Proc. Natl. Acad. Sci. USA
Vol. 77, No. 9, pp. 5312–5316, September 1980
Cell Biology

Local anesthetics inhibit induction of ornithine decarboxylase by the tumor promoter 12-*O*-tetradecanoylphorbol 13-acetate

(polyamines/lidocaine/tetracaine/procaine)

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Communicated by Howard A. Bern, June 12, 1980

ABSTRACT **The induction of ornithine decarboxylase (L-ornithine carboxy-lyase, EC 4.1.1.17) activity in mouse epidermal cells *in vivo* and *in vitro* occurs rapidly after exposure to the tumor promoter 12-*O*-tetradecanoylphorbol 13-acetate (TPA). This induction has characteristics of a cell surface receptor-mediated process. Local anesthetics modify a variety of cellular responses mediated by membrane receptors. When cultured mouse epidermal cells were exposed to the local anesthetics lidocaine, tetracaine, or procaine (0.1–1 mM), induction of the decarboxylase by TPA was inhibited by more than 90%. *In vivo*, lidocaine essentially abolishes the decarboxylase response of mouse epidermis when applied shortly after TPA. In contrast, local anesthetics have no effect on the enzyme's activity when added directly to the assay mixture and, in concert with TPA, have only a minimal effect on overall protein synthesis relative to controls. However, lidocaine has no effect on TPA-stimulated DNA synthesis *in vitro* (12-fold with or without lidocaine). Local anesthetics also markedly inhibit induction of the decarboxylase by ultraviolet light, which is probably not membrane mediated. Furthermore, in culture, lidocaine has only a small inhibitory effect on ornithine decarboxylase when given before TPA but is an effective inhibitor even when given up to 4–5 hr after the promoter, a time when decarboxylase activity has already increased. These findings suggest that local anesthetics, which are tertiary amines, do not act at the site of interaction of TPA and its putative receptor but may be acting specifically on polyamine biosynthesis. These drugs could be useful agents to determine the role of the polyamine pathway in tumor promotion.**

The induction of ornithine decarboxylase (Orn'ase; L-ornithine carboxy-lyase, EC 4.1.1.17) is a reproducible and sensitive cellular change which occurs in epidermis after exposure to phorbol ester tumor promoters (1, 2). A requisite role for Orn'ase induction in tumor promotion has not been defined. However, several observations suggest it may be a relevant response. These include the close correlation of promoter potency and ability to induce Orn'ase in mouse epidermis for phorbol esters (1–3) and the concomitant inhibition of phorbol ester-stimulated tumor promotion and Orn'ase induction by retinoids (4–6). Furthermore, Orn'ase induction is the only consistent marker *in vivo* and *in vitro* of a response to skin tumor promoters other than those of the phorbol ester series (3, 7). In contrast to these positive associations of Orn'ase and tumor promotion are reports of a dissociation of these events. Anti-inflammatory steroids are potent inhibitors of phorbol ester promotion of mouse skin tumors (8) but do not inhibit, and in fact enhance, the induction of Orn'ase (9). Mezerein, a diterpene ester structurally similar to phorbol esters, is a potent inducer of Orn'ase but an extremely weak tumor promoter for mouse skin (10). Taken together, these results suggest that Orn'ase induction may be necessary for tumor promotion but it is not sufficient to complete the process.

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The induction of Orn'ase of course is not unique to tumor promoters but is a response to several trophic stimuli (11). Many of these are mediated through plasma membrane or cytoplasmic receptors. A considerable body of evidence indicates that the cell surface is also a primary site of action of phorbol esters (12–15). Recent studies suggest that a specific membrane-associated receptor may exist for phorbol esters in avian and mammalian cells (16). To explore the role of a specific membrane-mediated pathway for Orn'ase induction by phorbol esters, we sought an inhibitor that might interrupt this process. Tertiary amine local anesthetics are a group of agents that can modify a variety of disparate cellular processes (17). In general, two common features emerge with regard to the mechanism of local anesthetic action. Most of the processes involve membranes, and calcium plays an important role in each process (18, 19). Because both cellular membranes and calcium (20) are involved in responses to phorbol esters, local anesthetics seemed to be ideal agents to study the role of these mediators in the induction of Orn'ase.

MATERIAL AND METHODS

Source of Chemicals. The tumor promoter 12-*O*-tetradecanoylphorbol 13-acetate (TPA) was obtained from Chemical Carcinogenesis (Eden Prairie, MN). Local anesthetics were initially a gift of Beverly Peterkofsky (National Cancer Institute) and subsequently purchased from ICN Pharmaceuticals (Plainview, NY). Labeled compounds were purchased from New England Nuclear as follows: DL-[1-^{14}C]ornithine monohydrochloride (42–54 Ci/mol; 1 Ci = 3.7×10^{10} becquerels), *S*-[1-^{14}C]adenosylmethionine (54 Ci/mol), L-[3,4,5-^{3}H]leucine (120 Ci/mmol), and [*methyl*-^{3}H]thymidine (5 Ci/mmol).

Treatment of Animals. Female BALB/c mice were supplied by the National Institutes of Health. Sencar mice were supplied by Thomas Slaga (Oak Ridge National Laboratory). Mice were used only in the resting phase of the hair cycle. Lidocaine and TPA were dissolved in acetone and applied as indicated in *Results*. At appropriate intervals after treatment, two mice per group were killed by cervical dislocation and epidermis was isolated by a heat–cold technique as described (21) and frozen at −70°C prior to enzyme assay.

Cell Culture. Epidermal cells from newborn BALB/c mice were isolated and cultured under conditions described (22). All experiments were performed on primary cultures that were plated at 3×10^6 cells per Falcon plastic 60-mm culture dish. TPA was dissolved in dimethyl sulfoxide and local anesthetics were dissolved in ethanol to give a final concentration of solvent in the medium of 0.1%. TPA and local anesthetics were gen-

Abbreviations: TPA, 12-*O*-tetradecanoylphorbol 13-acetate; Orn'ase, ornithine decarboxylase; AdoMet'ase, *S*-adenosyl-L-methionine decarboxylase; MI, mitotic index.

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erally applied simultaneously 24 hr after plating. In some cases, cells were pretreated with local anesthetics and in some experiments local anesthetics were applied after TPA. UV light (254 nm) is also an inducer of Orn'ase in mouse epidermis *in vitro* (23). Washed cell monolayers were irradiated by exposure to a germicidal lamp (General Electric G30T8, 30 W) at a distance of 48 cm and with a flux of 2–3 J m^{-2} sec^{-1} (Blak-Ray UV light meter, model J 225, Ultraviolet Products, San Gabriel, CA).

Biochemical Assays. Assays for Orn'ase activity and *S*-adenosyl-L-methionine decarboxylase (AdoMet'ase; *S*-adenosyl-L-methionine carboxy-lyase, EC 4.1.1.50) activity were performed on lysates of frozen cultures cells or in the 12,000 rpm supernate of extracts of three-times frozen and thawed, minced, and homogenized epidermis as described (2, 7). DNA synthesis was determined after a 1-hr pulse with [^{3}H]dThd as reported (9). Protein synthesis was assayed by measuring the incorporation of [^{3}H]leucine after a 1-hr pulse. Protein was determined by the method of Lowry as detailed by Layne (24). All assays were performed in duplicate on replicate samples from each experiment, and all experiments were performed at least twice.

RESULTS

Previous studies (2, 7, 9) had indicated that in cultured epidermal cells TPA induced a 10- to 20-fold increase in epidermal Orn'ase activity which peaked at 6–9 hr after exposure. When epidermal cells were exposed to 1 mM lidocaine in conjunction with TPA, Orn'ase induction was markedly inhibited (Fig. 1). For effective inhibition of Orn'ase activity, exposure to lidocaine was required after exposure to TPA. A 2-hr exposure to lidocaine, after which the medium was changed and the cells were exposed to TPA, resulted in a partial inhibition (data not shown). Lidocaine did not seem to be simply suppressing a response that TPA had initiated because removal of the anesthetic and the inducer (TPA) after 12 hr was not followed by an elevation of Orn'ase activity. Other local anesthetics (tetracaine and procaine) were just as effective at inhibiting TPA induction of Orn'ase as lidocaine (Fig. 2). This figure also demonstrates that local anesthetic effects are dose dependent.

Previous results from our laboratory have indicated that the regulation and characteristics of Orn'ase induction by TPA in epidermal cells *in vitro* are analogous to those events *in vivo* on mouse skin. When lidocaine was applied to mouse skin as a single exposure or as multiple exposures beginning shortly after exposure to TPA, the Orn'ase response was markedly reduced or abolished (Fig. 3*A*). A similar treatment also diminished the peak activity of TPA induction of AdoMet'ase by about 50% (Fig. 3*B*). The peak activity of this enzyme occurs at a later time after TPA exposure than Orn'ase and the residual activity could reflect a decrease in the effective lidocaine dose with time. However, the rise of AdoMet'ase activity 6 hr after exposure to TPA occurs at a time when lidocaine completely inhibits Orn'ase. Thus, the inhibitory effect may simply represent a decrease in available putrescine which may be crucial for the induction of AdoMet'ase.

Although local anesthetics are potent inhibitors of Orn'ase induction by TPA, it was possible that the inhibition resulted from a generalized cytotoxicity. At the doses used, local anesthetics did not produce morphological changes in epidermal cell culture during a 12-hr exposure. However, at higher concentrations or for longer exposures, cytoplasmic vacuoles could be seen in some cells, which disappeared upon removal of the anesthetics. Lidocaine, at a dose that effectively inhibited Orn'ase induction, had only a small effect on overall protein synthesis by itself and no effect on protein synthesis in TPA-treated cells when compared to TPA alone (which causes an average 20% decrease in incorporation of 1-hr-pulsed [^{3}H]-leucine at multiple points during the 8 hr studied). In a range of doses from 10^{-3} to 10^{-7} M, lidocaine did not affect Orn'ase

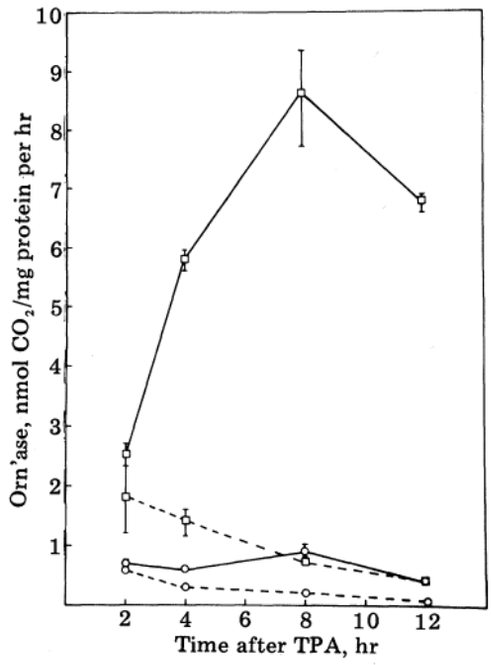

FIG. 1. Inhibition of TPA-induced Orn'ase activity by lidocaine. Epidermal cells were exposed simultaneously to TPA (16 nM) and lidocaine (1 mM) or to each agent individually. Cultures were washed and frozen at intervals and assayed for Orn'ase activity. □—□, TPA only; ○—○, TPA plus lidocaine; □---□, solvent control; ○---○, lidocaine control. The range of replicate Orn'ase determinations is indicated.

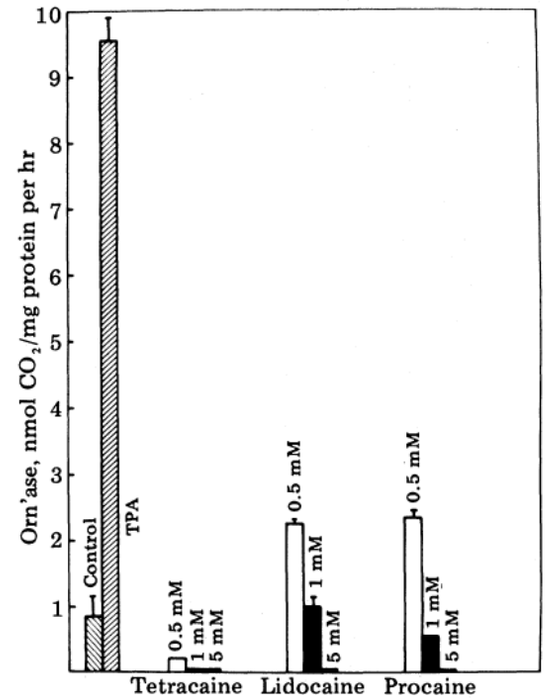

FIG. 2. Dose-dependent inhibition of TPA-induced Orn'ase by local anesthetics. Epidermal cells were treated with various doses of local anesthetics in concert with TPA (16 nM). Cultures were washed and frozen for Orn'ase assay after 8 hr. The range of replicate Orn'ase determinations is indicated.

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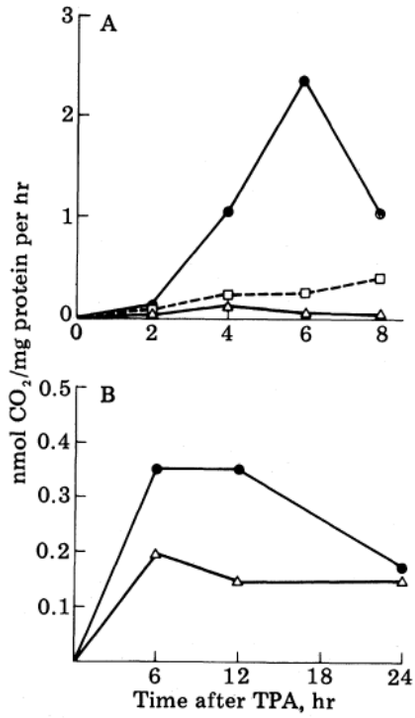

FIG. 3. (*A*) Inhibition of Orn'ase induction *in vivo*. Mice were treated and epidermis was removed at various times for Orn'ase assay. The lidocaine dose (4%, wt/vol) is that used clinically to provide local anesthesia. The initial lidocaine application was made 15 min after application of TPA. Subsequent applications were made every 2 hr. TPA-treated mice received acetone at the same time the anesthetic group received lidocaine. Each point represents results of duplicate assays run on epidermis from two mice assayed individually. Results varied by ±15% or less. Mice treated only with acetone or lidocaine had negligible Orn'ase activity. ●—●, TPA (5 μg); □---□, TPA plus one application of 4% lidocaine; △—△, TPA plus four applications of 4% lidocaine. (*B*) Inhibition of AdoMet'ase induction *in vivo*. Conditions were the same as in *A*. Orn'ase activity in the same cell lysates showed >95% inhibition in the lidocaine group (data not shown). ●—●, TPA (5 μg); △—△, TPA plus four applications of 4% lidocaine.

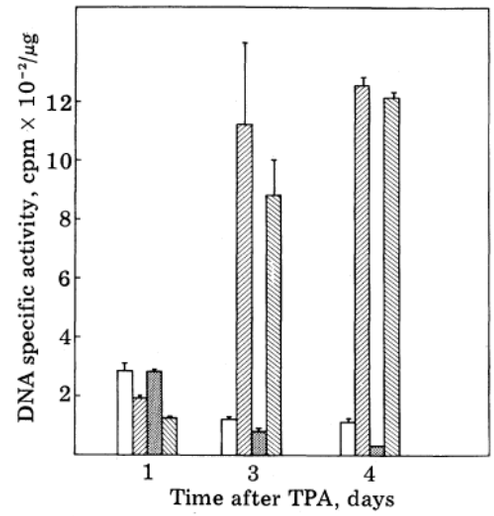

FIG. 4. Effect of lidocaine on TPA-stimulated DNA synthesis. Epidermal cells were exposed continuously to TPA and lidocaine. At the indicated times, cells were pulsed with [^{3}H]dThd and cultures were frozen and then assayed for incorporation as described (9). Medium was changed daily with freshly added lidocaine and TPA. □, 0.1% dimethyl sulfoxide; ▨, TPA (167 nM); ▫, lidocaine (1 mM); ▧, TPA plus lidocaine. The range of replicate determinations is indicated.

activity when added directly to the assay mixture (data not shown). Thus, the inhibitory influence of lidocaine appears to be specific for the induction of Orn'ase.

Because pretreatment of cells with local anesthetics was not as effective as treatment after TPA exposure, it was unlikely that local anesthetics acted by interfering with a "cellular cascade" triggered by TPA during its initial interaction at the cell surface. Thus, our initial hypothesis that local anesthetics would interfere at a plasma membrane site critical for TPA action did not fit the results. Further evidence against a general interruption of promoter action was obtained when lidocaine was tested as an inhibitor of another cellular response to TPA. In epidermal cell culture, phorbol esters induce a marked stimulation of DNA synthesis that is maximal 72–96 hr after treatment commences (2, 25). When epidermal cells were exposed to both TPA and lidocaine (Fig. 4), [^{3}H]dThd incorporation into DNA was stimulated to the same extent (12-fold) as observed for TPA alone. This suggested that local anesthetics did not generally interfere with the action of TPA.

UV light induces Orn'ase in mouse epidermis *in vitro* and appears to act by a mechanism that is partially distinct from that of TPA (26). The kinetics of UV light induction as well as other characteristics (26) suggest that the events prior to transcription and translation of enzyme may be unique for UV light whereas transcription and translation may be common to both UV light and TPA. Lidocaine was an extremely effective inhibitor of UV light-induced Orn'ase when given immediately after irradiation (Fig. 5). Other local anesthetics were equally potent as inhibitors of UV light-induced Orn'ase, and dose–response characteristics for UV light and TPA induction were similar (data not shown). These results further support the specificity of local anesthetics for polyamine biosynthesis and suggest that the action is on the common pathway of transcription and translation or on the production of Orn'ase antizyme (27).

The foregoing results indicated that local anesthetics were specific and effective inhibitors of polyamine biosynthesis but did not provide a mechanism for their action. Because induction by either UV light or TPA was inhibited, the pathway involving transcription or translation of enzyme was implicated as the inhibitory site. To provide further evidence that these later

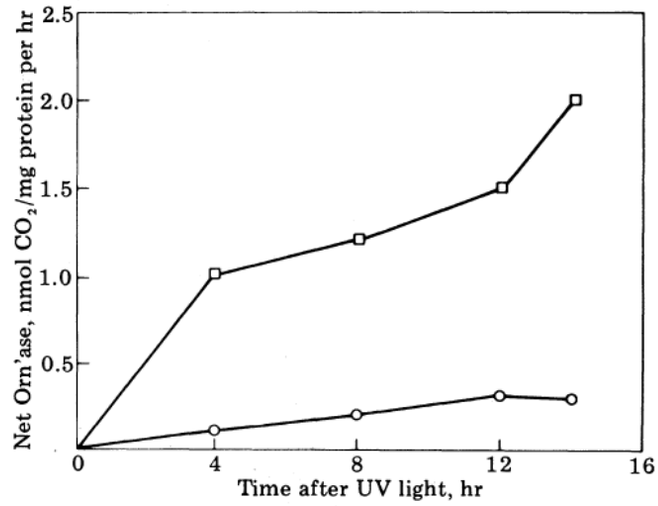

FIG. 5. Effect of lidocaine on UV light-induced Orn'ase. Epidermal cells were washed, exposed to UV light (254 nm; 10 J/m^2), and treated with either control medium or medium containing 1 mM lidocaine. Dishes were washed and frozen at time intervals and assayed for Orn'ase activity. Results represent net Orn'ase activity in which activity in sham-irradiated and solvent or lidocaine-treated cells was subtracted from the appropriate experimental group. □, UV light; ○, UV light plus lidocaine.

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events were likely to be involved, we compared lidocaine to other agents that inhibit at these sites of the induction pathway. Putrescine, the product of the Orn'ase-catalyzed decarboxylation of ornithine, is a primary diamine and inhibits Orn'ase either directly at the level of enzyme translation (28) or indirectly by inhibiting enzyme activity through induction of an antizyme (27). Cycloheximide is an effective translation inhibitor. Cells were treated with TPA, and lidocaine, putrescine, or cycloheximide was added to individual dishes at hourly intervals for up to 7 hr after exposure to TPA. Control groups received additions of solvent only at the times when experimental groups received inhibitors. Orn'ase activity in all groups was measured at the peak time of 8 hr. To follow the natural kinetics of induction, we also determined Orn'ase activity in parallel dishes exposed to TPA at zero time and frozen at hourly intervals thereafter. Fig. 6 presents results from these experiments. Cycloheximide completely suppressed Orn'ase activity when added up to 4 hr after TPA, a time when Orn'ase activity was already significantly elevated. Even up to 7 hr after exposure to TPA, a partial suppression of Orn'ase activity was obtained by addition of cycloheximide. This is in concert with our previous indication of a 60-min half-life for TPA-induced Orn'ase. Both putrescine and lidocaine were also effective inhibitors of induced Orn'ase activity for up to 5 hr after exposure to TPA. However, by 6–7 hr after exposure to TPA, the effectiveness of these agents diminished. The kinetics of inhibition were remarkably similar for putrescine and lidocaine and, in general, the shape of the inhibition curve paralleled that of cycloheximide. However, on a molar basis lidocaine was more effective than putrescine by about 10-fold.

DISCUSSION

Local anesthetics appear to be specific and extremely effective inhibitors of Orn'ase induction by TPA and UV light. For TPA this effect is observed in mouse skin *in vitro* and *in vivo*. The effect on induction by UV light *in vivo* has not been studied.

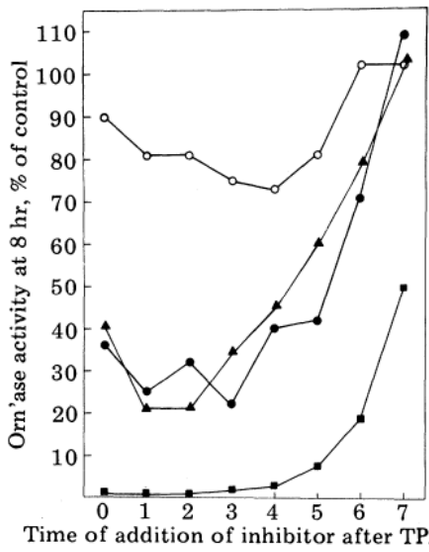

FIG. 6. Effect of addition of inhibitor after exposure to TPA. Epidermal cells were treated with TPA (16.7 nM) and lidocaine, putrescine, cycloheximide, or solvent (ethanol) was added at hourly intervals after TPA. All dishes were washed and frozen at the end of 8 hr and assayed for Orn'ase. Parallel dishes (not shown) were treated with TPA only and frozen at hourly intervals to determine the level of Orn'ase activity at the corresponding time for inhibitor addition. Results are expressed as the % inhibition produced at the time of each addition (but assayed at 8 hr) relative to the level of induction produced by TPA alone (assayed at 8 hr). ○, Solvent only; ■, cycloheximide (70 μM); ●, putrescine (10 mM); ▲, lidocaine (1 mM).

Orn'ase induction *in vitro* is inhibited in the absence of a significant suppressive effect by local anesthetics on protein or DNA synthesis. In fact, DNA synthesis *in vitro* is maximally stimulated by TPA even in the presence of the local anesthetic lidocaine. Thus, local anesthetics, along with corticosteroids (9) and retinoids (4), are agents that can dissociate the induction of Orn'ase from the stimulation of DNA synthesis.

The mechanism by which local anesthetics exert their suppressive effect on Orn'ase is unclear. They appear to act rapidly and are effective during the inductive response as demonstrated by the data in Fig. 6. Treatment before exposure to TPA is not as effective as treatment after exposure to TPA. These observations, combined with the inhibitory effect on UV light induction, suggest that local anesthetics do not act directly at a receptor site for TPA at the plasma membrane as anticipated from previous data on anesthetic effects on other trophic agents (29). A direct effect on the Orn'ase protein is unlikely because local anesthetics are inactive when added directly to the Orn'ase assay. Putrescine, a primary amine, is an inhibitor of Orn'ase induction, and the kinetics of this inhibition resemble those of lidocaine inhibition, at least when TPA is the inducer (Fig. 6). Other inducers have not been studied. Local anesthetics, of the type studied here, have in common a tertiary amine structure. It is conceivable that the tertiary amine structure of local anesthetics and the primary diamine or polyamine structure of the products of Orn'ase activity act similarly, most probably by inhibiting enzyme translation or enhancing antizyme synthesis. Alternatively, the anesthetic molecule could be modified intracellularly to resemble more closely the amine structure of putrescine. For any direct mechanism of action of local anesthetics, one would have to assume that the molecule is only transiently retained by the cell because pretreatment with anesthetics prior to TPA exposure had only a partial inhibitory effect. Lidocaine is a more potent inhibitor than putrescine. This difference may simply reflect preferential uptake of the anesthetic over the polyamine but, nevertheless, makes these agents valuable for further studies on the regulation of the Orn'ase enzyme.

Local anesthetics interfere with the structure and function of microtubules and microfilaments (30, 31). This action has been proposed as the basis for alterations produced by local anesthetics on membrane receptor mobility (31). The induction of Orn'ase by TPA appears to depend on the integrity of microtubules because colchicine and other microtubule-disrupting alkaloids inhibit the process in mouse skin (32). Several observations suggest that local anesthetics do not inhibit Orn'ase induction through microtubule disruption. Local anesthetics are more potent inhibitors of Orn'ase induction than the major microtubule-disrupting alkaloids yet produce only subtle changes in microtubules (30, 31) of some cell lines. Colchicine and similar agents have several generalized cellular effects, such as mitotic inhibition with accumulation of cells blocked in mitosis. Such effects are not observed in epidermal cells treated with local anesthetics. Experiments were performed to assess the mitotic index (MI) in epidermal cells treated with vinblastine, which acts as a mitotic inhibitor in these cells (32), and lidocaine. The MI for cells exposed to vinblastine (6 hr at 0.1 mM) was 39 ± 1/1000; exposure to lidocaine (6 hr at 1 mM) produced a MI of 5 ± 1/1000. Control cells had a MI of 12.5 ± 1.5/1000. Also, unlike lidocaine, microtubule-disrupting alkaloids required a relatively long period of exposure (2–24 hr) prior to exposure to TPA for effectiveness; exposures concurrent with or after exposure to TPA were ineffective. However, this could be related to the relatively slow rate of action of colchicine (33) whereas local anesthetics disrupt microtubules *in vitro* within a short time (30).

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Prostaglandins appear to be mediators of some of the actions of tumor promoters including promotion itself (34, 35), and inhibitors of prostaglandin synthesis interfere with TPA induction of Orn'ase in mouse skin (35). Prostaglandins may also be involved as mediators of the effects of UV light (36). Because local anesthetics inhibit prostaglandin synthesis in some systems (37), they could exert their inhibitory effect on Orn'ase induction indirectly, via the prostaglandin pathway. However, the most striking effect of prostaglandin inhibitors, such as indomethacin, in mouse skin is an inhibition of the proliferative response to TPA (38). Because local anesthetics do not affect this function of TPA, it is unlikely that they act by inhibiting prostaglandin synthesis.

Calcium may play a role in cellular responsiveness to phorbol esters (20, 39), and phorbol esters appear to sensitize cells to the effects of ionic calcium. Local anesthetics are potent antagonists of several calcium-dependent functions (19) and have marked effects on the intracellular distribution of calcium (40). However, there is no evidence to suggest that Orn'ase induction is calcium dependent in mouse epidermis. In fact, results from our laboratory indicate that the Orn'ase induction by TPA is greater when epidermal cells are grown at low extracellular calcium concentrations (ref. 41 and unpublished observations). Nevertheless, intracellular concentrations of calcium or calcium flux may be critical for both Orn'ase induction and phorbol ester action, and a mechanism involving calcium for local anesthetic effects cannot be ruled out.

Although the exact mechanism of action of local anesthetics in this study is unknown, a direct effect on a critical event in polyamine biosynthesis seems most likely rather than a more generalized effect. A calcium-mediated mechanism or microtubule disruption cannot be excluded, but a direct effect on a putative TPA receptor seems unlikely. It is not known whether inhibition of Orn'ase induction is related to the anesthetic properties of these agents, but the rapid onset of anesthesia would suggest that the Orn'ase inhibition is secondary. Nevertheless, these findings point out a previously unreported effect of local anesthetics that should be taken into consideration in studies of the pharmacology of these agents. Whatever the precise mechanism of Orn'ase inhibition, local anesthetics should be extremely valuable as a tool for further understanding the regulation of polyamine biosynthesis and for determining the role of Orn'ase induction in mouse skin tumor promotion.

We acknowledge the assistance and advice of Drs. Beverly Peterkovsky and Henry Hennings, the photographic work of Mr. Douglas Jones, and the secretarial assistance of Mrs. Margaret Green.

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