CALCIUM-DEPENDENT RELEASE OF ARACHIDONIC ACID FROM A MURINE EPIDERMAL CELL LINE INDUCED BY THE TUMOR PROMOTER TPA OR IONOPHORE A 23187

Dissection of ionophoretic and phospholipase A_2-stimulating activity

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1. Introduction

Mouse skin in vivo responds to injury and to a wide variety of chemical agents, including tumor promoting phorbol esters and the divalent cation ionophore A 23187, by inflammation and epidermal hyperproliferation (hyperplasia, [1,2]). The latter reaction depends on a short pulse of prostaglandin E_2 synthesis occurring in epidermis within 15 min of stimulation [2,3].

A rapid stimulation of prostaglandin synthesis by TPA is also observed in epidermal cell cultures, including the continuously-growing line HEL/30 [4–6]. This offers the possibility of studying the reaction in more detail.

TPA- and ionophore A 23187-induced prostaglandin synthesis is due to a phospholipase A_2-catalyzed release of arachidonic acid from phospholipids [7–13]. Here, we show that the TPA- and A 23187-induced arachidonic acid release in HEL/30 cells has an absolute requirement for extracellular Ca^{2+}, but is not coupled with a measurable uptake of $^{45}Ca^{2+}$ by the cells.

2. Materials and methods

[1-^{14}C]Arachidonic acid (56.3 mCi/mmol) and $^{45}Ca^{2+}$ (2.29 Ci/mmol) were purchased from New England Nuclear (Boston MA). The ionophore A 23187 was obtained from Calbiochem (Giessen); tetracaine, valinomycin, monensin, carbonyl cyanide, *m*-chlorophenylhydrazone (CCCP), and ethyleneglycol-bis-(β-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA) were obtained from Sigma (Munich). We are grateful to Professor Dr E. Hecker, German Cancer Research Center (Heidelberg) for supplying us with TPA and to Knoll-BASF AG (Ludwigshafen) for a gift of verapamil.

The murine epidermal cell line HEL/30 [4] was maintained in 10 cm (Falcon) plastic petri dishes in minimum essential medium with a 4-fold concentration of amino acids (4 × MEM), 2 mM Ca^{2+}, supplemented with 10% fetal calf serum (Seromed, Munich) and containing antibiotics (penicillin, 100 IU/ml; streptomycin, 100 μg/ml) at 30°C. All incubations were performed in a humidified atmosphere of 5% CO_2. The calcium of the fetal calf serum was removed by passing serum through a Chelex 100 column (Biorad). To obtain 'low calcium medium', this serum was mixed to 10% with Ca^{2+}-free 4 × MEM. The final Ca^{2+} content of the low calcium medium was 2×10^{-7} M as measured by atomic-absorption spectrophotometry.

For the experiments, 5×10^5 cells were plated into 35 mm plastic dishes (Falcon) and grown in 1.5 ml 4 × MEM containing 10% fetal calf serum (culture medium) at 34°C for 4 days, i.e., until confluency was reached (4×10^6 cells/dish). Culture medium was changed 24 h after plating. For the assays the culture medium was replaced by fresh culture medium containing either 0.5% acetone or ethanol, or a solution of various concentrations of TPA, ionophore A 23187, valinomycin, monensin and CCCP in acetone. For the experiments in media with defined [Ca^{2+}] the cells were washed with Dulbecco's PBS (without Ca^{2+} and Mg^{2+}) containing 1 mM EGTA (pH 7.4). The cells were then washed 3 times with PBS to remove EGTA and incubated in culture medium with defined Ca^{2+}-concentrations.

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To assay the release of arachidonic acid, the cells were prelabelled with 0.2 μCi/ml [1-^{14}C]arachidonic acid for 9–14 h, i.e., until reaching the plateau phase of the labelling kinetics. The assay for arachidonic acid released into the medium is detailed in [4].

For the $^{45}Ca^{2+}$-influx experiments, 10^5 cells were plated in each hole of 24 cluster multiwells (Costar) and grown as above. After 4 days, the cells were washed with Dulbecco's PBS (without Ca^{2+} and Mg^{2+}) containing 1 mM EGTA (pH 7.4). To remove the EGTA, the cells were washed with PBS 3 times and incubated with culture medium containing 2 μCi/ml $^{45}Ca^{2+}$ for various time intervals. Incubations were stopped by removing the medium and washing the cells 3 times with 20 mM $CaCl_2$ in isotonic NaCl. Cells were dissolved in 1 ml 0.1 M NaOH for 1 h at 37°C and 0.5 ml of the resulting solution was counted for radioactivity.

3. Results and discussion

When added to continuously-growing HEL/30 cells, both TPA and the ionophore A 23187 stimulated the release of arachidonic acid into the culture medium in a time- and dose-dependent manner after a lag phase of 10–15 min (fig.1). On a molar basis, A 23187 was 3-times more effective than TPA. Monovalent cation ionophores such as the sodium-ionophore monensin, the potassium-ionophore valinomycin, as well as the protonophore CCCP did not show such an effect (table 1). Therefore, the stimulation of arachidonic acid release may be due to the well known ability of A 23187 to facilitate the transport of divalent cations, most probably Ca^{2+}, across cell membranes [14].

The TPA- and A 23187-induced arachidonic acid release depended on the extracellular [Ca^{2+}] (fig.2). Whereas stimulation by A 23187 reached a plateau at physiological [Ca^{2+}] (~1 mM), the TPA-induced activation increased further at higher [Ca^{2+}]. The stimulatory effect of both drugs was completely inhibited when the normal culture medium was replaced by 'low Ca^{2+} medium' (10^{-7} M Ca^{2+}) or after complexing free Ca^{2+} by EGTA. The effect was re-established upon addition of Ca^{2+}, but not of Mg^{2+} (table 2).

Our data indicate that TPA as well as A 23187 induced a Ca^{2+}-dependent activation of phospholipase A_2 by facilitating the influx of extracellular Ca^{2+} into cells, rather than releasing Ca^{2+} from intracellular Ca^{2+}-pools [15]. To confirm this, influx studies with

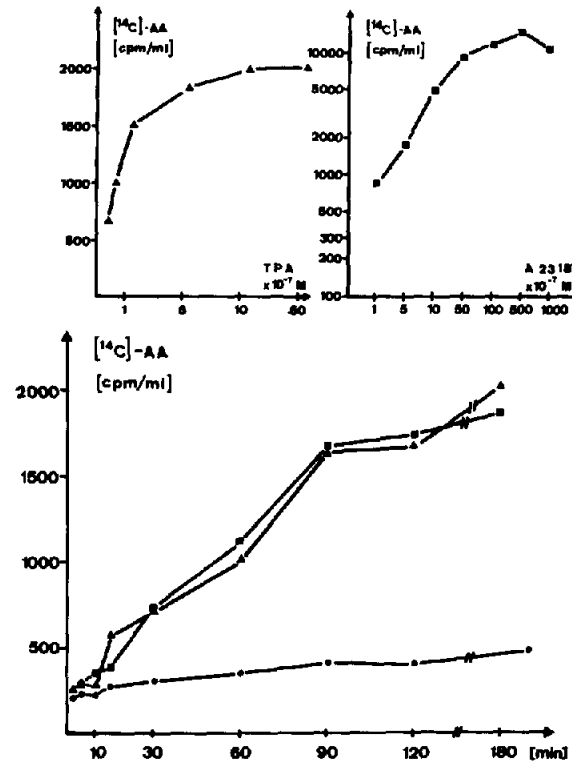

Fig.1. Time courses of the accumulation of arachidonic acid in the culture medium of HEL/30 cell treated with TPA and A 23187. Cultures prelabelled with [1-^{14}C]arachidonic acid were treated with 1.6 × 10^{-6} M TPA (▲), 5 × 10^{-7} M A 23187 (■) or acetone (●, controls) at zero time. At the times indicated, 1 ml culture medium was removed and processed as in [4]. Insert: dose–response curves for the effects of TPA and A 23187 on arachidonic acid release as measured after 90 min. Each point is the mean of 6 assays (duplicate assays from 3 different experiments), SD ≤ 15%.

Table 1
Stimulation of arachidonic acid release after addition of various ionophores

Compound	Dose (M)	Arachidonic acid release (cpm/ml medium)
Solvent		2014
A 23187	5 × 10^{-7}	4387
Monensin	10^{-6}	2349
Valinomycin	10^{-6}	1932
CCCP	10^{-6}	1826

Cultures prelabelled with [1-^{14}C]arachidonic acid were treated with A 23187, monensin, valinomycin and CCCP, dissolved in acetone or ethanol (final conc. 0.5%) or acetone and ethanol (controls) at zero time. After 90 min, the culture media were removed and processed as in [4]. Each value represents the mean of 5 assays, SD ≥ 15%

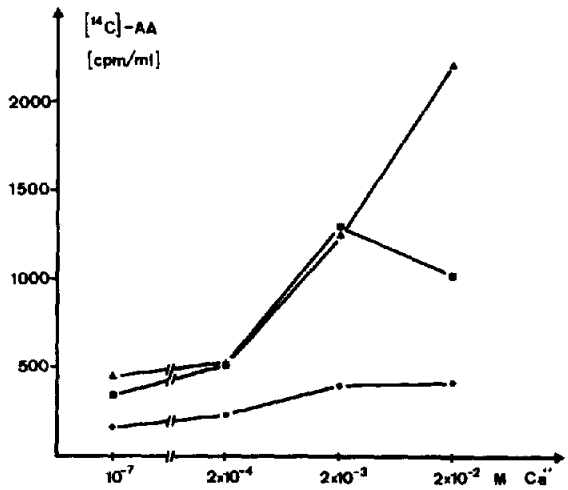

Fig.2. Effect on increasing $[Ca^{2+}]$ on the release of arachidonic acid into the culture medium of HEL/30 cells after treatment with TPA and A 23187. Cultures prelabelled with [1-^{14}C]arachidonic acid were washed free of Ca^{2+} (section 2) and treated with 1.6×10^{-6} M TPA (▲), 5×10^{-7} M A 23187 (■) or acetone (●, controls) in media containing increasing $[Ca^{2+}]$. After 90 min, 1 ml culture medium was removed and processed as in [4]. Each point is the mean of 3 dishes of 1 of 2 expt, SD ≤ 15%.

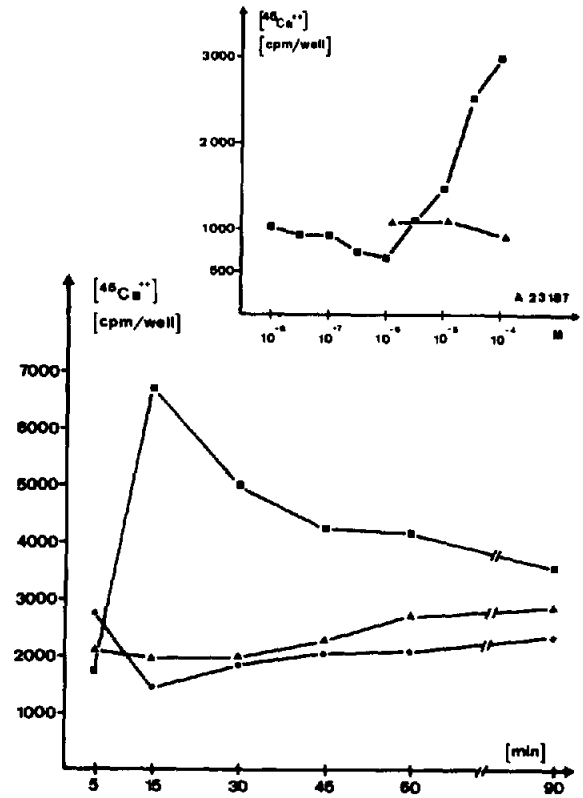

Fig.3. Effect of TPA and A 23187 on the Ca^{2+}-influx into HEL/30 cells. Cells were washed free of Ca^{2+} (section 2) and incubated with culture medium containing 2 μCi $^{45}Ca^{2+}$/ml and 1.6×10^{-6} M TPA (▲), 5×10^{-7} M A 23187 (■) or acetone (●, controls). After the times indicated, the incubation was stopped by removing media and determination of the $^{45}Ca^{2+}$-content of the cells as in section 2. Insert: dose–response curves for the effects of TPA and A 23187 on ^{45}Ca-influx into cells as measured after 15 min. Each point represents the mean of 4 assays of 1 of 3 expt, SD ≤ 15%.

$^{45}Ca^{2+}$ were carried out. The effects of TPA and A 23187 on the influx of $^{45}Ca^{2+}$ into HEL/30 cells are depicted in fig.3. At 10^{-5} M A 23187 elicited a sharp increase of Ca^{2+} influx, reaching a maximum after 15 min. Under steady-state conditions [16], i.e., after preincubation of HEL/30 cells with A 23187, addition of $^{45}Ca^{2+}$ led to the same results (not shown). When the dose of A 23187 was lowered to 10^{-6} M, no

Table 2
Effect of Ca^{2+} on the TPA- and A 23187-induced release of arachidonic acid (cpm/ml medium)

Treatment (dose)	Control medium	Low Ca^{2+}	Low Ca^{2+} + Mg^{2+}	Low Ca^{2+} + Ca^{2+}	EGTA	EGTA + Ca^{2+}
Acetone	570	380	360	480	470	890
TPA 1.6×10^{-6} M	1700	690	550	2330	520	2780
A 23187 5×10^{-7} M	1730	450	520	1350	510	3080

Cultures prelabelled with [1-^{14}C]arachidonic acid were washed free of Ca^{2+} (section 2) and incubated with normal culture medium, low Ca^{2+}-medium (without Mg^{2+}), low Ca^{2+}-medium supplemented with Mg^{2+} (2×10^{-7} M) or Ca^{2+} (2×10^{-3} M), normal medium supplemented with EGTA (2.5×10^{-3} M) or EGTA + Ca^{2+} (5×10^{-3} M), each containing 1.6×10^{-6} M TPA, 5×10^{-7} M A 23187 or acetone (controls) at zero time. After 90 min, the culture media were removed and processed as in [4]. Each value represents the mean of 4 assays of 1 of 2 expt, SD ≥ 15%

Table 3
Effect of tetracaine and verapamil on TPA- and A 23187-induced release of arachidonic acid (cpm/ml medium)

Treatment (dose)	Preincubation with		
	Acetone	Tetracaine (5×10^{-4} M)	Verapamil (5×10^{-4} M)
Acetone (control)	480	970	507
TPA (1.6×10^{-6} M)	1810	750	635
A 23187 (5×10^{-7} M)	2150	1380	570
A 23187 (10^{-5} M)	4150	1780	980

Cultures prelabelled with [1-^{14}C]arachidonic acid were preincubated with tetracaine or verapamil for 15 min. TPA or ionophore dissolved in acetone (final conc. 0.5%) or acetone (controls) were added at zero time. After 90 min, the culture media were removed and processed as in [4]. Each value represents the mean of 4 assays (duplicate assays from 2 different expt), SD $\leq$ 15%

Ca^{2+} influx was observed. Contrary to the ionophore, TPA did not evoke any $^{45}Ca^{2+}$ influx over the whole dose range tested.

Neither the phorbol ester nor the ionophore exhibited a measurable effect on Ca^{2+} influx at concentrations sufficient to stimulate arachidonic acid release. Since the latter reaction has an absolute requirement for extracellular Ca^{2+}, it may be concluded that the stimulation of phospholipase A_2 was due to a mobilization of cell surface-bound Ca^{2+} rather than to an increase of the cytoplasmic Ca^{2+} content. This conclusion was supported by the observation that stimulation of arachidonic acid release by either TPA or A 23187 could be prevented by verapamil or tetracaine (table 3). Both drugs are reported to interact with Ca^{2+} binding sites at cell surfaces [17,18].

TPA [7–9] as well as A 23187 [10–13] have both been shown to stimulate phospholipase A_2 activity not only in epidermis cells but also in other cell types. In the case of A 23187, this has been ascribed to the ionophoretic activity of the compound [14]. Our results do not confirm this assumption but support the results [19] that A 23187 is able to exert biological effects (in this case the activation of lymphocytes) at concentrations which do not alter the cytoplasmic Ca^{2+}-level. Moreover, the phorbol ester TPA did not show any ionophoretic effect even at high concentrations. This observation agrees with the finding that TPA is unable to stimulate the degranulation of mast cells in vitro [2], a process thought to be dependent on an increase of the cytoplasmic Ca^{2+} content. In addition, TPA-induced histamine release from polymorphonuclear leukocytes is provoked by secondary products such as arachidonic acid metabolites rather than by the phorbol ester itself [20]. Finally, in myoblasts TPA has even been found to inhibit Ca^{2+} influx [21]. These results indicate that TPA and A 23187 stimulate phospholipase A_2 activity in epidermal cells by means other than enhancement of Ca^{2+}-influx into the cytoplasm.

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