Journal of Supramolecular Structure
and Cellular Biochemistry 15:161–168 (1981)
Membrane Transport and Neuroreceptors 363–370

Cell Cycle Activation and Ouabain-Sensitive Ion Movements of 3T3 and C3H-10T½ Fibroblasts

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The introduction of either $PGF_{2\alpha}$ (10^{-7} M) or TPA (10^{-7}M) stimulated, ouabain-sensitive $^{86}Rb^+$ influx at 30 min in postconfluent 3T3-4 mouse fibroblast cultures by 117% and 124%, respectively. Both TPA and $PGF_{2\alpha}$ at these concentrations stimulated the incorporation of ^{3}H-TdR into DNA. TPA had the greatest stimulatory effect, which was similar to that obtained with 10% fetal calf serum. In accord with the idea that modulation of membrane processes such as Na^+/K^+ pump activity in fibroblasts may reflect important events related to the initiation of DNA synthesis, it was observed that in both 3T3-4 and C3H-10T½ cells there were parallel increases in ^{3}H-TdR incorporation and ouabain-sensitive $^{86}Rb^+$ influxes with 10^{-7} M TPA, whereas $PGF_{2\alpha}$ stimulated a significant increase in ^{3}H-TdR incorporation in 3T3-4 but not C3H-10T½ cells and only marginal increases in ouabain-sensitive $^{86}Rb^+$ influx in both. Therefore, although there appears to be a close correlation between Na^+/K^+ pump activation and subsequent S-phase entry following TPA stimulation, a similar correlation for $PGF_{2\alpha}$ cannot be confirmed.

Key words: phorbol ester prostaglandin $F_{2\alpha}$, $(Na^+ + K^+)$-ATPase, cell cycle activation

In postconfluent 3T3 mouse embryonic fibroblasts, tetradecanoyl phorbol acetate (TPA), $PGF_{2\alpha}$, as well as serum, purified growth factors, and vasopressin, stimulate ^{3}H-TdR incorporation into DNA. It has been proposed that one of the earliest changes produced in the cell leading to cycle activation is an increased ouabain-sensitive $^{86}Rb^+$ influx [1–6]. Ouabain itself also prevents the initiation of DNA synthesis after serum addition to postconfluent 3T3 cells [1]. This early increase in Na^+/K^+-pump-mediated activity by mitogenic agents raises the question as to the relationship of K^+ movements to other metabolic events that lead to initiation of rapid proliferation. In this regard, Dicker and

Abbreviations: TPA, 12-*o*-tetradecanoyl-phorbol-13-acetate; 4-*o*-Me-TPA: 4-*o*-methyl tetradecanoyl phorbol acetate; 4(α)-OH-PDD: 4(α)-OH phorbol-12,13-didecanoate; ^{3}H-TdR: ^{3}H-thymidine; PCA: perchloric acid.

Received April 21, 1980; accepted October 21, 1980.

0275-3723/81/1502-0161$02.50

Rozengurt [6] have suggested that stimulation of ion fluxes such as K^+ uptake may act as the possible convergence point of the pathway by which TPA and vasopressin act as mitogens.

Of particular interest is the comparison of TPA with mitogenic agents in the prostaglandin series. Rohrschneider and Boutwell [7] have pointed out that TPA bears a structural and functional relationship to polyunsaturated fatty acids, which are precursors of prostaglandins. In addition, Smythies et al [8] have proposed a tentative model receptor for prostaglandins and have suggested that TPA exerts its biological effects via prostaglandin receptor interaction. Although Furstenberger and Marks [9] have provided evidence that TPA is not a prostaglandin E-type agonist, Moroney et al [5] have demonstrated that TPA induces an early uptake of $^{86}Rb^+$ and $^{32}P_i$ in 3T3 cells in a manner analogous to that of $PGF_{2\alpha}$. $PGF_{2\alpha}$ was, therefore, the prostaglandin of choice for further study.

The availability of a cell type, namely the C3H-10T½ mouse fibroblast, in which TPA but not $PGF_{2\alpha}$ stimulated cell proliferation in preliminary studies, allowed the comparison of the stimulation of the (Na^+/K^+)-ATPase activity with cell cycle activation. Therefore, the effects of phorbol esters and prostaglandin $F_{2\alpha}$ on ^{3}H-TdR incorporation and ouabain-sensitive $^{86}Rb^+$ uptake were examined.

MATERIALS AND METHODS

Cell Culture

Cultures of 3T3-4 mouse fibroblasts or C3H-10T½ clone-8 mouse fibroblasts (passages 9–14) were used for all experiments and were maintained as previously described [5, 10] using Eagle's basal medium (BME) supplemented with 10% heat-inactivated fetal calf serum (GIBCO, NY). Experiments were carried out in replicate cultures in 60-mm diameter plastic petri dishes (n = 4) seeded in 5-ml aliquots at 500 cells/sq cm for measurement of ^{3}H-TdR incorporation at specific intervals or for measurement of $^{86}Rb^+$ uptake at 30 min after drug addition. The results are expressed in terms of mean values (± standard deviation) from quadruplicate plates. Stock cultures of 3T3-4 mouse embryonic fibroblasts were obtained from Dr. Howard Green, Massachusetts Institute of Technology, Cambridge, Mass, and C3H-10T½-8 were from Dr. John Bertram of this Institute.

^{3}H-TdR Incorporation Studies

Drugs were added to culture dishes containing 5 ml of medium using a 10-μl repeating automatic microsyringe (Hamilton). All drugs were prepared in 50% ethanol and control (untreated) cultures received equal volumes of solvent. Solvent additions did not exceed 10 μl/5 ml of culture fluid (18 mM). Incorporation of ^{3}H-TdR into the acid-insoluble fraction was measured following a 1-h pulse with ^{3}H-TdR (41 Ci/mmole) (Moravek, Calif) at 1 μCi/5 ml culture. Replicate cultures were pulsed every 3–5 h up to 28 h after drug addition.

Specific incorporation of ^{3}H-TdR into DNA was measured as follows: Cultures were removed from the incubator, the medium was removed by aspiration and the cell monolayer was gently washed twice with Earle's balanced salt solution (EBSS), and 5 ml of cold 1.5% PCA was added. The dishes were placed at 4°C for 12–24 h after which each culture was washed twice with fresh cold 1.5% PCA, scraped off with a rubber policeman, and collected in a 12-ml siliconized conical centrifuge tube with 2 ml of PCA on ice. Measurement of DNA was made as described by Kissane and Robbins [11]. After centrifugation (800–1,000g) for 5 min in a refrigerated centrifuge to form a uniform pellet, the supernatant was discarded and the pellet resuspended in 0.1 M potassium acetate in absolute ethanol

on ice. After centrifugation, the pellet was then resuspended in 2 ml of absolute ethanol and placed in a water bath at 60°C for 30 min. After centrifugation and discarding the supernatant, the pellet was air-dried at 40°C. At this time, 15 μl of 3,5-diaminobenzoate (300 mg/ml) was carefully placed upon the pellet in each tube. The tubes were then covered and placed in a water bath at 60°C for 30 min. Upon removal of the tubes, 2 ml of 6% PCA was added to each followed by brief vortex mixing. Total DNA was determined immediately by fluorescence measurement using a Perkin-Elmer 650-10S fluorescence spectrometer. The sample was then transferred to a 20-ml scintillation vial, and 10 ml of aqueous counting fluid was added followed by vigorous vortex mixing to ensure homogeneity. Final values were expressed as cpm of ^3H per fluorescent unit.

Autoradiographic analysis of nuclear labeling frequency was carried out by growing the cells in 60-mm plastic petri dishes and fixing the cells with 1.5% PCA (12–24 h, 4°C). After rinsing the dishes with water and finally with methanol, the dried culture dishes were coated with NTB-3 Nucleotrack Emulsion (Kodak) and developed after 6 days. Nuclear labeling indices were determined by counting both labeled and unlabeled nuclei in 25 random fields (approximately 2,000 cells total) on triplicate cultures and expressed as percent labeled nuclei (± standard error).

Studies of $^{86}Rb^+$ Movements

Uptake studies were carried out with postconfluent cultures 72 h after the last medium change. Cells were prepared for uptake studies by decanting the media, adding 5 ml of serum-free Hanks balanced salt solution (HBSS) (pH 7.25) that contained $^{86}Rb^+$ (New England Nuclear Corp, Mass) and the appropriate drug to be tested. As mentioned above, precautions were taken to minimize the solvent volume, maximum 10 μl/5.0 ml. Approximately 2 μCi $^{86}Rb^+$ was present in each dish. After incubation, the supernatant was removed and decanted. Trichloroacetic acid was added to rupture the cells, and the acid-soluble fraction counted for $^{86}Rb^+$. Experiments were carried out in quadruplicate plates. TPA was obtained from Dr. P. Borchert, University of Minn, and $PGF_{2\alpha}$ was a gift from Dr. J. Pike (Upjohn Co, Mich), and 4-α-OH-PPD was from Dr. E. Hecker (Heidelberg).

RESULTS

Comparison of the Effects of Prostaglandin $F_{2\alpha}$, TPA, and Serum on Ouabain-Sensitive $^{86}Rb^+$ Incorporation and ^3H-TdR Incorporation in 3T3-4 Cells

TPA bears a structural similarity to prostaglandin precursors [7], and since earlier results indicated that $PGF_{2\alpha}$ stimulated ouabain-sensitive $^{86}Rb^+$ uptake [5], the stimulatory effects of TPA and $PGF_{2\alpha}$ on $^{86}Rb^+$ uptake and ^3H-TdR were compared.

In an experiment described in Table I, the effects of TPA, $PGF_{2\alpha}$, and serum on $^{86}Rb^+$ uptake by 3T3-4 fibroblasts were examined in the presence and absence of ouabain (10^{-3} M). In either case, maximal stimulation was attained with serum, followed closely by TPA at a concentration of 10^{-7} M. This stimulation was largely ouabain-sensitive. $PGF_{2\alpha}$ gave an intermediate stimulation of $^{86}Rb^+$ uptake, and again this represented a largely ouabain-sensitive component.

When postconfluent quiescent cultures were stimulated by TPA 48–72 h after the last medium renewal, two changes in the kinetics of ^3H-TdR incorporation were observed: an initial but transient suppression between 8–13 h and a substantial increase in the incorporation rate between 13–28 h (Fig. 2). As also illustrated in Figure 1, $PGF_{2\alpha}$ was less active when compared with TPA but gave reproducible and significant increases in ^3H-

TABLE I. Effect of TPA, $PGF_{2\alpha}$, and Serum on Ouabain-Sensitive $^{86}Rb^+$ Uptake by 3T3-4 Fibroblasts†

Additions	Without ouabain	With ouabain (1 mM)
	% of Control	% of Control
None	100.0 ± 6.8	53.8 ± 7.8
TPA (10^{-7} M)	124 ± 7.1**	62.4 ± 8.2
$PGF_{2\alpha}$ (10^{-7} M)	117 ± 8.6*	56.6 ± 3.6
Fetal calf serum (10%)	140 ± 8.9**	67.1 ± 21.3

†The control values in this experiment were 59.5 ± 2.9 nmoles $^{86}Rb^+$ uptake per 5×10^5 cells × h.
*$P < 0.05$
**$P < 0.01$

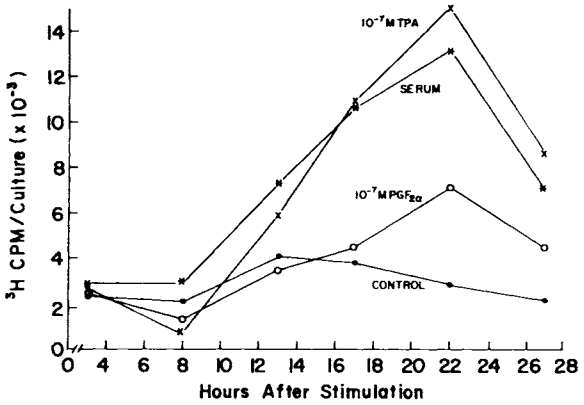

Fig. 1. Comparison of the effects of fresh serum (*), 10^{-7} M TPA (X), and 10^{-7} M $PGF_{2\alpha}$ (○) with untreated cultures (•) on ^{3}H-TdR pulse incorporation in postconfluent 3T3-4 cells 48 h after medium renewal. Measurements of ^{3}H-TdR incorporation into acid-insoluble material following 1 h exposure beginning at the indicated time after drug addition.

TdR incorporation at 10^{-7} M. The peak pulse incorporation was approximately 2.5 times that of control, substantially less than that obtained with either TPA (10^{-7} M) or fresh serum (10% v/v). Although transient suppression of incorporation could be observed with $PGF_{2\alpha}$ as shown here, such effects were not consistently observed nor did they exhibit any obvious dose dependency as found with TPA. In no experiment was serum observed to produce a suppression in the incorporation rate.

Comparison of TPA and $PGF_{2\alpha}$ Effects on $^{86}Rb^+$ Uptake in C3H-10T½ Cells; In Addition, the Effects of Stereoisomeric Homologs on $^{86}Rb^+$ Uptake

Next, the effects of TPA at varied concentrations on $^{86}Rb^+$ uptake by postconfluent C3H-10T½ fibroblasts were examined, and it was observed as seen in an experiment described in Table II, that optimal uptake occurred at concentrations of 10^{-8} M to 10^{-6} M. Also in Table II, the ability of TPA and $PGF_{2\alpha}$ to enhance $^{86}Rb^+$ uptake was compared

TABLE II. Effect of Concentration of TPA, 4-*o*-Me-TPA, and $PGF_{2\alpha}$ on $^{86}Rb^+$ Uptake by C3H-10T½ Cells†

Concentration	TPA (% of Control)	4-*o*-Me-TPA (% of Control)	$PGF_{2\alpha}$ (% of Control)
0	100.0 ± 2.2	–	–
10^{-8} M	139.0 ± 3.6**	99.6 ± 3.2	97.7 ± 4.7
1×10^{-7} M	139.7 ± 2.2**	103.2 ± 4.3	107.4 ± 7.3
3×10^{-7} M	141.2 ± 6.5**	102.9 ± 3.3	105.2 ± 2.2*
1×10^{-6} M	140.6 ± 2.7**	112.5 ± 2.7**	111.7 ± 2.9**

†The control value expressed in nmoles $^{86}Rb^+$ uptake per 5×10^5 cells × h was 96.0 ± 1.5.
*$P < 0.05$
**$P < 0.01$

TABLE III. Effect of TPA and $PGF_{2\alpha}$ on Ouabain-Sensitive $^{86}Rb^+$ Uptake in C3H-10T½ Fibroblasts

Ouabain (1 mM)	Control[a]	TPA (10^{-7} M)	$PGF_{2\alpha}$ (10^{-7} M)
		Experiment 1	
–	100.0 ± 4.7	130.5 ± 14.7*	110.8 ± 17.9
+	62.3 ± 7.2	68.2 ± 9.6	68.6 ± 9.3
		Experiment 2	
–	100.0 ± 4.3	128.6 ± 5.5**	112.1 ± 3.8*
+	67.7 ± 2.1	78.2 ± 3.6*	76.0 ± 2.4*

[a]The control values expressed in nmoles $^{86}Rb^+$ uptake per 5×10^5 cells × h for Experiment 1 were 92.8 ± 3.1; for Experiment 2, they were 70.9 ± 3.7.
*$P < 0.05$
**$P < 0.01$

with the inactive promoter, 4-*o*-Me-TPA. Only TPA gave a pronounced stimulation of $^{86}Rb^+$ uptake at concentrations of 3×10^{-7} M or below. At 10^{-6} M, both $PGF_{2\alpha}$ and 4-*o*-Me-TPA produced a small but significant increase in ^{86}Rb influx.

In Table III, two experiments are described in which the ouabain sensitivity of TPA- and $PGF_{2\alpha}$-induced responses in $^{86}Rb^+$ uptake were examined. Again in both experiments, TPA at 10^{-7} M produced a more pronounced enhancement of $^{86}Rb^+$ uptake. $PGF_{2\alpha}$ at 10^{-7} M was observed to stimulate a minor yet significant increase in $^{86}Rb^+$ uptake. The stimulation of $^{86}Rb^+$ uptake was largely ouabain-sensitive with approximately 9%–18% of the uptake for both TPA and $PGF_{2\alpha}$ being accounted for in ouabain-insensitive component.

In addition, the effects on $^{86}Rb^+$ uptake of a stereoisomeric phorbol ester analogue (inactive as a tumor promoter) were compared with those of TPA; 4-α-phorbol didecanoate (4α-PDD) allows assessment of surface activity as described previously [12]. The results obtained with 4α-PDD, its stereoisomer PDD and TPA are described in an experiment reported in Table IV. Again, TPA produced a pronounced stimulation of $^{86}Rb^+$ uptake, PDD produced an intermediate effect, and in contrast to what is observed with TPA and PDD, 4α-PDD produced little or no stimulation of $^{86}Rb^+$ uptake.

TABLE IV. Comparison of TPA and PDD With the Stereoisomeric 4α-PDD on $^{86}Rb^{+}$ Uptake by C3H-10T½ Cells*

Additions	nmoles $^{86}Rb^{+}$ uptake per test[a]	% of Control[a]
None	83 ± 2.8	100 ± 4.8
TPA (10^{-7} M)	111 ± 4.7	133.7 ± 7.2
4α-PDD	81 ± 0.6	97.5 ± 3.4
PDD	91 ± 4.9	110.0 ± 7.0

*The mean cell number per dish was 3.9×10^5.
[a]Mean ± standard error (n = 4).

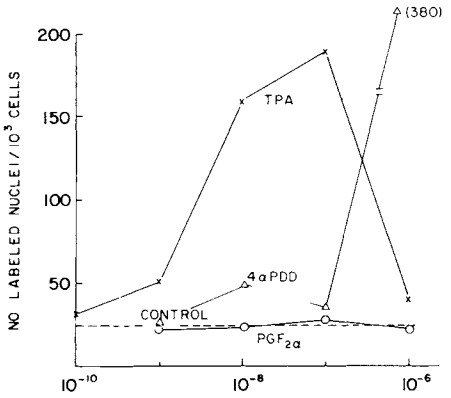

Fig. 2. The dose dependency of TPA (x), and $PGF_{2\alpha}$ (○) on the cumulative nuclear labeling index 20 h after drug addition in postconfluent quiescent 10T½ cells. Untreated cultures yielded fewer than 20 labeled nuclei per 10^3 cells (see Materials and Methods).

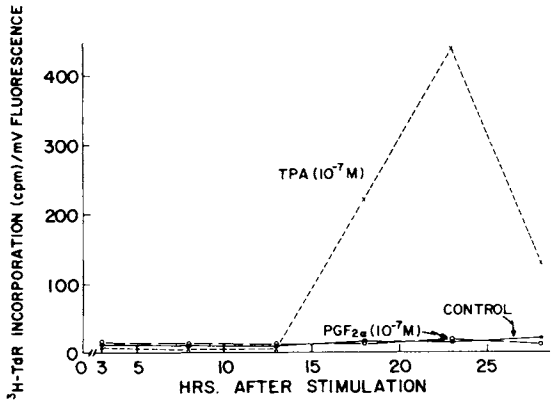

Fig. 3. Effect of TPA (x) and $PGF_{2\alpha}$ (○) on the specific incorporation of ^{3}H-TdR into acid-insoluble material in postconfluent quiescent 10T½ cultures. Cells were pulsed for 1 h with ^{3}H-TdR. Values are expressed in ^{3}H-TdR incorporation per fluorescent unit as measured by modification of the Kissane and Robbins method [11].

Effect of TPA and $PGF_{2\alpha}$ on ^{3}H-TdR Incorporation in C3H-10T½ Cells

The dose response curve of TPA and $PGF_{2\alpha}$ on ^{3}H-TdR incorporation into nuclei was examined by autoradiography. As described in an experiment reported in Figure 2, TPA in concentrations as low as 10^{-9} M increased the number of labeled nuclei at 20 h. The inactive tumor promoter analogue 4α-PDD exhibited no activity at 10^{-9}–10^{-7} M. At 10^{-6} M 4α-PDD did exhibit appreciable labeling activity. This is considered to be a toxic effect at a concentration that exhibits significant surface activity [12]. In contrast to the effects observed with TPA, $PGF_{2\alpha}$ at concentrations from 10^{-9}–10^{-6} M did not alter the number of labeled nuclei from control values.

As also seen in an experiment reported in Figure 3, TPA (10^{-7} M) but not $PGF_{2\alpha}$ enhanced the incorporation of ^{3}H-TdR into the acid-insoluble material by over 50-fold. Peak incorporation was observed at 20–24 h after TPA introduction.

DISCUSSION

It was observed that in 3T3 mouse fibroblasts, TPA and $PGF_{2\alpha}$ induce a parallel stimulation of ^{3}H-TdR incorporation into the acid-insoluble fraction and (Na^+/K^+)-ATPase activity as measured by an increase in ouabain-sensitive $^{86}Rb^+$ uptake. It is also noted that the stimulatory effects with $PGF_{2\alpha}$, although significant, are not as striking as that obtained with TPA or 10% fetal calf serum.

In contrast, in C3H-10T½ cells, $PGF_{2\alpha}$ does not alter ^{3}H-TdR incorporation into nuclei or the acid-insoluble fraction as does TPA. Further, the stimulation by $PGF_{2\alpha}$ of ouabain-sensitive $^{86}Rb^+$ uptake is marginal when compared with that of TPA. This marginal but significant stimulation of membrane transport by $PGF_{2\alpha}$ is not sufficient by itself to initiate the proliferative response. Nevertheless, modulation of membrane events such as the stimulation of the Na^+/K^+ pump activity in fibroblasts may be a necessary condition for initiation of associated biosynthetic events.

The effects of $PGF_{2\alpha}$ and TPA in these two lines of mouse embryonic fibroblasts differed qualitatively in several other aspects. For example, it was observed that the morphological changes induced by TPA in C3H-10T½ fibroblast cultures are not seen with $PGF_{2\alpha}$ additions. TPA but not $PGF_{2\alpha}$ caused marked alteration of cell shape from wide and flat to pleomorphic and highly refractile [14]. It was also noted that in contrast to TPA, which has been reported to stimulate cellular adhesion in several cell types including C3H-10T½ cells, similar responses to prostaglandins D_2, E_1, E_2, and $F_{2\alpha}$ were not observed for this cell type [14]. Further, TPA (10^{-8}–10^{-6} M) but not $PGF_{2\alpha}$ (10^{-8}–10^{-6} M) induces up to 50% increases in glycolysis with postconfluent C3H-10T½ cells in contrast to similar increases by TPA and $PGF_{2\alpha}$ observed in 3T3 cells [15].

A definitive answer to the role of ion movements in cell proliferation may require measurement within distinct regions or subpopulations of cells rather than studies of net differences over the entire cell population. In support of the idea that cellular domains are integral to cellular responses are current studies by the authors that provide evidence for nonrandom response patterns under conditions similar to those described in $^{86}Rb^+$ experiments [16]. Thus, a study of TPA-induced stimulation of cell cycle and associated trypsin-resistant adhesion in quiescent cell cultures revealed that responses are confined to distinct regions of the cell culture and that mitogenic responses are not normally distributed with respect to position on the culture dish. Thus, a spatial nonrandom statistical factor determining response to TPA has been found. This implies that a multidimensional analysis is necessary to finally determine the answers to the questions inferred from one-dimensional

analysis such as that presented here. In support of mutual interaction of cells in the pharmacology of TPA is recent evidence that TPA alters metabolic cooperativity between cells [17]. Therefore, based upon measurement of net $^{86}Rb^{+}$ influx, only TPA provides a clear correlation with cell cycle activation, whereas $PGF_{2\alpha}$ responses are equivocal at best. It is suggested that small net changes induced by $PGF_{2\alpha}$ may represent substantial increases in Na^{+}/K^{+} pump activity in a small number of cells. This is in accord with the percentage of labeled nuclei observed at 24 h since only 8%–12% of C3H-10T½ cells have labeled nuclei following 10^{-7} M TPA administration (Fig. 2). Similarly in 3T3 cells, approximately 15% of nuclei are labeled at 24 h after continuous incubation with TPA [18].

ACKNOWLEDGMENTS

The authors are indebted to John H. Hackney and Louise Bartels for their capable technical assistance.

This work was supported by a grant from the National Cancer Institute (CA-13784).

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