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INTRACELLULAR CALCIUM AND SKIN TUMOR PROMOTION: CALCIUM REGULATION OF THE INDUCTION OF EPIDERMAL ORNITHINE DECARBOXYLASE ACTIVITY BY THE TUMOR PROMOTER 12-O-TETRADECANOYLPHORBOL-13-ACETATE[1]

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Summary: The role of calcium in epidermal ornithine decarboxylase (ODC) induction by 12-O-tetradecanoylphorbol-13-acetate (TPA) was determined in adult mouse skin explants maintained in a serum-free Eagle's HeLa cell medium. Chelation of extracellular calcium by ethyleneglycol-bis-(β-aminoethyl ether) N,N'-tetraacetic acid (EGTA) prevented ODC induction by TPA, which could be resumed upon calcium restoration in the medium. Extracellular magnesium could not replace calcium for ODC induction by TPA. Concurrent incubation of skin pieces with a calmodulin inhibitor trifluoperzine (TFP) inhibited ODC induction. Furthermore, inclusion in the medium of lanthanum, which has a higher affinity for calcium-binding sites than calcium and displaces surface-bound calcium, inhibited ODC induction by TPA.

Among the numerous biochemical changes elicited following topical application of tumor promoter TPA to mouse skin, the induction of epidermal ODC is prominent (1,2). Evidence indicates that the induction of ODC activity by TPA and the accumulation of its product putrescine may play an important role in mouse skin tumor promotion (1-5).

The intracellular second messenger(s) for TPA-induced ODC activity is unclear (4,6-8). Unlike the role of cyclic nucleotides in other mammalian tissues (9), their role in the induction of ODC activity by TPA remains obscure (7,8). The role of microtubule proteins in the enzyme induction has been suggested from the findings that treatment of mouse skin with colchicine or other microtubule-disrupting agents prior to application

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of TPA suppressed ODC induction (6). We have shown that application of TPA to mouse skin leads to an enhanced accumulation of prostaglandins, which may play a role in ODC induction (4). As part of the study to elucidate the nature of the signal that leads to ODC induction by TPA, we found that skin explants incubated in calcium-deprived medium do not induce ODC activity in response to TPA. Furthermore, a calmodulin inhibitor inhibits ODC induction by TPA. The supporting data that calcium may be an intracellular second messenger for ODC induction by TPA are summarized in this communication.

Materials and Methods

Female Charles River CD-1 mice, 7-9 weeks of age, were housed and treated as described previously (4). The backs of the mice were shaved 3 to 4 days before experimentation, and only those mice not exhibiting hair regrowth during this period were used. TPA was purchased from Lifesystems Division LC Services Corporation, Woburn, MA. EGTA was obtained from Sigma Chemical Company, St. Louis, MO. Lanthanum chloride was purchased from Fischer Scientific Company, Fair Lawn, NJ. TFP was a generous gift from Dr. Frank L. Siegel, Waisman Center, University of Wisconsin, Madison, WI. DL-[1-^{14}C]ornithine hydrochloride (specific activity 49.9 mCi/mmol) was purchased from New England Nuclear, Boston, MA. TPA was added to the medium as ethanol solution; the final concentration of ethanol in the medium never exceeded 1%.

Skin was excised from the shaved backs of mice after cervical dislocation. Skin explants were incubated in a serum-free Eagle's HeLa cell medium as described previously (10), except subcutaneous fat and muscle were not scraped off. ODC activity in the soluble epidermal extracts was determined by measuring the release of $^{14}CO_2$ from DL-[1-^{14}C]ornithine hydrochloride (4).

Results

As described previously (10), adult mouse skin pieces remain viable and responsive to TPA addition when incubated in Eagle's HeLa cell medium. A typical time course of ODC induction by TPA in epidermis of incubated skin pieces is shown in Figure 1. Addition of 2.5 μM TPA in serum-free medium, which contains 1.82 mM Ca^{++} and 0.83 mM Mg^{++}, resulted in about a 200-fold increase in ODC activity. The peak activity occurred at about 8 hr; the enzyme activity returned to near basal level at about 18 hr.

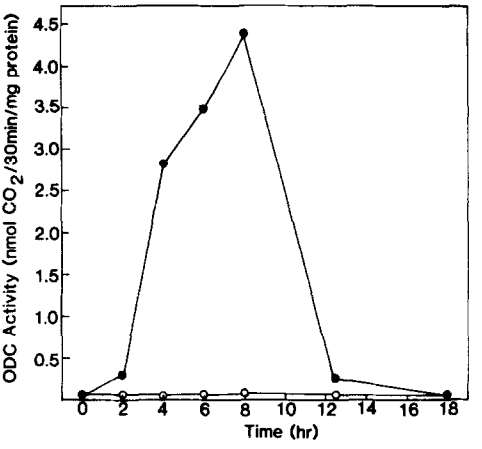

Figure 1. A time course of ODC induction by TPA in incubated skin pieces.

Skin pieces (4 from 4 mice) were incubated in 20 ml Eagle's HeLa cell medium containing ethanol (0-0) or 2.5 μM TPA in ethanol (●-●). Following incubation for the indicated length of time, epidermal ODC activity was determined. Each point represents the mean of determinations carried out from 4 skin pieces obtained from 4 mice.

The effect of chelation of extracellular calcium on ODC induction by TPA is shown in Figure 2. In this experiment, skin pieces were incubated in the concurrent presence of various concentrations of EGTA and 5 μM TPA. Epidermal ODC was determined 6 hr after incubation. Addition of 1.2 mM EGTA did not prevent ODC induction, whereas 3.6 mM EGTA resulted in an 85% inhibition of ODC induction by TPA (Figure 2). At any of the EGTA concentrations tested, pH of the medium remained essentially unaltered; pH of the medium in the presence of EGTA varied from 7.2 to 7.3.

Inhibition of ODC induction by the calcium chelating agent EGTA could be overcome by restoration of extracellular calcium in the medium (Figure 3). In this experiment, skin pieces were incubated in the concurrent presence of 6 mM EGTA and various concentrations of calcium in the medium containing 5 μM TPA; ODC activity was determined 6 hr after incubation. Addition of 6 mM EGTA resulted in 93% inhibition of ODC induction, and this inhibition was completely overcome by the inclusion of 7.2 mM calcium. In a separate

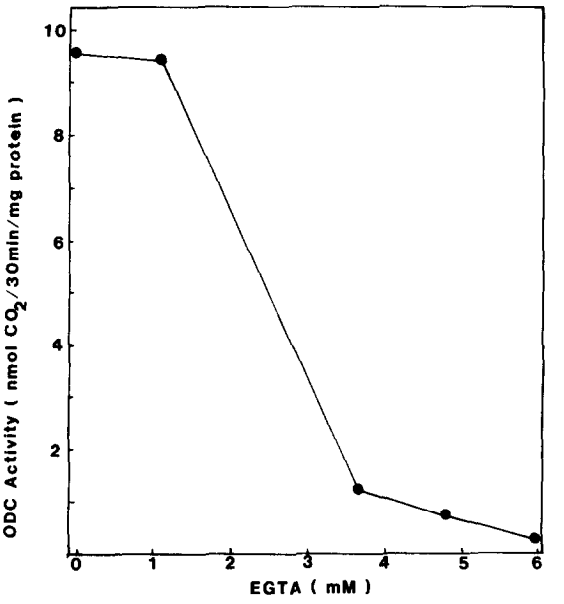

Figure 2. Effect of EGTA concentrations on ODC induction by TPA.

Skin pieces were incubated in the presence of the specified concentrations of EGTA and 5 μM TPA; epidermal ODC activity was determined 6 hr following incubation. Each point represents the mean of determinations carried out from 4 skin pieces obtained from 4 mice.

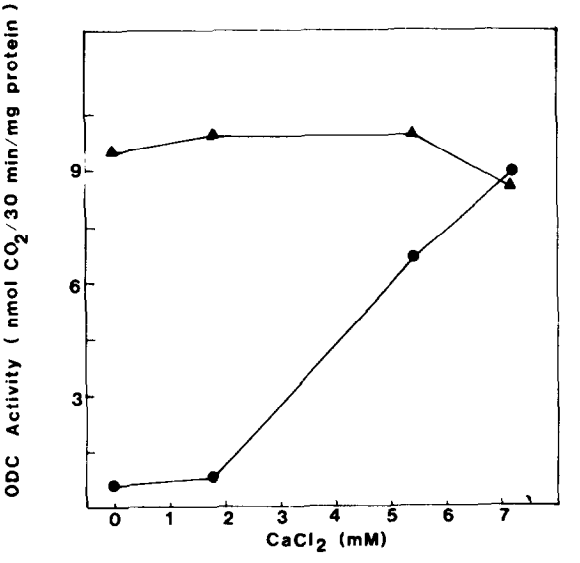

Figure 3. Effect of restoration of extracellular calcium on EGTA-caused inhibition of ODC induction by TPA.

Skin pieces were incubated in the presence (●) or absence (▲) of 6 mM EGTA in the medium containing 5 μM TPA and the indicated concentrations of calcium over and above the normal concentration of calcium (1.82 mM) in the medium. Epidermal ODC activity was determined 6 hr after incubation. Each point represents the mean of determinations carried out from 4 skin pieces obtained from 4 mice.

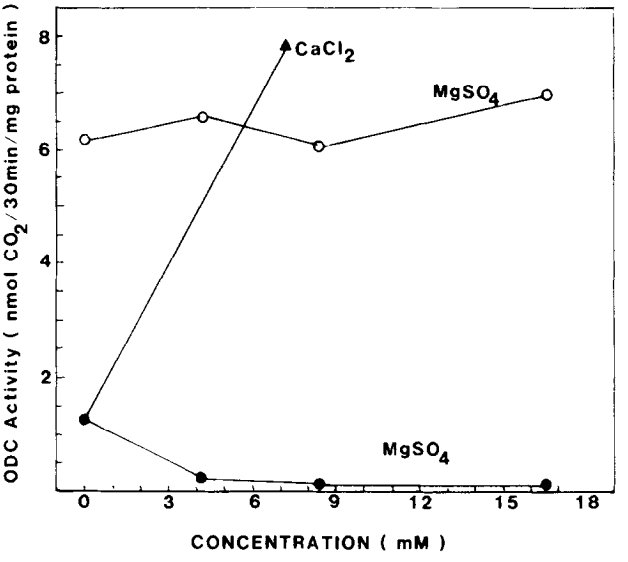

Figure 4. Effect of addition of extracellular Mg^{++} on EGTA-caused inhibition of ODC induction by TPA.

Skin pieces were incubated in 20 ml of medium containing 5 μM TPA and the following. Epidermal ODC activity was determined 6 hr after incubation.
○; medium contains no EGTA, but various concentrations of $MgSO_4$
●; medium contains 6 mM EGTA and various concentrations of $MgSO_4$
▲; medium contains 6 mM EGTA and 7.2 mM $CaCl_2$

experiment (Figure 4), addition of as much as 16.6 mM Mg^{++} failed to overcome the inhibition of ODC induction by EGTA.

Most of the physiological effects of calcium are mediated by its binding to a calcium-binding protein calmodulin (11,12). To further determine the role of calcium in ODC induction by TPA, the effect of TFP (a calmodulin inhibitor) on ODC induction was determined, and the results are shown in Figure 5. Incubation of skin pieces in the presence of 10 μM TFP did not affect ODC induction by 5 μM TPA. However, addition of 50 and 100 μM TFP resulted in 27 and 60% inhibition of enzyme induction respectively. In other experiments, 50 μM TFP inhibited ODC induction from 27 to 55%.

Lanthanum antagonizes the effects of calcium possibly by displacing surface-bound calcium as well as by blocking calcium influx (13). Consequently, the effect of addition of lanthanum chloride to the medium on ODC induction by 5 μM TPA was determined. $LaCl_3$ at 0.1 mM concentration did not inhibit

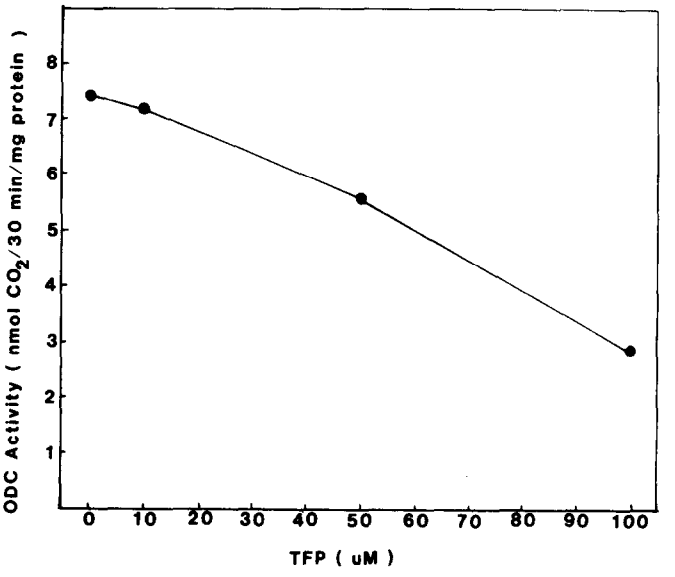

Figure 5. Effect of TFP on ODC induction by TPA.

Skin pieces were incubated in the concurrent presence of the indicated concentrations of TFP and 5 μM TPA; ODC activity was determined 6 hr after incubation.

enzyme induction, whereas a 71% inhibition was observed at 5 mM $LaCl_3$ concentrations; ODC activity in the absence of $LaCl_3$ was 13.75 and in the presence of 0.1 and 5 mM $LaCl_3$ was 14.15 and 4.05 nmol CO_2/30 min/mg protein, respectively.

Discussion

Ca^{++} regulates many cellular functions (14) and has been implicated as an intracellular second messenger for hormonal action (15). For many of its physiological effects, Ca^{++} requires the presence of a high-affinity calcium-binding protein termed calmodulin (11,12). There are indications (16-18) that calcium may play a role in changes triggered by the potent mouse skin tumor promoter, TPA. Thus, the mitogenic effects of TPA in nonmalignant fibroblasts or lymphocyte cultures require calcium (17). Furthermore, calmodulin inhibitor TFP inhibits basal as well as TPA-stimulated [^{3}H]choline incorporation into HeLa cell phospholipids (16). Here we

report that calcium may play a role in TPA-caused ODC induction, a phenotypic change considered to be an important component of the mechanism of tumor promotion by TPA (1-5).

Involvement of calcium in ODC induction by TPA is suggested from a number of findings summarized below.

I. EGTA binds to extracellular calcium with much higher affinity than Mg^{++}. Chelation of extracellular calcium presumably limits calcium influx which is required for ODC induction. This conclusion is supported by the results that EGTA-caused inhibition of ODC induction was completely overcome upon calcium restoration in the medium. (Figures 2, 3 and 4).

II. TFP, a phenothiazine class of antipsychotic drug, which inhibits the binding of calcium to calmodulin (19), inhibited the induction of ODC activity by TPA (Figure 5). These results indicate the role of calcium-calmodulin complex in the induction of ODC by TPA.

III. Inhibition of ODC induction by lanthanum further strengthens the possibility that calcium may be required for ODC induction.

In several cultured cells systems, calcium requirement has been indicated for ODC induction. Examples include serum induction of ODC activity in cultured aortic endothelial cells (20), rat astrocytoma cells cultured in the presence of fetal calf serum or agents that elevate cyclic AMP (21), and ODC induction in Chinese hamster ovary cells by asparagine or agents that elevate cellular cyclic AMP levels (22).

Although the results (Figures 2-5) presented are highly suggestive that Ca^{++} is the key element regulating ODC induction by TPA, the coordination of other cations such as Mg^{++} (23) is highly likely.

The molecular mechanism by which calcium regulates ODC induction by TPA is unknown. Calcium-calmodulin complex activates several enzyme systems such as protein kinases, phosphodiesterase, adenylate cyclase, thymidylate synthetase, transglutaminase, and phospholipase A_2 (11,12).

We have shown previously (4) that PGE_2 may play a role in ODC induction by TPA. The interrelation between Ca^{++}, PGE_2, and ODC induction has yet to be determined. It is possible that TPA treatment activates phospholipase A_2 by facilitating Ca^{++} transport, which in turn hydrolyzes phosphatidylcholine to produce arachidonic acid, a precursor for prostaglandin biosynthesis. In addition, it is highly likely that Ca^{++} may be directly involved via calcium-dependent protein kinase (24) in ODC induction by TPA.

In conclusion, the results presented indicate that calcium may be an intracellular signal that leads to ODC induction by TPA. Since TPA interaction with the plasma membrane is probably a primary target for TPA action and Ca^{++} manipulation modifies TPA interaction with membranes, it appears that the signal for ODC induction is transduced from membrane to nucleus and that Ca^{++} is the key element that plays a regulatory role. ODC induction by TPA is one of the important components of the mechanism of tumor promotion by TPA. Taken together, it is suggested that Ca^{++} may be an intracellular second messenger for tumor promotion by TPA, and the critical targets of Ca^{++} action are presently being investigated.

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