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STIMULATION OF HEXOSE UPTAKE IN RAT THYMIC LYMPHOCYTES BY PHORBOL ESTER. A ROLE FOR Ca^{2+} AND Na^+/H^+ EXCHANGE?

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SUMMARY. The tumor promoter 12-O-tetradecanoyl phorbol-13-acetate (TPA) stimulates hexose uptake into rat thymocytes. This study explores two possible messengers of this stimulation: changes in cytosolic $[Ca^{2+}]$, and activation of the Na^+/H^+ antiport. The cytosolic level of Ca^{2+}, determined by the fluorescence of quin-2, was elevated by TPA, and this rise required extracellular Ca^{2+}. In contrast, stimulation of hexose uptake was still observed in Ca^{2+} -free media even when cytoplasmic $[Ca^{2+}]$ was buffered with quin-2. TPA also raised the cytoplasmic pH, presumably through activation of the Na^+/H^+ exchange. However, replacement of extracellular Na^+ by N-methylglucamine$^+$ or choline$^+$ which prevents the cytoplasmic alkanization did not prevent stimulation of hexose uptake by TPA. Moreover, amiloride, at concentrations that inhibit Na^+/H^+ exchange in these cells, did not interfere with stimulation of hexose uptake by TPA. In conclusion, stimulation of hexose uptake by phorbol ester in rat thymocytes does not appear to be mediated by changes in cytosolic free Ca^{2+} or in the activity of the Na^+/H^+ antiport.

TPA[1] (12-O-Tetradecanoyl phorbol-13-acetate) is a potent tumor promoter and co-mitogen. As in the case of several growth-promoting agents (see 1), TPA stimulates sugar uptake into a variety of cell types (2-7). Stimulation is observed within the first hour of addition of the phorbol ester. The mitogenic capacity of TPA has been linked to its ability to stimulate hexose uptake (4). Therefore, it is important to determine the mechanism of this stimulation.

Recent observations indicate that phorbol esters induce mobilization of cell Ca^{2+} (8). This occurs presumably through stimulation of the turnover of phosphoinositides (9) and release polyphosphoinositol which in turn may

[1] Abbreviations used: TPA, 12-O-tetradecanoyl phorbol-13-acetate; EGTA, ethyleneglycol tetraacetate.

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release Ca^{2+} from intracellular stores into the cytoplasm (10). It has been suggested that Ca^{2+} ions may mediate stimulation of hexose transport by TPA, since chelation of extracellular Ca^{2+} with ethyleneglycol tetraacetate (EGTA) prevented the response in 3T3-mouse fibroblasts (5) and because Ca^{2+}-calmodulin inhibitors reduced the response in chick embryo fibroblasts (6). However, other authors have not found inhibition of the response with EGTA (3,7) and the effect of calmodulin inhibitors could be through their recognised action on protein kinase C (11). The latter has been identified with the phorbol ester receptor (12).

This study was undertaken to determine whether stimulation of glucose transport by TPA is mediated by changes in cytosolic Ca^{2+}. The effect of TPA on the concentration of free cytosolic Ca^{2+}, $[Ca^{2+}]_i$, was measured with the fluorescent intracellular indicator quin-2. In addition, given the high affinity of quin-2 for Ca^{2+}, the indicator can also function as an intracellular Ca^{2+}-chelator. This is particularly effective in cells suspended in Ca^{2+}-free media, in which quin-2 can dampen Ca^{2+}-transients caused by release of Ca^{2+} from intracellular stores. Quin-2 was used in this capacity to buffer potential changes in $[Ca^{2+}]_i$ during exposure to TPA.

A molecular event that occurs very early after addition of growth factors is activation of the Na^+/H^+ exchange system, resulting in cytoplasmic alkalinization in a variety of cell types (13). Recently, it was observed that TPA stimulates this system in a lymphoid cell line (14) and in rat thymocytes (15). Whereas activation of Na^+/H^+ exchange by TPA is observed within tne first minute, the increase in hexose transport rate is usually apparent several minutes after addition of the phorbol ester. Hence, it is conceivable that the activated Na^+/H^+ exchange could in turn stimulate hexose uptake. In this study we measured the activation of the Na^+/H^+ antiport with the fluorescent pH indicator bis-carboxyethyl carboxyfluorescein. The role of Na^+/H^+ exchange in stimulation of hexose transport by TPA was assessed by replacement of extracellular Na^+ with $choline^+$ or

N-methylglucamine$^+$, as well as by the use of amiloride, a specific inhibitor of the Na^+/H^+ antiport.

MATERIALS AND METHODS

TPA, 4α-phorbol 12,13 didecanoate and 4α-phorbol were purchased from Sigma and stock solutions in acetone or dimethyl sulfoxide were kept at -20°C. Quin 2-acetoxymethyl ester (quin 2-AM) was obtained from Lancaster Synthesis Ltd. 2-Deoxy-D-glucose and cytochalasin B were from Sigma Chemical Co. RPMI 1640 culture medium was from Gibco. [^{3}H]2-Deoxy-D-glucose was from New England Nuclear. [^{3}H]Quin-2 AM was from Amersham. Ionomycin was a generous gift from Squibb Laboratories. Amiloride was a gift from Dr. W. Dorian, Merck, Montreal. Bis-carboxyethyl carboxyfluorescein was synthesized by Dr. M. Ramjeesingh of this hospital, according to (16).

Suspensions of isolated thymocytes were prepared as in (17). Cell number and volume were determined in a Coulter-counter attached to a Channelyzer.

$[Ca^{2+}]_i$ was measured with the fluorescent indicator quin-2 by the method of Tsien et al. (18). Briefly, 5 x 10^7 cells/ml were incubated with 10 μM quin-2 AM for 30 min at 37° C. Extracellular ester was then removed by centrifugation followed by a wash and resuspension in RPMI-1640 culture medium. Aliquots of 3 x 10^6 cells were added into 1 ml of a medium containing 10 mM HEPES-Na (pH 7.3), 10 mM D-glucose, 140 mM NaCl, 3 mM KCl, 1 mM MgCl, and 0.5 mM EGTA, with 1.5 mM $CaCl_2$ where indicated. Fluorescence was recorded in a Perkin-Elmer 650-40 spectrofluorometer (excitation = 339 nm, emission = 490 nm). For calibration, maximum fluorescence (Fmax) was obtained by permeabilizing the cells to Ca^{2+} with 1 μM ionomycin, and minimum fluorescence (Fmin) was calculated as in (19) by the equation $Fmin = \frac{1}{6}(Fmax-FMn) + FMn$, where FMn is the fluorescence observed in the presence of ionomycin and 3 mM $MnCl_2$. From these values $[Ca^{2+}]_i$ was calculated from the observed fluorescence F according to the equation $[Ca^{2+}]_i = \frac{115(F-Fmin)}{Fmax - F}$. The intracellular concentration of quin-2 was calculated from the radioactivity present inside cells incubated with 10 μM [^{3}H]quin 2-AM (14.7 mCi/mmol).

Intracellular pH was measured with the fluorescent indicator bis-carboxyethyl carboxyfluorescein as described in (17).

The effect of TPA on $[Ca^{2+}]_i$ or on pH_i was determined by adding the phorbol ester directly to the cuvette containing cells loaded with either quin-2 or bis-carboxyethyl carboxyfluorescein, respectively. The concentration of TPA employed for spectroscopic measurements was usually 15 ng/ml, but higher and lower concentrations were also tested (see Results).

To determine the effect of TPA on hexose uptake, 1.3 x 10^8 cells/ml were incubated with 100 ng/ml TPA at 37°C for the time specified, in phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 0.5 mM $MgCl_2$, 5 mM L-Lactate, 9.3 mM Na-K phosphate, pH 7.3). Where indicated, this solution contained 1 mM $CaCl_2$ or 0.5 mM EGTA. Isoosmotic substitution of Na^+ was made by choline$^+$ of N-methylglucamine$^+$. The concentration of TPA used for stimulation of transport was higher than that used for the spectroscopic determinations described above, since (i) larger amounts of cells were incubated with the phorbol ester prior to transport determinations, and (ii) maximal stimulation of transport is observed with 100 ng/ml TPA (unpublished observation, see also ref. 7).

Measurements of 2-deoxy-D-glucose (0.1 mM) uptake were done by mixing 30 μl cell suspension (4 x 10^6 cells) and 30 μl of 0.2 mM [^{3}H] 2-deoxy-

D-glucose (33 μCi/ml) in phosphate buffered saline at room temperature. After 5 min, the cells were diluted with 60 μl ice-cold saline solution and pelleted through a mixture of 3:10 (v/v) corn oil (mazola):dibutyl phthalate. Cell pellets were suspended in 100 μl saline and counted by liquid scintillation. Non-carrier mediated uptake was calculated by addition of 5 μM cytochalasin B to the transport solution.

The results presented are the means ± S.E. of n number of determinations, or representative experiments.

RESULTS AND DISCUSSION

Fig. 1A shows a representative experiment depicting the level of $[Ca^{2+}]_i$ in the cytoplasm of rat thymocytes measured with quin-2. The basal level of $[Ca^{2+}]_i$ was 102 nM. Addition of TPA caused a progressive rise in $[Ca^{2+}]_i$. After 8 min of addition of 15 ng/ml TPA at 37°C, $[Ca^{2+}]_i$ reached a maximum value of 195 nM. In eight independent determinations, the basal level of 153 ± 11 nM rose to 221 ± 15 nM after addition of TPA. The same maximal elevations in $[Ca^{2+}]_i$ were obtained using 10, 15 or 100 ng/ml TPA (not shown). Fig. 1A also shows that the elevation in $[Ca^{2+}]_i$ occurred only in the presence of extracellular Ca^{2+}, suggesting that TPA induced an increase in Ca^{2+} permeability at the plasma membrane. A similar rise is produced by very high concentrations of TPA in rabbit neutrophils (20). In contrast to these observations, no change in $[Ca^{2+}]_i$ by the phorbol ester is detected in 3T3-fibroblasts (21) and a gradual decrease in $[Ca^{2+}]_i$ is observed in murine lymphocytes (22). The specificity of TPA to induce a rise in $[Ca^{2+}]_i$ was evidenced by the lack of effect of 4α-phorbol 12,13-didecanoate or of 4α-phorbol (see Table I). The latter two compounds are not active as comitogens or tumour promoters (23).

As in the case of fibroblasts (3-6), HeLa Cells (3), murine lymphocytes (7) and mouse epidermal cell lines (4), rat thymocytes increased their rate of hexose transport in response to TPA. A gradual increase was observed within the first hour after addition of the phorbol ester (Fig. 1B). In contrast, neither 4α-phorbol-12,13-didecanoate nor 4α-phorbol stimulated hexose uptake (Table I). Fig. 1C shows the effect of TPA on hexose uptake into rat thymocytes suspended in media containing or lacking Ca^{2+}. In the presence of 1 mM Ca^{2+}, a concentration of 100 ng/ml TPA stimulated hexose uptake by

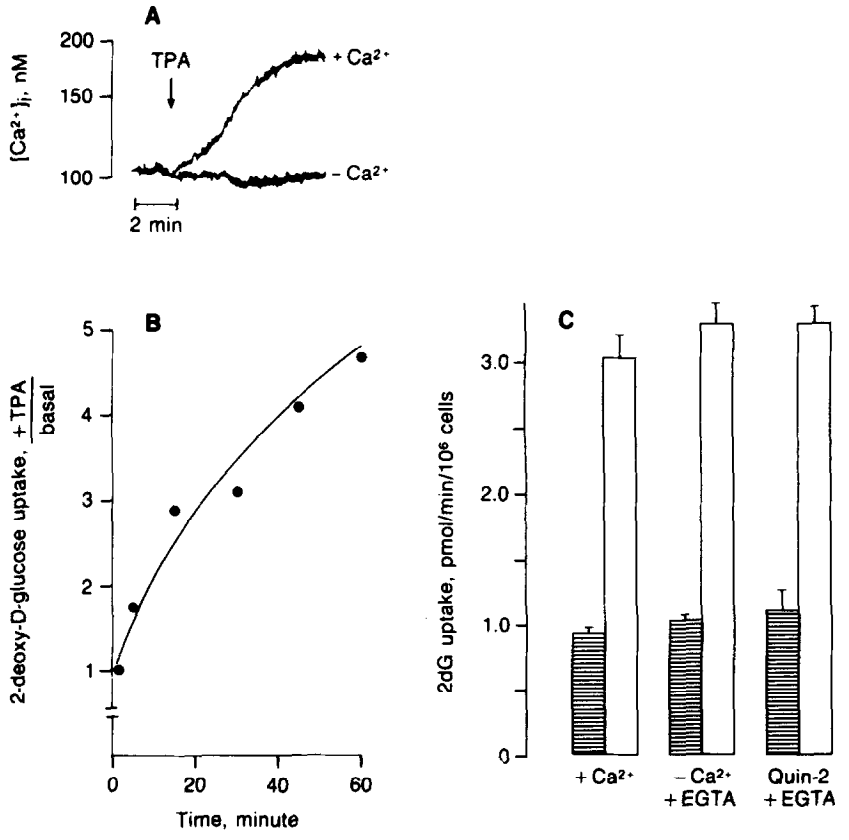

Fig. 1A Time course of the effect of TPA on $[Ca^{2+}]_i$. Quin-2 loaded cells were exposed to 15 ng/ml TPA in the presence or absence of Ca^{2+}, and the fluorescence of the indicator was recorded. See text for calibration.

Fig. 1B Time course of stimulation of hexose uptake by TPA. Thymocytes were incubated at 37°C in phosphate buffered saline containing 5 mM L-lactate in the presence or absence of 100 ng/ml TPA for the time indicated in the abscissa. Uptake of 2-deoxy-D-glucose (0.1 mM for 5 min) was then measured as described in Methods.

Fig. 1C Effect of Ca^{2+} on TPA-stimulation of hexose transport. Cells were incubated without (crossed bars) or with (open bars) 100 ng/ml TPA for 1 h as described under Methods, in the presence of 1 mM Ca^{2+}, 0.5 mM EGTA or 10 μM quin-2-AM. In the latter case, a 10 min pre-incubation with quin-2-AM preceded addition of TPA; incubation then proceded in the presence of phorbol ester and quin-2-AM. Uptake of 2-deoxy-D-glucose was next determined.

3.3-fold. Omission of extracellular Ca^{2+} and chelation of residual Ca^{2+} with 0.5 mM EGTA did not prevent stimulation of 2-deoxy-D-glucose uptake by this phorbol ester. This indicates that extracellular Ca^{2+} is not essential for stimulation of hexose uptake by TPA. Moreover, it suggests that changes in $[Ca^{2+}]_i$ are not involved in this stimulation, since no alteration in

Table I

Effect of Phorbol Derivatives on $[Ca^{2+}]_i$ and Hexose Uptake

Agent	$[Ca^{2+}]_i$ nM	2-deoxy-D-glucose uptake pmol/min/10^6 cells
-	150	1.34 ± 0.11 (3)
TPA	247	3.44 ± 0.08 (3)
4α-Phorbol 12,13-didecanoate	160	1.36 ± 0.05 (3)
4α-Phorbol	141	1.37 ± 0.14 (3)

Thymocytes were incubated without or with phorbol derivatives, under the conditions described in Methods. For $[Ca^{2+}]_i$ determinations, phorbol derivatives were added at a concentration of 24 nM (molar equivalent 15 ng/ml TPA) for 8 min. For hexose uptake determinations phorbol derivatives were tested at a concentration of 160 nM (molar equivalent of 100 ng/ml TPA) for 1 h.

$[Ca^{2+}]_i$ is caused by TPA in cells suspended in Ca^{2+}-free medium (Fig. 1A).

Notwithstanding, it is conceivable that TPA could induce release of Ca^{2+} from intracellular stores into the cytoplasm (as described in (9)) which would be manifested in cells devoid of quin-2, but which could be buffered in cells loaded with quin-2. Indeed, small transients in $[Ca^{2+}]_i$ may not be detected in quin-2 loaded cells given the high affinity of the indicator for the cation (Kd = 115 nM) and the comparatively high concentration of indicator accumulated in the cytoplasm (0.56 $\pm$ 0.09 mM, n = 4 under the experimental conditions of this study). Hence, thymocytes loaded with quin-2 suspended in Ca^{2+}-free medium constitute an ideal system in which to test if changes in $[Ca^{2+}]_i$ play a role in stimulation of hexose uptake by the phorbol ester. Fig. 1C shows that full stimulation of transport by TPA was still observed in cells loaded with quin-2 and suspended in Ca^{2+}-free medium.

Fig. 2A shows the rate of alkalinization of the cytoplasm of rat thymocytes following addition of TPA. Within 8 min, the intracellular pH rose from 7.20 to 7.38. This response, provoked by TPA but not by 4α-phorbol 12,13-didecanoate or 4α-phorbol (15), was totally prevented by 100 μM amiloride

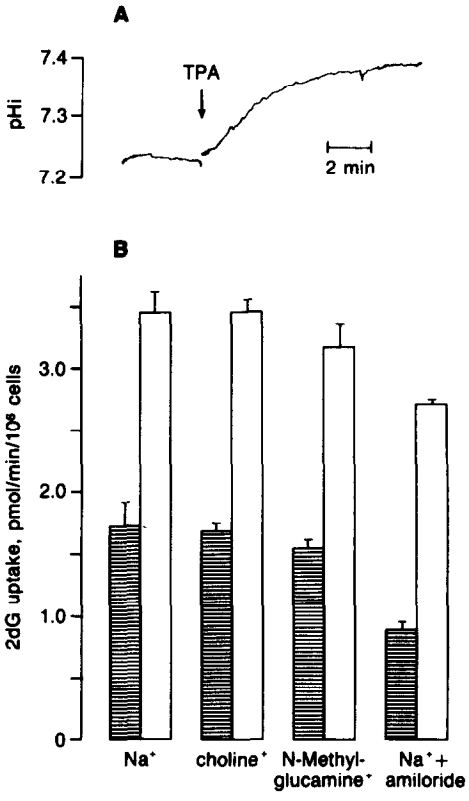

Fig. 2A Effect of TPA on intracellular pH. Cells were loaded with the fluorescent pH indicator bis carboxyethyl carboxyfluorescein and fluorescence was recorded and calibrated as in (17). The fluorescence trace of a typical experiment is depicted, before and after addition of 15 ng/ml TPA. The calculated values of pH_i are indicated.

Fig. 2B Effect of Na^+-replacement and of amiloride on stimulation of hexose uptake by TPA. Cells were incubated in the absence (crossed bars) or presence (open bars) of 100 ng/ml TPA in phosphate-buffered solutions containing 5 mM L-lactate and isotonic Na^+, choline$^+$, N-methylglucamine$^+$ or Na^+ with 300 µ amiloride. After 60 min at 37°C, 2-deoxy-D-glucose uptake was determined in the respective solutions.

or by substitution of Na^+ with choline$^+$ or N-methylglucamine$^+$ (not shown, see also ref. 15).

Fig. 2B analyses the possibility that activation of the Na^+/H^+ antiport by TPA could mediate stimulation of hexose transport. For this purpose, the activation of the antiport was prevented either by omission of extracellular Na^+ or by use of amiloride. In the experiment illustrated, the phorbol ester induced a 2.0-fold stimulation of hexose uptake when incubation was performed in the presence of 140 mM NaCl. Isoosmotic replace-

ment of Na^+ by choline$^+$ or by N-methylglucamine$^+$ did not affect significantly the stimulation of sugar uptake induced by the ester. The figure also shows that at a concentration of 300 μM, amiloride decreased the basal level of transport from 1.67 to 0.95 pmol/min/10^6 cells, yet full stimulation of transport was produced by the phorbol ester (2.8-fold). In addition, the ratio of hexose transport did not change in media of increasing alkalinity (not shown); the latter media have been shown to produce cytoplasmic alkalinization (24).

Taken together, the above results suggest that an increase in $[Ca^{2+}]_i$ is not likely to mediate stimulation of hexose uptake by TPA in rat thymocytes, since removal of extracellular Ca^{2+} and use of an intracellular chelator of Ca^{2+} do not interfere with the stimulation. Secondly, activation of the Na^+/H^+ antiport does not appear to mediate stimulation of hexose transport, since neither replacement of Na^+ by cations which are not substrates of the antiport nor addition of amiloride, an inhibitor of the ion exchange, could prevent the response of glucose transport to TPA. Instead, it is tempting to speculate that stimulation of glucose transport occurs directly through phosphorylation of membrane proteins by protein kinase C, or indirectly through a decrease in the concentration of cAMP caused by inhibition of adenyl cyclase by the phorbol ester (25).

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REFERENCES

1. Klip, A. (1982) Life Sci. 31, 2537-2548.
2. Driedger, P.E. and Blumberg, P.M. (1977) Cancer Res. 37, 3257-3265.
3. Lee, L.S. and Weinstein, I.B. (1979) J. Cell. Physiol. 99, 451-460.
4. Copley, M., Gindhart, T. and Colburn, N. (1983) J. Cell. Physiol. 114, 173-178.
5. Yamanishi, K., Nishino, H. and Iwashima, A. (1983) Biochem. Biophys. Res. Commun. 117, 637-642.
6. Yamanishi, K., Nishino, H. and Iwashima, A. (1983) Experientia 39, 1036-1037.
7. Nordenberg, J., Stenzel, K.H. and Norvogrodsky, A. (1983) J. Cell. Physiol. 117, 183-188.

8. Mottola, C. and Romeo, D. (1982) J. Cell Biol. 93, 129-134.
9. Kramer, C.M., Franson, R.C. and Rubin, R.P. (1984) Lipids 19, 315-323.
10. Berridge, M. (1984) Biochim. J. 220, 345-360.
11. Mori, T., Takai, Y. Minakuchi, R., Yu, B. and Nishizuka, Y. (1980) J. Biol. Chem. 255, 8378-8380.
12. Niedel, J.E., Kuhn, L.J. and Vandenbark, G.R. (1983) Proc. Natl. Acad. Sci. U.S.A., 80, 36-40.
13. Moolenaar, W.H., de Laat, S., Mummery, C.L. and van der Saag, P.T. (1982) Ions, cell proliferation and cancer, pp. 151-162, Academic Press, New York.
14. Rosoff, P.M., Stein, L.F. and Cantley, L.C. (1984) J. Biol. Chem. 259, 7056-7060.
15. Grinstein, S., Goetz, J., Cohen, S., Rothstein, A. and Gelfand, E. (1984) Proc. Natl. Acad. Sci. U.S.A., submitted.
16. Rink, T.J., Tsien, R.Y. and Pozzan, T. (1982) J. Cell Biol. 95, 189-196.
17. Grinstein, S., Cohen, S. and Rothstein, A. (1984) J. Gen. Physiol. 83, 341-369.
18. Tsien, R.Y., Pozzan, T. and Rink, T.J. (1982) J. Cell Biol. 94, 325-334.
19. Rink, T.J., Sanchez, A., Grinstein, S. and Rothstein, A. (1983) Biochim. Biophys. Acta 762, 593-596.
20. Sha'afi, R.I., White, J.R., Molski, T.F.P., Shefcyk, J., Volpi, M., Naccache, P.H. and Feinstein, M.B. (1983) Biochem. Biophys. Res. Commun. 114, 638-645.
21. Morris, J.D.H., Metcalfe, J.C., Smith, G.A., Hesketh, T.R. and Taylor, M.V. (1984) FEBS Lett. 169, 189-193.
22. Tsien, R.Y., Pozzan, T. and Rink, T.J. (1982) Nature 195, 68-71.
23. Shoyab, M. and Todaro, G.J. (1980) Nature 288, 451-455.
24. Deutsch, C., Taylor, J.S. and Wilson, D.I. (1982) Proc. Natl. Acad. Sci. U.S.A., 79, 7944-7948.
25. Heyworth, C.M., Whetton, A.D., Kinsella, A.R. and Houslay, M.D. (1984) FEBS Lett. 170, 38-42.