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THE GLUCOCORTICOID DEXAMETHASONE AND THE TUMOR-PROMOTING ARTIFICIAL SWEETENER SACCHARIN STIMULATE PROTEIN KINASE C FROM T51B RAT LIVER CELLS

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SUMMARY: Diacylglycerols, such as 1,2-diolein, and tumor-promoting phorbol compounds, such as TPA (12-O-tetradecanoyl phorbol-13-acetate), stimulate the Ca^{2+}/phospholipid-dependent protein kinase C from T51B rat liver cells, probably by sensitizing it to activation by Ca^{2+}, and they reduce the liver cells' content of EDTA-extractable (i.e., soluble) protein kinase C activity. Evidence is presented that indicates that the glucocorticoid, dexamethasone, and the tumor-promoting artificial sweetener, saccharin, also trigger a Ca^{2+}-dependent increase in the activity of the protein kinase C from T51B liver cells and reduce the cells' content of EDTA-extractable protein kinase C activity. However, these novel stimulators do not activate the enzyme by binding to the same site as diacylglycerols and TPA, although they do alter this site as indicated by an increase in the binding of the TPA analogue PDBu (phorbol 12,13-dibutyrate).

The archetypal tumor promoter, TPA (12-O-tetradecanoyl phorbol-13-acetate), seems to owe its several striking effects on cell function, such as the enabling of various normal cells to proliferate like tumor cells in Ca^{2+}-deficient medium (1-5) and the promotion of neoplastic transformation (6,7), to its ability to stimulate a Ca^{2+}/phospholipid-dependent protein kinase known as protein kinase C (8-11). Glucocorticoids and the artificial sweetener saccharin have long been known to share with TPA the ability to temporarily confer upon normal cells the neoplastic property of being able to proliferate in

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Ca^{2+}-deficient medium (3,12) and the former, though not known to be tumor promoters, seem to be related structurally to TPA (13) while the latter is known to be a tumor promoter (14). Therefore, we suspected that the synthetic glucocorticoid dexamethasone and saccharin might also share with TPA the ability to stimulate protein kinase C. Here we show that they do indeed stimulate protein kinase C from T51B rat liver epithelial cells, although they seem to do so differently from TPA.

MATERIALS AND METHODS

T51B rat liver epithelial cells were cultured as described previously (5). Briefly, cells were plated in 100 mm plastic Petri dishes in a medium consisting of 10% BCS (bovine calf serum from Colorado Serum Co., Denver, CO) and 90% BME (Eagle's basal medium from Flow Laboratories, Rockville, MD). The cultures were then grown to confluence at 37°C in an atmosphere consisting of 95% air and 5% CO_2. Two days after becoming confluent, the cells were removed from the dishes, homogenized in an EDTA buffer consisting of 20mM Tris-HCl (pH 7.4) and 2mM EDTA (ethylenediamine tetraacetic acid), and the homogenate was centrifuged at 105 000 xg for 30 min. at 4°C. The supernatant fluid, the EDTA extract, was used for the determination of EDTA-extractable protein kinase C activity and for the measurement of PDBu (phorbol 12,13-dibutyrate) binding to protein kinase C. It should be noted that it was not necessary to partially purify the enzyme in the EDTA extract before assaying its activity.

Protein kinase C activity was measured by adding 40 µl of EDTA extract to an assay mixture (270 µl final volume) containing 5 µmoles of Tris-HCl (pH 7.5), 1.25 µmoles of $MgCl_2$, 50 µg of histone H1 substrate (calf thymus type V-S of Sigma Chemical Co., St. Louis, MO), 2.5 nmoles of [γ-^{32}P]-ATP (1.0-1.5x10^5 cpm/nmole; from ICN, Irvine, CA), and $CaCl_2$, dexamethasone (from Merck, Sharpe and Dhome, Kirkland, PQ, Canada), 1,2-diolein (from Sigma Chemical Co.), sodium saccharin (from Dr. D. Stoltz, Health and Welfare Canada, Ottawa, ON, Canada), or TPA (from Sigma Chemical Co.) at the concentrations indicated in the appropriate figure legends. The reaction was allowed to proceed in the presence or absence of 10 µg of phosphatidylserine (from Avanti Polar Lipids, Inc., Birmingham, ALB) for 10 min. at 30°C and was then stopped by adding 2 ml of 20% trichloroacetic acid (TCA) and 50 µg of bovine serum albumin. The TCA-precipitable material was pelleted by centrifugation. The pellet was dissolved in 1N NaOH, re-precipitated with 20% TCA, centrifuged, and the final pellet was resuspended in 1N NaOH and added to a mixture of 1 ml of 0.1 N HCl and 10 ml of liquid scintillation cocktail 963 (New England Nuclear Corp., Boston, MA). The radioactivity from the phosphorylated histone H1 substrate was measured with a LKB 1217 Rackbeta liquid scintillation counter. Protein kinase C activity was the difference between the picomoles of ^{32}P incorporated into histone H1 per mg of protein during the 10-minute reaction period in the presence and absence of phosphatidylserine (15).

For PDBu binding assays, 40 µl of EDTA extract were added to a reaction mixture (270 µl final volume) containing 20mM Tris-HCl (pH 7.5), 10 µg of phosphatidylserine, 0.4mM $CaCl_2$, 50nM [20-^{3}H(N)]-PDBu (from New England Nuclear Corp.), bovine serum albumin (4 mg/ml), and

dexamethasone, 1,2 diolein, saccharin, or TPA at the concentrations indicated in Fig. 3. The reaction was allowed to proceed for 30 min. at 30°C. The reaction tubes were then put on ice and the bound PDBu was separated from free PDBu by rapid passage through Whatman GF/C glass fiber filters. The filters were oven-dried and placed into 10 ml of liquid scintillation cocktail 963 and the radioactivity from bound PDBu was measured in a LKB 1217 Rackbeta liquid scintillation counter with an efficiency of 36%. Non-saturable binding was measured in the presence of a 100-fold excess (5μM) of non-radioactive PDBu. The results were expressed as saturable binding (i.e., total PDBu binding minus non-saturable binding).

The protein contents of the EDTA extracts were determined by the method of Bradford (16) using bovine serum albumin as the standard.

RESULTS

Saccharin stimulated the protein kinase C activity in EDTA extracts of T51B cell homogenates (Fig. 1A) as well as the protein kinase C that had been partially purified from these extracts by FPLC chromatography on a Mono Q column (Fig. 1B). Under these conditions, the maximally effective saccharin concentration in both cases ranged between $5x10^{-6}$ and 10^{-4}M (Fig. 1).

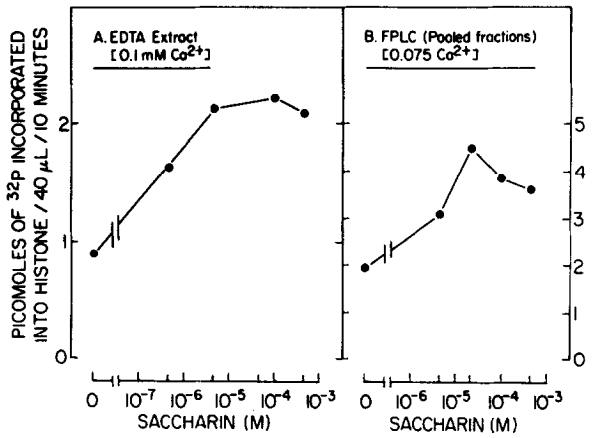

Fig. 1. The activation of protein kinase C by saccharin. The enzyme was assayed in the presence of phosphatidyl serine and suboptimal Ca^{2+} concentrations (0.1mM for A and 0.075mM for B) as described in Materials and Methods. It is the abilities of various concentrations of saccharin to increase protein kinase C activity above the activity in the presence of Ca^{2+} and phosphatidylserine that are shown. A. The effects of saccharin on protein kinase C activity in EDTA extracts. B. The effects of saccharin on protein kinase C that had been partially purified by FPLC chromatography on Mono Q columns (from Farmacia Fine Chemicals, Montreal, PQ).

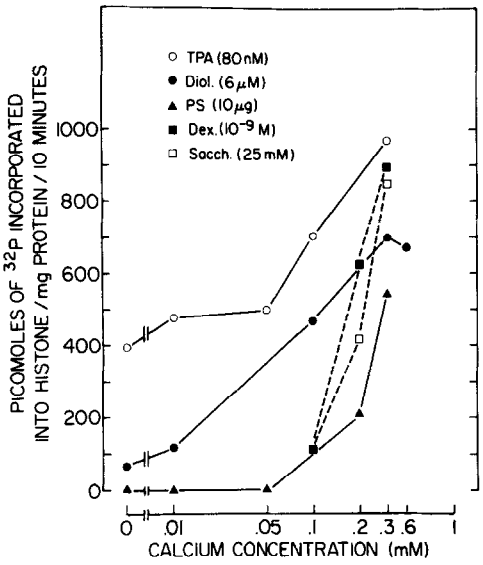

Fig. 2. The Ca^{2+}-dependence of the abilities of dexamethasone (Dex), 1,2-diolein (Diol), saccharin (Sacch) and TPA to stimulate protein kinase C in EDTA extracts of T51B cells. All assay mixtures contained 10 μg of phosphatidyl serine (PS) but different amounts of Ca^{2+}.

It is believed that diacylglycerols, such as 1,2 diolein, and tumor-promoting phorbol compounds, such as TPA, stimulate protein kinase C by sensitizing it to activation by Ca^{2+} (11,17,18). Indeed, from the data in Fig. 2, it appears that although TPA was most effective in the presence of 0.3mM Ca^{2+}, it was able to stimulate protein kinase C significantly even without supplementing the traces of Ca^{2+} remaining in the EDTA extracts. By contrast, neither 1,2 diolein, nor the optimally effective 25mM saccharin had TPA's ability to stimulate protein kinase C in the EDTA extract without added Ca^{2+}. Diolein significantly stimulated the enzyme only when the Ca^{2+} concentration exceeded 0.01mM while 25 mM saccharin needed more than 0.1mM Ca^{2+} to stimulate the enzyme (Fig. 2).

The question then arose as to whether saccharin stimulated protein kinase C by attaching to the enzyme's diacylglycerol/TPA-binding site. This was answered by measuring the ability of saccharin to affect the ability of the TPA analogue, PDBu, to bind selectively to a component of the cellular EDTA extract that co-purified with protein kinase C

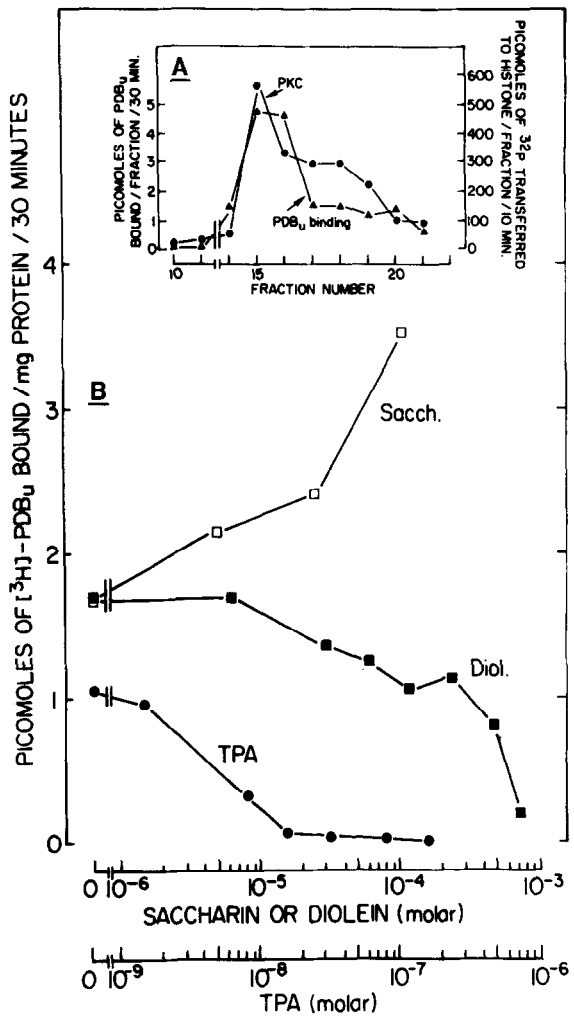

Fig. 3. The effects of 1,2-diolein (Diol), saccharin (Sacch) and TPA on the binding of PDBu (phorbol 12,13-dibutyrate) to protein kinase C. A. The copurification (during FPLC chromatography on a Mono Q column) of PDBu binding activity and protein kinase C activity from EDTA extracts of T51B cells. B. The effects of various concentrations of Diol, Sacch and TPA on PDBu binding to protein kinase C in EDTA extracts of T51B cells. The method of measuring PDBu binding is described in Materials and Methods.

activity during FPLC chromatography on a Mono Q column and was presumably protein kinase C (Fig. 3A). As expected, increasing concentrations of 1,2 diolein or TPA increasingly reduced and eventually completely prevented PDBu binding (Fig. 3B). By contrast, saccharin actually increased PDBu binding to protein kinase C (Fig. 3B). Therefore, saccharin did not stimulate protein kinase C by attaching to the enzyme's diacylglycerol/TPA-binding site.

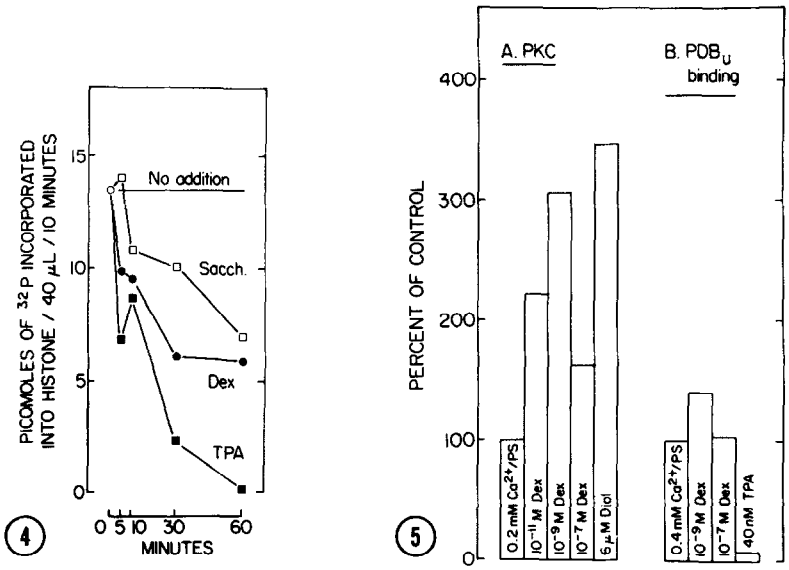

Fig. 4. Treatment of intact T51B liver cells with dexamethasone (Dex), saccharin (Sacch) or TPA lowers the cellular content of EDTA-extractable protein kinase C activity. T51B cell cultures were exposed to dexamethasone (10^{-7}M), saccharin (25mM), or TPA (80nM), the cells harvested at the indicated times, homogenized in EDTA buffer and the protein kinase C activity in the EDTA extracts was assayed in the presence of 0.3mM Ca^{2+} and 10 µg phosphatidylserine as described in Materials and Methods.

Fig. 5. The abilities of dexamethasone (Dex) to stimulate protein kinase C activity and affect the binding of PDBu to protein kinase C. A. The protein kinase C activity in EDTA extracts of T51B cells was measured in the presence of 0.2mM Ca^{2+} and 10 µg of phosphatidylserine. B. PDBu binding to the protein kinase C in EDTA extracts was measured (as described in Materials and Methods) in the presence of 0.4mM Ca^{2+} and 10 µg of phosphatidyl serine. The effects of dexamethasone on protein kinase C activity and PDBu binding to the enzyme are compared to those of 1,2-diolein (Diol) and TPA respectively.

TPA seems to cause protein kinase C to shift from a soluble to an insoluble state in several kinds of cell (10,19). It is not clear at this time whether this change is due to translocation of the enzyme from the cytosol to membranes as suggested by Kraft and Andersen (10) or to proteolytic activation of the enzyme as suggested by Tapley and Murray (20). A similar shift in soluble protein kinase C occurred in TPA-treated T51B cells as indicated by a rapid and large drop in the amount of EDTA-extractable enzyme activity during the first 60 min. of exposure to the drug (Fig. 4). Exposing T51B cells to the optimally effective 25mM saccharin also rapidly reduced the amount of

EDTA-extractable protein kinase C activity, but it did so less effectively than the optimally effective 80nM TPA (Fig. 4).

Dexamethasone (at concentrations from 10^{-11} to 10^{-7}M in the presence of 0.2mM Ca^{2+}) also stimulated protein kinase C (Figs. 2 and 5A). In fact, at the optimally effective 10^{-9}M, the steroid was as good a stimulator as the optimally effective 6μM 1,2-diolein (Figs. 2 and 5A). Like saccharin, dexamethasone needed much more Ca^{2+} to stimulate the enzyme than did 1,2-diolein or TPA (Fig. 2). Dexamethasone also did not compete with PDBu for the enzyme's diacylglycerol/TPA-binding site (Fig. 5B). In fact, the steroid, like saccharin, tended to increase PDBu binding to the enzyme (Fig. 5B).

DISCUSSION

We conclude from these observations that saccharin is another tumor promoter that stimulates protein kinase C, although it does not stimulate the enzyme by binding to the same site as the phorbol tumor promoters. We also conclude that the synthetic glucocorticoid dexamethasone (which to our knowledge has not been reported to be a tumor promoter) stimulates protein kinase C as we had guessed that it might from its structural resemblance to TPA (13). However, this steroid did not stimulate the enzyme by binding to the diacylglycerol/TPA-binding site, as it should have done if its structural resemblance to TPA were functionally meaningful. Thus, it seems possible from these preliminary observations that the protein kinase C from T51B rat liver cells might have at least two kinds of effector-binding site, one for diacylglycerols and tumor-promoting phorbol compounds and one (or more) for saccharin and steroids, the occupation of which activates the enzyme and at the same time increases the affinity of the diacylglycerol/TPA-binding site for its ligands. Finally, it now seems likely that regardless of its mechanism a stimulation of protein kinase C is the common basis of the abilities of such different compounds as glucocorticoid steroids, phorbol tumor

promoters and saccharin to enable non-neoplastic cells to replicate DNA and proliferate in the presence of normally inadequate Ca^{2+} concentrations (1-5,12).

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