Tumor promoters cause a rapid and reversible inhibition of the formation and maintenance of electrical cell coupling in culture

(tumor promotion/gap junction/cell-cell communication/phorbol ester)

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ABSTRACT The effect of tumor promoters on electrical coupling between human FL cells was investigated with a microelectrode technique. When a low concentration (100 ng/ml) of 12-O-tetradecanoylphorbol 13-acetate (TPA) was added to culture medium, only 6% of the cells showed electrical coupling after 5 hr, whereas in control medium more than 90% of the cells were coupled. In the presence of TPA, cell coupling remained suppressed for at least another 19 hr. When TPA was washed out from the culture medium, the cells commenced electrical coupling: 90% of the cells were coupled within 4 hr of the removal of TPA, a rate very similar to that of nontreated control cells. Therefore, TPA-mediated inhibition of cell coupling is reversible. When TPA was added to a culture in which more than 90% of the cells had already established electrical coupling, the percentage of coupled cells decreased to 6% within 8 hr, indicating that TPA can also diminish already established cell coupling. Inhibition of cell coupling also was achieved with other mouse skin tumor promoters—phorbol 12,13-didecanoate, ingenol dibenzoate, and mezerein—whereas nonpromoting derivatives—phorbol and 4 α -phorbol 12,13-didecanoate—showed no effect. None of these compounds changed the membrane potential, membrane resistance, or growth rate of FL cells. Thus, it appears that TPA and structurally-related tumor promoters specifically disturb the formation or function, or both, of cell-cell junctions, without significantly affecting the general properties of the surface membrane or the growth of FL cells.

The process of cancer induction, both in experimental animals and in human beings, is complex; that which leads ultimately to a malignant tumor is the interaction of target cells with endogenous and exogenous factors. Therefore, essentially all cancers may be multifactorial or multistage, or both, in their causation. The best defined multistage carcinogenesis model is “two-stage carcinogenesis” in mouse skin, in which at least two distinct phases, so-called initiation and promotion, can be clearly demonstrated (1–4).

In Berenblum-type two-stage mouse skin carcinogenesis, each stage can be elicited by a different type of compound: initiators (e.g., polycyclic hydrocarbons) and promoters (e.g., croton oil or phorbol ester) (for review, see refs. 1–4), and different mechanisms seem to be involved. The initiators of mouse skin tumors are usually complete carcinogens, which are carcinogenic by themselves when applied repeatedly or at high doses; owing to this fact, the study of the action of initiators has made considerable progress in the past decade. Most of the initiating carcinogens have also been shown to be mutagenic in bacterial and mammalian cell systems (5).

The mechanism by which a tumor promoter transforms the initiated (presumably mutated), latent cells into a visible tumor is still not clearly understood, however. Because there are cer-

tain limitations to studying the action of tumor promoters on mouse skin, many investigators now employ a simpler system—cell culture—as a model (6, 7). These studies have shown that the phorbol ester tumor promoters, as represented by 12-O-tetradecanoylphorbol 13-acetate (TPA), exert the following effects when administered to cultured cells (6, 7). They enhance cell transformation (8, 9), modulate cell differentiation (for review, see refs. 7, 10, and 11), and inhibit mixed lymphocyte reaction (12, 13); also they have pleiotropic biochemical effects (6, 7). It has been proposed that these promoters must interact with cell surface membranes in order to act (6, 7, 14, 15), but it is not understood how and by what means this results in their pleiotropic effects.

Among the various effects that TPA has on membranes, its interference with cell-cell communication is of particular interest (16–18) because this is considered to be directly involved in the regulation of cell proliferation (19–21), which in turn is thought to be an important feature in the process of tumor promotion (1–4, 10). It has recently been shown that TPA and other tumor promoters inhibit metabolic cooperation between cells in culture (16–18). Metabolic cooperation is the transfer between cells of relatively large molecules (19); it is a relatively complex process involving several biochemical reactions. Although such cell-to-cell transfer seems to be the result of cell-cell communication via junctions, there is no direct evidence for this hypothesis. Moreover, the absence of metabolic cooperation is not in itself a demonstration of junctional communication incompetence (19). The best test for the presence of junctional communication is to pass pulses of electric current through intracellular microelectrodes from the interior of a cell to an external medium and to measure the corresponding electrical potential in this cell and in a neighbor (19–21). We demonstrate here by means of such a method that the phorbol ester tumor promoters cause a rapid and reversible inhibition of junctional cell communication in human FL cells. Our results strongly suggest that TPA not only blocks the formation of gap junctions but also inactivates the function of already formed gap junctions.

MATERIALS AND METHODS

Chemicals. TPA, phorbol 12,13-didecanoate (PDD), 4 α -PDD, phorbol, and mezerein were obtained from P. Borchert, and ingenol dibenzoate from the late M. Kupchan. All chemicals were dissolved in dimethyl sulfoxide; the final concentration of solvent in the culture medium was 0.1%, and this concentration of solvent alone had no substantial effect on cell coupling or cell growth.

Cell Cultures. FL cells, epithelial cell strain from human amniotic membrane (22), were purchased from Dainippon

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Abbreviations: TPA, 12-O-tetradecanoylphorbol 13-acetate; PDD, phorbol 12,13-didecanoate.

Pharmaceutical, Osaka, Japan. The cells in logarithmic growth phase were dissociated with 0.02% EDTA, and suspensions of single cells were obtained by gentle treatment with a tissue grinder. The cells were aggregated mechanically by centrifugation as described by Johnson *et al.* (23), with a slight modification to obtain more paired cells, and seeded onto a cover glass coated with calf serum at a concentration of 2×10^4 cells per 18×18 mm cover glass. Under these conditions, 10–20% of the cells were paired. Only cell pairs were used for electrical cell coupling measurements. All cultures were made in Eagle's minimum essential medium supplemented with 10% (vol/vol) calf serum.

Electrical Measurements. The electrical measurements of intercellular coupling were carried out under an inverted phase-contrast microscope. The cultures were maintained at 37°C on the microscopic stage. Microelectrodes were filled with 3 M KCl, and the electrodes with tip resistance of 30–50 M Ω were used. Two microelectrodes were inserted into each of cell pairs. Inward pulse current (8×10^{-9} A for 200 msec) generated by a constant-current generator was passed through one of the electrodes with a bridge circuit. The bridge circuit used in this study is as described (24), except that 1000 M Ω impedance was used instead of 500 M Ω in order to have more accurate measurements. The resulting changes of the membrane potential were amplified, observed on an oscilloscope, and recorded with a pen recorder. The following information about electrical coupling was obtained: the percentage of electrically coupled cells and the input resistance. Because the coupling ratio of FL cells is very low (≈ 0.05), the input resistance was almost the same as membrane resistance when these two values were actually measured and compared. The minimum detectable electrotonic potential (V₂) obtained from the adjacent coupled cells by current injection was 0.25 mV in our experiments. The amount of this potential (V₂) response to constant current reflects the intercellular junction conductance in this two-cell system, provided that membrane resistance is constant (25).

RESULTS

Effect of TPA on Cell Coupling. FL cells that were mechanically paired and cultured in control medium showed rapid cell coupling: within 2.5 hr, more than 90% of the cells were coupled electrically, and the average electrotonic potential of these coupled cells was 3.7 ± 1.6 mV. The calculated coupling ratio of the paired FL cells is about 0.05. Neither the electrotonic potential nor the percentage of coupled cells changed over a further 24-hr culture, suggesting that cell coupling between FL cells was stable over our experimental period.

When low concentrations of TPA (10–100 ng/ml; 1.7 – 17×10^{-8} M) were added to the FL cell culture, cell coupling was almost completely inhibited: the percentages of coupled cells were less than 10% and 20% 24 hr after the addition of 100 ng and 10 ng of TPA per ml, respectively. Fig. 1 shows the dose-response curve of the effect of TPA on cell coupling; very little inhibition was observed with a concentration of 1 ng/ml (1.7×10^{-9} M).

Reversibility of TPA Effect on Cell Coupling. The actions of the phorbol ester tumor promoters are usually easily reversible both *in vivo* and *in vitro* (1–4, 6, 7, 10, 11). We investigated whether the inhibition of electrical cell coupling by TPA was also reversible when TPA was removed from the culture medium.

FL cells were incubated with TPA for 24 hr, during which cell coupling remained suppressed. Then, TPA was removed by thorough washing of the cell layers. There was a rapid increase in cell coupling after TPA removal: the percentage of coupled cells and their electrotonic potential were the same as

FIG. 1. Effect of increasing concentrations of TPA on electrical coupling of FL cells after a 24-hr incubation.

in control cultures within 4 hr of TPA removal (Fig. 2). This time course of the appearance of cell coupling is very similar to that of normal cell coupling after mechanical aggregation. These results show that the effect of TPA on cell coupling is reversible even after 24-hr continuous suppression of cell coupling and suggest that the inhibitory effect of TPA on cell coupling is not a mere result of toxic effect.

Effect of TPA on Established Cell Coupling. When TPA was added to a culture in which cell coupling was already established, the coupling was also blocked: addition of TPA (100 ng/ml) to coupled FL cells decreased the percentage of coupled cells to 35% 4 hr later and to less than 10% 8 hr later (Fig. 2). Thus, TPA not only inhibits the initiation of cell coupling but also destroys previously established cell-cell communication competence. It is not clear whether TPA actually destroys the structure of the gap junction or merely suppresses the function of existing junctions.

Effects of Various Phorbol Derivatives. The effects of various phorbol esters and related compounds on electrical coupling of FL cells are summarized in Table 1. PDD, a potent promoter of mouse skin tumors, inhibited the coupling to a similar extent as did TPA, while the nonpromoting derivatives phorbol and 4 α -PDD had no effect. A structurally related tumor promoter, ingenol 13,20-dibenzoate also inhibited the coupling but to a lesser extent than TPA or PDD. Mezerein usually has

FIG. 2. Effect of removal and readdition of 100 ng of TPA per ml on coupling between FL cells. Arrows indicate times of addition or removal of TPA. TPA was removed by carefully washing the cell layers three times with culture medium containing 10% calf serum. ○, Control; ●, TPA-treated cells.

Table 1. Effects of phorbol derivatives on FL cell coupling

Compound	Time after culture, hr	Membrane potential, -mV*	Input resistance, MΩ*	Electrotonic potential of coupled cells,† mV*	Coupled cells,‡ %
Control	5	17.8 ± 3.3	9.6 ± 2.8	3.7 ± 1.6	89 (16/18)
(0.1% Me ₂ SO)	24	16.7 ± 4.2	9.4 ± 2.1	3.5 ± 1.3	94 (17/18)
TPA	5	16.8 ± 3.2	8.7 ± 2.3	2.0	6 (1/15)
(100 ng/ml)	24	15.5 ± 3.9	8.3 ± 2.2	2.1 ± 1.3	6 (2/32)
Phorbol	5	17.4 ± 4.4	8.8 ± 2.8	4.1 ± 2.2	100 (15/15)
(100 ng/ml)	24	16.7 ± 3.1	9.2 ± 2.2	3.8 ± 1.2	88 (15/17)
4α-PDD	5	16.0 ± 4.4	8.5 ± 2.7	3.9 ± 2.8	80 (12/15)
(100 ng/ml)	24	18.3 ± 4.9	9.2 ± 2.7	3.8 ± 1.2	95 (19/20)
PDD	5	15.6 ± 2.4	8.5 ± 2.2	1.2 ± 0.8	9 (3/32)
(100 ng/ml)	24	15.8 ± 5.1	8.3 ± 2.7	1.0	5 (1/20)
Ingenol 13,20-dibenzoate	5	16.6 ± 2.9	8.7 ± 4.2	1.7 ± 1.2	32 (10/31)
(100 ng/ml)	24	15.8 ± 3.5	8.5 ± 2.5	3.0 ± 1.1	33 (5/15)
Mezerein	5	16.7 ± 3.1	9.0 ± 2.1	3.5 ± 1.6	93 (29/31)
(10 ng/ml)	24	16.0 ± 3.2	8.9 ± 2.2	4.4 ± 2.8	93 (28/30)
Mezerein	5	16.9 ± 4.2	8.6 ± 2.1	1.0	4 (1/24)
(100 ng/ml)	24	15.1 ± 4.3	8.3 ± 4.4	3.2 ± 1.9	11 (3/28)

Me₂SO, dimethyl sulfoxide.

* Mean ± SD.

† The electrotonic potential (V₂) was obtained by injecting the current (8×10^{-9} A pulse) into the adjacent cell.

‡ Indicated amounts of chemicals were added at the onset of culture. Numbers in the parentheses are coupled cell pair numbers/total measured cell pair numbers.

quantitatively similar effects to those of TPA at equimolar doses in cell culture systems (13, 26–29), but mezerein has been shown to be a weak tumor promoter on mouse skin (30, 31). As shown in Table 1, mezerein had approximately 1/10th the effect of TPA on FL cell coupling: to produce more than 90% inhibition of coupling, only 10 ng of TPA but 100 ng of mezerein were required per ml.

None of the compounds changed the membrane potential or input resistance of FL cells (Table 1), suggesting that tumor promoters do not affect the general membrane properties of FL cells but specifically affect cell coupling. Because input resistance measured here is essentially the same as membrane resistance, these results indicate that tumor promoters do not significantly affect nonjunctional membrane conductance and

that the inhibition of electrical cell coupling is due to the decrease in junctional conductance. Even with a high concentration of TPA (100 ng/ml), inhibition of cell coupling was not 100%. When the electrotonic potential of coupled cells that escaped the tumor promoter effect was measured, the value was significantly lower than those of normally coupled cells (Table 1). Therefore, it seems that the quality or quantity, or both, of the junctions of those cells that managed to couple in the presence of tumor promoters are significantly different from those formed in the absence of a tumor promoter.

Effect of TPA on the Growth and Morphological Characteristics of FL Cells. The growth rate and saturation density of FL cells were not affected by the addition of TPA (Fig. 3). This is in contrast to the effect of TPA on other types of cells: it transiently inhibits the growth of Friend erythroleukemia cells (28) and human lymphoblastoid cells (32), whereas the saturation density of several cell types is increased (33–35).

Significant morphological changes were seen when FL cells were cultured with TPA (100 ng/ml) and compared with control cultures (Fig. 4). Although control cells showed epithelial-like shape and forms and round island colonies with compactly packed cells, when TPA was added they became more fibroblastic and the colony shape was irregular (Fig. 4). Such morphological changes were not evident until about 8 hr after TPA addition; thus, it seems that inhibition of cell-cell communication by TPA precedes its effects on cell shape.

DISCUSSION

This study has demonstrated that TPA and related tumor promoters reversibly inhibit the formation of electrical cell coupling and that established cell coupling is also disconnected by TPA. For the six phorbol esters and related compounds tested, there was generally a good correlation between their tumor-promoting activities on mouse skin and their ability to inhibit the formation of coupling between FL cells.

The inhibition of electrical cell coupling by tumor promoters was obtained without significantly changing membrane poten-

FIG. 3. Effect of TPA on growth of FL cells. Cells (4.0×10^4) were seeded onto 60-mm Petri dishes with or without 100 ng of TPA per ml. Culture medium was changed every other day with fresh TPA, and the numbers of cells were counted on the days indicated. ○, Control culture (0.1% dimethyl sulfoxide only); ●, with TPA.

FIG. 4. Appearance of FL cells cultured in the absence (A) or presence (B) of 100 ng of TPA per ml for 24 hr.

tial and membrane resistance (measured as input resistance). Therefore, it is likely that tumor promoters decrease junctional conductance without significantly changing nonjunctional membrane conductance and general membrane properties.

Previous studies already have shown that these tumor promoting agents also inhibit metabolic cooperation between mouse epidermal cells and 3T3 cells (17) or between wild-type and hypoxanthine (guanine) phosphoribosyltransferase-deficient Chinese hamster V79 cells (16, 18). Although it is likely that such a metabolic cooperation phenomenon occurs through junctional communication between cells, the evidence for this hypothesis is totally circumstantial (19). It should be stressed that metabolic cooperation is the result of a relatively complex process involving several biochemical reactions, in which junctional cell-cell transfer is only one step in a chain. Therefore, the absence of metabolic cooperation is not in itself a demonstration of junctional communication incompetence (19). On the other hand, the measurement of electrical cell coupling used in our study is a direct means to demonstrate junctional communication (19, 25); the absence of electrical coupling is more informative than its occurrence because its absence means that neither small nor large molecules pass. Thus, we can now conclude that TPA and related tumor promoters reversibly block the junctional communication of human cells in culture. We observed similar inhibitory effects of TPA on communication between mouse 3T3 cells in culture (unpublished observations); therefore, the effect of TPA on junctional cell communication extends to a variety of cell types from different species. This is consistent with the findings of Blumberg's group (15, 36) that specific receptors for TPA are present on various types of cells.

It is likely that the block of junctional cell communication by TPA is preceded by cellular recognition of TPA by such specific receptors.

When TPA was removed from the culture medium, cell coupling was fully reestablished within 4 hr. Because 4 hr are required to establish cell coupling among cells never exposed to TPA, it is likely that the TPA does not completely destroy the apparatus for gap-junction formation, but merely suppresses the joining of the apparatus of two cells or their function. This is further supported by our own finding that TPA also inhibits the electrical coupling of cells with already established junctional communication (Fig. 2). It would be interesting to examine by electron microscopy whether the structural gap junction is altered or whether only its function is disturbed in the presence of TPA.

An alternative mechanism to direct interaction of TPA with cell membranes leading to inhibition of cell-cell communication might be an indirect one, such as alteration of the microenvironment of cells to modify cell coupling. It is well known that electrical cell communication is influenced by the intracellular Ca^{2+} content in the junctional area (21). Intracellular Ca^{2+} movement and fluxes of certain cells have been reported to be altered by TPA (37). Other investigators have reported that several biochemical effects exerted by TPA are significantly altered by extracellular Ca^{2+} concentrations (38–40). Thus, it is suggested that some of the effects of TPA are mediated through intracellular or extracellular Ca^{2+} concentrations; therefore, TPA may inhibit cell coupling by changing the intracellular and extracellular Ca^{2+} concentration balance.

We have shown that there is a good correlation between the mouse skin tumor-promoting ability of phorbol esters and their inhibitory effect on electrical cell coupling. It has already been reported that cell-cell communication, as measured by metabolic cooperation, is inhibited by promoting derivatives and not by nonpromoting derivatives of phorbol esters (16–18). We have observed that initiating (or complete) carcinogens, such as *N*-methyl-*N*-nitro-*N*-nitrosoguanidine, benzo(a)pyrene, and 7,12-dimethylbenz(a)anthracene cannot inhibit electrical coupling of FL cells (unpublished observations). Inhibition of cell-cell communication, as revealed by the electrical coupling method, is thus relatively specific for tumor-promoting phorbol esters. In the experiments with metabolic cooperation, non-phorbol-type tumor promoters also appeared to inhibit cell communication (18, 41). We are now studying the effects of such compounds on electrical cell coupling.

In two-stage carcinogenesis in mouse skin, tumor promoters play a key role in awakening latent tumor cells to form a growing tumor. The mechanism by which tumor promoters can recognize and activate such initiated cells is not understood clearly. Evidence obtained *in vivo* favors the idea that deviations of initiated cells from balanced cell proliferation to uncontrolled cell growth may be essential, although this biological effect is not in itself sufficient for tumor promotion (1–4). More recently, Slaga *et al.* (42, 43) have proposed a two-stage model of tumor promotion in mouse skin and suggested that cell proliferation is an important event in the second stage of tumor promotion. Evidence obtained *in vitro* also suggests that tumor promoters alter cell proliferation either by directly stimulating cell growth or by modulating terminal cell differentiation (10, 11). It is strongly suggested that junctional membrane channels are essential in the cell-to-cell transmission of molecules for the control of growth (43). Taken together, these data tempt one to speculate that tumor promoter-mediated inhibition of cell-cell communication might be an essential mechanism by which the initiated cells lose controlled growth among surrounding normal cells. Further studies are necessary to clarify whether the effect

of tumor promoters on cell-cell communication is important for tumor promotion.

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