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Communication

Inhibition of Intercellular Communication by Tumor-promoting Phorbol Esters[1]

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ABSTRACT

Cocultures were established of mouse epidermal cells (HEL/37) and mouse fibroblast cells (PG-19) deficient in the enzyme hypoxanthine-guanine phosphoribosyltransferase. Metabolic cooperation between the cocultured cells was detected as labeling of PG-19 cells on incubation of cocultures with [^{3}H]-hypoxanthine. The transfer of label from HEL/37 cells to PG-19 cells was inhibited by the tumor promoters 12-*O*-tetradecanoylphorbol-13-acetate (10^{-8} M) and phorbol-12,13-didecanoate (10^{-7} M) but not by nonpromoting derivatives of these phorbol esters. The inhibition was partially prevented by the antiinflammatory steroid fluocinolone acetonide, which is an antagonist of mouse skin tumor promotion, and by prolonged exposure of the cocultures to 12-*O*-tetradecanoylphorbol-13-acetate.

INTRODUCTION

In a recent communication (10), we demonstrated that low concentrations of tumor-promoting phorbol esters inhibited the transfer of nucleotides between mouse epidermal cells and 3T3 cells maintained in coculture. A similar inhibition of cell-cell communication by tumor promoters has been reported by Yotti *et al.* (14). In the former experiments, epidermal cells were prelabeled with [^{3}H]uridine and then cocultured with initially unlabeled 3T3 cells. The transfer of label from the epidermal cells to contacting 3T3 cells was detected by autoradiography. This method is believed to detect the transfer of labeled nucleotides between cells via gap junction structures (11). Because test compounds were added to culture medium at the time coculture was started, this technique could only measure the effect of promoters on the establishment of communication links between the 2 different cell types.

In the present paper, we extend the initial results and show that tumor promoters also inhibit the transfer of labeled molecules between cocultured cells with established communication links.

MATERIALS AND METHODS

Materials. The mouse epidermal cell line (HEL/37, used at passages 165 to 178) was maintained in culture as described before (10). The mouse fibroblastic line (PG-19), deficient in the enzyme hypoxanthine-guanine phosphoribosyltransferase (6), was obtained from Dr. R. Hope, Department of Genetics, University of Adelaide. PG-19 cells were maintained in Eagle's minimum essential medium supplemented with 10% fetal calf serum (Commonwealth Serum Laboratories, Melbourne, Australia) and antibiotics (penicillin, 100 IU/ml; streptomycin, 100 μg/ml). All incubations were conducted at 37° in a humidified atmosphere of 5% CO_2.

PDD,[3] 4α-PDD, and 4-*O*-methyl-TPA were obtained from P-L Biochemicals, Milwaukee, Wis., and TPA was obtained from Cambrian Chemicals, Croydon, England. [*G*-^{3}H]Hypoxanthine (specific activity, 0.435 or 1.2 Ci/mmol) and [*methyl*-^{3}H]thymidine (specific activity, 24 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, England. Fluocinolone acetonide was a gift from Syntex Labs. Inc., Palo Alto, Calif.

Coculture Experiments. The method made use of the mutant PG-19 cell line which lacks the enzyme hypoxanthine-guanine phosphoribosyltransferase and is unable to incorporate [^{3}H]hypoxanthine into nucleotides (6). HEL/37 cells and PG-19 cells were grown in coculture for sufficient time for the establishment of gap junctional links. The cocultures were then exposed to the test compounds for 1 hr before incubation with [^{3}H]hypoxanthine and analysis of the labeling pattern by autoradiography. PG-19 cells were labeled only if in contact with HEL/37 cells, and the effect of promoters on the extent of label transfer could be measured.

HEL/37 cells were grown as described (10) on glass coverslips in 35-mm plastic dishes. The cells were plated at a density of 1×10^5 cells/dish, and after 1 day of incubation at 37° the medium was removed and replaced with 1 ml of medium containing 1.5 μCi of [*methyl*-^{3}H]thymidine. Incubation was continued for 1 day further, and the cells were washed 5 times with 1 ml of medium containing 12.5 μM thymidine. PG-19 cells were harvested from confluent flasks by incubation with trypsin-EDTA (10), and 10^5 or 5×10^4 cells were added to each dish containing approximately 5×10^5 HEL/37 cells. The dishes were incubated at 37° for 4 hr to enable the establishment of cocultures and then were incubated for an appropriate time (usually 1 hr) with test compounds or with an equivalent volume of dimethyl sulfoxide. The medium was aspirated and replaced with 1 or 2 ml of medium containing [*G*-^{3}H]hypoxanthine (5 μCi/ml) and the test compound, and the dishes were incubated for 3 hr at 37°. The cells were washed with 1 ml of medium containing 0.1 mM hypoxanthine, and the coverslips were transferred to fresh dishes and washed a further 4 times. The coverslips were processed for autoradiography, as described before (10).

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[3] The abbreviations used are: PDD, phorbol-12,13-didecanoate; 4α-PDD, 4α-phorbol-12,13-didecanoate; 4-*O*-methyl-TPA, 4-*O*-methyl-12-*O*-tetradecanoyl-phorbol-13-acetate; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate.

PG-19 cells which were in visual contact with HEL/37 cells were scored under coded, blind conditions as labeled or unlabeled by comparison with randomly chosen areas free of cells. Labeled cells were defined as those containing at least double the background number of grains. Only PG-19 cells which were in contact with HEL/37 cells were labeled.

Each treatment group was comprised of at least 3 replicate slides, and 300 cells were scored on each slide. In all experiments reported, χ^2 analysis indicated homogeneity between the replicate slides of each treatment group. Consequently, comparisons between treatments were made with a 2 × 2 χ^2 analysis of the pooled data in each group. For convenience, the data are presented as the proportion of contacting PG-19 cells which were labeled.

Incorporation of [^{3}H]Hypoxanthine into Acid-soluble Nucleotides. Cocultures of HEL/37 and PG-19 cells, which had not been labeled with [^{3}H]thymidine, were incubated in medium containing dimethyl sulfoxide, 10^{-7} M TPA, or 10^{-7} M PDD. After 1 hr at 37°, the medium was aspirated and replaced with fresh medium containing test compounds and [^{3}H]hypoxanthine, as described above. After 3 hr incubation, the coverslips were washed as described above and finally washed 3 times with 0.9% NaCl solution. The acid-soluble and acid-insoluble radioactivity was measured as described before (10). A portion of the acid-insoluble residue that was solubilized in 0.1 M NaOH was used for the estimation of protein (9).

RESULTS AND DISCUSSION

In all experiments, HEL/37 cells and PG-19 cells were cocultured for 4 hr before treatment with test compounds. Separate experiments, using [^{3}H]uridine as described previously (10) or [^{3}H]hypoxanthine, demonstrated metabolic cooperation between the cells after this time. Thus, when HEL/37 cells were prelabeled for 3 hr with [^{3}H]hypoxanthine and then cocultured with PG-19 cells for 4 hr, 86% of contacting PG-19 cells were labeled. Hence, it can be concluded that any effect of promoters on the transfer of label was on cells with preexisting junctional channels.

Control experiments also established that no PG-19 cells were labeled when incubated alone with [^{3}H]hypoxanthine and that no PG-19 cells were labeled when incubated in coculture with HEL/37 cells prelabeled only with [^{3}H]thymidine. The thymidine-labeling procedure resulted in densely grained nuclei in more than 97% of the HEL/37 cells after autoradiography, which greatly assisted the unambiguous identification of the 2 cell types. Separate experiments established that preincubation with [^{3}H]thymidine did not influence the proportion of PG-19 cells scored as labeled, either in control cocultures or in the presence of test compounds (data not shown).

As shown in Table 1, in control cocultures about 90% of PG-19 cells in contact with HEL/37 cells were labeled. The tumor promoters TPA and PDD significantly decreased the proportion of labeled PG-19 cells. No inhibition was obtained with the nonpromoting or very weakly promoting derivatives 4-*O*-methyl-TPA and 4α-PDD. TPA was effective at a concentration of 10^{-8} M; no inhibition was observed at 10^{-9} M (Table 4) or at 10^{-10} M TPA (Table 1).

In these experiments, the promoters were present during the period of labeling with [^{3}H]hypoxanthine. Consequently, it was necessary to show that the decrease in labeled PG-19 cells was not due to an inhibition of [^{3}H]hypoxanthine incorporation into nucleotides in the HEL/37 cells. As summarized in Table 2, the highest concentration of TPA used (10^{-7} M) caused a small but significant decrease in the incorporation of [^{3}H]hypoxanthine into acid-soluble nucleotides. This decrease cannot explain the results shown in Table 1, as variation of the specific activity of the [^{3}H]hypoxanthine over a 4-fold range did not alter the proportion of contacting PG-19 cells which were scored as labeled, despite major changes in the labeling intensity of the HEL/37 cells (data not shown).

The inhibition of metabolic cooperation required the continuous presence of TPA. As shown in Table 3, few contacting PG-19 cells were labeled when cocultures were incubated for 1 hr with 10^{-8} M TPA and then with [^{3}H]hypoxanthine in fresh medium containing 10^{-8} M TPA for 3 hr (proportion of labeled cells, 0.30). However, the proportion of labeled PG-19 cells was significantly greater (χ^2 test) when the 1-hr exposure to TPA was followed by labeling in the absence of TPA (proportion of labeled cells, 0.81). The proportion of labeled cells was increased further to 0.90 when a further incubation period of 4 hr in the absence of TPA was included between the initial exposure to TPA and the 3-hr [^{3}H]hypoxanthine-labeling period (data not shown). This result shows that the TPA inhibition of transfer is reversible.

Prolonged incubation in the presence of TPA before labeling with [^{3}H]hypoxanthine rendered the cells insensitive to promoter-inhibition of metabolic cooperation (Table 3). This result was not due to breakdown of TPA by the cultured cells, since

Table 1

Effect of tumor promoters on metabolic cooperation in cocultures of HEL/37 and PG-19 cells

The transfer of label from HEL/37 cells to PG-19 cells was determined by autoradiography as described in "Materials and Methods." PG-19 cells in contact with HEL/37 cells were scored under coded, blind conditions as labeled or unlabeled.

Treatment	Proportion of labeled cells	Range of proportion of labeled cells on individual slides	χ^2[a] (vs. control)	p
Dimethyl sulfoxide control (9)[b]	0.89	0.87–0.92		
10^{-7} M TPA (3)	0.37	0.34–0.39	1025	<0.001
10^{-8} M TPA (6)	0.34	0.28–0.39	1493	<0.001
10^{-10} M TPA (3)	0.90	0.88–0.92	0.25	>0.50
10^{-7} M 4-*O*-methyl-TPA (3)	0.88	0.86–0.91	0.59	>0.30
10^{-7} M PDD (6)	0.41	0.35–0.45	1197	<0.001
10^{-7} M 4α-PDD (6)	0.90	0.86–0.93	1.19	>0.20

[a] Analysis was carried out on the pooled data for each treatment group.
[b] Numbers in parentheses, number of replicate slides examined for each treatment; 300 cells were scored on each slide.

Table 2

Effect of TPA and PDD on the labeling of the acid-soluble and acid-insoluble pools of HEL/37 and PG-19 cell cocultures by [^{3}H]hypoxanthine

Cocultures of HEL/37 cells and PG-19 cells were pretreated with dimethyl sulfoxide, 10^{-7} M TPA, or 10^{-7} M PDD for 1 hr and then incubated for a further 3 hr with medium containing the test substance and [G-^{3}H]hypoxanthine, as described in "Materials and Methods."

Treatment	Total dpm/μg protein (×10^{-2})	
	Acid-soluble pool	Acid-insoluble pool
Dimethyl sulfoxide	117 ± 4[a]	80 ± 3
10^{-7} M TPA	96 ± 4 ($p < 0.01$)	63 ± 3 ($p < 0.01$)
10^{-7} M PDD	111 ± 12 ($p > 0.6$)	75 ± 7 ($p > 0.5$)

[a] Mean ± S.E. of determinations carried out on 4 separate dishes.

fresh TPA was added during the 3-hr-labeling period. Although the mechanism for this phenomenon is not known, it is reminiscent of the "down regulation" frequently observed after the prolonged exposure of cultured cells to hormones, growth factors, and other ligands which interact with specific surface receptors (2, 4, 12). Exposure of HeLa cells to TPA has previously been shown to "desensitize" the cells to TPA inhibition of epidermal growth factor binding (7). It will be of considerable interest to determine whether this "desensitization" and the development of TPA-insensitive metabolic cooperation reported here have a common mechanism.

As shown in Table 4, the inhibitory effect of TPA on metabolic cooperation could be partly overcome by fluocinolone acetonide. This result was confirmed in a separate experiment. Fluocinolone acetonide is an antiinflammatory steroid and is a potent inhibitor of mouse skin tumor promotion (13). However, equivocal results were obtained with fluocinonide, another steroid-inhibitor of tumor promotion (13). In the 2 experiments carried out thus far (data not shown), fluocinonide partially overcame the TPA inhibition in one experiment and was inactive in the other (steroid concentration, 1 μg/ml). The reasons for these variable results are yet to be resolved.

Table 3

Effect of prolonged exposure to TPA on TPA inhibition of metabolic cooperation between HEL/37 and PG-19 cells

Cocultures of HEL/37 cells and PG-19 cells were exposed to 10^{-6} M TPA for 1 hr, washed 3 times, and incubated in fresh medium containing 10^{-6} M TPA for varying times (0, 4, 8, and 20 hr). The cells were washed and incubated for 3 hr in fresh medium containing [^{3}H]hypoxanthine and either TPA or dimethyl sulfoxide as described in "Materials and Methods." Three replicate slides were examined for each treatment; at least 300 cells were scored on each slide.

Total time of exposure to TPA before labeling period (hr)	TPA present during labeling period	Proportion of labeled cells	Range of proportion of labeled cells on individual slides
1	–	0.81	0.78–0.84
1	+	0.30	0.29–0.31
5	+	0.44	0.41–0.47
9	+	0.74	0.72–0.75
21	+	0.88	0.87–0.90

Table 4

Effect of fluocinolone acetonide on the TPA-inhibition of metabolic cooperation between HEL/37 and PG-19 cells

Coculture conditions were as described in the legend to Table 1. Where appropriate, fluocinolone acetonide (1 μg/ml) was added to the culture medium at the same time as Me_2SO[a] or 10^{-6} M TPA. Three replicate slides were examined for each treatment; 300 cells were scored on each slide.

Treatment	Proportion of labeled cells	Range of proportion of labeled cells in individual slides	χ^2	p
Me_2SO	0.95	0.95–0.96		
Me_2SO + fluocinolone acetonide	0.96	0.96–0.97	0.67 (*vs.* Me_2SO)	>0.3
10^{-6} M TPA	0.45	0.43–0.46	585 (*vs.* Me_2SO)	<0.001
10^{-6} M TPA	0.94	0.93–0.95	0.89 (*vs.* Me_2SO)	>0.3
10^{-6} M TPA + fluocinolone acetonide	0.71	0.68–0.73	130 (*vs.* 10^{-6} M TPA)	<0.001

[a] Me_2SO, dimethyl sulfoxide.

The present data confirm that tumor-promoting phorbol esters markedly inhibit metabolic cooperation between cocultured mammalian cells (10). A similar conclusion has recently been reached by Yotti *et al.* (14). These authors demonstrated that promoters were able to rescue 6-thioguanine-resistant cells cultured together with 6-thioguanine-sensitive cells. Further, the present data shows that promoters reversibly inhibit transfer between cells with already established communication channels. The proposal that promotion involves the disruption of intercellular communication (10, 14) is strengthened by the observation that wounding some cell types results in electrical uncoupling of the normal and injured cells (1). As with metabolic cooperation, electrical coupling between cells probably occurs via gap junctions (8). This result is of interest, since wounding is known to be a promoting stimulus (3, 5).

Although it is probably reasonable to conclude that the effect of promoters on intercellular communication is yet another manifestation of the interaction of promoters with the surface of cells (see Ref. 7 for references), the precise mechanism and the physiological significance of the phenomenon remain to be determined.

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