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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-β1 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 $[Ca^{2+}]_i$.

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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.

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-^{14}C] 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

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/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.

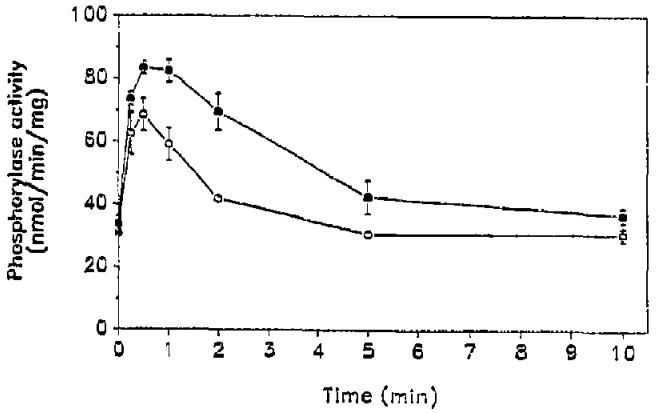

Fig. 1. Effect of TGF-β1 on phosphorylase *a* activity in hepatocytes and dependency on external calcium. Hepatocytes were preincubated at 37°C for 40 min in Krebs–Ringer bicarbonate buffer containing 1.33 mM $CaCl_2$, and then centrifuged at 50 × *g* for 70 s and resuspended in Krebs–Ringer bicarbonate 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.

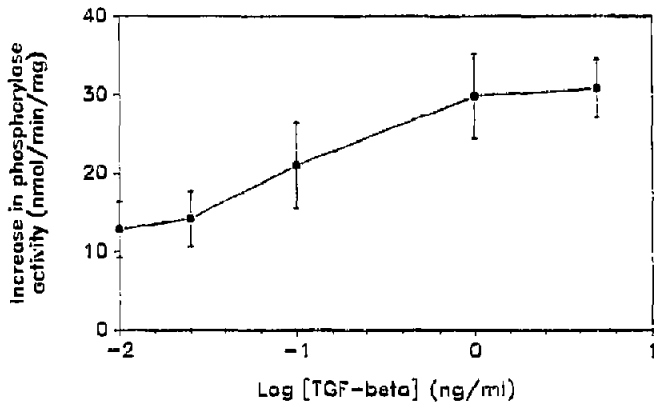

Fig. 2. Dose–response curve for rate of activation of phosphorylase in hepatocytes by TGF-β1. Time courses for activation of phosphorylase in hepatocytes incubated in Krebs–Ringer bicarbonate buffer containing 1.33 mM $CaCl_2$, by concentrations of TGF-β1 from 0.01 to 5 ng/ml were established by removing 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.

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

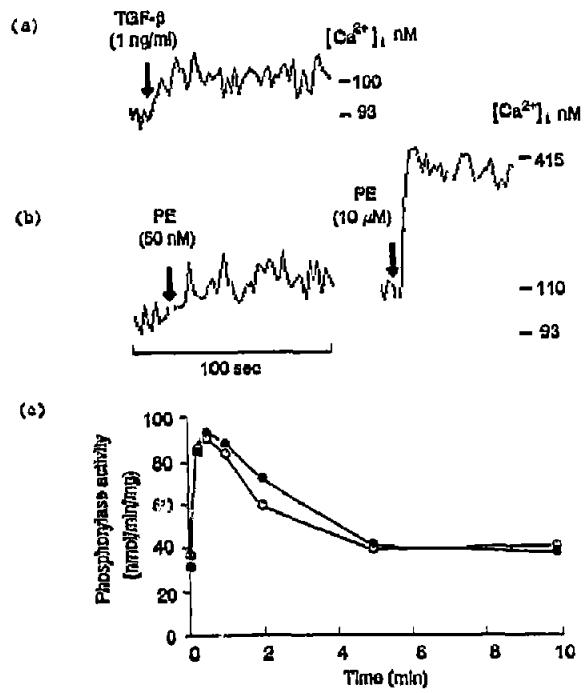

Fig. 3. Comparison of changes in cytosolic Ca^{2+} concentration elicited by TGF-β1 and by low and high concentrations of phenylephrine (PE), with corresponding changes in phosphorylase *a* activity. Panels (a) and (b) show the changes in fluorescence emission at 410 nm in indo-1-loaded hepatocytes, 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, ○), 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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REFERENCES

[1] Massague, J. (1990) Annu. Rev. Cell Biol. 6, 597–641.
[2] Roberts, A.B. and Sporn, M.B. (1990) in: Handbook of Experimental Pharmacology: Peptide Growth Factors and Their Recep-

tors (Sporn, M.B. and Roberts, A.B., Eds.) vol. 95/I, pp. 419–472, Springer-Verlag, Heidelberg.
[3] Barnard, J.A., Lyons, R.M. and Moses, H.L. (1990) Biochim. Biophys. Acta 1032, 79–87.
[4] Assoian, R.K., Komoriya, A., Meyers, C.A., Miller, D.M. and Sporn, M.B. (1983) J. Biol. Chem. 258, 7155–7160.
[5] Roberts, A.B., Anzano, M.A., Lamb, L.C., Smith, J.M. and Sporn, M.B. (1981) Proc. Natl. Acad. Sci. USA 78, 5339–5343.
[6] Moses, H.L., Branum, E.L., Proper, J.A. and Robinson, R.A. (1981) Cancer Res. 91, 2842–2848.
[7] Nakamura, T., Tomita, Y., Hirai, R., Yamaoka, K., Kaji, K. and Ichihara, A. (1985) Biochem. Biophys. Res. Commun. 133, 1042–1050.
[8] Carr, B.I., Hayashi, I., Branum, E.L. and Moses, H.L. (1986) Cancer Res. 46, 2330–2334.
[9] McMahon, J.B., Richards, W.L., delCampo, A.A., Song, M.H. and Thorgeirsson, S.S. (1986) Cancer Res. 46, 4665–4671.
[10] Russell, W.E. (1988) J. Cell Physiol. 135, 253–261.
[11] Frolik, C.A., Wakefield, L.M., Smith, D.M. and Sporn, M.B. (1984) J. Biol. Chem. 259, 10995–11000.
[12] Tucker, R.F., Branum, E.L., Shipley, G.D., Ryan, R.J. and Moses, H.L. (1984) Proc. Natl. Acad. Sci. USA 81, 6757–6761.
[13] Cheifetz, S., Like, B. and Massague, J. (1986) J. Biol. Chem. 261, 9972–9978.
[14] Wakefield, L.M., Smith, D.M., Masui, T., Harris, C.C. and Sporn, M.B. (1987) J. Cell Biol. 105, 965–975.
[15] Segarini, P.R., Roberts, A.B., Rosen, M.D. and Seyedin, S.M. (1987) J. Biol. Chem. 262, 14655–14662.
[16] Cheifetz, S., Bassols, A., Stanley, K., Ohta, M., Greenberger, J. and Massague, J. (1988) J. Biol. Chem. 263, 10783–10789.
[17] Boyd, F.T. and Massague, J. (1989) J. Biol. Chem. 264, 2272–2278.
[18] Laiho, M., Weis, F.M.B. and Massague, J. (1990) J. Biol. Chem. 265, 18518–18524.
[19] Laiho, M., Weis, F.M.B., Boyd, F.T., Ignotz, R.A. and Massague, J. (1991) J. Biol. Chem. 266, 9108–9112.
[20] Massague, J., Cheifetz, S., Boyd, F.T. and Andres, J.L. (1990) Ann. NY Acad. Sci. 593, 59–72.
[21] Lin, H.Y., Wang, X.-F., Ng-Eaton, E., Weinberg, R.A. and Lodish, H.F. (1992) Cell 68, 775–785.
[22] Lopez-Casillas, F., Cheifetz, S., Doody, J., Andres, J.L., Lane, W.S. and Massague, J. (1991) Cell 67, 785–795.
[23] Wang, X.-F., Lin, H.Y., Ng-Eaton, E., Downward, J., Lodish, H.F. and Weinberg, R.A. (1991) Cell 67, 797–805.
[24] Kramer, I.M., Koorneef, I., deVries, C., deGroot, R.P., deLaat, S.W., van den Eijnden-van Raaij, A.J.M. and Kruijer, W. (1991) Biochem. Biophys. Res. Commun. 175, 816–822.
[25] Kramer, I.M., Koorneff, I., deLaat, S.W. and van den Eijnden-van Raaij, A.J.M. (1991) EMBO J. 10, 1083–1089.
[26] Muldoon, L.L., Rodland, K.D. and Magun, B.E. (1988) J. Biol. Chem. 263, 18834–18841.
[27] Rodland, K.D., Muldoon, L.L. and Magun, B.E. (1990) J. Invest. Dermatol. 94, 33S–40S.
[28] Meijer, A.J., Gimpel, J.A., Deleeuw, G.A., Tager, J.M. and Williamson, J.R. (1975) J. Biol. Chem. 250, 7728–7738.
[29] Gilboe, D.P., Larson, K.L. and Nuttall, F.Q. (1972) Anal. Biochem. 47, 20–27.
[30] Uhing, R.J., Janski, A.M. and Graves, D.J. (1979) J. Biol. Chem. 254, 3166–3169.
[31] Tsien, R. and Pozzan, T. (1989) Methods Enzymol. 172, 230–262.
[32] Grynkiewicz, G., Poenie, M. and Tsien, R.Y. (1985) J. Biol. Chem. 260, 3440–3450.
[33] Williamson, J.R., Cooper, R.H., Joseph, S.K. and Thomas, A.P. (1985) Am. J. Physiol. 248, C203–C216.
[34] Joseph, S.K., Coll, K.E., Thomas, A.P., Rubin, R. and Williamson, J.R. (1985) J. Biol. Chem. 260, 12508–12515.
[35] Thomas, A.P., Alexander, J. and Williamson, J.R. (1984) J. Biol. Chem. 259, 5574–5584.