Effects of protein kinase modulators on transferrin receptor expression in human leukaemic HL-60 cells

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Abstract The mRNA of transferrin receptor (TfR) is constitutively expressed in proliferating human leukaemic HL-60 cells. Treatment of HL-60 cells with phorbol 12-myristate 13-acetate (PMA), a protein kinase C (PKC) activator, or dibutyryl-cyclic AMP (dbcAMP), a protein kinase A (PKA) activator, resulted in a 90% decrease in the level of TfR mRNA. Inhibition of TfR mRNA expression induced by 10 nM PMA and 100 μM dbcAMP was abolished by prior incubation of cells with 0.1–1.0 μM GF109203X, a PKC-specific inhibitor, and 1–10 μM H-89, a PKA-specific inhibitor, respectively. The blocking effects of GF109203X and H-89 were dose-dependent and complete at the highest concentrations of the inhibitors used. Although treatment of cells with GF109203X or H-89 alone did not alter the constitutive expression of TfR mRNA, incubation of cells with 30–100 nM staurosporine, a wide-spectrum protein kinase inhibitor, resulted in suppression of the constitutive expression of TfR mRNA in a dose-dependent manner. These results suggest that (i) the down-regulation of TfR mRNA expression during the differentiation of HL-60 cells can be mediated by activation of either PKC or PKA; (ii) the constitutive expression of TfR mRNA in proliferating HL-60 cells is staurosporine-sensitive and is probably maintained by protein kinase(s) other than PKC and PKA.

Key words: Transferrin receptor; Protein kinase; Bisindolylmaleimide (GF109203X); Isoquinolinesulfonamide (H-89); Staurosporine; HL-60

1. Introduction

The transferrin receptor (TfR), which is constitutively expressed in the proliferating normal and malignant cells, has been considered as a proliferation marker in a number of cellular systems [1,2]. Cessation of cell growth or terminal differentiation results in down-regulation of TfR expression [3]. The human promyelocytic leukaemia cell line HL-60 is a model for studying this aspect of cellular regulation as these cells can be induced to differentiate into either granulocytes by retinoic acids and dibutyrl-cyclic AMP (dbcAMP) or monocytes/macrophages by 1,25-dihydroxyvitamin D_3 and phorbol 12-myristate 13-acetate (PMA) [4–7]. Upon induction of differentiation by these agents, the expression of TfR mRNA and the protein were decreased [3,8,9]. Since PMA and dbcAMP are able to modulate the expression of TfR, participation of protein kinase C (PKC) and protein kinase A (PKA) are implicated, although no direct evidence for this action has been demonstrated. In this study, we investigate the role of protein kinases in the regulation of TfR expression in HL-60 cells by using GF109203X, a PKC-specific inhibitor [10], H-89, a PKA-specific inhibitor [11], as well as staurosporine, a wide-spectrum protein kinase inhibitor [12]. We demonstrate that the down-regulation of TfR mRNA expression in HL-60 cells induced by PMA and dbcAMP was completely blocked by GF109203X and H-89, respectively. Although treatment of cells with GF109203X or H-89 alone did not alter the constitutive expression of TfR mRNA, incubation of cells with staurosporine resulted in suppression of the TfR mRNA level. These pharmacological evidences suggest that the down-regulation of TfR mRNA expression during the differentiation of HL-60 cells can be mediated by activation of either PKC or PKA. On the other hand, the constitutive expression of TfR mRNA in proliferating HL-60 cells is staurosporine-sensitive and is probably maintained by protein kinase(s) other than PKC and PKA. The effectiveness of these protein kinase inhibitors in elucidating the cellular mechanisms involved in cell growth and differentiation of HL-60 cells are also revealed.

2. Materials and methods

2.1. Chemicals, probes and cell culture

GF109203X, H-89 and staurosporine were purchased from Calbiochem (San Diego, CA). PMA and dbcAMP were from Sigma (St. Louis, MO). The cDNA probes for human TfR (pcDTR1), human actin (HHCI89) and HL-60 cell line were obtained from the American Type Culture Collection (Rockville, MD). HL-60 cells were maintained in RPMI 1640 with 10% fetal calf serum. The TfR expression increased significantly after they were subcultured [3]. To minimize this effect during the experiments, the cells were washed and cultured at 5×10^5 cells/ml in the same medium with reduced serum (1%) for 24 h before the addition of drugs. Under this condition, DNA synthesis was maintained and the TfR expression of the control culture remained constant during the course of experiments.

2.2. Northern blot analysis

Total RNA was isolated according to the method of Chomczynski and Sacchi [13]. Equal amounts of samples (10 μg) were size-fractionated on 1% formaldehyde-agarose gel, transferred to Hybond-N membrane (Amersham) and hybridized with labeled probes according to procedures of Sambrook and Maniatis [14].

2.3. [^{3}H]Thymidine incorporation

At the last 4 h of incubation with drugs, the cells were pulsed labeled with 1μCi [^{3}H]thymidine (5 Ci/mmol). After that, the cells were washed with phosphate-buffered saline and lysed with 0.1% Triton X-100 before incubated with 5% trichloroacetic acid. The acid-insoluble material was collected on glass fiber filter and measured by liquid scintillation counting.

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Abbreviations: TfR, transferrin receptor; HL-60, human promyelocytic leukaemic HL-60 cells; PMA, phorbol 12-myristate 13-acetate; dbcAMP, dibutyryl-cyclic AMP; GF109203X, 3-[1-(3-dimethylaminopropyl)-indo-3-yl]-3-(indoly-3-yl)-maleimide; H-89, *N*-[2-((3-(4-Bromophenyl)-2-propenyl)-amino)-ethyl]-5-isoquinolinesulfonamide.

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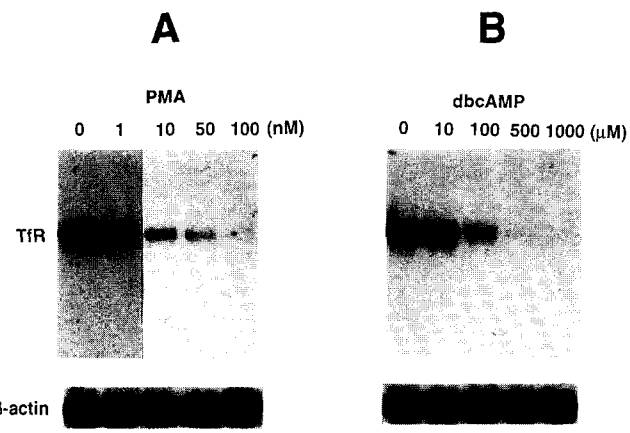

Fig. 1. Effects of PMA and dbcAMP on TfR mRNA expression in HL-60 cells. Cells were treated with indicated concentrations of PMA (A) or dbcAMP (B) for 24 h. The TfR mRNA level was determined by Northern blot hybridization.

3. Results

As shown in Fig. 1, the mRNA of TfR was constitutively expressed in the proliferating HL-60 cells. Treatment of these cells with PMA, a PKC activator, or dbcAMP, a PKA activator, for 24 h resulted in a 90% decrease in TfR mRNA level. The concentrations of these two activators required to inhibit half-maximally TfR mRNA expression (IC_{50}) were about 10 nM and 100 μM, respectively. By contrast, treatment of cells with 1 μM GF109203X, a PKC-specific inhibitor, or 10 μM H-89, a PKA-specific inhibitor, did not modulate the expression of TfR mRNA (Fig. 2A,B). Nevertheless, inhibition of TfR mRNA expression induced by PMA (10 nM) could be abolished by preincubating the cells with GF109203X (0.1–1.0 μM) (Fig. 2A). As a negative control, this PKC inhibitor at the previous concentrations used was ineffective in nullifying the inhibitory action of dbcAMP (100 μM) (Fig. 2A). Similarly, prior addition of H-89 (1–10 μM) to the cells abrogated the effect of dbcAMP (100 μM) but not that of PMA (10 nM) (Fig. 2B). At the highest concentrations used, both GF109203X and H-89 fully inhibited the action of PMA and dbcAMP on TfR mRNA expression, respectively.

The effect of these protein kinase modulators on DNA synthesis was also investigated. [^{3}H]Thymidine incorporation was markedly suppressed by PMA (10 nM) or dbcAMP (100 μM) but not affected by GF109203X (1 μM) or H-89 (10 μM). However, preincubation of cells with GF109203X or H-89 reversed the inhibitory action of PMA and dbcAMP, respectively (Table 1).

The effect of staurosporine, a wide-spectrum protein kinase inhibitor, on TfR mRNA expression was also tested. At concentrations that are commonly used for the inhibition of protein kinases (1–100 nM), staurosporine did not mimic the effect of GF109203X on PMA-treated cells or H-89 on dbcAMP-treated cells (not shown). However, incubation of cells with 30–100 nM of staurosporine alone resulted in a suppression of mRNA level of TfR in a dose-dependent manner. At the highest concentration of staurosporine used, more than 90% of the TfR mRNA expression was inhibited (Fig. 3). Also, within this range of concentrations of staurosporine, DNA synthesis, but not cell survival, was inhibited (data not shown).

4. Discussion

GF109203X and H-89 are novel and specific inhibitors for PKC and PKA, respectively. In this study, we show that GF109203X and H-89 completely blocked the down-regulation of TfR mRNA expression in HL-60 cells induced by PMA and dbcAMP, respectively. These results suggest that activation of either PKC or PKA may be necessary or perhaps sufficient to induce down-regulation of TfR gene expression in HL-60 cells. It should be noted that PMA-modulated responses have been attributed to either activation or down-regulation of PKC (loss of the protein kinase activities through proteolysis upon prolonged treatment of PMA) [15–20]. The present results indicate that the down-regulation of TfR mRNA expression is unlikely mediated by the depletion of PKC activities upon prolonged treatment of PMA, otherwise GF109203X would not have reversed the inhibitory effect of PMA. As for cAMP-modulated responses, although many experimental evidences have indicated that PKA activation was required, certain studies have implicated that PKA-independent mechanisms may also play a role in some cases ([21] and references therein). Our experiments using H-89 as a PKA-specific inhibitor demonstrate that the effect of dbcAMP on TfR mRNA expression is PKA-dependent.

The effects of PMA and dbcAMP on the down-regulation of TfR gene expression in leukaemic cells were shown to be mediated at transcription level [9,22]. It is noteworthy that the promoter sequence of the TfR gene contains a TRE/CRE-like element as revealed by sequence homology and DNAase footprinting [23]. Conceptually, this element is recognized by transcription factor(s) AP-1(Jun) and/or CREB which are activated by PKC and/or PKA [24]. In fact, we have demonstrated that an increase in AP-1 binding to the TRE/CRE-like element was associated with the down-regulation of TfR expression during HL-60 cell differentiation induced by PMA [25]. Consistent to PKC-/AP-1-modulated gene expression model, this binding activity could be abolished by preincubation of the cells with GF109203X (data not shown).

The constitutive expression of transferrin receptor is associated with active DNA synthesis in proliferating HL-60 cells. Our results indicate that both GF109203X and H-89 did not affect the constitutive expression of TfR mRNA and also DNA

Table 1
Effects of GF109203X and H-89 on the PMA- and dbcAMP-induced inhibition of DNA synthesis

Treatment	[^{3}H]Thymidine incorporation (% Control ± S.D., n = 3)
PMA (10 nM)	8 ± 1
GF109203X (1 μM)	96 ± 6
GF109203X (1 μM) + PMA (10 nM)	77 ± 15
dbcAMP (100 μM)	38 ± 5
H-89 (10 μM)	81 ± 19
H-89 (10 μM) + dbcAMP (100 μM)	78 ± 15

Cells were treated with the indicated agents for 24 h. DNA synthesis was determined by [^{3}H]thymidine incorporation. The mean incorporated count of the control culture was 2400 cpm/10^6 cells.

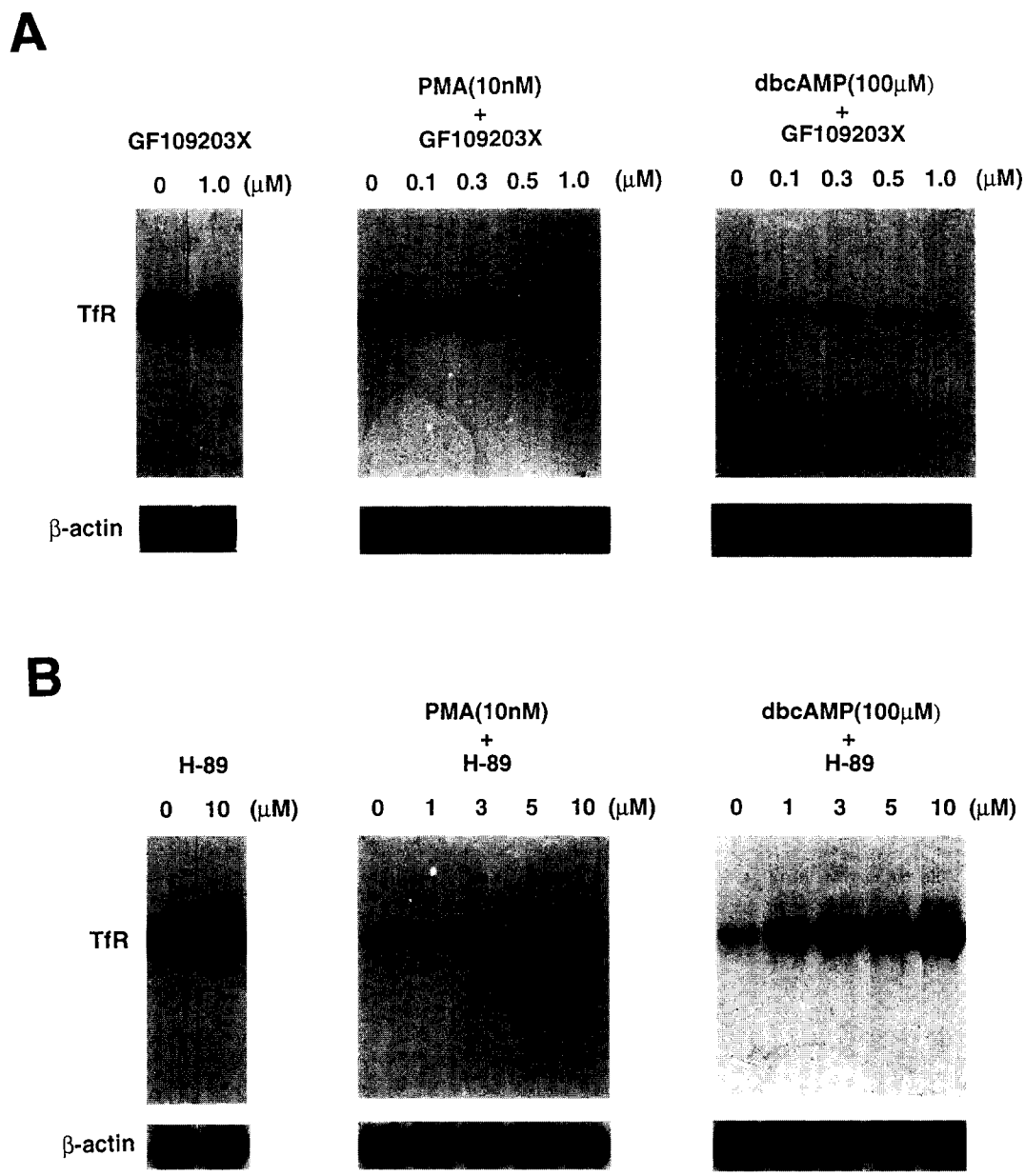

Fig. 2. Effects of GF109203X and H-89 on the PMA- and dbcAMP-induced down-regulation of TfR mRNA expression in HL-60 cells. Cells were preincubated with indicated concentrations of GF109203X (A) or H-89 (B) for 30 min and PMA (10 nM) or dbcAMP (100 μM) was then added for 24 h. The TfR mRNA level was determined by Northern blot hybridization.

synthesis even at high concentrations (at least 70 times of the in vitro IC_{50} for PKC [10] or PKA [11]). Thus, it is unlikely that the proliferation-associated processes of HL-60 cells are maintained by the basal activity of PKC and PKA. This also indirectly reveals that in contrast to other protein kinase inhibitors which inhibit cell growth or even induce cell death of HL-60 cells at concentrations used for inhibition of protein kinases [26,27], GF109203X and H-89 are relatively non-cytotoxic and presumably more specific in protein kinase inhibition.

Staurosporine is a potent inhibitor of PKC. However staurosporine also inhibits a wide spectrum of protein kinases ([27] and references therein). It can inhibit both PKC and PKA with close in vitro IC_{50} (3 and 15 nM, respectively) [12,28]. Contrary to our expectation, this inhibitor, used within the range of concentrations which could inhibit PKC and PKA, did not block the down-regulation of TfR mRNA expression induced by PMA or dbcAMP [28]. The study from Matsui and associates shows that H-7, a serine/threonine protein kinase inhibitor, caused only subtle reversal on the suppressive effect of PMA on TfR expression [29]. The reasons for these observations are still unclear. The possibility exists that these inhibitors may act on protein kinases or other cellular machinery which are responsible for the constitutive expression of TfR. In fact, we found that the constitutive expression of TfR mRNA was suppressed by staurosporine. It is possible that the expression of TfR mRNA in proliferating cells is maintained by certain protein kinases (other than PKC and PKA) that are inhibited by staurosporine. Of course, we cannot rule out that the effect of

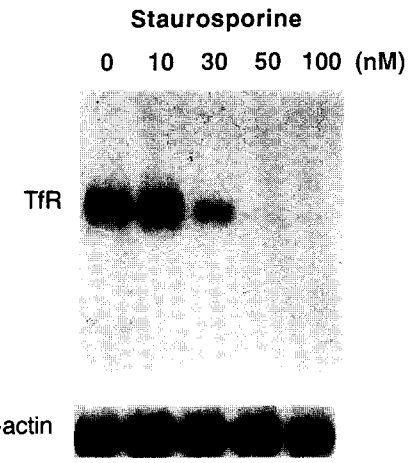

Fig. 3. Effect of staurosporine on the constitutive expression of TfR mRNA in HL-60 cells. Cells were treated with indicated concentrations of staurosporine for 24 h. The TfR mRNA level was determined by Northern blot hybridization.

staurosporine on TfR expression may be due to non-specific perturbation other than the inhibition of protein kinases. Nevertheless, this result demonstrates that staurosporine may be an useful pharmacological tool for studying the constitutive expression of TfR or other proliferation-associated processes in HL-60 cells.

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