptor dephosphorylation as opposed to the plain detection of net receptor phosphorylation and it does not require the destruction of cell integrity for measurement. This assay was employed to evaluate the characteristics of PDGF receptor dephosphorylation in Swiss 3T3 cells.

Swiss 3T3 cells exhibit a pronounced density-dependent inhibition of cell growth and can be mitogenically stimulated by a wide variety of growth factors including PDGF. Increased membrane-associated PTPase activity [19] and increased mRNA expression of the transmembrane PTPase mRPTPσ [20] have been observed before in density-arrested Swiss 3T3 cells, suggesting that PTPase activity might contribute to generation of the cell density-dependent growth arrest. Such growth attenuation might involve negative modulation of growth factor receptor autophosphorylation. We therefore investigated the effect of growth state and growth factor treatment on the activity of PDGF receptor dephosphorylating PTPases. As shown in Fig. 1, no difference could be observed when rapidly growing and resting Swiss 3T3 cells were compared with respect to PDGF receptor dephosphorylation rate. Also, pretreatment of the cells with the mitogenically very potent combination of EGF (10 ng/ml) and insulin (1 μg/ml) for 20 min, 2 h or 24 h had no effect (not shown). Thus, the previously observed increases in unidentified PTPase activity [19] as well as in the mRPTPσ level by growth arrest [20] seem not to affect PDGF receptor dephosphorylation under our assay conditions. Pretreatment of the cells for 2 h with PDGF, however, attenuated the subsequently observed receptor dephosphorylation rate (Table 1) to 41%. PDGF treatment could inactivate or reduce the expression of PTPases which participate in receptor dephosphorylation. In this context it is interesting to note that mRNA expression of mRPTPα, a transmembrane PTPase which is dominantly expressed in Swiss 3T3 cells [20], becomes reduced after 2 h PDGF treatment. The latter correlation of reduced PDGF receptor dephosphorylation rate and reduced mRPTPα expression level might suggest that mRPTPα participates in PDGF receptor dephosphorylation. This hypothesis is currently being tested in our laboratory.

Regulation of cell membrane-associated PTPase activity towards synthetic substrates via Ca^{2+}- and cAMP-dependent pathways has been observed in African green monkey kidney (CV-1) cells [21]. We therefore examined whether elevation of intracellular Ca^{2+} by ionophore treatment could lead to modulated PDGF receptor dephosphorylation. Indeed, A23187 treatment significantly accelerated PDGF receptor

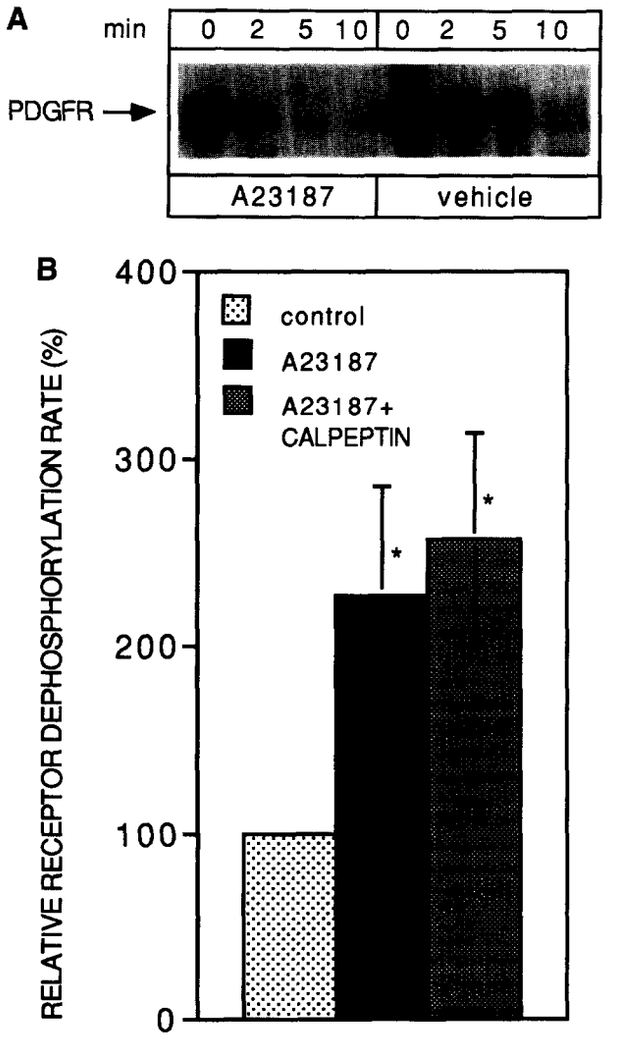

Fig. 2. Accelerated PDGF receptor dephosphorylation in A23187-treated Swiss 3T3 cells. (A) Confluent Swiss 3T3 cells were treated for 5 min in serum-free medium with 1 μM A23187 or solvent vehicle, as indicated. Then, PDGF receptor dephosphorylation was measured. The result of a typical experiment is shown. Densitometric analysis of the blot revealed the following band intensities (untreated/A23187-treated, in arbitrary units): 0 min, 164/147; 2 min, 105/34; 5 min, 42/9; 10 min, 20/8: The relative dephosphorylation rate was calculated as 214% in the presence of A23187 using the time point 2 min, as described under Section 2. (B) Dephosphorylation rate of PDGF receptor in untreated cells (control) or cells treated with 1 μM A231876 for 5 min or with 40 μM calpeptin for 10 min and thereafter with A23187, as indicated. Means ± S.D. of four independent experiments are shown. $^*p < 0.05$ for difference from control.

dephosphorylation (Fig. 2A,B). Likewise, thapsigargin, another agent elevating intracellular Ca^{2+} levels, enhanced PDGF receptor dephosphorylation (Table 1). A23187 has been shown in platelets to release the endoplasmic PTPase PTP1B into the cytoplasm via calpain-mediated cleavage of the PTPase catalytic core from its anchor peptide in the endoplasmic reticulum membrane [22]. PTP1B is expressed in Swiss 3T3 cells, albeit at a relatively low level [20]. However, calpeptin, a potent calpain inhibitor which is known to block PTP1B release in platelets, had no effect on the A23187-induced activation of PDGF receptor-directed PTPases in Swiss 3T3 cells (Fig. 2B). Thus, PTP1B is unlikely to mediate the effect of elevated intracellular Ca^{2+} concentration observed by us. In the search for another mechanism, we evaluated the effect of PKC modulators on PDGF receptor dephosphorylation. Neither acute nor prolonged treatment of the Swiss 3T3 cells with phorbol 12-myristate-13-acetate nor treatment with the PKC inhibitor calphostin C had consistent effects on PDGF receptor dephosphorylation (not shown). Further experiments are required to assess the possible involvement of other Ca^{2+}-activated protein kinases such as Ca^{2+}/calmodulin-dependent protein kinases [23] or protein kinase PYK2 [24] in the observed regulation.

We were interested to determine if the increase in intracellular Ca^{2+} would also increase the bulk of cellular PTPase activity. Extracts were prepared from cells treated with vehicle, PDGF or A23187 or both and the PTPase activity was tested against phosphorylated Raytide. The latter substrate has been shown to become dephosphorylated by a wide spectrum of structurally divergent PTPases. In contrast to what we had observed with PDGF receptor dephosphorylation, the ionophore A23187 did not increase but rather decreased the total PTPase activity detected in this assay (Fig. 3) whereas short-term PDGF treatment had no significant effect. Thus, the rise of intracellular Ca^{2+} seems to accelerate PDGF receptor dephosphorylation with some specificity since it has an opposite effect on the bulk cellular PTPase activity. A similar decrease in total cellular PTPase activity has been observed in A23187-treated HER14 cells [25]. Also in agreement with our results is the observation that pretreatment of different fibroblast cell lines with A23187 or high extracellular Ca^{2+} concentration diminishes subsequent PDGF receptor autophosphorylation [26].

Table 1
Modulation of PDGF receptor dephosphorylation in intact Swiss 3T3 cells by various agents

Effector	Concentration	Time of treatment	PDGF receptor dephosphorylation rate (% of control ± S.D.)
PDGF-BB	10 ng/ml	2 h	41 ± 13**
Thapsigargin	30 nM	30 min	139 ± 4*
Forskolin	100 μM	15 min	143 ± 29*
IBMX	5 mM	1 h	189 ± 19*

Confluent Swiss 3T3 cells were treated with the listed agents at the concentrations shown for the indicated times at 37°C in serum-free medium. Then, PDGF receptor dephosphorylation rates were evaluated by immunoblotting and densitometric analysis as described under Section 2. The dephosphorylation rate in the respective controls was 100%. The receptor phosphorylation level at the start of the dephosphorylation experiment was similar in treated and untreated cultures except for PDGF pretreatment, where the signal was reduced to 50–60% of that in control cells in most experiments. Means ± S.D. are representative of at least 4 independent determinations. $^{**}p < 0.01$, $^{*}p < 0.05$.

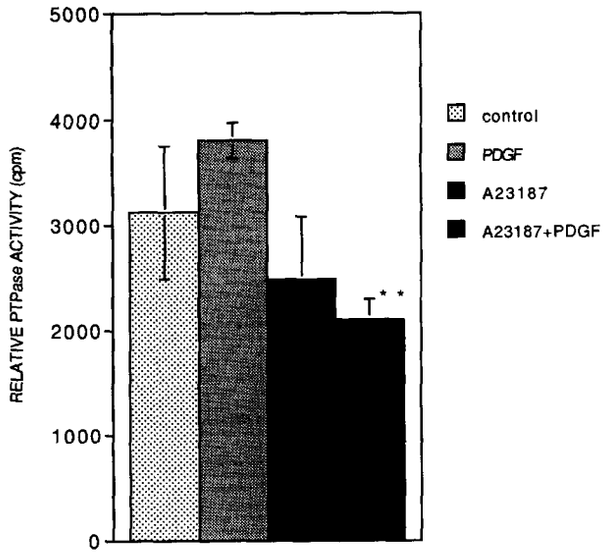

Fig. 3. Effect of PDGF and the Ca^{2+} ionophore A23187 on total cellular PTPase activity in Swiss 3T3 cells. Confluent Swiss 3T3 cells were treated for 5 min in serum-free medium with A23187 or vehicle and were then stimulated with 100 ng/ml PDGF for 5 min or not, as indicated. Cell extracts were prepared and analyzed for total PTPase activity with [^{32}P]Raytide as a substrate using 1 μg lysate protein per assay. In the inset, linearity of the assay is shown for the range of 0.5–2 μg lysate protein. $^{**}p < 0.01$ for difference from respective control without A23187 treatment.

We further tested the effect of modulators of the cellular cAMP level on PDGF receptor dephosphorylation. Treatment with forskolin or isobutylmethylxanthine accelerated PDGF receptor dephosphorylation (Table 1), albeit to a lesser extent than with A23187.

We have shown here that elevation of intracellular Ca^{2+} and cAMP can activate PDGF receptor-directed PTPases. To our knowledge, this is the first example demonstrating the regulation of cellular PTPase activity monitoring directly physiological substrate dephosphorylation in intact cells. An obvious explanation for the effects we observed would be the activation of Ca^{2+}- and cAMP-dependent protein kinases which in turn activate the PDGF receptor-directed PTPase(s). Evidence is accumulating that phosphorylation of PTPases can regulate their catalytic activity [12,27–30]. Modulation of receptor dephosphorylation by intracellular Ca^{2+} and cAMP levels might present an important level of regulation of receptor signaling activity and will help to identify the involved PTPases.

References

[1] Ullrich, A. and Schlessinger, J. (1990) Cell 61, 203–212.
[2] Fantl, W.J., Johnson, D.E. and Williams, L.T. (1993) Annu. Rev. Biochem. 62, 453–481.
[3] Charbonneau, H. and Tonks, N.K. (1992) Annu.Rev. Cell Biol. 8, 463–493
[4] Pot, D.A. and Dixon, J.E. (1992) Biochim. Biophys. Acta 1136, 35–43.
[5] Lammers, R., Bossenmaier, B., Cool, D.E., Tonks, N.K., Schlessinger, J., Fischer, E.H. and Ullrich, A. (1993) J. Biol. Chem. 268, 22456–22462.
[6] Böhmer, F.D., Böhmer, S.A. and Heldin, C.H. (1993) FEBS Lett. 331, 276–280.

[7] Møller, N.P.H., Møller, K., Lammers, R., Kharitonenkov, A., Hoppe, E., Wiberg, F., Sures, I. and Ullrich, A. (1995) J. Biol. Chem. 270, 23126–23131.
[8] Kulas, D.T., Goldstein, B.J. and Mooney, R.A. (1996) J. Biol. Chem. 271, 748–754.
[9] Kulas, D.T., Zhang, W.R., Goldstein, B.J., Furlanetto, R.W. and Mooney, R.A. (1995) J. Biol. Chem. 270, 2435–2438.
[10] Feng, G.S. and Pawson, T. (1994) Trends Genet. 10, 54–58.
[11] Yi, T.L., Mui, A.L.F., Krystal, G. and Ihle, J.N. (1993) Mol. Cell. Biol. 13, 7577–7586.
[12] Vogel, W., Lammers, R., Huang, J. and Ullrich, A. (1993) Science 259, 1611–1614.
[13] Tomic, S., Greiser, U., Lammers, R., Kharitonenkov, A., Imyanitov, E., Ullrich, A. and Böhmer, F.D. (1995) J. Biol. Chem. 270, 21277–21284.
[14] Ek, B. and Heldin, C.-H. (1982) J. Biol. Chem. 257, 10486–10492.
[15] Berti, A., Rigacci, S., Raugei, G., Degl'Innocenti, D. and Ramponi, G. 1994 FEBS Lett. 349, 7–12.
[16] Mooney, R.A., Freund, G.G., Way, B.A. and Bordwell, K.L. (1992) J. Biol. Chem. 267, 23443–23446.
[17] Böhmer, F.D., Böhmer, A., Obermeier, A. and Ullrich, A. (1995) Anal. Biochem. 228, 267–273.
[18] Kovalenko, M., Gazit, A., Böhmer, A., Rorsman, C., Ronnstrand, L., Heldin, C.H., Waltenberger, J., Böhmer, F.D. and Levitzki, A. (1994) Cancer Res. 54, 6106–6114.
[19] Pallen, C.J. and Tong, P.H. (1991) Proc. Natl. Acad. Sci. USA 88, 6996–7000.
[20] Celler, J.W., Luo, X.M., Gonez, L.J. and Böhmer, F.D. (1995) Biochem. Biophys. Res. Commun. 209, 614–621.
[21] Brautigan, D.L. and Pinault, F.M. (1991) Proc. Natl. Acad. Sci. USA 88, 6696–6700.
[22] Frangioni, J.V., Oda, A., Smith, M., Salzman, E.W. and Neel, B.G. (1993) EMBO J. 12, 4843–4856.
[23] Braun, A. and Schulman, H. (1995) Annu. Rev.Physiol. 57, 417–445.
[24] Lev, S., Moreno, H., Martinez, R., Canoll, P., Peles, E., Musacchio, J., Plowman, G., Rudy, B. and Schlessinger, J. (1995) Nature 376, 737–745.
[25] Errasfa, M. and Stern, A. (1993) Eur. J. Pharmacol. 247, 73–80.
[26] Epstein, R. (1995) Cell. Signalling 7, 377–388.
[27] Flint, A.J., Gebbink, M.F., Franza, B.J., Hill, D.E. and Tonks, N.K. (1993) EMBO J. 12, 1937–1946.
[28] Schievella, A.R., Paige, L.A., Johnson, K.A., Hill, D.E. and Erikson, R.L. (1993) Cell Growth Differ. 4, 239–246.
[29] Garton, A.J. and Tonks, N.K. (1994) EMBO J. 13, 3763–3771.
[30] Den Hertog, J., Sap, J., Pals, C., Schlessinger, J. and Kruijer, W. (1995) Cell Growth Differ. 6, 303–307.