Rapid and Reversible Inhibition of Junctional Communication by Tumor Promoters in a Mouse Cell Line[1]

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ABSTRACT

HEL-37 cells rapidly transferred microinjected fluorescein between contacting cells. This transfer was strongly inhibited by tumor-promoting phorbol esters and mezerein but not by non-promoting derivatives. The onset of the inhibition occurred after 5 to 10 min of exposure to the test compound and was complete within 20 min. Using a wash-off procedure, it was demonstrated that inhibition of dye transfer by phorbol-12,13-dibutyrate was fully reversed by 100 min, after a lag of 40 to 60 min. The recovery of dye transfer competence was blocked by puromycin. Both 12-O-tetradecanoylphorbol-13-acetate and monensin caused elevated Na^+ levels in HEL-37 cells, but the latter compound did not inhibit dye transfer. It was concluded that the promoter inhibition of transfer was not a consequence of Na^+-induced increase in cytoplasmic Ca^{2+} concentration.

INTRODUCTION

Since the original observations (22, 35), a number of reports have confirmed that phorbol ester and other tumor promoters inhibit junctional communication between cultured mammalian cells (9–11, 17, 24, 29–31, 33). It was originally proposed (22, 35) that permeable junctions between normal and initiated cells allowed the interchange of regulatory molecules which limited clonal expansion of the initiated cells and consequent expression of the transformed phenotype. This hypothesis remains an attractive one, although it has proved difficult to obtain direct evidence for a regulatory role of junctional transfer in carcinogenesis.

Most recent data suggest that the phorbol ester promoters interact with specific, high-affinity membrane receptors (Ref. 8; see Ref. 18 for later references) leading to a range of cell surface-associated changes (3, 4, 6, 32). These early membrane alterations are followed by changes in the synthesis of specific proteins, altered proliferative rates in some cell types, and modified terminal differentiation patterns (3, 4, 6, 32). Clearly, an understanding of the mechanism of promoter inhibition of junctional communication requires information on the rapidity of its onset relative to other surface changes and on the rate at which junctional competence is recovered following promoter removal from its binding sites. These questions have been approached in the present study by measuring the effect of promoters on the transfer of microinjected fluorescein between contacting mouse epidermal cells in culture.

MATERIALS AND METHODS

Materials. The mouse epidermal cell line HEL-37 (used at Passages 151 to 170) was maintained in culture as described previously (22). All incubations were carried out at 37° in a humidified atmosphere of 5% CO_2 in air.

Eagle's minimal essential medium and fetal calf serum were obtained from the Commonwealth Serum Laboratories, Melbourne, Australia. TPA,[3] PDD, and 4α-PDD were obtained from P-L Biochemicals, Milwaukee, Wis., and PDBU, phorbol-12,13-diacetate, and mezerein were from Life Systems, Newton, Mass. Monensin was obtained from Calbiochem-Behring Corp., La Jolla, Calif. The Ca^{2+}-ionophore A23187 was a gift from Eli Lilly and Co., Indianapolis, Ind. Fluorescein, Lucifer Yellow CH, puromycin, and cycloheximide were all from the Sigma Chemical Co., St. Louis, Mo.

[methyl-³H]Choline (specific activity, 80 Ci/mmol) was obtained from New England Nuclear, Boston, Mass.

Methods. For microinjection experiments, cells were plated at a density of 0.3 to 0.45×10^6 cells/35-mm dish and used 24 hr later (final cell number, 0.6 to 1.0×10^6). The dishes were transferred to the heated stage (37°) of an inverted Zeiss IM35 fluorescence microscope equipped with an interference blue excitation filter (band width, 450 to 490 nm), a dichromatic beam splitter (510 nm), and a barrier filter (LT520). Cells were injected pneumatically using a Leitz micromanipulator and glass micropipets (tip diameter, 2 to 3 μm) containing 10 mM fluorescein in reverse Dulbecco's phosphate-buffered saline (13, 27). The medium used in these experiments was prefiltered through a 0.45-μm Millipore filter to prevent blockage by debris, and micropipets were loaded using negative pressure. After injection of 6 to 10 cells in separate clones, the area was flushed with medium. The number of contacting cells containing detectable fluorescein was counted 2 min after injection (recipient cells). All scoring was done under "blind" conditions. Occasionally, the extent of dye spread was recorded photographically, using Kodak Ektachrome 400 ASA film. All treatments were added to the culture dishes in DMSO solvent (final concentration, 0.1%); DMSO alone was added to control dishes. For each treatment, the data obtained from at least 2 separate dishes were pooled.

In experiments to determine intracellular Na^+ and K^+, incubations were carried out in Eagle's minimal essential medium (0.5 to 1×10^6 cells/dish). After incubation, cells were washed with 0 mM $MgCl_2$ at 4° (5 × 1.5 ml) and lysed in 1 ml of 15 mM LiCl-0.2% Triton X-114 (37°; 30 min). Samples were briefly centrifuged, and Na^+ and K^+ were measured using a Corning 430 flame photometer.

The release of radioactivity from HEL-37 cells (0.6×10^6/dish) prelabeled with [³H]choline (0.5 μCi/ml) was carried out as described previously (15).

RESULTS

When HEL-37 cells were injected with fluorescein, there was a rapid spread of fluorescence to contacting cells. Although

[3] The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; PDD, phorbol-12,13-didecanoate; PDBU, phorbol-12,13-dibutyrate; DMSO, dimethyl sulfoxide.

transfer could be detected within 10 sec of injection, the number of recipient cells containing dye was routinely scored after 2 min. As shown in Chart 1, preincubation of cultures with the tumor promoters TPA, PDBU, and PDD or with the potent second stage promoter mezerein caused a marked decrease in the number of contacting cells which were labeled with dye over a 2-min period. Preincubation with solvent (DMSO) or with the non- or weakly promoting phorbol esters phorbol-12,13-diacetate and 4α-PDD for up to 30 min did not inhibit dye transfer (Chart 1). The inhibitory effect of the promoting phorbol esters and mezerein was not immediate but showed an apparent lag of between 5 and 10 min. A dose-response curve for TPA is shown in Chart 2; 50% inhibition was obtained at a concentration of about 10 nM.

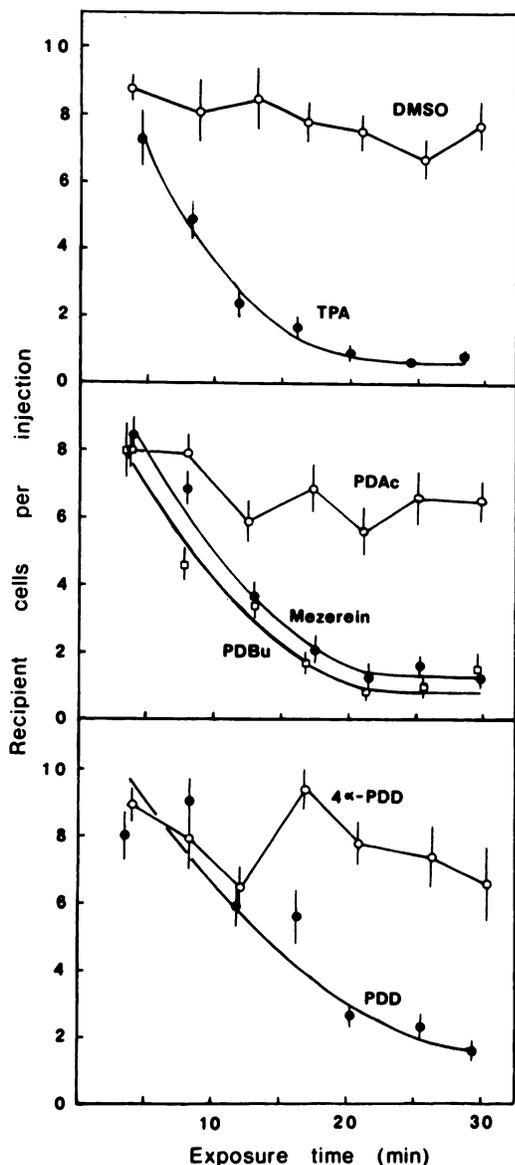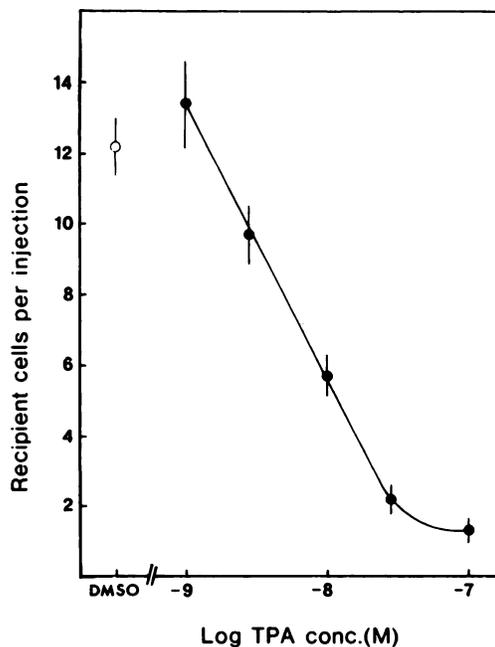Leakage of injected fluorescein has been reported from some

Chart 2. Effect of TPA concentration on the transfer of fluorescein between HEL-37 cells. Cultures were exposed to DMSO (O) or TPA (●) for 20 min before microinjection of fluorescein as described in "Materials and Methods." Each *point* is the mean of 10 to 15 separate injections. *conc.*, concentration; *bars*, S.E.

Chart 1. Effect of phorbol esters and mezerein on fluorescein transfer between HEL-37 cells. Cultures were exposed to DMSO or the test compound (10^{-7} M) for varying times before microinjection of fluorescein as described in "Materials and Methods." Each *point* is the mean of 11 to 27 separate injections. *PDAc*, phorbol-12,13-diacetate; *PDBU*, PDBU. *Bars*, S.E.

cultured cells (28). This cannot lead to an "apparent" transfer in the absence of an active cellular uptake of fluorescein, and injection of dye onto the exterior surface of cells did not result in cellular staining. Extensive transfer was also observed when cells were injected with Lucifer Yellow. This dye has been reported to leak from cells only very slowly following injection (28). Transfer of Lucifer Yellow was also sensitive to inhibition by TPA (data not shown).

Experiments were also carried out to determine how rapidly the ability to transfer fluorescein was regained following removal of the phorbol ester. This work was done with PDBU, because it has been established that this phorbol derivative can be readily removed by washing the cells. The washing procedure described previously (16) removed about 95% of bound PDBU, as determined in experiments with [^{3}H]PDBU (data not shown). Cells which had been preexposed to 100 nM PDBU for 20 min and then washed gradually regained dye-transfer competence after a lag of between 40 and 60 min (Chart 3). One explanation for this lag is that transfer was inhibited by the residual bound PDBU (about 5%) in washed cells, which was then slowly released during continued incubation. On the basis of previous studies (23), it can be calculated that HEL-37 cells reach 5% of maximal PDBU binding at a ligand concentration of about 2 nM. Preincubation of cultures with 2 nM PDBU for 20 min caused no decrease in fluorescein transfer between cells (results not shown). Consequently, the data in Chart 3 suggest that removal of PDBU from its binding sites leads to a delayed recovery of the ability to transfer fluorescein.

As shown in Table 1, the recovery of the ability to transfer fluorescein between cells was blocked by puromycin (10 μg/ml). At this concentration, puromycin inhibited the incorporation of [^{3}H]leucine into acid-insoluble material by 85% (data not shown). Puromycin caused a small decrease in fluorescein transfer after 120 min (Table 1) but completely inhibited the recovery of transfer
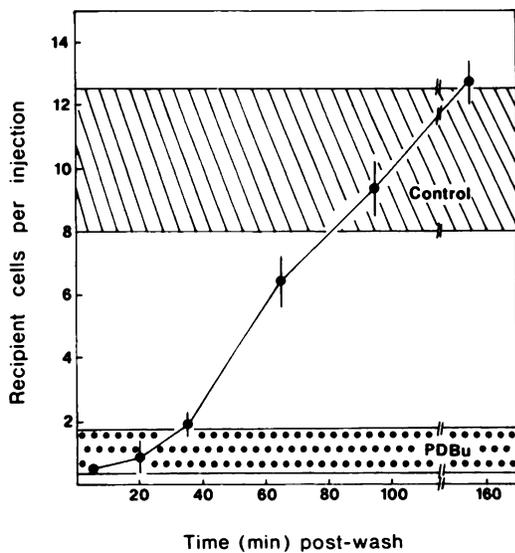
Chart 3. Reversal of PDBU inhibition of fluorescein transfer between HEL-37 cells. Cells were preexposed to PDBU (10^{-7} M) for 20 min, washed as described before (14), and incubated for varying times in medium containing DMSO (●) or PDBU (10^{-7} M; ▥). The DMSO control (▨) and PDBU control (▦) data indicate the range of values obtained in each instance. Other *data points* are the means of 10 to 15 separate injections. *Bars*, S.E.

Table 1

Effect of puromycin on the recovery of fluorescein transfer competence in cells preexposed to PDBU

Cells were preincubated for 20 min with DMSO or 10^{-7} M PDBU and then washed as described (16). Cultures were then incubated in complete medium containing DMSO or 10^{-7} M PDBU in the presence or absence of puromycin (10 μg/ml), and dye transfer was measured after 5 and 120 min. Results are expressed as the mean of 10 to 15 separate injections.

Prewash treatment	Postwash treatment	Recipient cells/injection	
		5 min	120 min
DMSO	DMSO	13.3 ± 1.3^a	10.5 ± 0.9
DMSO	Puromycin	11.8 ± 0.7	7.5 ± 0.9
PDBU	PDBU	0.7 ± 0.3	1.3 ± 0.6
PDBU	DMSO	0.9 ± 0.4	9.8 ± 0.9
PDBU	Puromycin	0.9 ± 0.3	0.7 ± 0.3

^{a} Mean ± S.E.

in cells preexposed to PDBU. Incubation of cultures with cycloheximide (5 μg/ml) for 120 min caused a complete inhibition of dye transfer (data not shown).

As reported before for cocultures of HEL-37 and PG-19 cells (10), prolonged exposure of HEL-37 cells to TPA rendered the cells insensitive to the inhibition of transfer. Thus, the number of recipient cells per injection when cells were exposed to 10^{-7} M TPA for 17 hr, washed, and incubated with fresh TPA for 20 min was 9.8 ± 1.1 (S.E.), compared with 13.2 ± 1.1 for DMSO controls. A value of 2.5 ± 0.6 recipient cells was obtained when cells were incubated with DMSO for 17 hr and then with 10^{-7} M TPA for a further 20 min before injection. It is not yet known if this desensitization is accompanied by a decrease in the number of phorbol ester binding sites on the HEL-37 cells.

A number of experiments were carried out in an attempt to correlate the time course for the TPA-induced decrease in junctional permeability with other early membrane changes induced by promoters. For example, TPA leads to a rapid activation of the Na$^+$-K$^+$-ATPase as measured by uptake of ^{86}Rb, and this is associated with an elevated internal concentration of Na$^+$ (7). This provides a potential mechanism for decreasing junctional

transfer, because Na$^+$ can increase mitochondrial release of Ca^{2+} (5), which, in turn, may act as a negative regulator of junctional permeability (26). In the present experiments (Table 2), TPA caused a significant increase in intracellular Na$^+$ at 10 min ($p < 0.01$) but not at 20 min ($p > 0.2$). Incubation with the monovalent ionophore monensin resulted in elevated Na$^+$ at both 10 and 20 min ($p < 0.1$ and 0.05, respectively). The results shown in Table 2 were confirmed in at least one replicate experiment. However, although TPA decreased the extent of dye transfer between cells, no such effect was obtained with monensin (Table 2). We conclude that the TPA-inhibition of transfer is unlikely to be mediated solely via an elevated intracellular Na$^+$ concentration. Similarly, we could obtain no evidence that fluorescein transfer between HEL-37 cells was sensitive to Ca^{2+}, because incubation of cells with the Ca^{2+}-ionophore A23187 (2 μM) for 20 min caused no decrease in the transfer of dye to recipient cells. Thus, values of 12.7 ± 1.4 and 10.2 ± 0.8 recipient cells were obtained in DMSO- and A23187-treated cultures, respectively ($p > 0.1$).

One of the most rapid cellular responses to phorbol ester promoters is enhanced phosphatidylcholine turnover, initiated by phospholipase C (14, 15, 21). As shown in Chart 4, release of radioactivity from HEL-37 cells prelabeled with [^{3}H]choline was stimulated by TPA within 2.5 min, compared with a lag of 5 to 10 min before promoter inhibition of dye transfer is marked. Although these results suggest that the enhanced turnover of phosphatidylcholine and decreased junctional transfer are not directly coupled, the dye-transfer assay may not be sufficiently sensitive to detect small changes.

DISCUSSION

The present results demonstrate that phorbol ester promoters and mezerein cause a rapid (5 to 10 min) decrease in the spread

Table 2

Effect of TPA and monensin on Na$^+$ and K$^+$ levels and fluorescein transfer in HEL-37 cells

All determinations were carried out as described in "Materials and Methods." Na$^+$ and K$^+$ values are expressed as the mean of 3 or 4 replicate determinations. Fluorescein transfer data are expressed as the mean of 10 to 15 separate injections.

Treatment	Preincubation (min)	Na$^+$ (μmol/10^6 cells)	K$^+$ (μmol/10^6 cells)	Recipient cells/injection
Zero time		4.7 ± 0.5^a	45.7 ± 3.8	
DMSO	10	4.2 ± 0.6	41.6 ± 1.7	
TPA (10^{-7} M)^b	10	9.5 ± 0.7	36.7 ± 0.7	
Zero time		5.3 ± 0.8	43.1 ± 1.3	
DMSO	10	12.1 ± 3.0	45.4 ± 2.3	
Monensin (5 μg/ml)	10	20.4 ± 1.3	32.5 ± 0.7	
Zero time		4.0	33.5 ± 1.0	
DMSO	20	5.0 ± 1.0	35.5 ± 1.3	
TPA (10^{-7} M)	20	7.0 ± 1.0	32.5 ± 1.0	
Monensin (5 μg/ml)	20	21.5 ± 5.6	26.5 ± 1.0	
DMSO	10			12.4 ± 0.8
Monensin (5 μg/ml)	10			11.9 ± 1.5
DMSO	20			11.7 ± 0.9
Monensin (5 μg/ml)	20			12.3 ± 1.1
TPA (10^{-7} M)	10			6.1 ± 1.3
TPA (10^{-7} M)	20			1.8 ± 0.6

^{a} Mean ± S.E.
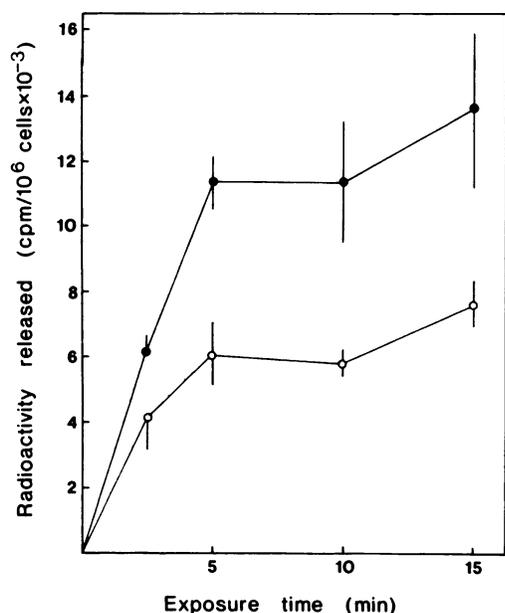^{b} Numbers in parentheses, concentration.

Chart 4. Effect of TPA on the release of radioactivity from HEL-37 cells preincubated with [^{3}H]choline. Radioactivity release from preloaded cells (15) was measured in the presence of DMSO (O) or 10^{-7} M TPA (●). Each *point* is the mean of 4 replicate dishes. *Bars*, S.E.

of microinjected fluorescein between cultured HEL-37 cells. With each active derivative tested, a maximum response was achieved after 20 min of exposure. The data are not sufficiently precise to decide whether a significant lag occurs or whether the decrease in dye-transfer ability occurs progressively over about 20 min. It is clear, however, that the effect is not an immediate consequence of receptor occupancy and that it presumably follows from earlier events triggered by promoters. The general characteristics of the promotor inhibition of dye transfer are common to a range of cultured cell lines, and results similar to those presented in this paper have been obtained with PG-19, Swiss albino 3T3, and L-6 myoblast cells (data not shown).

It is of considerable interest that recovery of the capacity for the cell-to-cell transfer of fluorescein following PDBU wash-off showed a pronounced lag (40 to 60 min). This is in direct contrast to the essentially immediate cessation of promoter-stimulated phospholipid turnover following PDBU removal (16). In addition, recovery was blocked by puromycin, suggesting that new protein synthesis was required. One interpretation of these data is that promoters cause the internalization and degradation of gap junctions, with recovery dependent on protein synthesis. This interpretation is only cautiously presented, however, both because of the rapidity of the responses and because it is likely that protein synthesis inhibitors can have nonspecific effects on junctional transfer. For example, cycloheximide alone caused a complete inhibition of dye transfer in the present experiments and has been reported to variably modify junctional permeability between mouse C1-1D cells (2).

Both the internalization of gap junctions (20) and the dispersal of junctional particles (19) have been proposed as mechanisms to explain the hormone-stimulated disappearance of gap junctions. Although we have no evidence that promoters decrease the number of junctions in HEL-37 cells, Yancey *et al.* (34) have reported that TPA causes a decreased incidence of gap junctions between Chinese hamster V-79 cells. These authors exposed

cells to TPA for 18 hr, so it is not possible to decide whether the disappearance of junctions correlates with the initial decline in permeability, which is likely to occur much earlier.

As well as inhibiting junctional transfer, TPA has also been shown to cause an increase in the permeability of epithelial cell tight junctions (25), suggesting that the promoter may have a more generalized effect on cell-to-cell interactions. However, the time of exposure to TPA required to increase tight junction premeability (1 to 2 hr) was considerably longer than that required to inhibit junctional transfer in the present experiments (5 to 10 min), and the 2 phenomena may have quite different mechanisms. It would be useful to study the effects of TPA on both gap junction and tight junction permeability in the same cell line.

Ca^{2+} has been widely proposed as a physiological regulator of junctional permeability (26), and it is therefore important to determine whether changes in Ca^{2+} fluxes or in the intracellular distribution of Ca^{2+} are responsible for the promoter inhibition of cell-to-cell transfer. We do not believe this to be a likely mechanism, because transfer between HEL-37 cells was not blocked by the Ca^{2+}-ionophore A23187. In addition, a possible Na^+-enhancement of mitochondrial Ca^{2+} release (5) is probably not involved in modifying junctional permeability, because there was no correlation between internal Na^+ levels and dye transfer. Thus, although both TPA and monensin increased the internal Na^+ concentration of HEL-37 cells, the latter compound did not inhibit the transfer of fluorescein between cells.

Although it is possible that the microinjection procedure itself modifies the phorbol ester effect on junctional communication, the results obtained are generally consistent with those obtained using noninvasive methods (10, 11, 17, 24, 29–31, 33). Two recent studies have also used microinjection to demonstrate decreased transfer following infection of fibroblasts with avian sarcoma virus (1) or treatment of primary epithelial cells with TPA (12). The microinjection procedure has a number of advantages over previously used techniques in studies to determine the mechanism of the promoter effect. Rapid changes in junctional permeability can be detected, making detailed correlations with other membrane events possible. In addition, the microinjection technique is less likely to produce ambiguous results with test compounds, which may interfere with the isotope incorporation methods used previously (10, 11, 22).

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