Transforming growth factor-β1 rapidly activates phosphorylase in a calcium-dependent manner in rat hepatocytes

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Transforming growth factor-β1 (TGF-β1) rapidly activated phosphorylase in isolated rat hepatocytes (half-maximal rate of activation with approximately 0.1 ng/ml). Removal of Ca^{2+} from the external medium just before TGF-β1 addition markedly attenuated phosphorylase activation. TGF-β1 (1 ng/ml) produced a small increase in $[Ca^{2+}]_i$ (approximately 10% increase after 30 s), which appears sufficient to account for phosphorylase activation. These observations indicate that activation of the TGF-β signal transduction system in hepatocytes is linked with a small increase in $[Ca^{2+}]_i$ and external Ca^{2+} may contribute in part to this increase.

Transforming growth factor-β1; Phosphorylase; Calcium; Hepatocyte; Signal transduction; Growth factor

1. INTRODUCTION

The transforming growth factor-beta (TGF-β) family comprises at least five closely related polypeptides which modulate growth and differentiation in a variety of cell types [1–3]. The prototype, now designated TGF-β1, was initially purified from human platelets [4] and exists in the biologically active form as a homodimeric peptide of 25 kDa. Although originally identified by its ability to induce anchorage-independent growth in fibroblasts [5,6], TGF-β1 has subsequently been shown to be a potent inhibitor of the growth of epithelial cells, including hepatocytes [7–10]. The mechanisms whereby TGF-β1 and other TGF-β family members regulate cell growth are presently unknown, but appear to be mediated by specific cell surface receptors. Three types of TGF-β receptors have been identified [11–16]; types I and II are glycoproteins thought to be directly involved in the TGF-β signal transduction process, while receptor III (betaglycan) appears to serve some other function [17–20]. The type II receptor contains a serine/threonine kinase activity in the cytoplasmic domain [21], unlike the type III receptor [22,23].

The intracellular events which follow receptor binding of TGF-β are not well defined. TGF-β1 causes rapid phosphorylation of a number of nuclear proteins in

mink lung CC164 cells [24], one of which is identified as the cyclic AMP responsive element binding protein [25]. It has been reported that TGF-β1 increased inositol 1,4,5-trisphosphate levels and promoted Ca^{2+} influx in Rat-1 cells [26,27], but these effects occurred after several hours and were blocked by actinomycin D. The present study was undertaken to examine the early effects of TGF-β1 on isolated rat hepatocytes; evidence is presented for a rapid increase in phosphorylase *a* activity which appears to be mediated through a small increase in $[Ca^{2+}]_i$.

2. MATERIALS AND METHODS

2.1. *Materials*

TGF-β1 prepared from porcine platelets, was purchased from R & D Systems (Minneapolis, MN, USA); collagenase D was from Boehringer Mannheim (Indianapolis, IN, USA); fatty acid free bovine serum albumin (PENTEX, fraction V) was from Miles Diagnostics (Kankakee, IL, USA); indo-1/AM was from Molecular Probes (Junction City, OR, USA); α-D-[U-¹⁴C] glucose-1-phosphate was from DuPont/NEN (Boston, MA, USA). All other reagents were of analytical grade.

2.2. *Hepatocyte preparation*

Isolated hepatocytes were prepared by collagenase perfusion of intact liver from fed, male, Sprague–Dawley rats (175–250 g), as described by Meijer et al. [28].

2.3. *Phosphorylase measurements*

Hepatocytes were incubated (7.5 mg dry weight/ml) in Krebs–Ringer bicarbonate buffer containing 15 mM glucose and 0.2% (w/v) fatty acid-free bovine serum albumin (medium A). After shaking for 30 min at 37°C in a water bath under an atmosphere of O_2/CO_2 (95:5), 200 μl aliquots were rapidly mixed with 200 μl aliquots of buffer containing 50 mM MES, pH 6.1, 150 mM NaF, 20 mM EDTA, 0.4 M sucrose, 0.025% (v/v) Triton X-100 and 5 mM dithiothreitol, and immediately frozen in liquid N_2. Samples were stored at −80°C until

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Abbreviations: TGF-β, transforming growth factor beta; $[Ca^{2+}]_i$, cytosolic free calcium concentration; indo-1/AM, 1-[2 amino-5-(6-carboxyindol-2-yl)phenoxy]-2-(2′-amino-5′-methylphenoxy)ethane-*N,N,N′,N′*-tetraacetic acid, penta-acetoxymethylester.

assayed for phosphorylase a by the procedure of Gilboe et al. [29]. Total phosphorylase activity was measured in the presence of 5 mM AMP and 10% (v/v) ethylene glycol dimethyl ether [30].

2.4. Cytosolic free Ca^{2+} measurements using indo-1

Hepatocytes were preincubated for 10 min at 37°C (10 mg dry weight/ml) in medium A in a shaking water bath. Indo-1/AM was added (4 μM final concentration) and incubation continued for a further 15 min. Hepatocytes were centrifuged at 50 × g for 1 min, resuspended at the same concentration in fresh medium A, re-centrifuged, and resuspended in medium A at a concentration of 25 mg dry weight/ml. Intracellular Ca^{2+} measurements were performed using an Hitachi F2000 fluorescence spectrophotometer, with excitation at 355 nm, and fluorescence emission at 410 nm and 480 nm was monitored alternately every 0.5 s. Indo-1-loaded hepatocytes (6 mg dry weight/ml) were preincubated in a stirred cuvette maintained at 32°C, for 3 min prior to addition of growth factor or agonist. For calibration purposes, sequential additions of EGTA (3 mM), Tris base (10 mM), Triton X-100 (0.1%, v/v), and excess $CaCl_2$ (5 mM) were made [31]. $[Ca^{2+}]_i$ was calculated after correction for autofluorescence, according to the formula [32]:

$$[Ca^{2+}]_i = K_d(R-R_{min})/(R_{max}-R) \times S_{f2}/S_{b2}$$

where K_d (250 nM) is the dissociation constant for Ca^{2+} binding to indo-1, R_{max} and R_{min} are the fluorescence ratio values under saturating and Ca^{2+}-free conditions, respectively, and S_{f2}/S_{b2} is the ratio of fluorescence values for Ca^{2+}-free/Ca^{2+}-bound indicator at 480 nm.

3. RESULTS

As shown in Fig. 1, TGF-β1 (1 ng/ml) caused a rapid increase in hepatocyte phosphorylase a activity, with maximal activation (2.5-fold increase relative to control) occurring between 30 s and 1 min, followed by a return towards control values. Total hepatocyte phosphorylase activity (i.e. a and b forms) was essentially unchanged by TGF-β1; values of 88.4 ± 3.7 nmol/
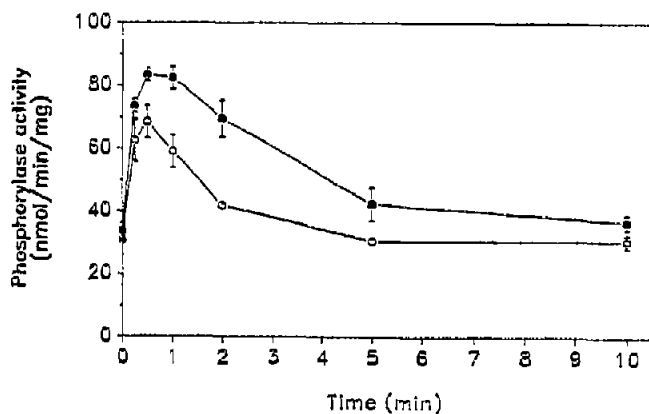

Fig. 1. Effect of TGF-β1 on phosphorylase a activity in hepatocytes and dependency on external calcium. Hepatocytes were preincubated at 37°C for 40 min in Krebs-Ringer bicarbonate buffer containing 1.33 mM $CaCl_2$, and then centrifuged at 50 × g for 70 s and resuspended in Krebs-Ringer bicarbonate buffer containing $CaCl_2$ or in buffer without added $CaCl_2$. The centrifugation step was repeated followed by resuspension of the cells. Cells were subsequently incubated either in the presence (●) or absence (○) of $CaCl_2$ for 5 min, before addition of TGF-β1 (1 ng/ml). Aliquots were removed for phosphorylase assay at the times indicated. Results shown are means ± S.E.M. for 4 separate experiments.
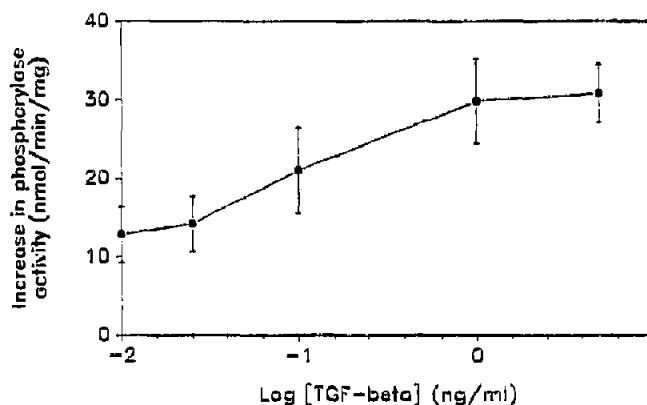

Fig. 2. Dose-response curve for rate of activation of phosphorylase in hepatocytes by TGF-β1. Time courses for activation of phosphorylase in hepatocytes incubated in Krebs-Ringer bicarbonate buffer containing 1.33 mM $CaCl_2$, by concentrations of TGF-β1 from 0.01 to 5 ng/ml were established by removing samples for phosphorylase measurement from cell suspensions before, and at various times (10, 20, 30 s) after addition of the particular concentration of TGF-β1. The initial rates of activation of phosphorylase, 10 s after TGF-β1 addition, are plotted as a function of TGF-β1 concentration. Values shown are means ± S.E.M. for data from 4 separate experiments.

min/mg and 93.0 ± 3.0 nmol/min/mg (mean ± S.E.M. for 4 separate experiments) were observed before, and 2 min following TGF-β1, respectively. When TGF-β1 was added to hepatocytes which had been washed twice and incubated for 5 min in buffer with no added $CaCl_2$, there was a rapid activation of phosphorylase (Fig. 1) but the maximal extent of this activation and the duration were reduced relative to the response observed in the presence of external calcium.

Fig. 2 shows initial rates of activation of phosphorylase, measured 10 s after addition of TGF-β1, plotted as a function of TGF-β1 concentration. Phosphorylase activation was observed at TGF-β1 concentrations as low as 0.01 ng/ml, and the rate of activation was maximal at 1 ng/ml. This concentration range is similar to that previously reported for inhibitory effects of TGF-β1 on epidermal growth factor-induced DNA synthesis in hepatocytes [10].

The increase in phosphorylase a by TGF-β1 and its dependency on external Ca^{2+} (Fig. 1) may indicate receptor-activated increases in cytosolic Ca^{2+} from intracellular stores and entry of external Ca^{2+}, as is well established for α_1-agonists and vasopressin [33,34]. Accordingly, the effect of TGF-β1 on hepatocyte $[Ca^{2+}]_i$ was examined using cells loaded with the Ca^{2+}-sensitive dye, indo-1 [32]. As shown in Fig. 3a, TGF-β1 (1 ng/ml) caused an increase in the 410 nm fluorescence emission signal, corresponding to a calculated change in $[Ca^{2+}]_i$ from 93 nM (mean value determined over a 60 s period prior to TGF-β1 addition) to a value of approximately 100 nM within 30 s after TGF-β1 addition. Overall, in 6 determinations from three hepatocyte preparations, treatment with TGF-β1 (1 ng/ml) for 30 s increased
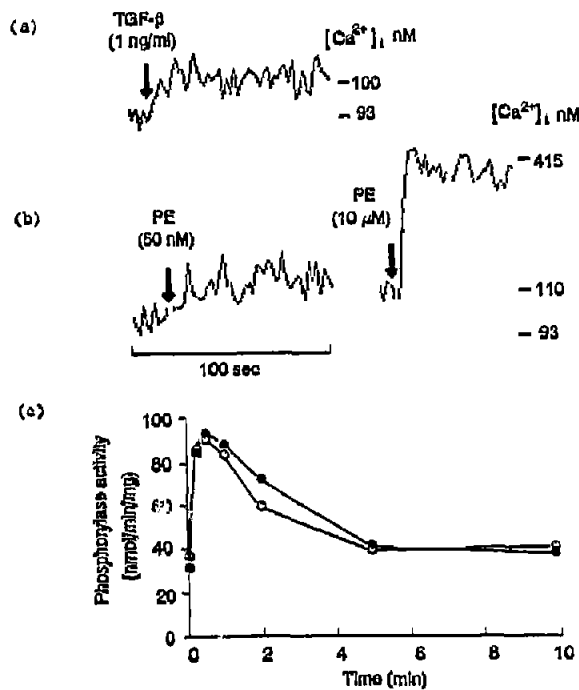
Fig. 3. Comparison of changes in cytosolic Ca^{2+} concentration elicited by TGF-β1 and by low and high concentrations of phenylephrine (PE), with corresponding changes in phosphorylase a activity. Panels (a) and (b) show the changes in fluorescence emission at 410 nm in indo-1-loaded hepatocytes, following addition of TGF-β or phenylephrine, respectively. In panel (b) the indicated addition of PE were made to the same cuvette approximately 3 min apart; part of the intervening trace was deleted for presentation. Calculated intracellular Ca^{2+} concentrations [Ca^{2+}]$_i$, are as indicated. Panel (c) shows changes in phosphorylase activity at the indicated times following addition of either TGF-β (1 ng/ml, o), or PE (50 nM, ●).

[Ca^{2+}]$_i$ from 112 ± 8 nM to 126 ± 9 nM (means ± S.E.M. for control and TGF-β1-treated cells, respectively). The increase in [Ca^{2+}]$_i$ was significant ($P < 0.01$) when analyzed by the Paired Student's t-test. The relevance of this relatively small change was assessed by determining whether the α_1-adrenergic agonist, phenylephrine, could, at a suitably low concentration, elicit a similar activation of phosphorylase with a correspondingly small increase in [Ca^{2+}]$_i$. It is well established that α_1-adrenergic agonists promote phosphoinositide hydrolysis causing mobilization of Ca^{2+} from intracellular stores and entry of external Ca^{2+} [33,34]; the increase in [Ca^{2+}]$_i$ activates phosphorylase kinase which in turn, promotes conversion of phosphorylase b to the a form. Fig. 3b indicates that a low concentration of phenylephrine (50 nM) caused a small increase in [Ca^{2+}]$_i$ concentration after 30 s, of similar magnitude (approximately 10% increase in duplicate determinations) to that observed with TGF-β1 in Fig. 3a. A higher concentration of phenylephrine (10 μM) added subsequently to the low concentration as shown in Fig. 3b caused a larger increase in [Ca^{2+}]$_i$ as expected. It appears however from the data shown in Fig. 3c, that phenylephrine (50 nM), was capable of activating phosphorylase to the same

extent observed with TGF-β1 (1 ng/ml), suggesting that the relatively small increase in [Ca^{2+}]$_i$ in response to TGF-β1 is sufficient to account for the activation of phosphorylase.

4. DISCUSSION

The present results demonstrate that TGF-β1 produces a rapid increase in phosphorylase a activity in hepatocytes (Fig. 1), and the concentration range over which such activation occurs (Fig. 2), is similar to the concentration range over which TGF-β1 inhibits epidermal growth factor-induced DNA synthesis in rat hepatocytes [10]. The change in phosphorylase activity is rapid (maximal by 30 s) and is one of the earliest observed changes in a cellular response to TGF-β1.

Evidence is presented in Fig. 3a for a small increase in [Ca^{2+}]$_i$ in hepatocytes exposed to TGF-β1 for 30 s. Furthermore, removal of external Ca^{2+} from the medium just prior to TGF-β1 addition, markedly attenuates the phosphorylase activation (Fig. 1), suggesting that entry of external Ca^{2+}, as well as mobilization of Ca^{2+} from internal stores, may contribute to the elevation of cytosolic Ca^{2+} underlying phosphorylase activation. Entry of external Ca^{2+} into the hepatocytes is known to be required for a sustained activation of phosphorylase in response to phenylephrine and vasopressin [33,34]. Although the increase in [Ca^{2+}]$_i$ elicited by TGF-β1 is relatively small, phenylephrine, whose ability to promote changes in intracellular Ca^{2+} levels and activate phosphorylase is well established [33,34], was capable of increasing hepatocyte phosphorylase activity to the same extent as TGF-β1, with a similarly small increase in [Ca^{2+}]$_i$ (Fig. 3). This finding is consistent with previous data comparing vasopressin dose-response curves for phosphorylase activation and increase in [Ca^{2+}]$_i$ in hepatocytes, which suggested that phosphorylase kinase can be activated at very low Ca^{2+} levels [35].

In summary, the ability of TGF-β1 to promote rapid activation of hepatocyte phosphorylase activity, associated with a small increase in [Ca^{2+}]$_i$, represents one of the earliest reported intracellular effects of TGF-β1. The rapidity of these changes indicates a close coupling with events occurring upon binding of TGF-β1 to the receptor, and elucidation of the mechanisms responsible for these changes in [Ca^{2+}]$_i$ should therefore provide insight into the TGF-β1 signal transduction system.

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