# Tachyphylaxis in 12-0-Tetradecanoylphorbol Acetate- and Arachidonic Acid-Induced Ear Edema

JOHN M. YOUNG, PH.D., BONNIE M. WAGNER, B.S., AND DOREEN A. SPIRES, M.S.

Institute of Biological Sciences, Syntex Research, Palo Alto, California, U.S.A.

12-0-Tetradecanoylphorbol acetate (TPA) applied to mouse ears rapidly induces an edema which is maximal by 6 hr but has substantially waned by 24 hr. (This is in contrast to many inflammatory agents that cause a prolonged edema lasting many days.) Reapplication of TPA at 16–24 hr will not provoke a second edematous response although increased erythema is evident. Arachidonic acid (AA) applied to mouse ears (4 mg) provokes an even more rapid edema which is maximal at 1 hr and has substantially waned by 6 hr. Reapplication of AA at 3–24 hr also will not provoke a second edematous response although, again, increased erythema does result. Pretreatment of ears with AA results in inhibition of the edema response to subsequent application of TPA, and TPA pretreatment moderately inhibits a subsequent response to AA. TPA-induced edema can be delayed by agents such as naproxen, an inhibitor of AA cyclooxygenase. In contrast, AA-induced edema is inhibited only by agents, such as phenidone, that inhibit both cyclooxygenase and lipoxygenase. The data suggest that the edemas result from interaction of the products of the cyclooxygenase and lipoxygenase pathways of AA metabolism. The lack of secondary edema response appears to be related to the inability of TPA or AA to reinduce vascular permeability. The effect is specific to AA and TPA; responses to xylene or anthralin are unaffected by TPA or AA pretreatment. It is postulated that the tachyphylactic effects observed involve lipoxygenase metabolites of AA.

In the search for novel therapeutic agents useful in the topical treatment of inflammatory skin conditions, considerable emphasis must be placed on the routine screening of compounds in animal models. With respect to known classes of agents such as topical corticosteroids whose mechanism of action is fairly well understood [1] and whose clinical efficacy is virtually guaranteed, any animal model yielding relative potency estimates serves well [2], although occasional pitfalls are encountered [3]. Under this circumstance we are not normally concerned with the mechanisms involved in the model inflammation, nor even with the relevance of the model to human skin inflammation. However, with respect to novel agents, whose efficacy and mechanism of action are unknown, the relevance and properties of the animal model must be given due consideration so that false-positive or negative results do not lead us too far astray.

One of the more commonly used animal models is that of

rodent ear edema induced by application of croton oil [2] or its active constituent 12-0-tetradecanoylphorbol acetate [4]. This latter compound, TPA, is under active investigation as a tumor promoter, as an inducer of epidermal hyperplasia, and as an effector or actuator in a multitude of in vitro systems. The processes involved in the induction of inflammation by topical application of this compound are not well understood, although the Merck group [5] has amassed considerable evidence that elevated levels of the hydroperoxy endoperoxide, $PGG_2$, formed from arachidonic acid (AA), are associated with edema induction.

Our own interest in the mechanisms involved in TPA-induced inflammation was stimulated by the above considerations, as well as the observation that TPA-induced ear edema was short-lived in comparison with that induced by other phlogistic agents such as retinoic acid, oxazolone, and anthralin. While TPA-induced edema could be prolonged by using higher doses, reapplication of the usual dose of 1 µg/ear, after a 24-hr interval, did not result in reinduction of edema. We report here our studies on a phenomenon which we interpret as a specific tachyphylaxis to induction of vascular leakage which may involve products of AA metabolism.

## MATERIALS AND METHODS

### Animals

Female Swiss Webster mice, 5–8 weeks old, were obtained from Simonsen Labs, Gilroy, California and held under standard conditions for 1 week prior to use, with food and water ad lib. Animals were randomly assigned to treatment groups of 8, housed together in a cage.

### Chemicals

12-0-Tetradecanoyl phorbol acetate was from Chemical Carcinogens, Eden Prairie, Minnesota; anthralin (USP, > 95%) from City Chemical Corp., New York, New York; all-trans-retinoic acid, arachidonic acid, and oxazolone from Sigma Chemical Co., St. Louis, Missouri. Compounds were prepared as solutions in reagent-grade acetone just prior to application.

### Treatments

Test materials dissolved in acetone were delivered to the right ears only of mice by means of an automatic microliter pipet. Volumes of 10 µl were delivered to each of the inner and outer surfaces of the ear. This volume spread evenly over the whole surface of the ear with minimal drainage to the base and onto the skull and neck.

### Edema Measurements

For ear thickness determinations, an Oditest dial caliper gauge, 0–0.8 inches, with gradations of 0.001 inches modified to increase the contact surface area and reduce the tension, was applied to the tip of the ear. Ear thickness was recorded in units of 0.001 inches. In order to minimize variation due to technique, measurements throughout an experiment were performed by a single investigator. This method allows edema development in individual animals to be followed through time without the need for anesthesia. In order to provide a more objective assessment of edema at critical time points, appropriate experiments were repeated in which groups of animals were killed by cervical dislocation, the ears immediately removed with sharp scissors, and 8 mm diameter plugs were obtained from the tip with a biopsy punch. The plugs were immediately weighed to the nearest 0.1 mg. In both cases, the means ± standard deviation were calculated for each treatment group (N = 8).

*Histologic Procedures*

At appropriate intervals after application of the inflammatory agents, ears from groups of 4 mice were removed, fixed in neutral buffered formalin, paraffin embedded and sectioned at 7 μm. Slides were stained with hematoxylin-eosin or with Wright's buffered differential stain.

## RESULTS

*Comparative Time Course of TPA-Induced Ear Edema*

When 1 μg TPA is applied to the ears of mice, vasodilatation becomes apparent by 1.5 hr, and edema maximizes at 6 hr, waning thereafter to near control levels at 24 hr (Fig 1). Although the edema has substantially resolved at 24 hr, the ears are still visibly vasodilated and hyperemic, as shown previously by Hecker et al [6]. Inflammatory cells are still present at 24 and 48 hr (Table I). This rapid resolution of edema in the TPA-
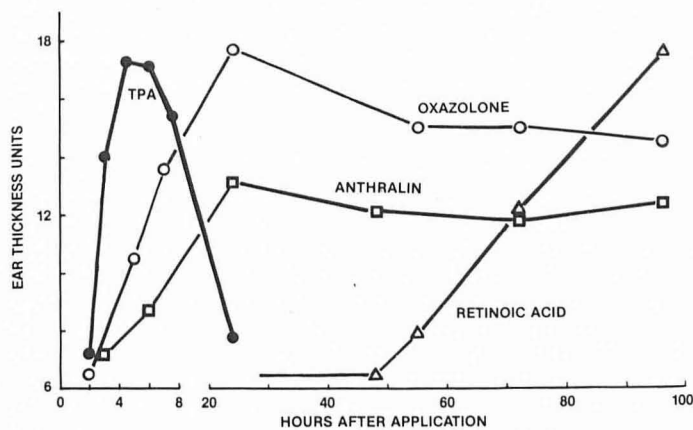
FIG 1. Time course of mouse ear edema response to topically applied agents. The agents were applied in 20 μl acetone at doses of: TPA, 1 μg (●); oxazolone, 1 mg (○); anthralin, 200 μg (□), and retinoic acid, 200 μg (△). The oxazolone response was obtained in animals sensitized by prior topical application to abdominal skin.

FIG 2. Mouse ear edema response to TPA, 1 μg (●) or anthralin, 200 μg (○) 24 hr after initial application of 1 μg TPA. *Vertical bars* indicate standard deviations of treatment groups differing with a significance of $p < 0.05$ or better.

inflamed ear is dose dependent since application of 10 μg/ear results in a much prolonged response and eventual necrosis. Doses lower than 1 μg produced consistently less edema, with the threshold of detectable edema occurring at 0.1 μg. This is in contrast to the much lower doses that produce erythema but no edema [6]. Consequently, unless otherwise stated, all applications of TPA were made at the 1 μg/ear level.

In contrast to the inflammatory reactions produced by anthralin, retinoic acid, or oxazolone in sensitized animals (Fig 1) the response to TPA appeared to be peculiarly short-lived, at least with respect to edema.

*Tachyphylaxis to Repeat Applications of TPA*

In view of the short-lived edema response to a single application of TPA, several attempts were made to boost or prolong the response by reapplying TPA between 16 and 24 hr. Although ears treated with TPA 24 hr previously (Fig 2) showed residual edema and erythema, reapplication of TPA resulted in increased vasodilatation and erythema but substantially less edema than anticipated. This tachyphylaxis appeared to be specific to TPA since application of the unrelated agents, anthralin (Fig 2) or xylene (not shown), resulted in return of maximal edema.

The tachyphylactic response to TPA was examined with respect to dose and time as shown in Fig 3. Ears were initially treated with either the optimal dose (1 μg) or the threshold dose (0.1 μg) of TPA and then treated with 1 μg TPA at 24 hr (Fig 3A). Animals initially receiving the threshold dose responded maximally to the second dose; animals initially receiving the optimal dose of TPA responded minimally. In a series of experiments using initial doses of 0.1–2 μg TPA, the edema response to 1 μg TPA at 24 hr was inversely proportional to the amount of edema produced by the first dose.

If the second application of TPA was delayed until 48 hr (Fig 3B), maximal edema, not tachyphylaxis, was observed. When ears were treated sequentially with TPA at 0, 24, and 48 hr (Fig 3C), maximal edema was produced after the third dose in spite of the fact that these same animals had failed to respond to the second dose at 24 hr.

TABLE I. *Histologic Examination of Inflammatory Ear Reactions to TPA and to AA*

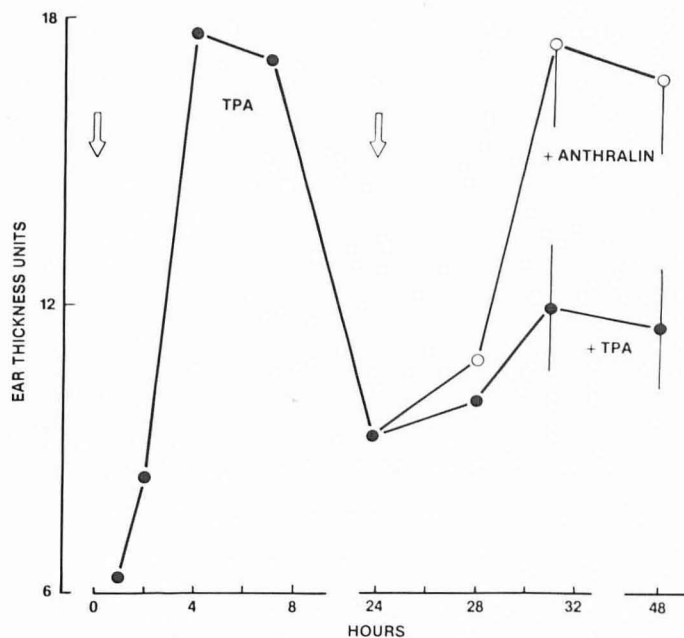| Hours post application | TPA, 1 μg/ear | AA, 4 mg/ear |
|---|---|---|
| 1 | Slight vasodilatation | Marked vasodilatation, dermal edema<br>Inflammatory cells in vessels and tissue |
| 3 | Marked vasodilatation, variable dermal edema<br>Inflammatory cells in vessels and tissue | Marked vasodilatation, slight reduction in dermal edema<br>Decrease (?) in cellular infiltrate |
| 6 | Vessels dilated and occluded with inflammatory cells<br>Marked edema and cell infiltrate in dermal interstitium | Substantial reduction of edema and cell infiltrate compared with 1 hr |
| 24 | Vasodilatation, slight edema<br>Masses of degenerating cells in dermis<br>Epidermal mitoses and mild hyperplasia | Slight vasodilatation, minimal edema<br>Infrequent accumulations of inflammatory cells beneath stratum corneum<br>Frequent epidermal mitoses but minimal hyperplasia |
| 48 | Cell infiltrate in dermis still marked<br>Occasional epidermal abscesses<br>Moderate to marked epidermal hyperplasia | Inflammatory cells absent<br>Frequent epidermal mitoses but only slight hyperplasia |

developed (Fig 4). However, when the applied dose was increased to 0.4 mg and 4 mg, a threshold and maximal edema, respectively, were produced (Fig 4). The notable feature of AA-induced edema was the rapidity of onset and the even more rapid waning compared with TPA. Because of the high dose of AA used, it was necessary to demonstrate that nonspecific toxic effects were minimal. The results of histologic studies comparing AA- with TPA-induced inflammation are given in Table I. One hour after AA application, ear sections showed vasodilatation, dermal edema, and masses of polymorphonuclear and large mononuclear cells, not only in blood vessels, but already in the dermal interstitium. By 3 hr the number of inflammatory cells appeared to be decreased although this could not be quantitated because of the reduction in edema. At 6 hr, the reduction in edema and cellular infiltrate were obvious. During this period no epidermal changes suggestive of necrosis were detected. By 24 and 48 hr, it was difficult to find any inflammatory cells; epidermal mitoses were frequent, but by comparison with TPA-treated ears there was little, if any, epidermal hyperplasia. By comparison, oleic acid at 20 mg/ear produced a slight ear reddening, but no significant edema over a 48-hr observation period.

### Arachidonic Acid-Induced Tachyphylaxis

Since AA appeared to mimic the inflammatory effects of TPA, but with an accelerated time course, the effect of AA pretreatment on TPA-induced edema was examined. When AA (4 mg/ear) was applied 2 hr prior to TPA (Fig 5A), the response to the latter was moderately but significantly diminished. If the time between AA and TPA treatments was extended to 20 hr (Fig 5C), suppression of TPA-induced edema was virtually complete. As shown in the previous experiments (Fig 3A), the degree of tachyphylaxis induced by AA is dependent on the dose used initially. When ears were pretreated with a suboptimal dose of AA (1 mg/ear), only partial inhibition of TPA-induced edema occurred (Fig 5C).

Arachidonic acid was found to be very effective in causing tachyphylaxis to itself. In a series of time course experiments, unresponsiveness to a second application of AA could be demonstrated over the interval of 2–30 hr. Figure 6 illustrates the complete suppression of AA-induced edema 24 hr after the initial application. At this time, the AA-pretreated ears show a slight residual erythema and they become intensely erythema-
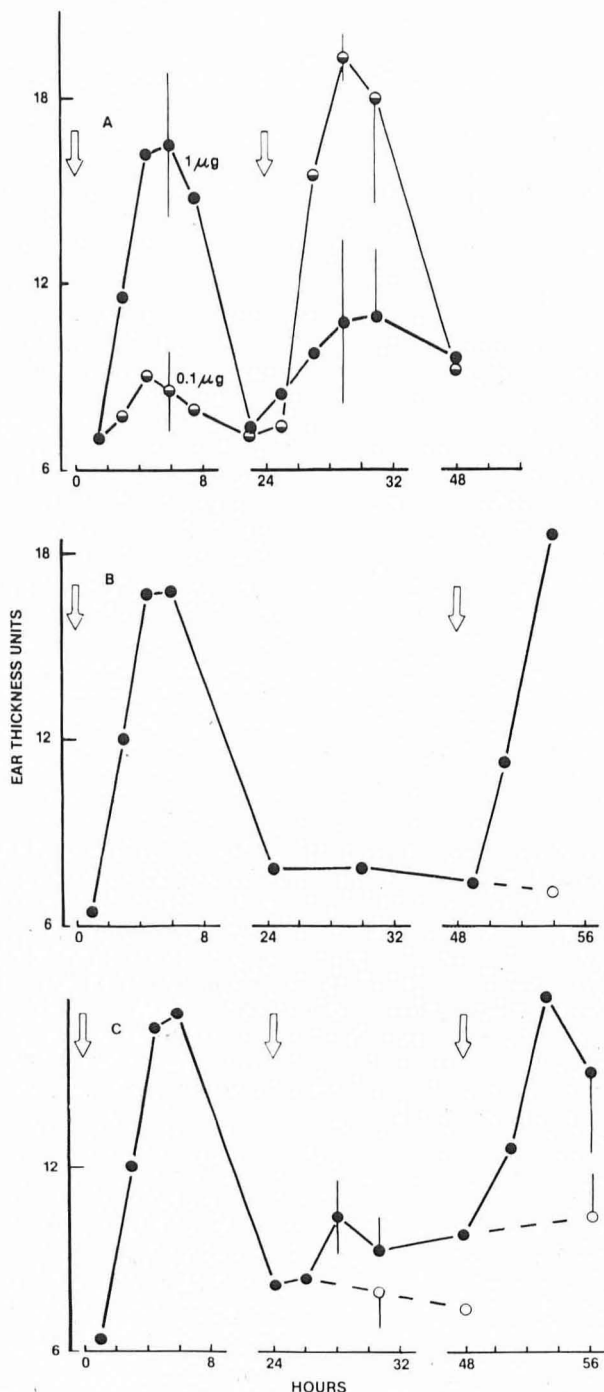


FIG. 3. Mouse ear edema responses to repeated application of TPA. A, Ears were treated with 0.1 $\mu$g (◐) or 1 $\mu$g (●) TPA at 0 hr, and 1 $\mu$g TPA was applied at 24 hr. B and C, After the initial dose of 1 $\mu$g TPA, ears were treated with either 1 $\mu$g TPA (●) or acetone (○) at the times indicated by the *arrows*.

### Arachidonic Acid-Induced Ear Edema

In order to explore the role of AA release and metabolism in TPA-induced edema and subsequent tachyphylaxis, the effects of topical application of prostaglandins and AA on the mouse ear were examined. At doses of 10–40 $\mu$g/ear, $PGE_1$ and $PGE_2$ caused marked vasodilatation within 30 min, but the ears did not become edematous; $PGF_{2\alpha}$ had no observable effect. Pretreatment of ears with these prostaglandins did not alter the inflammatory reaction produced by TPA.

When AA was applied at 40 $\mu$g/ear, an intense vasodilatation and erythema were observed within 5 min, but again no edema
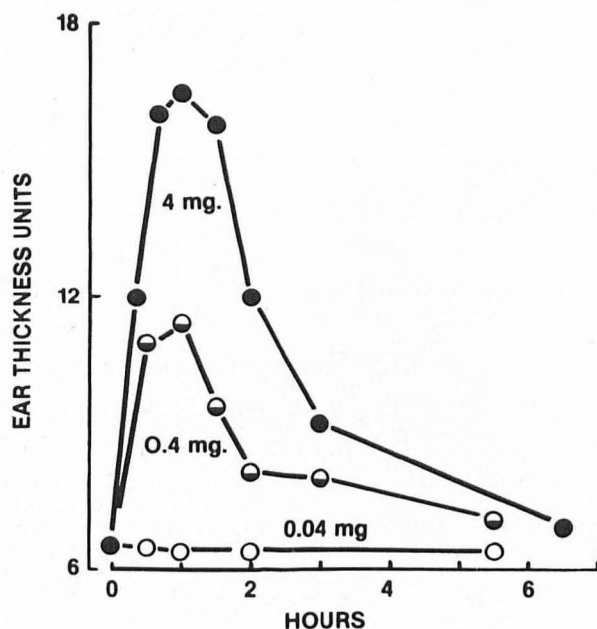


FIG. 4. Mouse ear edema response to doses of topically applied AA. Ears treated with 0.04 mg (○) showed marked vasodilatation.
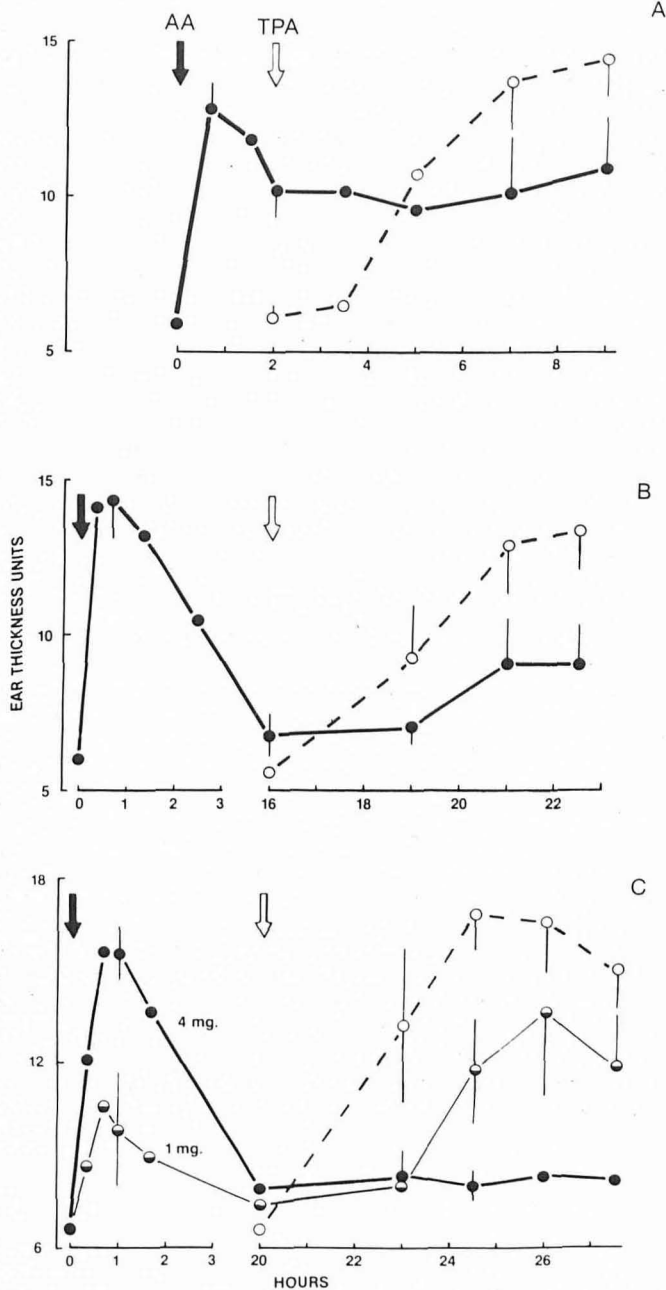
FIG 5. Mouse ear edema response to 1 μg TPA applied 2 hr (*A*) or 16 hr (*B*) or 20 hr (*C*) after AA at 4 mg (●) or 1 mg (◑). Control animals (○) received acetone prior to TPA treatment.

tous in response to the second AA application. Again, the effect is fairly specific since both xylene and anthralin are capable of inducing edema in AA-pretreated ears.

In order to determine whether the tachyphylaxis was a local or systemic effect, a series of experiments was performed in which mice received an initial dose of AA on either the left or the right ear. At intervals between 2 and 24 hr, all groups including appropriate acetone controls were dosed a second time on the right ears only. In every case, the effect was observed to be entirely local; pretreatment of the right ear had no effect on the subsequent response of the left ear.

*Effect of TPA Pretreatment on AA-Induced Edema*

In a series of experiments in which TPA-pretreated ears were dosed with AA 16–24 hr later, there was a consistent and significant depression of the expected AA response, but the

tachyphylaxis was less dramatic than in the previous cases. Since TPA-treated ears still show significant but reduced edema up to 24 hr, the question arises as to what the appropriate control groups should be. If acetone- and TPA-pretreated ears are compared for their response to AA, significant tachyphylaxis amounting to about 30% was observed in only 2 out of 3 experiments and inhibition was restricted to measurements made 30 and 60 min after AA application. The results could be interpreted as a delay and then prolonged response to AA in TPA-pretreated animals. However, if comparisons were made to animal groups which were TPA pretreated and then acetone dosed, thus taking into account the residual TPA-induced edema, TPA pretreatment appeared to produce a 40–60% reduction in the subsequent response to AA over observation periods of up to 6 hr.

*Effect of Nonsteroidal Antiinflammatory Agents on TPA- and AA-Induced Edema*

Since the above results suggested that AA release and subsequent metabolism were important in the observed phenomena and since it had been shown that TPA-induced edema could be prevented by nonsteroidal antiinflammatory agents [4,7,8], we examined several agents for their effects on both TPA- and AA-induced edema. While we have not confirmed the specificity of action in the mouse ear, the agents were chosen based on literature reports of relative inhibitory activity against arachidonate cyclooxygenase and lipoxygenase. Naproxen and indomethacin are relatively specific inhibitors of cyclooxygenase [9] although inhibition of peroxidase [10] has recently been reported. In contrast, nordihydroguaiaretic acid [11] and phenidone [9] inhibit both cyclooxygenase and lipoxygenase, with slight specificity for the latter. These relative specificities have been confirmed at least for mouse epidermal homogenates [12].

As can be seen in Table II, TPA-induced edema is significantly inhibited by all 4 agents, although the degree of inhibition is dependent on the time of measurement. Thus, for example, naproxen delays the onset of maximal edema, but under the conditions described this apparent inhibition disappears with time. It should be noted that these inhibitory effects are observed only if the compounds are applied within 30 min before or after TPA. While an exhaustive pharmacologic anal-
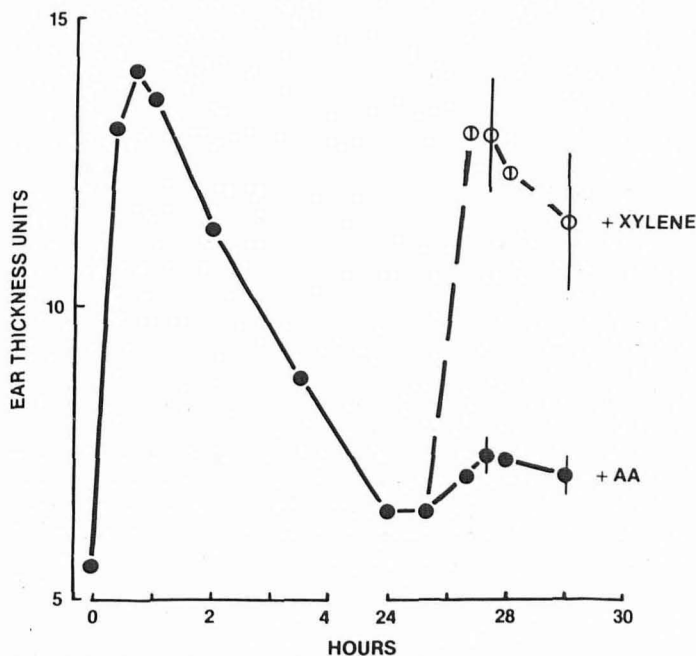


FIG 6. Mouse ear edema response to 20 μl xylene or 4 mg AA in acetone applied 24 hr after initial treatment with AA.

TABLE II. *Inhibitory Effects of Anti-inflammatory Agents on TPA-
and AA-Induced Edema*

| Compound | Dose/ear (mg) | % Inhibition of ear plug weight | | | |
|---|---|---|---|---|---|
| | | TPA edema[a] | | | AA edema[b] |
| | | 4 hr | 6 hr | 8 hr | |
| Naproxen | 2 | 61 | 43 | 33 | 0 |
| | 1 | | 35 | | — |
| | 0.5 | | 21 | | — |
| Indomethacin[c] | 2 | | 42 | | 24[d] |
| | 1 | | 37 | | — |
| | 0.5 | | 11 | | — |
| Nordihydroguaiaretic acid | 2 | 36 | 55 | 50 | 43 |
| | 1 | | 14 | | 9 |
| Phenidone | 2 | 59 | 27 | 31 | 63 |
| | 1 | | — | | 63 |
| | 0.5 | | — | | 26 |

[a] Compounds were applied 15 min after TPA; ear plug weights were determined at times indicated after TPA.

[b] Compounds were applied 30 min before AA; ear plug weights were determined 1 hr after AA.

[c] Tested against a TPA dose of 2.5 $\mu$g/ear.

[d] In 2 repetitions of this assay, inhibitions of 17% and 27% were obtained, but neither was significant ($p > 0.05$).

ysis of the TPA inflammation is beyond the scope of this paper, the results are consistent with a major role for AA metabolites in the production of edema.

Neither naproxen nor indomethacin were consistently active in the AA-induced edema, although the vasodilatation and erythema at 15 min were partially suppressed. Significant inhibition of AA-induced edema could be achieved only with agents that possessed both cyclooxygenase and lipoxygenase inhibitory activity, as exemplified by phenidone and nordihydroguaiaretic acid.

## DISCUSSION

The rapid resolution of TPA-induced ear edema in comparison with other model inflammations stimulated our investigation of the possible mechanisms involved. The demonstration of a specific unresponsiveness with respect to edema induction suggested either a depletion of inflammatory mediators or a tachyphylaxis to such mediators. On the basis of the following considerations it appeared that AA metabolites might be involved in edema induction by TPA as well as the subsequent resolution and unresponsiveness: (1) For both in vivo [13,14] and in vitro [15,16] situations, TPA causes the release from phospholipid pools and subsequent metabolism of AA. (2) TPA-induced ear edema can be inhibited or at least modified by agents whose presumed mechanism of action is inhibition of AA metabolism [7,8].

The demonstration that topically applied AA induced an even more abrupt and short-lived edema followed by a specific unresponsiveness to both AA and TPA provides strong support for the role of AA metabolites in these phenomena. Even though relatively high doses of AA were used in this study, histologic and gross observations exclude nonspecific toxicity effects. While the participation of other inflammation mediators cannot be excluded at this time, recent observations concerning the actions of various arachidonate metabolites provide the basis for a tentative explanation of our results.

In the ear reaction to TPA, vasodilatation is apparent within 1–2 hr and lasts for as long as 48 hr. This is consistent with an early [13] and prolonged [14] production of vasodilatory prostaglandins, as shown for mouse dorsal skin after TPA treatment.

The increase in erythema in response to reapplication of TPA or AA is consistent with stimulation of prostaglandin production to even greater levels. Prostaglandins (and $PGI_2$) do not by themselves cause edema, but by increasing blood flow they enhance the effects of agents causing vascular permeability such as complement-derived products, bradykinin [17] and the leukotrienes [18,19]. The tachyphylactic effects which we have described are related to vascular permeability changes and not to vasodilatation or blood flow changes. Further studies are needed to determine which mediators of vascular permeability are involved in the TPA- and AA-induced ear edemas and whether the effects observed are the result of mediator depletion or a true tachyphylaxis. In view of the inhibitory effects of phenidone and nordihydroguaiaretic acid on both inflammations (Table II), we are proceeding on the assumption that lipoxygenase products are responsible for inducing vascular leakage in these instances.

## REFERENCES

1. Feldman D, Funder JW, Edelman IS: Subcellular mechanisms in the action of adrenal steroids. Am J Med 53:545–560, 1972
2. Tonelli G, Thibault L, Ringler I: A bioassay for the concomitant assessment of the antiphlogistic and thymolytic activities of topically applied corticosteroids. Endocrinology 77:625–634, 1965
3. Young JM, Wagner BM, Fisk RA: Topical betamethasone 17-valerate is an anticorticosteroid in the rat. Br J Dermatol 99:665–673, 1978
4. Kuehl FA, Humes JL, Egan RW, Ham EA, Beveridge GC, Van Arman CG: Role of prostaglandin endoperoxide $PGG_2$ in inflammatory processes. Nature 265:170–173, 1977
5. Kuehl FA, Humes JL, Ham EA, Dougherty HW: Inflammation: the role of peroxidase-derived products, Advances in Prostaglandin and Thromboxane Research, vol 6. Edited by B Samuelson, PW Ramwell, R Paoletti. New York, Raven, 1980, pp 77–86
6. Hecker E, Immich H, Bresch H, Schairer HV: Uber die Wirkstoffe des Crotonols, VI. Entzundungsteste am Mauseohr. Z Krebsforsch 68:366–374, 1966
7. Van Arman CG: Antiinflammatory drugs. Clin Pharmacol Ther 16:900–904, 1974
8. Swingle KF, Reiter MJ, Schwartzmiller DH: Comparison of croton oil and cantharidin induced inflammations of the mouse ear and their modification by topically applied drugs. Arch Int Pharmacodyn Ther 254:168–176, 1981
9. Baumann J, Bruchhausen FV, Wurm G: Flavonoids and related compounds as inhibitors of arachidonic acid peroxidation. Prostaglandins 20:627–639, 1980
10. Siegel MI: Effect of non-steroidal antiinflammatory drugs on arachidonic acid metabolism. Headache 21:264–271, 1981
11. Hammarstrom S, Falardeau P: Resolution of prostaglandin endoperoxide synthase and thromboxane synthase of human platelets. Proc Natl Acad Sci USA 74:3691–3695, 1977
12. Bedord CJ, Wagner BM, Young JM: Assay of cyclo-oxygenase and lipoxygenase activities in mouse tail skin epidermis. Clin Res 30:115A, 1982
13. Furstenberger G, Marks F: Early prostaglandin E synthesis is an obligatory event in the induction of cell proliferation in mouse epidermis *in vivo* by the phorbol ester TPA. Biochem Biophys Res Commun 92:749–756, 1980
14. Ashendel CL, Boutwell RK: Prostaglandin E and F levels in mouse epidermis are increased by tumor-promoting phorbol esters. Biochem Biophys Res Commun 90:623–627, 1979
15. Levine L, Hassid A: Effects of phorbol-12,13-diesters on prostaglandin production and phospholipase activity in canine kidney (MDCK) cells. Biochem Biophys Res Commun 79:477–484, 1977
16. Hammarstrom S, Lindgren JA, Marcelo C, Duell EA, Anderson TF, Voorhees JJ: Arachidonic acid transformations in normal and psoriatic skin. J Invest Dermatol 73:180–183, 1979
17. Wedmore CV, Williams TJ: Control of vascular permeability by polymorphonuclear leukocytes in inflammation. Nature 289:646–650, 1981
18. Bray MA, Ford-Hutchinson AW, Smith MJH: Leukotriene B₄: an inflammatory mediator *in vivo*. Prostaglandins 22:213–222, 1981
19. Peck MJ, Piper PJ, Williams TJ: The effect of leukotrienes C₄ and D₄ on the microvasculature of guinea pig skin. Prostaglandins 21:315–321, 1981