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Epidermal growth factor (EGF), tumor promoter 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and calcium ionophore A23187 increase cytoplasmic free calcium and stimulate arachidonic acid release and PGE_2/6-keto $PGF_{1\alpha}$ production in cultured porcine thyroid cells

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Epidermal growth factor (EGF), 12-*O*-tetradecanoylphorbol 13-acetate (TPA) and calcium ionophore A23187 increase cytoplasmic free calcium ($[Ca^{2+}]_i$) and stimulate arachidonic acid release and production of PGE_2 and 6-keto $PGF_{1\alpha}$, an end metabolite of PGI_2, in cultured porcine thyroid cells. Addition of EGF, TPA or A23187 to the cells loaded with fura-2, a fluorescent Ca^{2+} indicator, causes an immediate increase in $[Ca^{2+}]_i$, which is the earliest event after mitogen stimulation. This $[Ca^{2+}]_i$ response occurs immediately, reaching a maximum within several seconds. EGF, TPA and A23187 stimulate arachidonic acid release and PGE_2 and 6-keto $PGF_{1\alpha}$ production; the maximum effects are obtained after 2–4 h incubation. EGF, TPA and A23187 increase $[Ca^{2+}]_i$ and then stimulate arachidonic acid release and PG production.

Epidermal growth factor; 12-*O*-Tetradecanoyl 13-acetate; Ionophore A23187; Ca^{2+}; Fura-2; Prostaglandin; (Thyroid)

1. INTRODUCTION

Epidermal growth factor (EGF) and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) are potent mitogens for thyroid cells [1]. In the search for putative intracellular mitogenic signals, attention has been focused on the earliest detectable events following the stimulation by growth factors. The initial consequences of growth factor stimulation include an increase in $[Ca^{2+}]_i$ [2,3] and prostaglandin (PG) production [4]. It is of interest to determine whether EGF and TPA increase $[Ca^{2+}]_i$ in the thyroid gland. We therefore studied the effects of EGF and TPA on $[Ca^{2+}]_i$ and report that EGF and TPA do in fact increase $[Ca^{2+}]_i$. EGF and TPA stimulate the production of PGE_2 and 6-keto $PGF_{1\alpha}$, an end metabolite of PGI_2, in the thyroid gland [4]. This PG production occurs immediately after stimulation. A further question concerns which event is the earlier, i.e. an increase in $[Ca^{2+}]_i$ or PG production after mitogen stimulation. This report shows that an increase in $[Ca^{2+}]_i$ is the earliest event after mitogen stimulation in the thyroid gland.

EGF and TPA stimulate PG production [4]. However, the mechanism of this process is not known. After our initial report [4], we subsequently found that the Ca^{2+} ionophore A23187 also stimulates PG production. This raised the possibility that EGF- and TPA-stimulated PG production is mediated by an increase in $[Ca^{2+}]_i$.

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Thus, we studied the effects of EGF, TPA and A23187 on $[Ca^{2+}]_i$, arachidonic acid release and PG production. This is the first report to show that EGF, TPA and A23187 increase $[Ca^{2+}]_i$ and stimulate arachidonic acid release and PG production. The results indicate that EGF, TPA and A23187 stimulate PG production through an increase in $[Ca^{2+}]_i$. An increase in $[Ca^{2+}]_i$ is the earliest event after mitogen stimulation and occurs immediately after stimulation.

2. MATERIALS AND METHODS

2.1. *Thyroid cell culture*

Thyroid cells were obtained from porcine thyroid glands as described [5]. Freshly isolated cells were suspended (3×10^6 cells/ml) in Eagle's minimum essential medium (EMEM) supplemented with 1% fetal calf serum and antibiotics (penicillin, 200 U/ml; streptomycin, 50 μg/ml). Cells were cultured as a suspension at 37°C in a 95% air:5% CO_2 water-saturated atmosphere.

2.2. *$[Ca^{2+}]_i$ measurement by fura-2*

Isolated thyroid cells were cultured for 16 h and the cells then loaded with fura-2, as in [6]. Fura-2 AM (1 μM final concentration) was added to the EMEM buffered with 10 mM Hepes (pH 7.4). The cells were incubated for 45 min under the culture conditions. Under these conditions, fura-2 AM permeates the cells, is hydrolyzed to fura-2, and binds cytoplasmic free calcium. To remove the unincorporated probe, cells were centrifuged for 3 min at 500 × *g*, and washed in PBSG-Hepes solution, consisting of 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.45 mM $CaCl_2$, 0.49 mM $MgCl_2$, 5.6 mM glucose and 10 mM Hepes [12]. The cells were washed twice and resuspended in the same buffer (10^6 cells/ml). The cell suspension was transferred to a thermostatted quartz cuvette (37°C), for measurement of calcium concentrations ($[Ca^{2+}]_i$) in a Hitachi F-3000 fluorimeter (Hitachi, Tokyo). After reaching a steady state, EGF, TPA or A23187 was added to the cuvette using a syringe. Fluorescence

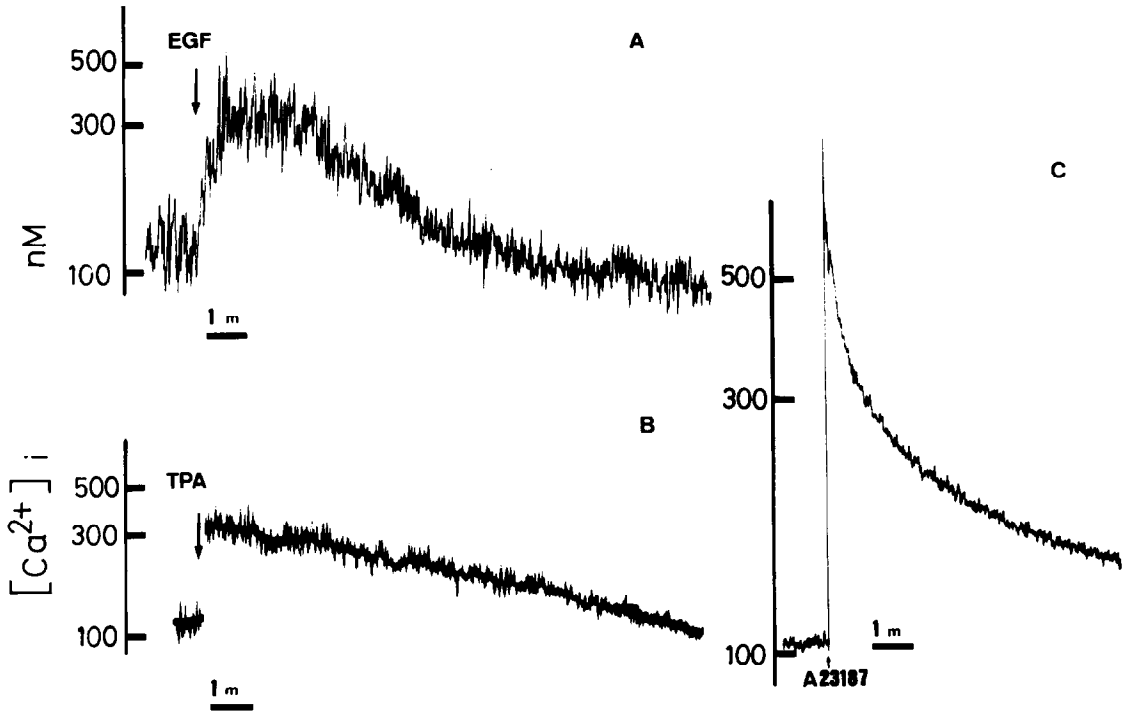

Fig.1. EGF (A), TPA (B) and Ca^{2+} ionophore A23187 (C) induced rise in $[Ca^{2+}]_i$. The fura-2-loaded cells were stimulated by 10^{-10} M EGF (A), 10^{-10} M TPA (B) or 10^{-7} M A23187 (C) as indicated by an arrow. Horizontal bar (1 m) = 1 min.

was recorded with excitation and emission wavelengths of 340 and 505 nm, respectively. $[Ca^{2+}]_i$ was determined as described by Grynciewicz et al. [6].

2.3. *Arachidonic acid release*

Isolated thyroid cells were cultured for 16 h and then cells incubated with [1-^{14}C]arachidonic acid (4 μCi/ml) (70.2 Ci/mmol, New England Nuclear, Boston, MA). After 16 h, i.e. in the plateau phase of the labeling kinetics, the cells were washed twice with the medium and then suspended in EMEM supplemented with 0.5% fetal calf serum. To measure arachidonic acid release, 3 ml of the cell suspension (3×10^6/ml) were incubated with EGF, TPA or A23187 under the culture conditions. After appropriate time intervals, 1 ml medium was removed, acidified to pH 4 with 0.1 N HCl and extracted twice with 4 and 2 ml ethyl acetate, respectively. After vacuum evaporation of the solvent the residue was chromatographed on silica gel plate 60 F (Merck, Darmstadt). The upper phase of isooctane/water/ethyl acetate/acetic acid (50:100:110:20) was used as a solvent and the plate was developed. Reference spots were visualized with iodide vapor. Arachidonic acid spots were scraped and the radioactivities were determined using a liquid scintillation counter.

2.4. *Prostaglandin production*

Isolated thyroid cells were cultured for 16 h and then washed. After washing, cells were incubated in the absence or presence of EGF, TPA or A23187 under the culture conditions. After several hours of incubation, cells and medium were collected and the concentrations of PGE_2 and 6-keto $PGF_{1\alpha}$, an end metabolite of PGI_2, were measured, as reported in [8].

2.5. *Materials etc.*

Murine EGF was obtained from Collaborative Research (Waltman, MA), TPA and A23187 from Sigma (St. Louis, MO), TSH from Armour Pharmaceutical (Phoenix, AZ), and fetal calf serum and Eagle's MEM (EMEM) from Flow Laboratories (Irvine, Scotland). All other chemicals were of the highest purity available commercially. Experiments were conducted at least 4 times. Typical data and the final concentrations of EGF, TPA and A23187 are shown in the text and figures. Student's t-test was used for statistical evaluations.

3. RESULTS

3.1. *EGF-, TPA- and A23187-induced increases in $[Ca^{2+}]_i$*

The cells were loaded with fura-2. The basal $[Ca^{2+}]_i$ was estimated as 106 ± 10 nM (mean ± SE, n = 20). Addition of 10^{-10} M EGF, 10^{-10} M TPA or 10^{-7} M A23187 resulted in a prompt increase in $[Ca^{2+}]_i$ (fig.1). The increase was immediate with no detectable lag period. A peak in $[Ca^{2+}]_i$ was observed within several seconds after addition of EGF, TPA or A23187, followed by a gradual decrease. Graded doses of EGF, TPA or A23187 increased $[Ca^{2+}]_i$ and the peak $[Ca^{2+}]_i$ concentrations after stimulation are shown (fig.2). The effect of EGF or TPA on $[Ca^{2+}]_i$ was observed at 10^{-11} M ($P < 0.05$) and was maximal at 10^{-8} M.

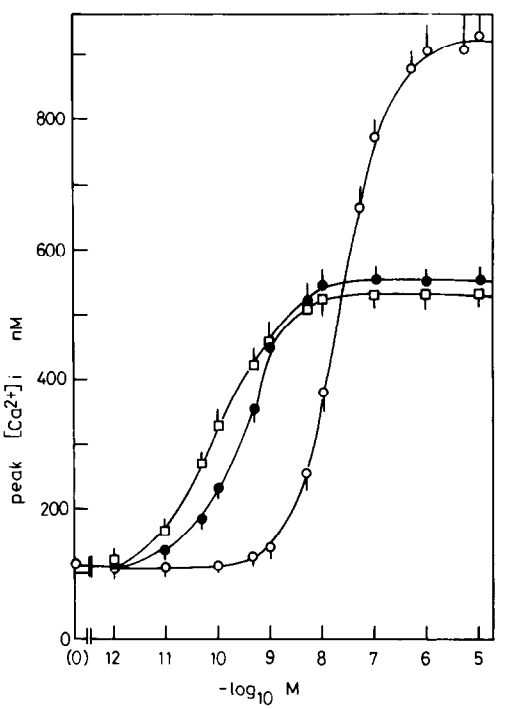

Fig.2. Effects of graded doses of EGF (●), TPA (□) and A23187 (○) on $[Ca^{2+}]_i$. Fura-2-loaded cells were stimulated with graded doses of EGF (●), TPA (□) and A23187 (○) and the peak $[Ca^{2+}]_i$ values are shown. Results shown are the mean of four sets of experiments using different cell preparations.

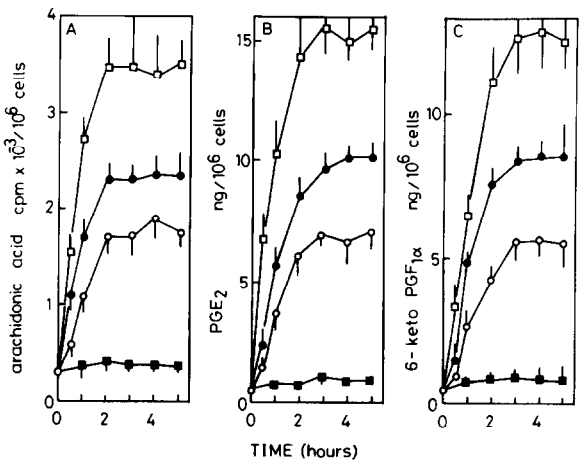

Fig.3. Effects of EGF (●), TPA (□) and A23187 (○) on arachidonic acid release (A), and PGE_2 (B) and 6-keto $PGF_{1\alpha}$ (C) production. Isolated thyroid cells were cultured for 16 h and then washed. After washing, cells were incubated in the absence (■) or presence of 10^{-8} M EGF (●), 10^{-8} M TPA (□) or 10^{-6} M A23187 (○). After 0–5 h incubation, arachidonic acid release (A), and PGE_2 (B) and 6-keto $PGF_{1\alpha}$ (C) production were measured. Each point is the mean ± SE of 3–5 determinations.

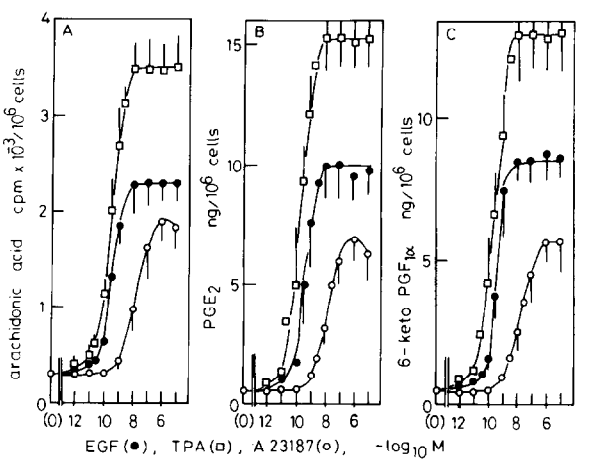

Fig.4. Effects of graded doses EGF (●), TPA (□) and A23187 (○) on arachidonic acid release (A), and PGE_2 (B) and 6-keto $PGF_{1\alpha}$ (C) production. Isolated thyroid cells were cultured for 16 h and then washed. After washing, cells were incubated in the presence of graded doses of EGF (●), TPA (□) or A23187 (○) for 2 (A) or 4 h (B,C). Then arachidonic acid release (A), and PGE_2 (B) and 6-keto $PGF_{1\alpha}$ (C) production were measured. Each point is the mean ± SE of 3–5 determinations.

The effect of A23187 on $[Ca^{2+}]_i$ was evident at 5×10^{-9} M ($P < 0.05$) and was maximal at 10^{-6} M. The A23187-stimulated maximal increase in $[Ca^{2+}]_i$ was higher than that of EGF or TPA.

3.2. *EGF-, TPA- and A23187-stimulated increases in arachidonic acid release and PG production*

Addition of 10^{-8} M EGF, 10^{-8} M TPA and 10^{-6} M A23187 stimulated arachidonic acid release and PGE_2 and 6-keto $PGF_{1\alpha}$ production (fig.3); maximum arachidonic acid release and PG production were obtained after 2 and 3–4 h incubation, respectively. Graded doses of EGF, TPA or A23187 stimulated arachidonic acid release and PG production (fig.4); 10^{-11} M EGF, 10^{-11} M TPA and 5×10^{-9} M A23187 stimulated arachidonic acid release and PG production significantly ($P < 0.05$), with maxima being obtained with 10^{-8} M EGF, 10^{-8} M TPA and 10^{-6} M A23187. The magnitude of increases in arachidonic acid release and PG production was the greatest with TPA and the least with A23187.

4. DISCUSSION

EGF, TPA and A23187 increase $[Ca^{2+}]_i$ and stimulate arachidonic acid release and PGE_2 and 6-keto $PGF_{1\alpha}$ production in cultured porcine thyroid cells. An increase in $[Ca^{2+}]_i$ is the earliest event after EGF, TPA or A23187 stimulation. EGF, TPA and A23187 stimulate arachidonic acid release and PGE_2 and 6-keto $PGF_{1\alpha}$ production through an increase in $[Ca^{2+}]_i$.

EGF and TPA are potent mitogens for thyroid cells [1]. EGF and TPA stimulate PG production, which was thought to be one of the early processes of thyroid cell proliferation [4]. However, we found that the earliest event to occur in response to EGF or TPA is an increase in $[Ca^{2+}]_i$, which stimulates arachidonic acid release and PG production.

EGF and TPA stimulate PG production in the thyroid gland [4]. However, the underlying mechanism remains unknown. In the thyroid gland A23187 has been believed to increase $[Ca^{2+}]_i$ and actually does so. A23187 also stimulates arachidonic acid release and PG production. EGF and TPA increase $[Ca^{2+}]_i$ and subsequently stimulate arachidonic acid release and PG produc-

tion. These results support the concept that the EGF-, TPA- or A23187-stimulated increase in $[Ca^{2+}]_i$ induces arachidonic acid release and PG production. In the thyroid gland, it was reported that stimulation of endogenous phospholipase A_2 caused the release of arachidonic acid from phosphatidylinositol and then increased PG production [9]. Phospholipase A_2 seems to be stimulated by calcium [10]. The EGF-, TPA- and A23187-stimulated increases in $[Ca^{2+}]_i$ activate phospholipase A_2, which stimulates arachidonic acid release and PG production.

EGF and TPA stimulate thyroid cell proliferation [1]. The earliest event to occur in response to EGF and TPA thus far reported is an increase in $[Ca^{2+}]_i$. However, it is not known how an increase in $[Ca^{2+}]_i$ stimulates cell proliferation. The stimulation of arachidonic acid release and PG production by an increase in $[Ca^{2+}]_i$ raised the possibility that EGF- and TPA-stimulated cell proliferation is mediated by some PGs. This possibility is currently under investigation in our laboratory. Stimulation of proliferation by some PGs has been reported in other systems [11].

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